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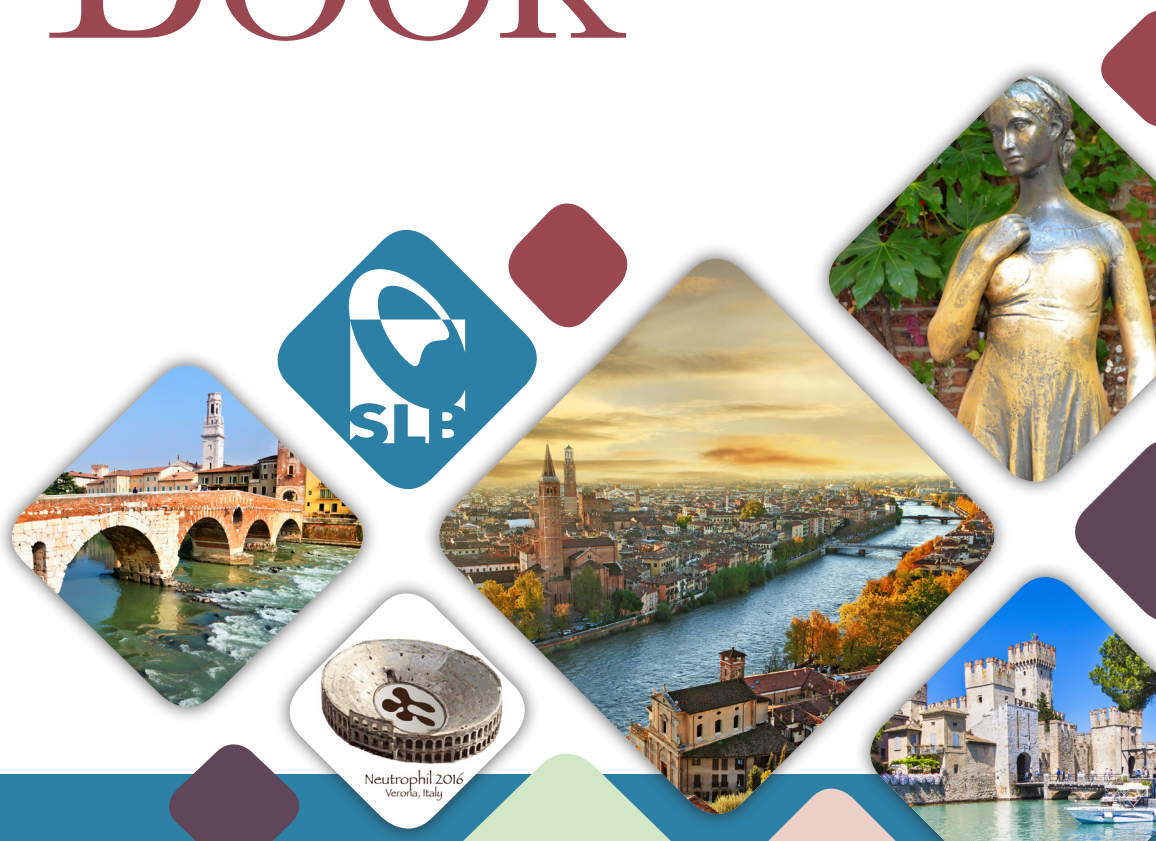
Abstract Book



1

Micro Scale in Vitro Models to Study Neutrophil Behavior: Past, Present and Future

David J. Beebe, *University of Wisconsin*

Microfluidics is now a mature field. The basic physics of microfluidics are now well described and a plethora of components, devices and systems have been demonstrated. The study of cell migration (specifically neutrophils) was one of the first uses of microfluidics in cell biology research. I will review our history of developing and applying micro scale phenomena and technology to enable studies of neutrophil function. While our original focus was to enable and improve basic studies of cell migration, our current focus is multifold. First, we strive to make the systems simple and, thus, more accessible and useable by a broader community. Second, we have begun to apply the systems in studies involving human subjects to, for example, understand immune system regulation in response to various lifestyle factors (e.g. caffeine, exercise, alcohol, meditation). Third, we have begun to build organotypic models that more accurately recapitulate structure/function relationships to study neutrophil trafficking. These models include engineered blood vessels within complex 3D microenvironments to study neutrophil migration (and reverse migration) in various contexts. While, the use of micro scale devices to study leukocyte biology has a long history (actually predating the field of microfluidics), the next decade appears to be primed to begin to reap the full benefits of these now well understood technologies in furthering our understanding of leukocyte biology.

2

On-Chip Phenotypic Analysis of Leukocyte Inflammatory Recruitment in Acute and Chronic Diseases

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Neutrophil arrest and migration on inflamed endothelium involves tethering via selectins and ligation of G-protein coupled receptors, both signaling mechanisms can elicit a conformational shift in the b2-integrins (CD11/CD18) to a high-affinity and clustered state that determines the strength and lifetime of bond formation with ICAM-1. Cytoskeletal adapter proteins Kindlin-3 and Talin-1 anchor clustered b2-integrins to the cytoskeleton and facilitate the transition from arrest to diapedesis. This process represents a gatekeeper mechanism that regulates the rate and number of neutrophils that across endothelium and gain access to inflamed tissue. We recently reported that tensile force acts on LFA-1 (CD11a/CD18) bonds inducing their colocalization with Orai1, the predominant membrane store operated Ca2+ channel that cooperates with the endoplasmic reticulum to elicit cytosolic flux. Employing custom fabricated microfluidic flow channels combined with total internal reflection fluorescence microscopy, we applied defined shear stress to low- or high affinity LFA-1 and imaged the spatiotemporal regulation of bond formation with Kindlin-3 recruitment and Ca2+ influx. We will present a model by which neutrophils use focal adhesions as mechanosensors that convert shear stress–mediated tensile force into local bursts of Ca2+ influx that catalyze cytoskeletal engagement and protrusive force to drive a migratory phenotype.

3

Microfluidic Chambers to Screen Neutrophil Killing of Aspergillus Fumigatus in the Presence of Novel Bifunctional Small Molecules

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The contribution of human neutrophils to the protection against fungal infections by *Aspergillus fumigatus* is essential but not fully understood. Whereas healthy people can inhale spores of *A. fumigatus* without developing disease, neutropenic patients and those receiving immunosuppressive drugs have a higher incidence of invasive aspergillosis. We present a novel microfluidic platform in which the interactions between human neutrophils and *A. fumigatus* were observed in real time, at single-cell resolution, in precisely controlled microenvironments. The design of the microfluidic platform requires neutrophils to migrate along a migration channel to reach nanoliter-sized chambers, where they subsequently interact with *A. fumigatus*.

Time-lapse imaging allows the recording of neutrophil phenotypes including chemotaxis, NET release, swarming, and phagocytosis, while simultaneously monitoring *A. fumigatus* growth rates. The platform technology enabled the robust screening of neutrophil trafficking towards and killing of *A. fumigatus* in the presence of novel bifunctional small molecules (Cloudbreak technology, C-001 and C-016). The Cloudbreak technology (C-001 and C-016) is a bifunctional small molecule utilizing a targeting moiety (antifungal agent) that binds to the cell wall of *A. fumigatus* and is conjugated to an effector moiety (chemotactic peptide fMLP) that attracts and activates neutrophils, directing them to the site of infection and priming them for killing. We found that neutrophils alone are weakly attracted to and modestly control the growth of *A. fumigatus* hyphae (49%). C-001 [100 nM] produced a high influx of neutrophils and reduced hyphal growth to 5%. Chambers containing C-016 [10 nM] plus neutrophils were reduced to <1% hyphal growth. C-016 is significantly more effective at priming neutrophils for effective fungal killing than controls – fMLP or anti-fungal agent. Neutrophils from patients receiving immunosuppressive treatment after kidney transplantation were less effective against the fungus than those from healthy donors and broader heterogeneity exists between patients, compared with healthy individuals. The fungal killing capacity of neutrophils isolated from immunosuppressed patients was significantly increased in the presence of C-001 and C-016 ($P<0.01$). Bifunctional small molecules represent promising immunotherapies for the treatment of aspergillosis or bacterial and viral infections and further study of these agents is warranted. The microfluidic chamber device will accelerate the study of dynamic host-pathogen interactions and will facilitate the development of immunomodulating therapies. Currently, we are working on developing integrated multi-sensing platforms to measure cytokine secretion rates and to detect levels of ROS at the single cell level in the context of infection and inflammation. The microfluidic platform developed in this study may eventually become a useful tool for measuring the ability of neutrophils from patients to mount effective immune responses against fungi or other pathogens *in vitro*. Such tools may be able estimate the risk for infections for each patient, and have implications for the faster diagnosis and start of treatment during infections.

4

Rapid Microfluidic Immune Cell Migration Analysis for Biologically Oriented and Clinically Oriented Research

Francis Lin, *University of Manitoba*

Directed cell migration is a complex cellular function that critically mediates a broad range of physiological and pathological processes such as immune response and cancer metastasis. Chemical concentration gradient is an important guidance cue for many cell types, particularly the diverse immune cell types. Microfluidic devices can precisely configure cellular microenvironments and therefore have been increasingly employed to investigate the mechanism of immune cell migration and chemotaxis. Recently, microfluidics-based immune cell migration studies have also shown promise for enabling clinical diagnostic applications. In this talk, I will discuss our recent work in the development of new microfluidic devices to study the biological mechanisms of human blood neutrophil migration and chemotaxis. Some of these studies are connected to disease models such as chronic kidney disease and fibrosis. In addition, I will discuss the development of an integrated microfluidic cell migration system to enable rapid all-on-chip human neutrophil migration analysis for assessing chronic lung disease.

5

Application of Machine Learning Algorithms to Identify Sepsis-Specific Neutrophil Migration Signatures

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Sepsis affects more patients than breast, lung, and prostate cancer combined, and is responsible for up to half of US hospital mortalities. Treatment of

sepsis is the single largest expenditure in US hospitals, but is misdiagnosed in one third of cases. Early diagnosis of sepsis is crucial, with mortality rates increasing by 10% for every 6 hours that treatment is delayed. Current diagnostic approaches are complicated by clinical overlap with systemic inflammatory responses and long culture periods, and provide low predictive power (AUROC = 0.72). Dysregulation of neutrophil biology, including an increase in circulating numbers, is a key characteristic of sepsis that has long provided diagnostic value. Recently, more detailed *in vitro* analyses have also identified sepsis-specific changes to neutrophil function and behavior, including altered phagocytic activity and chemotaxis. However, most traditional *in vitro* assays of neutrophil function only provide endpoint readouts and do not facilitate detailed measurement of complex migration phenotypes.

In this study, we designed a microfluidic device to assess complex behavioral phenotypes of neutrophils migrating spontaneously from a drop of whole blood. The device consisted of a simple maze that allowed measurement of multiple aspects of neutrophil migration, better mirroring what can be observed *in vivo*.

Application of machine-learning approaches to complex neutrophil migration datasets identified key behaviors that segregated with septic and pre-septic patients groups. Integration of these characteristics into a scoring strategy allowed diagnostic and predictive identification of sepsis in patients with AUROC values greater than 0.95. This represents an enormous improvement upon current approaches, and provides a new diagnostic tool that could significantly impact patient outcomes.

6

Staphylococcus Aureus Enterotoxin A Induced Pulmonary Inflammation is Accompanied by Distinct Changes in Cell Populations within the Lung

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Staphylococcus aureus is a part of the normal human flora colonizing the skin and nasopharyngeal regions, but under pathological conditions it can also represent one of the most common etiologies of sepsis. The abnormal immune response occurring during sepsis can lead to tissue damage, organ dysfunction, and death. Enterotoxins such as *S. aureus* enterotoxin A (SEA) are thought to play major causative roles in the mechanism of injury. SEA directly crosslinks MHC II and specific T cell receptor Vβ chains, triggering oligoclonal T cell activation and excessive immune response. We showed previously in a mouse model that SEA inhalation induced a rapid, systemic immune response followed by local inflammation in lung tissue. Exposed mice presented with alveolitis resembling acute lung injury, which often occurs septic patients. The purpose of this study was to further characterize the resulting pulmonary inflammation and to determine the mechanism leading to increased lung permeability. Following SEA inhalation, mouse lung tissue was examined by confocal microscopy. We first inspected the lung for SEA binding and found that SEA was bound predominantly to alveolar macrophages; however, SEA could no longer be detected 16 h after inhalation. Clusters of SEA-specific T cells, granulocytes, and active caspase 3-positive apoptotic cells were apparent by 48 h after inhalation. The mechanism by which SEA induces increased lung permeability was investigated using mass cytometry (CyTOF) to detect subtle changes in cell populations present in the lung. Pulmonary tissue was stained with panels of 16 surface and 8 intracellular markers and analyzed using a mass cytometer. ViSNE maps were generated characterizing discrete cell populations, which were then examined for changes in marker expression patterns. T cells from SEA-exposed mice showed greater expression of pSTAT3, pSTAT5 and pS6 but lesser expression of IκB. $CD11c^{+}$SIGLEC F^{+} alveolar macrophages and $CD11c^{+}$MHC II^{+} dendritic cells obtained from SEA-treated mice had greater expression of MHC II, pSTAT1 and pSTAT3 compared to vehicle control. Finally, $CD45{-}CD31^{+}$ endothelial cells had greater levels of pSTAT1 and MHC II following SEA inhalation compared to vehicle. Flow cytometric analysis showed that total endothelial cell numbers were significantly reduced 48 h after SEA inhalation. Isolation of RNA from sorted endothelial cells revealed reduced expression levels of *Nos3* (iNOS) and *Cdh5* (VE-cadherin) in SEA-exposed mice relative to vehicle control. These findings demonstrated that SEA inhalation leads to dynamic changes in cell populations within the lung and in particular, transcriptional and signaling changes in

endothelial cells. Future studies will focus on defining how the adaptive immune system integrates signals with the innate immune system to give rise to endothelial cell injury. Understanding SEA-induced pulmonary inflammation may unravel new therapeutic options and help improve outcomes for patients with sepsis and acute lung injury related to sepsis.

7

Agents Differentiating Hl-60 Cells toward Granulocyte-Like Cells Determine Their Ability to Release Neutrophil Extracellular Traps

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Release of neutrophil extracellular traps (NETs) by granulocytes, although firstly described over a decade ago, remains a poorly understood phenomenon. Notably, studying the functions of neutrophils have been constrained by the facts that these cells live shortly, can easily become preactivated during the isolation and are a difficult object to genetic modifications using current techniques. Thus, the investigations into neutrophil biology could benefit from the availability of a cell line model well resembling the functions of peripheral blood neutrophils. The purpose of our study was to find a compound most effectively differentiating HL-60 (human promyelocytic leukemia) cells toward GLC able to release NETs. HL-60 cells purchased from Sigma Aldrich were cultured in RPMI 1640 + 10% fetal bovine serum and antibiotic/antimycotic in 5% CO2 humidified air at 37ºC. Human peripheral blood neutrophils were obtained by density gradient centrifugation from buffy coats of healthy blood donors. HL-60 cells were differentiated to GLC by treatment with ATRA (1μM), DMSO (1,25%) or DMF (70 mM) for 5 days. Cell differentiation was assessed morphologically by May-Grünwald-Giemsa staining and by evaluating CD11b and CD14 expression by flow cytometry. NETs formation was stimulated with PMA (phorbol 12-myristate 13-acetate; 100nM) or CI (calcium ionophore A23187; 4μM) for 3h. NETs release was measured with a fluorometer using extracellular DNA binding dye Sytox Green. NETs formation was also assessed qualitatively with fluorescent microscope after staining for DNA and myeloperoxidase. The processes important for NETs release: reactive oxygen species (ROS) production and citrullination of histone 3 were assessed respectively: fluorometrically after loading the cells with 4 ug/ml DHR and by Western blotting after incubation with anti-CitH3 antibody. Flow cytometry revealed, that ATRA differentiated HL60- cells most effectively toward granulocyte-like cells (% of CD11b+ cells: ATRA-97.8%±1.3%, DMSO-91.3%±2.0%, DMF-90.8±2.9%), lack of CD14 expression indicated that no treatment induced monocytic differentiation. Regardless to differentiating compound used, GLC were morphologically similar to peripheral blood neutrophils. We found poor release of NETs in ATRA-differentiated(d) HL-60 cells, moderate in DMSO-dHL-60 cells and the best in DMF-dHL-60 cells. Moreover, we found higher NETs release in CI-stimulated than in PMA-stimulated DMSO- and DMF-dHL60 cells. Similarly to peripheral blood neutrophils, both PMA and CI induced oxidative burst in DMSO- and DMF-dHL-60 cells. Conversely, ATRA-dHL-60 cells were unable to release ROS after stimulation with CI. We observed an increase in the level of CitH3 in DMSO- and DMF-, but not in ATRA-dHL-60 cells in an unstimulated state. Citrullination of histone 3 in these cells further increased after stimulation with CI (significant increase) and PMA (slight increase). Interestingly, the level of citH3 in ATRA-dHL60 cells after stimulation with PMA and CI did not increase and was even lower than in undifferentiated HL-60 cells stimulated with these compounds. We concluded, that when studying NETs biology , DMF is the best stimulus for HL-60 cells differentiation. Conversely, ATRA-dHL-60 cells are not recommended for these studies.

8

Imaging the Interaction of the Cytosolic Subunits of the NADPH Oxidase in Live Cells – from Quantitative Fret Imaging to 3D Model

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The phagocyte NADPH oxidase (NOX2) is a key enzyme of the immune system generating superoxide anions, which are precursors for other reactive oxygen species. Dysfunctions of NOX2 are associated with numerous diseases and thus detailed knowledge about its regulation is needed. This oxidase is composed of five subunits, the membrane-bound gp91phox and p22phox and the cytosolic p47phox, p67phox, and p40phox. The latter are assumed to be in a ternary complex that translocates together with the small GTPase Rac to the membranous subunits during activation. Our aim was to discover and to characterize specific interactions of the cytosolic subunits of NOX2 in live cells using a Förster Resonance Energy Transfer (FRET) based approach. Since FRET depends on the distance between two fluorophores, it can be used to reveal protein-protein interactions non-invasively by studying fluorescent protein (FP) tagged subunits. The cytosolic subunits of NOX2, p40phox, p47phox and p67phox were tagged with a FRET pair of FPs on the N-terminus or the C-terminus and expressed in COS7 cells. FRET was measured by fluorescence lifetime imaging microscopy (FLIM), because it allows a direct determination of the apparent and molecular FRET efficiency, which contains both qualitative and quantitative information about the interaction and the structure of the interacting proteins. Fluorescence cross correlation spectroscopy (FCCS) is a completely independent method that is not based on distances like FRET but on the observation of the co-diffusion of the FP-labelled subunits when they move across a small confocal observation volume inside the cells. Together, our FRET-FLIM and FCCS data allowed us in a first step to discover heterodimeric interactions between all cytosolic subunits in live cells. They also indicate that each cytosolic subunit is fully bound to its partners provided that the partners are present in sufficient quantity. From the FRET-FLIM results we were able to extract structural information about the interactions and to compare them with available structural data obtained from in vitro studies using small angle X-ray scattering (SAXS). The information from FRET-FLIM was coherent with the SAXS data. We then aligned the available structures leading to the first 3D-model of the cytosolic complex of the NADPH oxidase in the resting state in live cells.
In conclusion, we developed a quantitative FRET-FLIM approach that is not only able to distinguish between specific and unspecific protein-protein interactions in live cells, but gives also information about the structural organisation of the interacting proteins. We can propose for the first time a 3D-model of the cytosolic complex of the NADPH oxidase covering the in vitro as well as the live cell situation.

9

Chlorinated Flavonoids Are Effective Modulators of Hypochlorous Acid Production, Both in Isolated Human Neutrophils and Whole Blood

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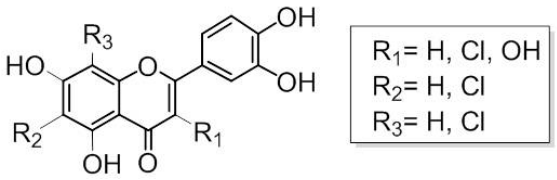
Flavonoids are bioactive molecules found in a wide variety of plants and fruits and are important components of the human diet. These compounds display many biological activities and have been suggested as effective antioxidant and anti-inflammatory agents.
During the complex and orchestrated inflammatory cascade, neutrophils play a crucial role and are responsible for the production of various inflammatory mediators and reactive species, namely hypochlorous acid (HOCl), generated by myeloperoxidase. HOCl is known to be the major oxidant agent produced by activated neutrophils, with a long lifetime and potent microbicidal and cytotoxic properties. It was previously shown that flavonoids are able to react with HOCl, forming stable mono and dichlorinated products. Taking into account the lack of information about the biological

activities of chlorinated metabolites of flavonoids, our aim was to investigate the effect of synthetic chlorinated flavonoids and their parent compounds (Figure 1), quercetin and luteolin, in the modulation of HOCl production. For that purpose, this study was performed in two different cellular models: in isolated human neutrophils and in human whole blood, to resemble, as close as possible, the *in vivo* physiologic state.

The oxidative burst measurement was undertaken by applying a fluorescent technique, in which the HOCl - induced oxidation of aminophenyl fluorescein (APF) was monitored in phorbol myristate acetate (PMA) - activated cells. It was observed that, in general, chlorinated flavonoids were efficient modulators of HOCl production in the two cellular models tested. In human neutrophils, 3-chloro-3',4',5,7-tetrahydroxyflavone and 6-chloro-3',4',5,7-tetrahydroxyflavone were the most active compounds. In whole blood, quercetin, luteolin and 6-chloro-3',4',5,7-tetrahydroxyflavone had the highest activities. Even though the tested flavonoids were more efficient in the study with isolated neutrophils, the whole blood model should be considered as a preferential tool in this kind of assays since it mimics the physiologic environment, not excluding important blood elements that may take part in the immune response.



Acknowledgements

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10

The Importance of Being "Pure" Neutrophils

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In the last years, we have developed a simple procedure to isolate neutrophils at > 99.6 % purity from either whole blood or buffy coats[1] (herein defined as Neu). Neu incubated with 5 μM R848 (a TLR8 ligand) were then shown to produce IL-6 and TNFα, at maximal levels after an overnight incubation. Under the same experimental conditions, however, Neu neither express IL-10 or interferon-stimulated genes (ISG) mRNA, nor produce IL-10[2].

Recently, one-step isolation kits designed to guarantee a rapid isolation of highly pure neutrophils from the blood have become commercially available. We thus isolated neutrophils by using the "MACSexpress® Neutrophil isolation kit" and the "EasySep™ direct human neutrophil isolation kit" (herein defined as, respectively, wbNeuM and wbNeuE) to check their capacity to express genes and produce cytokines in comparison to Neu. As a result, we found that both wbNeuM and wbNeuE expressed notable levels of IL-10, IFIT1 and ISG15 mRNA, as well as released detectable amounts of IL-10, when incubated with R848 for 20 h. Similarly, wbNeuM and wbNeuE, but not Neu, treated with R848 produced elevated levels of TNFα and IL-6 already after 4 h of incubation. Furthermore, both wbNeuM and wbNeuE expressed IFIT1 and ISG15 mRNAs upon incubation for 20 h with 50 μg/mL poly(I:C) (a TLR3 ligand), while Neu did not, in the latter case consistent with the notion that human neutrophils lack TLR3.

Hence, we investigated the precise purity of wbNeuM, wbNeuE and Neu by flow cytometric analysis focusing on thirteen different cell markers. While Neu confirmed to be 99.7 % pure, both wbNeuM and wbNeuE, although 98.9 ± 0.5 % and 97.5 ± 1 % pure, respectively, were found to be reproducibly contaminated by eosinophils (0.29 ± 0.37 % for wbNeuM and 0.53 ± 0.61 % for wbNeuE) and slan^{+}CD16^{+}-monocytes (0.33 ± 0.16 % for wbNeuM and 0.22 ± 0.19 % for wbNeuE).

Because slan^{+}CD16^{+}-monocytes display a well-known capacity to produce elevated amounts of cytokines when stimulated by TLR ligands, we set up a protocol to remove them from wbNeuM and wbNeuE. Notably, wbNeuM completely lost the capacity to produce IL-10, as well as to express IFIT1 and ISG15 mRNA in response to either R848 or poly(I:C) after slan^{+}CD16^{+}-monocyte removal. In addition, both wbNeuM and wbNeuE lost the capacity to express or produce IL-6 when stimulated with R848 for 4 h, again after slan^{+}CD16^{+}-monocyte depletion. On the other hand, slan^{+}CD16^{+}-monocyte-depletion was less effective for wbNeuE, particularly for IFIT1 and ISG15 transcription, indicating that other contaminating cells are responsible for the mRNA expression of IFN stimulated genes. Our findings support the notion that we have been always recommending, namely that is absolutely mandatory to use highly purified populations of neutrophils when gene expression and/or neutrophil-derived cytokines are investigated.

1 Davey, M. S. et al. (2011) Nat. Immunol.; 11:1017-8
2 Zimmermann, M. et al. (2015) Nat. Commun.; 6:6061

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Cathelicidins Have Direct Antiviral Activity against Respiratory Syncytial Virus in Vitro and Protective Function in Vivo in Mice and Humans.

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Respiratory syncytial virus (RSV) is a leading cause of respiratory tract infection in infants, causing significant morbidity and mortality. No vaccine or specific, effective treatment is currently available. A more complete understanding of the key components of effective host response to RSV, and novel preventative and therapeutic interventions, are urgently required.
Cathelicidins are host defence peptides, primarily expressed by neutrophils and epithelial cells, with key microbicidal and modulatory roles in innate host defence against infection. Here we demonstrate that the human cathelicidin LL-37 mediates an antiviral effect on RSV via direct damage to the viral envelope, disrupting viral particles and decreasing virus binding to, and infection of, epithelial cells. Delivery of exogenous LL-37 is protective in vivo in a murine model of pulmonary RSV infection, demonstrating maximal efficacy when applied concomitantly with virus. Furthermore, endogenous murine cathelicidin, induced by infection, has a fundamental role in protection against disease following infection with RSV. Finally, higher nasal levels of LL-37 are associated with protection in a healthy human adult RSV infection model. These data lead us to propose that cathelicidins are a key, non-redundant component of host defence against airway infection with RSV; functioning as a first point of contact “antiviral shield”, and having additional later phase roles in minimising the severity of disease outcome. Consequently, cathelicidins represent an inducible target for preventative strategies against RSV infection and may inform the design of novel therapeutic analogues for use in established infection.

12

YKL-39 is Expressed in Tumour-Associated Macrophages, Stimulates Monocyte Migration and Reversely Correlates with Hematogenous and Lymphatic Metastasis in Human Breast Cancer

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Introduction. Human chitinase-like proteins are considered as a novel biomarker of cancer, inflammation and tissue remodeling. The role of YKL-39 and its associations with tumor progression has not been studied until now. The aim of study was to examine the intracellular trafficking

pathways, and secretion mode of YKL-39 in M2 macrophages, to explore the effect of YKL-39 on monocytes migration; and correlation of YKL39 levels with breast cancer progression. Methods. CD14+ monocytes were isolated from buffy coats, cultured with IL-4, TGF-beta, dexamethasone and M-CSF to obtain M2 macrophages. YKL-39 expression level was measured by RT-PCR. The intracellular distribution of YKL-39 was checked by IF staining and confocal microscopy. YKL-39 secretion was measured by ELISA assay. Monocyte migration was performed with a trans-well system. The correlations of YKL-39 with metastasis in human breast cancer were verified by RT-PCR, IHC and IF staining. Results. YKL-39 gene level was strongly up-regulated with IL4/TGF-beta in human macrophages: on day 6 (13.4 fold, $p<0.05$) and day 12 (62.2 fold, $p<0.05$) (n=6). YKL-39 was detected in the TGN, p62lck positive late endosomes, Lamp-1 positive lysosomes and CD63 positive secretory lysosomes. The extracellular secretion of YKL-39 (1.51 ng/ml) was detected on day 12. Recombinant YKL-39 significantly enhanced the migration of monocytes by 1.9 fold ($p<0.01$) in 1 hour, and reached to 4.9 fold ($p<0.01$) in 3 hours. In patients with breast cancer, low levels of YKL39 correlated with a higher frequency of lymphatic metastasis ($p=0.036$, n=74) and hematogenous metastasis ($p=0.0337$, n=74). The pre-dominant TAM phenotypes were YKL-39 +/ stabilin-1+ (73%, n=10) and CD68+/YKL39+ (25%, n=10).Conclusions. YKL-39 is expressed in tumor-associated macrophages in human breast cancer, is secreted via lysosomal pathway, stimulates migration of monocytes and its expression levels have a reverse correlation with hematogenous and lymphatic metastasis in human breast cancer.

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Role of Chemokine CXCL17 in Macrophage Homeostasis and Infection

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Chemokines belong to a superfamily of secreted proteins which function in the trafficking and activation of different types of leukocytes. CXCL17, one of the last chemokines to be identified, is a novel one-of-a-kind chemokine since it contains only three of the four conserved cysteine residues. Therefore, it cannot adopt a canonical chemokine fold suggesting a unique structure and so its function must also be unique. It is constitutively expressed in various mucosal tissues suggesting a role in homeostasis. In order to characterize CXCL17 function, we generated a homozygous CXCL17 knockout (KO) mice. Interestingly, these mice show no overt phenotype, which is in sharp contrast to knocking out other homeostatic chemokines that are embryonically lethal. Our studies to date indicate that the KO mice have reduced basal levels of large peritoneal macrophages suggesting that CXCL17 could play an important role in the strategic positioning of immune cells for surveillance. In a model of acute bacterial infection, we observed a decrease in macrophages but no change in neutrophil levels. Surprisingly, we observed a significant decrease in several inflammatory cytokines and chemokines. Our results suggest that CXCL17 plays a highly specialized pro-inflammatory role in bacterial infections. Our planned studies on characterizing the signaling pathways linking and establishing the crosstalk and the spatiotemporal relationship between CXCL17 and leukocyte trafficking should result in describing why CXCL17 is one-of-a-kind chemokine.

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Natural Nitration of CXCL12 Reduces Its Signaling Capacity and Chemotactic Activity in Vitro and Abrogates Intra-Articular Lymphocyte Recruitment in Vivo

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The chemokine CXCL12/stromal cell-derived factor-1 is important for leukocyte migration to

lymphoid organs and inflamed tissues and is involved in tumor development. In vitro, CXCL12 activity is strongly regulated by proteolytic processing. However, limited information is available on in vivo posttranslationally modified CXCL12. Therefore, natural CXCL12 from stromal cells stimulated with leukocytes and inflammatory agents was purified. CXCL12 with a nitration on Tyr7, designated [3-NT^7]CXCL12, was detected. CXCL12 and [3-NT^7]CXCL12 were chemically synthesized to evaluate the biological effects of this modification. [3-NT^7]CXCL12 recruited b-arrestin 2, phosphorylated the kinases Akt and ERK1/2 and bound to glycosaminoglycans and the G protein-coupled chemokine receptor CXCR4 similar to CXCL12. However, it showed a reduced ability to enhance intracellular calcium concentrations, to generate inositol triphosphate and to induce monocyte and lymphocyte chemotaxis in vitro. Moreover, nitrated CXCL12 failed to induce in vivo extravasation of lymphocytes to the joint. In summary, nitration on Tyr7 is a novel natural posttranslational regulatory mechanism on CXCL12 under inflammatory conditions which may affect the CXCR4-mediated inflammatory and tumor-promoting activities of CXCL12.

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Agonists of the ß-Hydroxybutyrate Receptor (HCA2) Increase the Chemotactic Response of Bovine Neutrophils

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An elevated concentration of the ketone body β-hydroxybutyrate (BHB) in dairy cattle during lactation is associated with an increased incidence of inflammatory diseases, such as metritis and mastitis. However it remains unclear how BHB affects the inflammatory response in dairy cows. BHB was identified as endogenous ligand of the Hydroxycarboxylic acid receptor 2 (HCA2 or GPR109A), a G protein-coupled receptor. HCA2 also is activated by nicotinic acid (NA), a lipid lowering drug. However, recent studies suggest that HCA2 activation modulates the inflammatory response in human macrophages, monocytes and neutrophils, where this receptor is highly expressed. Neutrophils are undoubtedly the major effectors of acute inflammation and are characterized by their ability to direct migration to the site of infection or inflammation; a process called chemotaxis. Because neutrophil chemotaxis is critical to many inflammatory diseases in humans and cattle, chemoattractant receptors are subjects of exhaustive study. HCA2 expression and the response of specific ligands on bovine immune cells have not been previously studied. In this study, we show HCA2 receptor mRNA expression in bovine neutrophils. Besides treatment with MK-1903 and nicotinic acid, two HCA2 selective full agonists elicited a transient rise of intracellular Ca^{2+} levels that were concentration dependent, suggesting that bovine neutrophils express a functional HCA2 receptor. On the contrary, the structurally related compound Nicotinamide, which does not bind to HCA2 receptor had no effect on Ca^{2+} levels. We also observed that BHB, MK-1903 and NA but not nicotinamide increased bovine neutrophil chemotaxis. Also, HCA2 agonists activate crucial intracellular pathways in neutrophil chemotaxis such as PLC, AKT, p38 and AMPKα. In summary, these results contribute to our knowledge about novel modulatory HCA2 mechanisms in the innate immune system that could be involved in various pathologies in cattle.

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Staphylococcus Aureus Proteases Inactivate Galectin-3 Immunoregulatory Functions and Increase the Severity of Skin Infection

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BACKGROUND: Galectin-3, a β-galactoside-binding human lectin, has significant impact on the outcome of inflammation and infection, and is able to activate antimicrobial functions of neutrophils. Galectin-3 carries a proteolytically sensitive domain which, when cleaved by bacterial proteases, generates a carbohydrate-binding fragment that is inert with regard to cellular activation. *Staphylococcus aureus* expresses four major proteases that are emerging as virulence factors: SspA, SspB, ScpA, and Aur. We hypothesized that these proteases can cleave galectin-3 during infection, and thereby affect disease outcome. We thus investigated the ability of *S. aureus* proteases to process galectin-3 and the impact of such activity on neutrophil activation and *in vivo* skin infection. METHODS: Proteolytic cleavage of galectin-3 in culture supernatants from *S. aureus* strain 8325-4 and protease-lacking mutants was characterized by immunoblotting, and the impact of galectin-3 cleavage on neutrophil NADPH-oxidase activation was assessed by isoluminol-enhanced chemiluminescence. The importance of galectin-3 and bacterial galectin-3-cleaving proteases *in vivo* was investigated in a murine skin infection model, by measurement of lesion sizes and bacterial load. Also, the localization of galectin-3 within healthy or *S. aureus*-infected skin was characterized by immunohistochemistry. RESULTS: The staphylococcal protease SspB was potently cleaving galectin-3, abolishing the neutrophil-activating capacity of the lectin. In healthy murine skin, galectin-3 was strongly associated to the skin squamous and adnexa epithelial cells, as well as to tissue macrophages and dendritic cells. Upon infection with SspB-expressing *S. aureus*, infiltrating macrophages were strongly positive for galectin-3, as were the bacteria, while necrotic tissue had lost all galectin-3. When comparing the lesion sizes induced by protease-expressing bacteria with that of protease-deficient bacteria, SspB-expressing *S. aureus* generated significantly greater lesion sizes, but only in galectin-3-expressing mice. CONCLUSIONS: *S. aureus* is able to cleave galectin-3 by the action of secreted proteases, and thereby inactivate the immunoregulatory functions of the lectin. The combination of galectin-3 and the galectin-3-cleaving protease SspB increases the severity of *S. aureus* skin infection, which suggests that galectin-3-modification is an active function within *S. aureus* virulence.

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Gro-α Induce Neutrophils Chemotaxis via the Pure Chemotactic Receptor CXCR2

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Neutrophil chemotaxis is induced by a variety of stimuli, either from endogenous sources or from invading pathogens, via G-protein coupled receptors (GPCRs) on the neutrophil surface. Typically, ligation to the GPCRs also leads to neutrophil activation, e.g., granule mobilization and production of reactive oxygen species from the NADPH-oxidase. The chemokine IL8 has been shown to induce signaling in neutrophils via the GPCRs CXCR1 and CXCR2. CXCR2 is also a receptor for several other chemoattractants and in this study we have investigated the CXCR2 agonist Gro-α, a chemokine known to attract neutrophils, but less is described about how it affects other neutrophil functions. We evaluated chemotaxis and expression of surface markers on human neutrophils, and as the transmigration process is calcium dependent, we also monitored intracellular calcium levels. We found that similar to IL8, Gro-α induced *in vitro* chemotaxis and intracellular calcium transients in neutrophils, but the Gro-α induced signal was completely blocked by a CXCR2 antagonist which was not the case for IL8.This suggests that in opposite to IL8, Gro-α only induce signaling via CXCR2. Furthermore, stimulation with IL8 lead to granule mobilization with increased expression of complement receptor (CR) 3 on the neutrophil surface while Gro-α treated cells did not upregulate surface CR3 . The neutrophil NADPH-oxidase was activated by stimulation of IL8, but Gro-α was incapable of inducing superoxide production. In conclusion, we describe clear differences in activation patterns between IL8 and GRO-α, and found that the latter triggered chemotaxis without any further activation of the neutrophils. We suggest

that CXCR2 is a pure chemotactic receptor that induces chemotaxis but no additional cell activation.

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Neutrophil Extracellular Traps in Abdominal Aortic Aneurysm

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Objective:
Neutrophils are known to play an essential role in the formation of abdominal aortic aneurysms (AAAs) since neutrophil depletion leads to inhibition of experimental AAA development in rodents. Furthermore, markers of neutrophil activation are elevated in patients with AAA. The formation of so-called neutrophil extracellular traps (NETs) is a process of extreme neutrophil activation and death involving histone modification (citrullination) and DNA expulsion to entrap pathogens. Recently, NETs have been implicated in thrombotic processes. We hypothesized that NETs may be associated with AAA development and NET markers are increased in circulation of AAA patients. We further investigated whether NET parameters are more sensitive than regular neutrophil activation markers in AAA detection.
Methods:
Aortic tissue and peripheral venous blood were collected from 20 AAA patients scheduled for surgical repair. 21 age and sex matched healthy individuals served as controls. Markers of neutrophil activation like elastase, myeloperoxidase (MPO) and neutrophil gelatinase associated lipocalin (NGAL) were measured in plasma by ELISA. In comparison, parameters of NET formation, free DNA-histone complexes and citrullinated histone H3, were evaluated. Differences between groups were calculated by Mann-Whitney-U-test and diagnostic marker potential was assessed by ROC analysis (AUC=area under the curve). Furthermore, neutrophils and NETs were detected in AAA tissue and associated intraluminal thrombus (ILT) by immunofluorescence microscopy.
Results:
AAA patients had significantly elevated plasma MPO levels of median 13.0 vs. 6.7 ng/ml (P<0.001; ROC AUC=0.828). In contrast, NGAL and elastase showed no significant difference between AAA patients and healthy controls. Free DNA/histone complexes were significantly higher in AAA patients with 45.8 vs. 28.9 relative units (P=0.036; ROC AUC=0.689). Neutrophils with citrullinated histones were readily detectable in the ILT associated with AAA.
Conclusion:
This is the first report on the association of NET markers with AAA. While free DNA/histone complexes in plasma seem to be less sensitive as diagnostic tool than the standard neutrophil activation marker MPO, NET parameters like circulating citrullinated histones are currently under investigation and may be of particular interest in the prediction of AAA progression and rupture.

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Multifactorial Effects of Nitro-Oleic Acid on Macrophage Mediated Cardiovascular Inflammation

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Chronic inflammation can lead to severe pathologies and tissue dysfunction and possibilities of its regulation are nowadays intensively studied. Nitro-fatty acids are endogenous molecules with immuno-regulatory potential generated in the adaptive response of organisms to oxidative stress. Currently, they are suggested as highly promising compounds for treatment of diseases associated with immune homeostasis deregulation. The purpose of our study was to characterize the effects of nitro-oleic acid (OA-NO2) in regulation of macrophage action responsible for initiation of chronic inflammatory responses in endothelium and point to a novel

therapeutic strategy to treat distinct macrophage-induced inflammatory diseases.
The effect of OA-NO2 was tested in vitro employing RAW 264.7 macrophages, bone marrow-derived macrophages, and endothelial cells (MS-1, HUVEC) activated or differentiated with various stimuli. In vitro experiments were further expanded by in vivo observations using mouse model of angiotensin-II induced cardiomyopathy.
Our results showed that physiologically-relevant concentrations of OA-NO2 significantly regulated macrophage action in different inflammation-related pathologic processes. Firstly, we characterized the immunomodulatory effect of OA-NO2 on macrophage polarization to pro-inflammatory and immuno-regulatory subsets. We demonstrated that OA-NO2 significantly blocked the LPS-induced activation of transcription factors STAT1, NF-kB and MAPKs as well as production of pro-inflammatory mediators, including reactive oxygen and nitrogen species and cytokines TNF-α, IL-6, IL-1β and TGF-β in pro-inflammatory macrophages. On the other hand, in IL-4-stimulated macrophages, OA-NO2 inhibited STAT6 activation and arginase-1 expression and regulatory macrophage phenotype development. Moreover, we demonstrated that OA-NO2 effectively influences the process of macrophage differentiation induced by growth factors GM-CSF/M-CSF and thus regulates their activation phenotype during inflammation.
Further, we characterized OA-NO2 effects on endothelium response to macrophage derived cytokines with consequent fibrosis development arising from vessel inflammation in different tissues. OA-NO2 prevented pathological activation of endothelial cells by reduction of pro-inflammatory cytokines and chemokines (RANTES, IL-6, GM-CSF and MCP5) production and adhesive molecules (ICAM-1) expression. Accordingly, OA-NO2 can prevent transformation of endothelial cells to the pro-fibrotic phenotype (α-SMA and fibroblast-specific protein 1) through blocking of endothelial-mesenchymal transition triggered by TGF-β produced by macrophages.
Regulation of macrophage polarisation and anti-fibrotic effects of OA-NO2 were verified by in vivo experiments.
Our study provided the unique results showing the protective effects of OA-NO2 in over-activation of macrophages and ability to alternate pro-inflammatory feedback of endothelial cells in during cardiovascular inflammation. Following main signalling pathways, we helped to clarify molecular mechanism of nitro-lipids action. OA-NO2 plays an important role in regulation of chronic inflammation and pathologies of vascular endothelium. Therefore, we can conclude that nitro-fatty acids are highly promising drugs for treatment of chronic inflammation-accompanied diseases.

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S100A9 Potentiates the Activation of Neutrophils by the Etiological Agent of Gout, Monosodium Urate Crystals.

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Introduction and rationale: Gout is one of the most painful types of arthritis, and its prevalence is increasing worldwide. It arises when the body mounts an acute inflammatory reaction against monosodium urate crystals (MSU).During the symptomatic phase, a large number of neutrophils are recruited to the affected joint where they are activated by MSU to release a panoply of inflammatory molecules (eg: IL-1beta, S100 proteins), degradative enzymes and oxygen radicals. These activated neutrophils perpetuate the inflammatory reaction and cause damage to the surrounding tissues. Neutrophil-derived mediators and neutrophils themselves, are thus attractive therapeutic targets to dampen inflammation during gout attacks.
Several lines of evidence strongly suggest that the protein S100A9 plays a role in the pathogenesis of gout. The silencing of its expression in vivo reduces MSU-induced inflammation. Moreover, S100A9 is expressed throughout the synovium of gout patients. Its expression is particularly enriched around blood vessels. Moreover, synovium and sera of gout patients contain elevated levels of S100A9 heterodimerized to S100A8.
Although extracellular S100A9 induces several functional responses in human neutrophils, it remains unknown how neutrophils respond to MSU in the presence of extracellular S100A9. S100A9 alone, induces the shedding of L-selectin, an increase in the expression and activation of cell-surface Mac-1, degranulation, adhesion to fibrinogen

and chemotaxis of neutrophils. In contrast, it is unable to directly induce cytokine production and its ability to produce radical oxygen species (ROS), remains to be confirmed. S100A9 also enhances neutrophil phagocytosis and bactericidal activity. Hypothesis: Since S100A9 is highly expressed around blood vessels, we hypothesized that S100A9 primes human neutrophils for enhanced responsiveness to MSU.
Objective: In this study, we investigated the priming effect of S100A9 on various well characterized, MSU-induced neutrophil effector functions. We also explored less well-known response of neutrophils to MSU, namely, changes in metabolism. Methods: Human neutrophils were incubated with S100A9 prior to activation with MSU. The mobilization of intracellular calcium was determined with Fura-2AM. Glycolysis and the oxygen consumption rate were determined in real-time with the XF96 extracellular analyzer. The former was determined by measuring the extracellular acidification rate (ECAR), and the latter by measuring the oxygen consumption rate (OCR).IL-1 and IL-8 production were determined by ELISA and signaling pathways were studied by Western blot analysis.
Results: S100A9 enhances several MSU-induced neutrophil effector functions including calcium mobilization, glycolysis, oxygen consumption as well as IL-1 and IL-8 production. We also show that S100A9 enhances the activation of signalling pathways known to underlie the response of neutrophils to MSU. Of note, is the ability of S100A9 to induce glycolysis in human neutrophils. Conclusion: Our observations suggest that S100A9 promotes the inflammatory reaction in gout by enhancing the MSU-induced activation of neutrophils. The priming effect of S100A9 may be explained, in part, by its ability to enhance known MSU associated signalling pathways and by inducing glycolysis. The latter may also underlie the priming of neutrophils by S100A9 for functional responses towards other stimuli. The impact of this discovery on other auto-inflammatory and autoimmune pathologies remains to be investigated.

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F-Actin-Rich Contractile Endothelial Pores Prevent Vascular Leakage during Leukocyte Diapedesis through Local RhoA Signalling

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During immune surveillance and inflammation, leukocytes exit the vasculature through transient openings in the endothelium without causing plasma leakage. However, the exact mechanisms behind this intriguing phenomenon are still unknown. Here we report that maintenance of endothelial barrier integrity during leukocyte diapedesis requires local endothelial RhoA cycling. Endothelial RhoA depletion*in vitro*or Rho inhibition*in vivo*provokes neutrophil-induced vascular leakage that manifests during the physical movement of neutrophils through the endothelial layer. Local RhoA activation initiates the formation of contractile F-actin structures that surround emigrating neutrophils. These structures that surround neutrophil-induced endothelial pores prevent plasma leakage through actomyosin-based pore confinement. Mechanistically, we found that the initiation of RhoA activity involves ICAM-1 and the Rho GEFs Ect2 and LARG. In addition, regulation of actomyosin-based endothelial pore confinement involves ROCK2b, but not ROCK1. Thus, endothelial cells assemble RhoA-controlled

contractile F-actin structures around endothelial pores that prevent vascular leakage during leukocyte extravasation.

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Endothelial TIM-1 Triggers Neutrophil Adhesion under Inflammatory Conditions and Contributes to the Induction of Autoimmunity

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BACKGROUND. Neutrophils are the first line of defense against invading pathogens and they have long been viewed as the final effector cells of an acute inflammatory response. However, recent evidence has demonstrated a role for neutrophils in chronic inflammation, particularly in autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS). Importantly, during EAE, neutrophils represent the first leukocyte subset adhering to the blood-brain barrier and favoring immune cell invasion of the central nervous system (CNS), but the molecular mechanisms controlling neutrophil trafficking in the CNS during EAE are largely unknown. The aim of this project was to investigate a potential role for endothelial T cell immunoglobulin and mucin domain-containing molecule-1 (TIM-1) in neutrophils trafficking under inflammatory conditions.
RESULTS. Using under flow adhesion assays, we found that immobilized TIM-1 in capillary tubes captured neutrophils under physiological flow conditions in vitro, with most cells undergoing immediate firm arrest. Strikingly, adhered neutrophils rapidly displayed spreading after arrest, suggesting a prompt neutrophil activation following interaction with immobilized TIM-1. To identify TIM-1 ligands on neutrophil surface, we performed under flow experiments using blocking antibodies or neutrophils deficient for several adhesion molecules. Surprisingly, our data showed that neutrophils are captured on TIM-1 under flow through engagement of mucin P-selectin glycoprotein ligand-1 (PSGL-1) and integrin leukocyte function-associated antigen-1 (LFA-1), suggesting that TIM-1 may directly interact with both PSGL-1 and LFA-1 on neutrophils, or that TIM-1-PSGL-1 interaction transactivates integrins promoting subsequent integrin-dependent neutrophil activation and arrest. Immunofluorescence studies showed constitutive TIM-1 expression on the surface of both brain- and cardiac-derived endothelial cells, and we also detected TIM-1 on the vascular endothelium of inflamed spinal cord (SC) pial venules during EAE, in particular at disease pre-onset and onset phases. Importantly, anti-TIM-1 blocking antibody inhibited neutrophil adhesion on endothelial cells in vitro as well as neutrophil attachment in CNS vessels in intravital microscopy studies on exposed SC during EAE. Finally, our results demonstrate that anti-TIM-1 treatment at disease pre-onset phase characterized by high neutrophil infiltration of the CNS significantly inhibited EAE development. CONCLUSIONS. Our data suggest a pivotal role for endothelial TIM-1 in controlling neutrophil trafficking under inflammatory conditions and that targeting TIM-1 may represent an attractive therapeutic approach in autoimmune diseases such as MS.

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X-Linked Neutropenia Caused by Overactive Mutations in the Wiskott-Aldrich Syndrome Protein Renders Neutrophils Hyperactive

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The gene encoding the Wiskott-Aldrich syndrome protein (WASp) is highly expressed and upregulated during neutrophil maturation. Gain-of-function mutations in WASp that destroys the auto-inhibited conformation cause a rare form of X-linked neutropenia (XLN). Although neutrophil granulocytes are clearly affected in XLN patients, it remains largely unknown what role WASp plays in neutrophil development and function. Here, we generated two new mouse models that express the

XLN patient mutations WASp-L272P and WASp-I296T and compared neutrophils from these mice to WASp-deficient neutrophils. We found that several neutrophil functions, such as chemotaxis, phagocytosis, adhesion, degranulation, and reactive oxygen species (ROS) production were regulated by WASp. Surprisingly, while WASP-deficient neutrophils exhibited defective actin polymerization, intracellular ROS production, phagocytosis, and chemotaxis, XLN neutrophils showed increased polymerized actin, intracellular ROS, phagocytosis rate,and chemotaxis. In the competitive setting of bone marrow chimeric mice, WASp-deficient neutrophils had a selective disadvantage when competing with wildtype cells. In contrast, XLN neutrophils had an advantage over wildtype neutrophils in entering peripheral tissues in naïve mice and under sterile inflammation. These data indicate that there are unique requirements for the presence and activation status of WASp in neutrophils and that activating mutations in WASp render neutrophils hyperactive.

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Live Cell Time-Lapse Imaging Allows Quantification of Netosis and Dissection of the Pathways Involved in Cell Death and Chromatin Decondensation

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Objective: Neutrophil extracellular traps (NETs) and the cell death process associated with their release (NETosis) have been studied using a variety of stimuli. It remains unclear whether different NET-inducing stimuli operate through a common pathway. Furthermore, it remains unclear which pathways are important for cell death versus DNA decondensation, the two hallmarks of NETosis. Methods: We used a novel quantitative live cell imaging strategy to explore biochemical pathways involved in NETosis. The live cell platform allowed investigation of both the death and chromatin decondensation components of NETosis. Healthy control neutrophils and neutrophils from a Chronic Granulomatous Disease patient were stimulated with phorbol myristate acetate (PMA), Candidal hyphae and pathologically relevant crystals including monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD) and silica. Live cell and fixed fluorescence microscopy using DNA binding dyes and cell vitality markers enabled dissection of the kinetics of cell death and decondensation. Results: NETosis induced by crystal stimuli occurred more rapidly than that induced by PMA and Candida, the latter two stimuli demonstrating a characteristic latency prior to NETosis. Chromatin decondensation occurs post-mortem in crystal stimulated neutrophils but prior to cell death in PMA and Candida treated neutrophils. NADPH oxidase function assessed chemically and genetically was required for PMA- and Candida-induced NETosis and cell death. MSU and CPPD induced robust neutrophil reactive oxygen species generation but this was not required for crystal-induced NETosis. Histone degradation during NETosis was evident with all stimuli tested and was inhibited by neutrophil elastase inhibitor (311616A). Neutrophil elastase inhibition reduced DNA decondensation with crystal and PMA-induced NETosis but did not alter the kinetics of cell death. Conclusion: Pathologically relevant crystals trigger neutrophil cell death and chromatin decondensation using distinct upstream biochemical pathways with neutrophil elastase as the final common mediator of chromatin decondensation.

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Cracking the Neutrophil Transcription Paradox: Transcriptional Firing Drives Netosis

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Background and rationale: The relevance of transcription in neutrophils has remained a paradox. It is well established that neutrophils respond quickly to stimuli and die within minutes to hours. Why would a short-lived cell such as neutrophil transcribe its geneome? We hypothesized that genome-wide transcription decondenses chromatin necessary to drive neutrophil extracellular trap (NET) formation or NETosis.
Methods: Human neutrophils from healthy donors were purified and induced to undergo Nox-dependent (by PMA) or Nox-independent (by calcium ionophore A23187) NETosis. Transcriptomic analysis was conducted for unstimulated or NETotic neutrophils at different

time points (30, 60 min; 3 conditions, 2 time points, 3 dornors; 18 transcriptomes). Kinase specific genome-wide transcription was analysed by Human Affymetric array, and GeneSpring, Ingenuity and GeneGo Metacore softwares. Nuclear morphology, citrulination of histone and NETs were analyzed by flourescence microscopy. Effects of transcription and translation inhibitors on NETosis was determined by Sytox Green plate reader assays and flourescence microscopy.

Results: We have identified that the transcriptional activity reflects the degree of DNA decondensation occurring in both Nox-dependent and Nox-independent NETosis. Genome-wide transcription starts earlier in the rapid Nox-independent NETosis than Nox-dependent NETosis. Transcriptomics analyses show that NETosis-specific kinase cascades differentially activate transcriptional firing in these two types of NETosis. Inhibitors of transcription, but not translation, suppress both types of NETosis. In particular, promoter melting and DNA unwinding are important transcription steps necessary to drive NETosis. Immunofluorescence microscopy indicates that extensive chromatin-wide citrullination of histones occurs only in Nox-independent NETosis. Hence, citrullination of histone could contribute to the rapid transcription-mediated DNA decondensation observed in Nox-independent NETosis. However, blocking transcription suppresses both types of NETosis, without affecting the reactive oxygen species production that is necessary for anti-microbial functions of neutrophils.

Conclusions: Therefore, we assign a novel function for transcription in neutrophils: Transcriptional firing, regulated by NETosis-specific kinases, drives NETosis.

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Expression of Lewis-A Glycans on PMN Augments Function by Increasing Transmigration

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Pathogen-triggered neutrophil (PMN) recruitment is critical for innate immunity, but aberrant PMN influx is also implicated in the pathogenesis of numerous inflammatory diseases of the gut and lungs. Fucosylated terminal glycans from the Lewis glycan family, such as Lewis-x (Le^x) and Sialyl Lewis-x, have previously been implicated in the regulation of important PMN functions, including Selectin-mediated PMN trafficking across the vascular endothelium. While such glycans based on the type 2 sequence (Galb1-4GlcNAc-R) are abundant on PMNs, the presence of type 1 Galb1-3GlcNAc-R glycans required for the expression of Lewis-a (Le^a) have not yet been reported. Here, we conclusively demonstrate by immunoblotting and immunofluorescence analyses that Le^a is in fact expressed in human PMNs. Specific Le^a glycan recognition by anti-Le^a mAbs was verified using glycan array technology. In addition PCR and immunoblotting analyses revealed robust PMN expression of both a ß1-3 Galactosyltransferase and an a1/4 Fucosyltransferase, glycosyltransferase enzymes required for Le^a synthesis. We further report that antibody mediated ligation of PMN expressed Le^a increases both PMN chemotaxis across collagen and migration across model intestinal epithelia. As would be expected treatment of PMN from individuals deficient in a1/4 fucosylation with anti-Le^a mAbs did not result in changes in neutrophil trafficking. These results identify, for the first time, expression of Le^a by human PMN and demonstrate its relevance to PMN trafficking within the intestine. We propose that PMN Le^a represents a new target for regulating intestinal innate immunity and modulating inflammation in diseases where dysregulated PMN influx is associated with bystander tissue damage. Furthermore, differential responses of human PMN to physiological ligands of Le^a (based on an individuals Lewis phenotype) may provide insights into the mechanisms linking specific Lewis phenotypes with differential susceptibilities to bacterial infections, viral infections and coronary heart disease.

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Neutrophil Deposition of Microparticles onto Inflamed Epithelium; A New Mechanism to Disrupt Epithelial Cell-To-Cell Adhesions and Promote Transepithelial Migration

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Neutrophil (PMN) migration across epithelial monolayers is a hallmark of many inflammatory mucosal disorders. While it is well established that *en-masse* tissue infiltration by PMNs can cause tissue damage, mechanisms involved in this process are not yet well defined. The current work describes a new mechanism whereby deposition of membrane-derived microparticles (PMN-MPs) by PMNs onto intestinal epithelial cells (IECs) during transepithelial migration (TEM) results in loss of epithelial cadherins, leading to epithelial damage and increased tissue infiltration by PMNs. We demonstrate that PMN-MPs secreted by activated, transmigrating PMNs display high levels of matrix metalloproteinase 9 (MMP-9). Importantly, MMP-9 associated with the PMN-MP surface was confirmed to have high enzymatic activity and to mediate profound effects on the integrity of epithelial monolayers. Isolated PMN-MPs efficiently bound to IECs, leading to cleavage of desmoglien-2 (Dsg-2) and disruption of epithelial cell-to-cell adhesions. Furthermore, PMN-MP binding to intestinal epithelium *in-vitro* and *in-vivo* significantly enhanced PMN TEM. In both settings these effects were specific to MMP-9 and were reversed in the presence of specific pharmacological inhibitors. Finally we demonstrate that while Dsg-2 negatively regulates PMN TEM, activity of PMN-derived, but not IEC-derived MMP-9 was essential for proper trafficking of PMNs across epithelial layers *in-vitro* in a transwell assays and *in-vivo* in ligated intestinal loop preparations. Thus, our findings implicate PMN-MPs in exacerbated, PMN-mediated inflammation and epithelial damage as observed in inflammatory disorders of mucosal surfaces.

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Anti-Mouse Collagen XVII Human Immunoglobulin a Antibodies Induce FcαRI - Dependent Neutrophil Migration in a Novel Mice Model of LABD

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Introduction

Immunoglobulin A (IgA) is the prevalent antibody class at mucosal surfaces and plays an important role in mucosal defense. IgA can activate neutrophils, as these cells express a receptor for IgA, the FcαRI. We have demonstrated that cross-linking of FcαRI by IgA complexes induced neutrophil recruitment and release of the chemoattractant leukotriene B4 (LTB4) leading to amplification of neutrophil migration. Our hypothesis is that excessive neutrophil activation by IgA–FcαRI interactions play a harmful role in autoimmune diseases and lead to tissue damage, such as in Linear IgA Bullous Disease (LABD). LABD is a chronic autoimmune skin blistering disease characterized by the presence of anti-collagen XVII IgA autoantibodies and high neutrophil influx.

We have now developed a novel mouse model to investigate the role of FcαRI and IgA-induced tissue damage in vivo.

Materials and Methods

A hybridoma, producing human IgA (hIgA) directed against mouse collagen XVII (anti-mCOL17 hIgA) was generated. To determine the in vivo role of FcαRI in IgA-induced neutrophil migration, FcαRI transgenic mice (expressing human FcαRI on neutrophils) were crossbred with LysEGFP mice (that have green fluorescent neutrophils). Anti-mCOL17 hIgA mAbs were injected in the ears of these mice, and intravital imaging was performed 48 hours later. Furthermore, FcαRI transgenic mice were crossbred with hIgA knock-in mice to investigate tissue damage after injection of anti-mCOL17 hIgA antibodies in the ears of these mice.

Results

Anti-mCOL17 hIgA were able to bind to the basement membrane of mouse skin cryosections and these antibodies were able to activate neutrophils in vitro. After injection of anti-mCOL17 hIgA in the ears of these mice, intravital imaging showed activation and high numbers of neutrophils in the blood vessels, which was absent when the vehicle was injected as well as in control non-transgenic mice. Staining cryosections with GR-1 confirmed the presence of a high neutrophil influx in response to anti-mCOL17 hIgA, which was not seen in non-transgenic mice.

Importantly, minimal activation of neutrophils was observed when mice were injected with a FcαRI blocking antibody.

We further analyzed whether IgA-induced neutrophil migration results in tissue damage and blister formation after frequent injection of anti-mCOL17 hIgA antibodies in our FcαRI/hIgA mice model. We were able to see differences in recruitment of neutrophils and tissue damage/blister formation in the ears. Low numbers of neutrophils were seen in the ears injected with vehicle in hIgA and FcαRI/IgA mice. Importantly, minimal neutrophil influx was seen in the ears injected with anti-mCOL17 hIgA antibodies in hIgA mice, compared to the massive recruitment of neutrophils in the ears of FcαRI/hIgA mice.

Conclusions

Anti-mCOL17 hIgA antibodies induce neutrophil activation and migration in vivo. We were able to prevent neutrophil recruitment by blocking the IgA receptor FcαRI. Currently we are investigating whether we can reduce inflammation induced by activated neutrophils by blocking FcαRI on neutrophils. These results indicate that IgA-induced neutrophil activation and migration is dependent on FcαRI in vitro and in vivo. Blocking IgA-FcαRI interactions may be a therapy for IgA-induced blistering diseases.

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Targeting ADAM17 Increases Neutrophil Recruitment and Reduces Bacterial Spread during Sepsis

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Sepsis is a severe systemic inflammatory response to bacterial infection. Due to prolonged stays in the ICU, complex therapies, and concerns of antibiotic-resistant microbes, sepsis remains a serious medical issue. The cause of sepsis is heterogeneous and its clinical features are diverse, and so determining central regulators for effective host-directed therapies has been a considerable challenge. It is well established that sepsis impairs leukocyte and endothelial function. A rapid and robust recruitment of circulating neutrophils at sites of infection is critical for preventing bacterial spread, and it is well established that this process is greatly impaired during sepsis. The proteolytic activity of a disintegrin and metalloprotease-17 (ADAM17) is induced in the cell membrane of leukocytes upon their activation, and this regulates the release of various pro-inflammatory factors as well as the surface density of an assortment of receptors important for neutrophil effector functions, including their recruitment. We show that conditional knockout mice lacking ADAM17 in all leukocytes have a survival advantage when subjected to polymicrobial sepsis. Bacteremia and circulating pro-inflammatory cytokines, key determinants of sepsis severity, were significantly reduced in conditional ADAM17 knockout mice during sepsis. The cecal bacterial microbiota and load were similar in unmanipulated conditional ADAM17 knockout and control mice, yet the peritoneal spread of bacteria was significantly reduced in conditional ADAM17 knockout mice following sepsis induction. This was associated with an amplified recruitment of neutrophils at the infectious locus. Taken together, our findings suggest that systemic ADAM17 activation is a pivotal mechanism during sepsis that tips the balance between efficient and impaired neutrophil recruitment. We are currently examining the therapeutic efficacy of ADAM17 targeting after sepsis onset in preclinical studies.

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Phosphoinositol-3-Phosphate Acts as a Timer for ROS Production in the Phagosome by Controlling P67phox Accumulation

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Neutrophils participate in the host defense via pathogen phagocytosis and microbial killing by producing reactive oxygen species (ROS) in the phagosome. The ROS are produced by the NADPH oxidase (NOX2). The NADPH oxidase is activated when the cytosolic subunits of NOX2 ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$) and Rac assemble with the membrane subunits ($gp91^{phox}$ and $p22^{phox}$) at the phagosomal membrane. $P67^{phox}$ triggers the electron flow from NADPH to O2-. $P40^{phox}$ has a phosphatidylinsositol-3phosphate (PI3P) binding domain. The role of $p40^{phox}$ in the oxidase complex is still unclear. Recent studies have revealed that the PI3P may play an important role in locally boosting phagosomal NADPH oxidase activity through its binding to the $p40^{phox}$ NADPH oxidase subunit. We aim at investigating the importance of PI3P in phagosomal ROS production and the recruitment of the $p67^{phox}$ and $p40^{phox}$ subunits.

We found that VPS34 IN1, a specific inhibitor of the PI3Kinase, decreases the ROS production both in differentiated PLB-985 cells and human neutrophils. The general PI3Kinase inhibitor wortmannin drastically decreases the time of presence of PI(3)P, $p40^{phox}$ and $p67^{phox}$ at the phagosome, which all left the phagosome at the same time. An increase in PI(3)P at the phagosome, triggered by siRNA against the PI3Kinase associated protein Rubicon and/or the PI3phosphatase MTM1, increases ROS production inside the phagosome and extends the accumulation of $p67^{phox}$ at the phagosome. Furthermore, the down-regulation of PI(3)P at the phagosomal membrane, by overexpression of MTM1, prevents the ROS production and the accumulation of $p67^{phox}$.

In conclusion, PI(3)P sustains ROS production in the phagosome by allowing $p40^{phox}$ and $p67^{phox}$ to stay at the phagosomal membrane. Our data suggest that $p40^{phox}$ works as a late adaptor controlled by PI(3)P to maintain $p67^{phox}$ in the NADPH oxidase complex, PI(3)P acts as a timer for NADPH oxidase assembly.

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Transforming Growth Factor-ß-Activated Kinase 1 (TAK1) is Required for Human FcγRIIIb - Induced Neutrophil Extracellular Trap (NET) Formation

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Neutrophils (PMN) are the most abundant leukocytes in the blood. PMN migrate from the circulation to sites of infection, where they are responsible for antimicrobial functions. PMN use phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) to kill microbes. Several stimuli, including bacteria, fungi, and parasites, and some pharmacological compounds such as PMA are efficient inducers of NETs. Antigen-antibody complexes are also capable of inducing NET formation. Recently, it was reported that FcγRIIIb crosslinking induced NET formation similarly to PMA stimulation. Direct crosslinking of FcγRIIA or integrins did not promote NET formation. FcγRIIIb-induced NET formation presented a different kinetics from PMA-induced NET formation, suggesting differences in signaling. Because FcγRIIIb also indeces a strong activation of ERK and nuclear factor Elk-1, and the transforming growth factor-β-activated kinase 1 (TAK1) has recently been implicated in the ERK signaling, in the present report, we explored the involvement of TAK1 in the signaling pathway activated by FcγRIIIb leading to NET formation. FcγRIIIb was stimulated by specific monoclonal antibodies and NET formation was evaluated in the presence or absence of pharmacological inhibitors. The antibiotic LL Z1640-2, a selective inhibitor of TAK1 prevented FcγRIIIb-induced, but not PMA-induced NET formation. Both PMA and FcγRIIIb crosslinkng induced phosphorylation of ERK. But, LL Z1640-2 only inhibited the FcγRIIIb-mediated activation of ERK. Also, only FcγRIIIb, similarly to transforming growth factor-β-induced TAK1 phosphorylation. A MEK (ERK kinase) specific inhibitor was able to prevent ERK phosphorylation induced by both PMA and FcγRIIIB. These data show for the first time that FcγRIIIB crosslinking activates TAK1 and that this kinase is required for triggering the MEK/ERK signaling pathway to NETosis.

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Immobilized Immune Complexes Induce the Formation of Neutrophil Extracellular Traps (NETS)

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Background: One important antimicrobial effector mechanism of neutrophils is the release of neutrophil extracellular traps (NETS), which are composed of chromatin, histones and antimicrobial proteins and contribute to pathogen containment. However, NETs have also been suggested to play a pathophysiological role in autoimmune diseases, which are characterized by the presence of autoimmune complexes. In several autoimmune diseases pathogenic immune complexes are formed on the extracellular matrix and thus are immobilized. We report, that immobilized immune complexes induce the release of NETs from primary human neutrophils.
Methods: Human blood neutrophils were incubated with immobilized HSA/anti HSA immune complexes (iIC) and the ability to generate reactive oxygen species (ROS) and NETs was investigated. The contribution of various ROS was assessed by using inhibitors of ROS-generating pathways and the antioxidants L-ascorbic acid and 5-ASA. Roles of Fc-gamma receptors (FcγR) and the integrin Mac-1 were determined by incubation with blocking antibodies. Intracellular signaling pathways engaged downstream of the receptors were investigated by use of specific inhibitors and western blotting. Results: Treatment of human neutrophils with iIC induced the release of NETs. Pre-incubation of neutrophils with TNF-α significantly increased iIC-induced NET production. We could show that iIC induce NETs in a ROS-dependent manner via activation of NADPH-oxidase (NOX) and myeloperoxidase. The activation of iIC induced oxidative burst was shown to depend on stimulation of both FcγRIIa and FcγRIIIb, whereas only FcγRIIIb is sufficient for iIC-induced NET release. Mac-1 blocking also abolished NET formation. This suggests that FcγRIIIb, that lacks an intracellular domain, can signal in association with Mac-1. As intracellular signaling pathways involved we identified the tyrosine kinases Src/Syk pathway. We could show that the Src/Syk-pathway downstream regulates the PI3K/Akt-, p38MAPK- and ERK 1/2-pathways upon iIC stimulation. ROS do not mediate the activation of Akt, p38MAPK and ERK 1/2 since treatment with NOX inhibitor DPI, in contrast to the Src-inhibitor PP2, prior to iIC stimulation did not affect their phosphorylation.

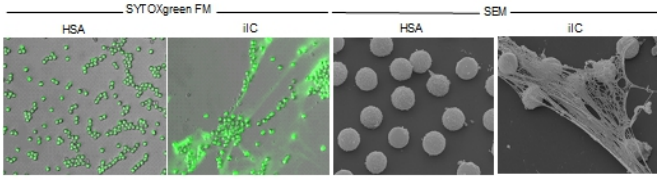

Figure1: Immobilized immune complexes induce the formation of NETs.

Fluorescence microscopy (FM) and scanning electron mircroscopy (SEM) images of neutrophils incubated on HSA-coated and iIC-coated surfaces. For FM cells were stained with SYTOXgreen and overlay of brightfield and fluorescence is shown. Conclusion: Our data show for the first time that iIC induces ROS-dependent NET formation in human neutrophils, which is enhanced in the presence of the pro-inflammatory mediator TNF-α. This indicates that an (auto-) inflammatory milieu, characterized by iIC and pro-inflammatory cytokines is highly potent regarding the induction of NETs in the absence of microbial stimuli. Thus we conclude that NETs contribute to pathology in autoimmune inflammatory disorders associated with surface-bound immune complexes.

Data published in:
Behnen M, Leschczyk C, Möller S, Batel T, Klinger M, Solbach W, Laskay T: Immobilized Immune Complexes Induce Neutrophil Extracelluar Trap Release by Human Neutrophil Granulocytes via FcγRIIIB and Mac-1. Journal of Immunology 2014; 193:1954-1965

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HS1 Deficiency Impairs Neutrophil Activation and Extravasation

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Neutrophil extravasation is a critical step in innate immunity in response to tissue injury or invading pathogens. The interaction of neutrophils with the endothelium requires activation of adhesion molecules such as the β2-integrin LFA-1 leading to firm adhesion, polarization and intraluminal crawling to the actual site of diapedesis. These steps alsorequire coordinated cytoskeletal remodeling. The cortactin homologue in leukocytes, hematopoietic cell-specific lyn substrate (HS1) is an actin-binding protein (ABP) that regulates actin dynamics and may thus be relevant in the regulation of leukocyte extravasation. Analyzing the CXCL1-stimulated cremaster by intravital microscopy, we found that leukocyte adhesion and extravasation was strongly inhibited. This was accompanied by an increased rolling velocity pointing to a disturbed transition from rolling to firm adhesion in the absence of HS1. Moreover, CXCL1-induced activation of the small GTPases Rac1 and Rap1 was strongly inhibited leading to disturbed actin remodelling and aberrant pseudopod formation. In response to inflammation, HS1 interacted with the ABPs Wiskott-Aldrich syndrome protein (WASP), WASP-interacting protein (WIP) and vasodilator-stimulated phosphoprotein (VASP). Interestingly, these processes were dependent on PKA since PKA inhibition blocked chemokine-induced Rap1 activation and interaction with the mentioned ABPs. Absence of HS1 likely prevents the formation of this signalling complex thus explaining aberrant actin polymerization and impaired neutrophil extravasation. The importance of PKA for HS1-mediated support of extravasation was corroborated by the fact that PKA activation increased whereas inhibition reduced transmigration of WT neutrophils but not of HS1-KO neutrophils. Our results are the first in vivo evidence that HS1 is crucial for orchestrating the molecular events in neutrophils during inflammation that mediate extravasation.

Group V Secreted Phospholipase A2 Mediates the Production of Angiogenic and Anti-Angiogenic Factors from Human Neutrophils

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Background

Angiogenesis, the formation of new blood vessels from preexisting ones, plays a prominent role in chronic inflammatory disorders and tumors. This process is sustained by the coordinated production of several angiogenic factors including Vascular Endothelial Growth Factors (VEGFs) and Angiopoietins (Angs). Secreted phospholipases A_2 ($sPLA_2$) are multivalent mediators involved in inflammatory diseases and tumors. Human neutrophils (PMNs) are both a source and a target of $sPLA_2$s. These cells release group V $sPLA_2$ and can be activated by $sPLA_2$s to release CXCL8. We have investigated the role of group V $sPLA_2$ in the production of angiogenic factors from PMNs.

Methods

VEGF-A, -B, -C, -D and Angs (Ang1 and Ang2) expression was evaluated by RT-PCR in highly purified (>99%) PMNs. Release of VEGF-A, VEGF-A_{165b}, Ang1 and CXCL8 was evaluated by ELISA. $sPLA_2$ activity was measured by EnzChek® PLA_2 Assay Kit.

Results and Conclusions

PMNs constitutively express mRNAs for the proangiogenic molecules VEGF-A_{165}, VEGF-B_{167}, VEGF-B_{186}, and Ang1. mRNA for VEGF-A_{121}, VEGF-A_{189}, VEGF-C, VEGF-D, and Ang2 was not detected. PMNs also expressed mRNA for the anti-angiogenic factor VEGF-A_{165b}. *In vitro* stimulation of PMNs with increasing concentrations (0.1 to 5 mg/ml) of human recombinant group V $sPLA_2$ (hGV) caused the release of VEGF-A, Ang1 and CXCL8. hGV also induced the release of VEGF-A_{165b}. hGV-induced release of VEGF-A was

significant after 15 min ($p<0.01$) and progressively increased up to 6 hours. Preincubation (30 min, 37°C) of hGV with Me-Indoxam (1 mM), which blocks M-type receptor-mediated effects of $sPLA_2$s, abolished the release of VEGF-A, Ang1 and CXCL8 but not that of VEGF-A_{165b}. The release of VEGF-A_{165b} was reduced by preincubation (37°C, 30 min) of neutrophils with P11 (100 nM) and/or TCS 2314 (100 nM), antagonists of integrin receptors (anb3 and a4b1, respectively). These results indicate that hGV induced the production of both angiogenic and anti-angiogenic factors from PMNs by different receptor-mediated mechanisms. Activation of PMNs by fMLF induced the release of hGV as well as of VEGF-A and CXCL8. Preincubation (10 min, 37°C) of PMNs with Me-Indoxam (1 mM) before stimulation with fMLF (1 mM) inhibited (≈ 55%) the release of VEGF-A and CXCL8. These results are compatible with the hypothesis that endogenous hGV may be involved in fMLF induced release of VEGF-A and CXCL8.

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The Role of T-Cell Lymphoma Invasion and Metastasis-Inducing Protein 1 (Tiam1) in Neutrophils.

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Rac GTPases have an important role in regulating the cytoskeleton and are involved in many neutrophil functions such as adhesion, spreading, chemotaxis, phagocytosis, reactive oxygen species (ROS) formation and degranulation.

We have investigated the functional importance of the Rac-activator Tiam1 in a variety of neutrophil functions. We have shown that Tiam1 is expressed in mouse neutrophils and is absent in neutrophils from Tiam1 -/- mice. Neutrophils from Tiam1 -/- mice primed with GM-CSF and TNF-alpha show an increase in adhesion in response to f-Met-Leu-Phe (fMLP) stimulation, however neutrophil spreading is unaffected. fMLP-stimulated chemotaxis is impaired in neutrophils from Tiam1 -/- mice primed with GM-CSF and TNF-alpha, and Tiam1 -/- neutrophils have a reduced ability to turn in response to a change in direction of fMLP stimulation. ROS formation to a variety of stimuli in unprimed and primed Tiam1 -/- neutrophils is unaffected. Finally, a significant defect in neutrophil recruitment to the peritoneum upon thioglycollate challenge is observed in Tiam1 -/- mice.

In conclusion, Tiam1 has an important role in neutrophil function by regulating a specific subset of Rac-dependent responses, and further characterisation of these responses in progress.

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Regulation of Neutrophil Responses by P-Rex and Norbin

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Phosphatidylinositol 3, 4, 5-trisphosphate-dependent Rac exchanger 1 (P-Rex1) is a guanine nucleotide exchange factor (GEF) for the RAC family of small GTP-binding proteins (GTPases). It catalyses the active conformation of Rac GTPases, thus regulating many different cell responses, including morphology and motility. P-Rex1 activation is controlled synergistically by PIP_3, the lipid product of PI3K activity, and by the Gbg, subunits of heterotrimeric G proteins.

A screen carried out recently by the Welch lab identified a new binding partner of P-Rex1, the GPCR adaptor protein Norbin. This study revealed a novel mechanism of regulation of P-Rex1 by Norbin, where Norbin is an important regulator of P-Rex1 subcellular localization and a direct stimulator of the P-Rex1 Rac-GEF activity (Pan D. et al, 2016, JBC). The study showed furthermore that Norbin is expressed in neutrophils. In order to assess the functional importance of the P-Rex1/Norbin interaction in neutrophils, we generated two new genetically-modified mouse strains: a strain with a conditional Norbin deletion in myeloid cells and a strain with combined Norbin and P-Rex1 deficiency.

Using these new mouse strains, we found that isolated Norbin-deficient neutrophils show increased ROS production, adhesion, spreading and polarity upon stimulation of the GPCR fPR1 (fMLP receptor). Under some conditions, the Norbin deficiency was found to override functional impairments caused by the P-Rex1 deficiency. These data indicate that Norbin plays an important

functional role in neutrophils. Ongoing experiments are aimed at investigating whether Norbin acts by modulating P-Rex1 dependent Rac activity and whether it affects P-Rex1 dependent neutrophil recruitment during inflammation *in vivo*.
The characterization of neutrophil responses through the analysis of mouse strains deficient in Norbin, P-Rex1 or both, *in vitro* as well as *in vivo*, will enable us to fully understand the importance of Norbin in neutrophils and its interaction with P-Rex1 for the ability of neutrophils to fight bacterial and fungal infections.

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Rac-GEFS Activate Discrete Spatiotemporal Pools of the Small GTPase Rac to Elicit Specific Neutrophil Responses

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Rac is a small GTPase that can be activated by numerous guanine-nucleotide exchange factors (Rac-GEFs) to control a wide range neutrophil functions, including adhesion, migration, degranulation, phagocytosis, ROS and NET formation. An important question in this field is how the many different types of Rac-GEFs can activate Rac to elicit specific neutrophil responses. Intramolecular fluorescence resonance energy transfer (FRET) probes are a useful tool for studying Rac signalling. However, use of these probes in neutrophils has been limited because these cells are terminally differentiated, short-lived and difficult to manipulate to express exogenous proteins. We recently developed a reporter mouse strain that expresses a FRET reporter for Rac activity, Raichu-Rac, and used it to define spatiotemporal patterns of Rac activity during the adhesion and migration of primary mouse neutrophils (Johnsson A-K et al, 2014, Cell Rep). To determine if different neutrophil Rac-GEFs can activate specific spatiotemporal pools of Rac, we crossed this Rac-activity reporter strain with mouse strains deficient in neutrophil Rac-GEFs such as Prex1, Vav1 and Dock2. By correlating the Rac-GEF dependence of spatiotemporal Rac activity pools with the ability of neutrophils to undergo adhesion, spreading, chemotaxis and phagocytosis, we were able to identify how different Rac-GEFs elicit specific neutrophil responses by activating discrete subcellular pools of Rac. Further work aims to determine the dependence of these Rac-GEF mediated pools of Rac activity on specific adhesion molecules and signalling pathways.

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RIPK1-Dependent NET Formation Requires MLKL and PAD4

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Neutrophil extracellular trap (NET) formation can generate short-term functional anuclear cytoplasts and trigger the loss of cell viability. Whether regulated non-apoptotic cell death pathways stimulate NETosis is poorly studied. By developing methods to engage receptor-interacting protein kinase-3 (RIPK3) and mixed lineage kinase domain-like (MLKL) in mouse and human neutrophils, we demonstrate that the kinase activity of receptor-interacting protein kinase-1 (RIPK1) induces MLKL clusters at the membrane and peptidylarginine deiminase 4 (PAD4)-dependent hypercitrullination of histone H3 leading to NET formation. NETs prevented *S.aureus* replication *ex vivo* and mice lacking RIPK3 or MLKL were sensitive to *S.aureus*. Loss of RIPK3 and MLKL prevented NET formation but stimulated Caspase-8-dependent cell death, suggesting that the activation of Caspase-8 or RIPK3/MLKL by the kinase activity of RIPK1 was dependent on substrate availability.

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Blockade of Neutrophil Adhesion Has Therapeutic Effect in Animal Models of Alzheimer'S Disease

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Alzheimer's disease (AD) is the most common neurodegenerative disorder and is characterized by a progressive decline of cognitive functions. The

neuropathological features of AD include amyloid beta (Aβ) deposition, intracellular neurofibrillary tangles derived from the cytoskeletal hyperphosphorylated tau protein, amyloid angiopathy, the loss of synapses, and neuronal degeneration. In the last decade, inflammation has emerged as a key feature of AD, but most studies have focused on the role of microglia-driven neuroinflammation mechanisms. Vascular inflammation and a dysfunctional blood-brain-barrier (BBB) have been implicated in the pathogenesis of AD. However, the role of leukocyte trafficking mechanisms in the induction of neuropathological changes and memory deficit in AD is unclear. We have recently demonstrated that neutrophils infiltrate the AD brain and contribute to the induction of cognitive deficit and neuropathological changes in animal models of AD. The aim of our study was to investigate the role of neutrophil integrins in the pathogenesis of disease in animal models of AD. Two-photon laser-scanning microscopy (TPLSM) experiments in the brain of mice with AD-like disease showed that LFA-1 integrin blockade prevents neutrophil adhesion in brain vessels, extravasation, and inhibits intraparenchymal motility, suggesting a key role for this integrin in neutrophil recruitment in AD. Notably, the blockade of neutrophil trafficking by an anti-LFA-1 antibody rescued cognitive deficits in AD-like mice. 3xTg-ADx*Itgal*$^{-/-}$mice lacking LFA-1 integrin showed improved memory in cognitive tests compared to wild-type animals, suggesting that LFA-1 integrin contribute to the induction of cognitive deficit in AD mice. These findings were supported by neuropathological studies showing a lower density and activation state of microglia and a reduction of amyloid beta deposition and tau hyperphosphorylation in 3xTg-AD mice deficient of LFA-1 integrin compared to aged-matched controls. Moreover, the blockade of alpha4 integrins, which represent an alternative mechanism for neutrophil migration during inflammatory conditions, clearly improved memory function in AD mice and the restoration of cognition was maintained also at later time points, suggesting that therapeutic blockade of leukocyte adhesion during early stages of disease provides a long-term beneficial effect on cognition in older mice. In addition, neuropathological studies performed in mice treated with an anti-alpha4 antibody, showed reduction of amyloid beta deposition, tau hyperphosphorylation, microglial activation and restoration of synaptic proteins compared to animals treated with a control antibody. Collectively, our results suggest that pharmacological targeting of integrins controlling leukocyte trafficking may represent a new therapeutic strategy to address AD.

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Impaired Neutrophil Extracellular Traps (NETs) Release in Children with Acute Leukemias

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Neutrophils have been recognised as the front-line fighters of the innate immune system. One of their weapon is mechanism named NETosis, created to destroy bacteria, fungi, parasites and even some of the viruses. In this process neutrophilic granulocytes release a web-like structure outside the cells, composed mainly of deoxyribonucleic acid, histones and enzymes derived from their granules. Emergent construction is known as neutrophils extracellular traps (NETs), and in some cases is equivalent of the cell death. Insufficient releasing of NETs or formation them as a defective structure, leads to increases susceptibility to contamination with different pathogens. In children with acute leukemias serious, life-threatening infections remain a major cause of morbidity and mortality. Therefore the goal of our study was to verify of the contribution of NETs to infectious complications of acute leukemias in children.

Blood for analysis was collected from 30 leukemic children at the time of the diagnosis and from 10 healthy donors. Neutrophils after isolation, were seeded on 96-well plate or Lab-Tek slides, and activated with well-known NETs stimulator - PMA (phorbol-12-myristate-13-acetate) or treated only by cell medium – RPMI (negative control). Extracellular DNA, as a marker of NETs formation, was quantitated by fluorometry using a fluorescent dye. Furthermore, construction of this structure was assessed by using fluorescent microscopy.

Averages of extracellular DNA release after 3 – hours incubation with PMA were 9317 ± 182 RFU (relative fluorescent units) for children with acute

leukemia and 15382 ± 499 RFU for healthy children. Differences between these two groups were statistically significant. Comparison of unstimulated samples between these groups resulted in averages 3001 ± 57 RFU for children with acute leukemias and 2658 ± 86 RFU for healthy children. The differences observed between negative controls were statistically unsignificant.
In our investigation we presented that neutrophils isolated from blood derived from children with acute leukemia (ALL/AML) demonstrated lower expression of extracellular traps formation than neutrophils derived from blood of healthy children. We hope that this observation will be the first step to explain the marked susceptibility of these patients to recurrent life-threatening bacterial and fungal infections, and contribute to find solution of this problem.

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Role of Receptor CXCR2 in the Pathogenesis of Experimental Septic Arthritis

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Introduction: Septic Arthritis is the joint disease which occurs when pathogens invade the joint causing infection. The main microorganism involved in that pathology is *Staphylococcus aureus*. The disease results in high mortality and morbidity, about 50% of patients have irreversible loss of joint function. The most prominant cell involved in immune response to bacterial infections is the neutrophil. This cell is the first to arrive at the site of inflammation, thereby helping to combat infection by means of several enzymes and mediators. The CXC chemokines that signals via CXCR2 activate neutrophils and promote their adhesion to the endothelium. The objective of this work was to investigate the role of chemokine receptor CXCR2 in inflammation caused by *S. aureus* in an experimental model of septic arthritis. **Methods and Results:** Experimental septic arthritis was induced by intra-articular injection of *S. aureus* (10^7 CFU; 10 μL) in C57/Bl6 mice (5-6 mice/group). Mice were treated orally with an alosteric inhibitor (DF2156A) of CXCR2 receptor 1 h after the injection of bacteria and daily for 7 days or treated intra-articularily every two days for 7 days. The oral treatment with DF2156A presented decreased number of total cells and neutrophils into the inflamed joint when compared to non-treated arthritic mice. This reduced cellular migration in DF2156A-treated mice was associated to a lower TNF-α and IL-1β in inflamed tissue. Furthermore, DF2156A-treated mice had decreased articular damage and reduced hypernociception compared to vehicle-treated mice. On the other hand, DF2156A-treated group showed slightly increased in bacterial load at the joint when compared with non-treated mice. In a similar manner, the local treatment with DF2156A increased the bacterial load compared to non-treated mice. Purified human neutrophils stimulated with CXCL8 increased the killing of *S. aureus* compared to non-stimulated neutrophils. **Conclusions:** The blockage of CXCR2 prevents the accumulation of neutrophils in the joint and decreases the articular inflammation, tissue damage and dysfunction following *S. aureus* infection. However, CXCR2-binding chemokines are very important to control the bacterial load by neutrophils. Thus, the use of CXCR2 antagonists in the context of *S. aureus*-induced septic arthritis must be carefully investigated to avoid the loss of bacterial control. **Financial support**: CAPES, CNPq, FAPEMIG and FWO

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Nuclear Segmentation of Neutrophils and Migration of Neutrophils through Narrow Pores; Cause or Consequence?

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Neutrophils have to pass narrow pores when they extravasate and patrol for pathogens in the extracellular matrix. The current dogma states that migration through narrow pores is favored by the characteristic segmented nucleus of neutrophils. A bulky nucleus is thought to be a limiting factor in cell migration (Wolf *et al.*, 2013). Therefore, we tested the hypothesis that immature neutrophils, characterized by a band-shaped nucleus, would migrate less efficiently than mature neutrophils, recognized by a segmented nucleus (2-3 lobes). Hypersegmented neutrophils (≥4 lobes)

would in turn migrate most efficiently. These three subtypes of neutrophils with different nuclear segmentation appear in the blood during acute inflammation (Pillay *et al.*, 2012).
We evoked acute inflammation by systemic challenge of healthy volunteers with bacterial lipopolysaccharide (LPS) and FACS-sorted the three neutrophil subtypes. We imaged migration of subtypes in dense collagen matrices in custom glass chambers (as described in Wolf *et al.*,2013). Additionally, transendothelial migration was studied in a transwell system, as a model for extravasation. In Fluoroblok™ transwells, the kinetics of the transmigrating neutrophil subtypes were recorded in real-time. Before and after transendothelial migration, the expression of cell surface markers and nuclear segmentation were determined.
In 3D collagen matrices as well as during transendothelial migration, the type of nuclear morphology was not associated with a difference in migration capacity or speed. In parallel, we tested the hypothesis that nuclear morphology could also be a *consequence* of migration through narrow pores. However, nuclear segmentation was not altered after transendothelial migration or during two hours of migration in collagen.
In contrast to the current dogma, nuclear segmentation does not facilitate neutrophil migration either in a matrix or through an endothelial monolayer. Nuclear (hyper)segmentation may be important for other functions of the neutrophils, or may be a consequence of separate differentiation of neutrophil subtypes.

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High Affinity ß2 Integrins Inhibit Neutrophil Recruitment during Acute Murine *Pseudomonas aeruginosa* Pneumonia

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Pneumonia is a significant burden to healthcare as the second leading cause of mortality worldwide. *Pseudomonas aeruginosa* is one of the most common Gram-negative bacteria to cause nosocomial pneumonia in immunocompromised patients. Evidenced through neutropenic patients being predisposed to respiratory infection, neutrophils are critical for lung immunity and promote bacterial clearance. To carry out these effector functions neutrophils are first required to traffic from the blood to tissues, and, for lung immunity, to the airspaces. Neutrophil recruitment from the systemic circulation occurs predominantly in post-capillary venules, which involves a well-studied neutrophil signaling cascade for β2 integrins transferring from a low to a high affinity state. With respiratory infections, neutrophils are sequestered within the alveolar capillary bed independent ofβ2 integrins, and then pass into the interstitium and airspaces. In response to certain infectious stimuli, β2 integrins retain sequestered neutrophils within the pulmonary capillary and play a role in migration into the airspaces. This study expands this knowledge using a technique to quantify the intravascular, interstitial and airspace fractions of the murine lung in order to look at the role of β2 integrin activation in neutrophil trafficking during acute *P. aeruginosa*-induced pneumonia. Talin-1 and Kindlin-3 are involved in activating β2 integrins to their intermediate and high affinity states. Tissue analysis of neutrophil trafficking in mixed chimeric mice suggests that talin-1 and Kindlin-3 knockout neutrophils exit the pulmonary capillaries and enter the interstitial spaces more efficiently than their wild-type counterpart. Pharmacological inhibition of high affinityβ2 integrins through the small molecule XVA143 increases the number of neutrophils entering the pulmonary interstitial spaces and airspaces during acute pseudomonal pneumonia. Bacterial clearance within the airspaces in vivo was not significantly impacted by XVA143 treatment, despite enhanced neutrophil recruitment. XVA143 attenuated neutrophil phagocytosis of *P. aeruginosa*, possibly contributing to unimproved clearance in vivo. This study highlights the role of high affinityβ2 integrins in restricting neutrophils from exiting the pulmonary vasculature during acute *P. aeruginosa* respiratory infection, which may provide a target to augment host defense against pneumonia in immunocompromised patients.

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Competition with High Affinity Glycosaminoglycan Binding Peptides Inhibits Neutrophil Dependent Inflammation

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The CXC chemokines CXCL9 and CXCL12gamma are characterized by a long, highly positively charged COOH-terminal region, absent in most other chemokines. To investigate the role of the COOH-terminal region of CXCL9 and CXCL12gamma, several COOH-terminal peptides were chemically synthesized. These peptides display high affinity for glycosaminoglycans (GAGs) with Kd's in the low nM range and compete with functional intact chemokines for GAG binding. The COOH-terminal peptides don't signal through the classical G protein-coupled chemokine receptors (GPCR) nor act as GPCR antagonists. Moreover, they do not influence the neutrophil chemotactic activity of CXCL8 in vitro. Based on the GAG binding data, an anti-inflammatory role for these peptides may be expected in vivo. Intravenous injection of site-specifically labeled peptides showed staining on the endothelial surface of blood vessels. Simultaneous intravenous injection of the GAG-binding peptides with CXCL8 injection in the peritoneum or joint diminished CXCL8-induced neutrophil extravasation. Analogously, monosodium urate crystal-induced neutrophil migration to the tibiofemural articulation, a murine model of gout, is highly reduced by intravenous injection of CXCL9(74–103). In addition, the peptides significantly inhibited neutrophil extravasation in response to locally administrated bacterial toll-like receptor agonists LPS and peptidoglycan. These data show that chemokine-derived peptides with high affinity for GAGs may be used as anti-inflammatory peptides. By competing with several active neutrophil attractants for binding and immobilization on GAGs, these peptides may lower local chemokine presentation on the endothelium and disrupt the generation of a chemokine gradient. Thereby the peptides prevent chemokines from properly performing their chemotactic function. Such high affinity GAG-binding peptides may serve as a lead molecules for further development of inhibitors of inflammation based on their interference with interactions between multiple chemoattractants and GAGs.

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The Small Molecule CR3 (CD11B/CD18) Agonist Leukadherin-1 is Protective of the Endothelial Barrier Disruption When Challenged by Neutrophils Obtained from Critically Ill Patients.

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During critical illness, whether from trauma, sepsis, or invasive surgery, the vascular integrity is disturbed leading to edema, hypotension and, in severe cases, multisystem organ failure. This endothelial dysfunction is mediated in part by neutrophil-to-endothelial interactions driven by endothelial activation and expression of ICAM, neutrophil activation by pathogen- and damage-associated molecular patterns, and subsequent neutrophil transmigration through the endothelium. The β2 integrin receptor, CR3 (Complement Receptor 3, CD11b/CD18, αMβ2), on the neutrophil surface plays a key role in both adhesion and diapedesis. Leukadherin-1 (LA-1) is a small molecule CR3 agonist that binds to the alpha I domain of CR3 and allosterically stabilizes the receptor in a high affinity conformation with open headpiece, while decoupling the receptor from some of its intracellular signaling. LA-1 has been found to increase neutrophil cell adhesion, decrease chemotaxis, and decrease transendothelial migration. While animal models have suggested an anti-inflammatory effect of this molecule, the impact of increased neutrophil adherence to an activated endothelium on endothelial integrity and barrier function has not been investigated. Human umbilical vein endothelial cell (HUVEC) monolayers were generated and Electrical Cell-substrate Impedance sensing (ECIS) was used to

quantify barrier disruption over time after neutrophil adhesion. Blood was obtained from healthy volunteers and patients in the surgical or trauma intensive care unit at Rhode Island Hospital. Neutrophils were isolated by dextran sedimentation and allowed adhere to TNFα-activated endothelial monolayers before additional fMLF stimulation. Results from a combined ICU population including trauma patients, sepsis patients, and surgical patients found neutrophils from critically ill patients caused significantly more endothelial damage than those from healthy donors. Neutrophils obtained from healthy donors and activated by fMLF *ex vivo* had significantly less endothelial disruption when treated with LA-1 than untreated neutrophils. Patient neutrophils treated with LA-1 showed significantly less damage that untreated neutrophils from the same population. LA-1 treated patient neutrophils had a reduction in endothelial damage to levels indistinguishable from neutrophils from healthy donors.

LA-1 treatment attenuated loss of endothelial barrier function in response to activated neutrophils from both critically ill patients and from healthy volunteers. This result identifies CR3 as a strong therapeutic target in treatment of endothelial dysfunction and warrants further investigation into applications for the anti-inflammatory effects of LA-1 in situations of critical illness. (Supported by NIH HL25265 and T32GM065085).

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The ADP-Ribosyl Transferase Activity of Exos from *P. aeruginosa* Targets Ras to Inhibit Reactice Oxygen Species Production in Human Neutrophils

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Pseudomonas aeruginosa is a major cause of acute infections, such as hospital-acquired pneumonia, blood stream infections, and microbial keratitis, as well as chronic lung infections in cystic fibrosis patients. Like many Gram-negative bacterial pathogens, P. aeruginosa relies on a type III secretion system (T3SS) to directly inject effector proteins into the cytoplasm of host cells. These effectors paralyze normal cellular functions, thereby enabling successful establishment of infection. Neutrophils are the first responders in bacterial infections. They are also the primary target of injection by the T3SS in early stages of P. aeruginosa infections. Injection of two effectors, ExoS and ExoT, promotes survival of P. aeruginosa during establishment of the infection. These effectors prevent killing of the invading bacteria by neutrophils recruited to the site of the infection. ExoS and ExoT are bifunctional effectors that contain distinct GTPase activating protein (GAP) and ADP-ribosyltransferase (ADPRT) activities. The increased survival of P. aeruginosa in murine models of pneumonia and keratitis, as well as survival in neutrophil in vitro, can be attributed entirely to ADPRT activities of these two proteins. Given that neutrophils are the first and predominant immune cell present in the lungs, blood stream, and cornea during in vivo infection, investigating the effect of ExoS and ExoT on primary neutrophils is critical to understanding P. aeruginosa pathogenesis. Using peripheral blood human neutrophils from healthy volunteers, we found that the injection of ExoS or ExoT into the cytoplasm of PMNs result in inhibition of reactive oxygen species (ROS) production. Here, we show for the first time that P. aeruginosa targets the Ras-mediated PI3K signaling cascade that is responsible for the assembly of NADPH oxidase complex which leads to ROS production. Specifically, in human neutrophils, ExoS and ExoT, prevent the phosphorylation of the PI3K associated regulatory kinase Akt and the cytosolic NADPH oxidase component p40phox thereby rendering both inactive and preventing ROS production. Importantly, preventing ROS production by neutrophils leads to increased survival of P. aeruginosa in vivo and in vitro. Our in vitro studies revealed that ExoS targets Ras for ADP-ribosylation in human neutrophils. ExoS had been shown previously to ADP-ribosylate Ras in epithelial cells at either Arg41 or Arg128. Intracellular delivery of a mutated Ras (R41Kh), which is unable to be ribosylated at Arg41, rescued ROS production in neutrophils infected with P. aeruginosa. This increase in ROS production was accompanied by a decrease in intracellular survival of P. aeruginosa in human neutrophils harboring Ras (R41K). Together, our data indicate that P. aeruginosa utilizes its T3SS to inject ExoS into the neutrophil cytoplasm which directly targets Ras. Riboyslation of Ras at Arg41 leads to the inhibition of ROS

production and, therefore, increased intracellular survival within the neutrophil.

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Src Family Kinases in Monosodium-Urate Crystal-Induced Inflammatory Responses

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Background: Deposition of monosodium urate (MSU) crystals in the joints or other tissues is a hallmark in the pathogenesis of gout, which is known to be mediated by neutrophils besides monocytes and macrophages. Although MSU crystal-mediated signal transduction is in the focus of recent investigations, the molecular mechanism is only partially characterized. In this study, we investigated the role of Src family kinases in MSU crystal induced myeloid cell activation.
Methods: Bone marrow isolated neutrophils from wild type and triple Src family kinases-deficient (Hck–/–Fgr–/–Lyn–/–) mice were stimulated by different concentrations of MSU crystals followed by the analysis of superoxide production and cytokine release. The process of crystal phagocytosis was investigated by videomicroscopy and flow cytometry in neutrophils derived from wild type and Src family kinases-deficient mice or in human neutrophils treated by the Src-inhibitor dasatinib or vehicle control.
Results: The MSU crystal-induced superoxide release and cytokine production were dramatically impaired in Src family kinases-deficient or in dasatinib-treated neutrophils. Cytochalasin D, which is an actin polimerization blocker that can effectively inhibit phagocytosis, strongly reduced the superoxide release of neutrophils activated by urate crystals. Both in the presence of cytochalasin D or dasatinib, the crystal phagocytosis of human neutrophils was abrogated. Neutrophils with genetic deficiency of Src family kinases also failed to phagocytose the MSU crystals.
Conclusions: The Src family kinases Hck, Fgr and Lyn play an indispensable role in MSU crystal-induced superoxide and cytokine production in neutrophils. The activation of neutrophils by urate crystals require the Src kinase-dependent phagocytosis of the crystals. Identification of these novel players in urate crystal-induced intracellular signaling pathways in neutrophils leads to a better understanding of the pathogenesis of gout and may help to develop novel therapeutic strategies in MSU crystal- associated inflammatory diseases.

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Effect of Ly6G Ligation on Neutrophil Extravasation Varies with the Dependence of Migration on Beta2 Integrins

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BACKGROUND: Ly6G is a GPI-linked murine neutrophil surface protein of unknown function that is frequently targeted for depletion and labeling studies. Ligation via specific antibody abrogates neutrophil migration in IgG-dependent experimental K/BxN arthritis but not in some other systems, and migration in Ly6G-null mice is intact. We sought to understand whether the association of Ly6G with beta2 integrins could account for these apparently discordant findings.
METHODS: We employed CD18-null mice to define the contribution of integrins to neutrophil migration in peritonitis and lung inflammation. To isolate the role of neutrophil beta2 integrins, we employed simultaneous adoptive transfer of WT and CD18-null neutrophils to animals subjected to inflammation in lung or peritoneum using either sterile (IL-1beta) or infection-like (LPS or *E. coli*) stimuli. We then administered the anti-Ly6G antibody 1A8 to test the impact of Ly6G ligation as a function of neutrophil surface integrins.
RESULTS: Unlike K/BxN arthritis, which is strictly dependent on CD18 expression, beta2 integrins play

a reduced role in experimental inflammation in lung or peritoneum. Co-engrafted WT and CD18-/- neutrophils migrated equally to bronchial irritation induced by either LPS or IL-1beta, and administration of 1A8 had no effect on neutrophil recruitment. In peritoneum, WT and CD18-/- neutrophils migrated equally to *E. coli*, but integrin-sufficient neutrophils demonstrate a distinct if partial advantage when inflammation was induced via IL-1beta. Correspondingly, in mice subjected to peritoneal inflammation after co-engraftment with WT and CD18-/- neutrophils, Ly6G ligation had no effect on the migration of either cell population in *E. coli* peritonitis but selectively impaired entry of integrin-sufficient neutrophils in IL-1beta peritonitis. CONCLUSIONS: Neutrophil migration exhibits variable dependence on beta2 integrins, depending not only on site but also on inflammatory stimulus. Impairment of migration through Ly6G ligation is selective for beta2 integrin-expressing neutrophils and occurs only under conditions where beta2 integrins contribute to neutrophil extravasation. The mechanism by which Ly6G ligation alters integrin-dependent migration, and the implications of this mechanism for the endogenous function of Ly6G, remain to be determined.

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Physiological Stimuli Induce ROS-Independent NETosis Using PAD4 and Discrete Signaling Pathways.

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In addition to being professional phagocytes, neutrophils produce a wide spectrum of inflammatory cytokines, which shape both the innate and adaptive immune responses. More recently, neutrophils were shown to extrude decondensed chromatin, thus forming NETs (neutrophil extracellular traps). These structures immobilize pathogens, thus preventing their spreading, but also feature antimicrobial molecules that efficiently kill various microorganisms. NETs were additionally shown to participate in the pathogenesis of autoimmune and inflammatory disorders.
Despite the importance of NETs, the molecular mechanisms underlying their formation, as well as the upstream signaling pathways involved, are only partially understood. Likewise, current methodological approaches to quantify NETs suffer from significant drawbacks, not the least being the inclusion of sometimes abundant nonspecific fluorescent signal. In this study, we developed a quantification method based on a novel, cell-impermeable fluorescent polymer that only binds extruded NETs – not intracellular DNA or membrane-bound proteins. This new approach allows for a straightforward, reliable, standardized quantification of NETosis, and was applied to decipher the upstream signaling pathways controlling the phenomenon. In neutrophils activated with various physiological stimuli, we confirmed that inhibition of the Syk or PI3K pathways blocks NETosis, and found that it does so by preventing chromatin extrusion from activated neutrophils. We also observed that inhibition of the TAK1, p38 MAPK or MEK pathways partially prevent NETosis, albeit without preventing chromatin extrusion – only filament generation seems to be affected. This indicates that extrusion does not necessarily lead to filament formation, and that the latter step can be regulated separately. By contrast, inhibiting PKC or Src family kinases failed to prevent NETosis in activated neutrophils. Finally, we revisited the issue of whether NET generation is ROS-dependent. Inhibition of the NADPH oxidase expectedly prevented NETosis in response to PMA, but was found to be largely ROS-independent in response to several soluble physiological agonists. Conversely, inhibition of PAD4 potently prevented NETosis by all stimuli tested. In summary, we describe a reliable method to quantify NETosis, that can be used to identify new molecular targets for therapeutic intervention in pathologies known to feature NET involvement.

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Temporal Dynamics of Hepatic CD4+FoxP3+ Tregs following Chronic CCL4-Induced Liver Fibrosis

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Objective: Wound healing is a naturally occurring physiologic response; however, upon excessive exposure to toxicants (chronic alcohol abuse, CCl_4) or persistent injury (viral infections, nonalcoholic steatohepatitis), fibrosis occurs. During fibrosis, hepatic stellate cells (HSCs) become activated and deposit extracellular matrix, hepatocytes are damaged, and hepatic nonparenchymal cells (NPCs) promote tissue injury. Both the progression and resolution of fibrosis depends on the interactions of NPCs, including HSCs, resident leukocytes, and infiltrating leukocytes. Regulatory T cells (Tregs) are key contributors to the resolution phase of inflammation and maintain immunological tolerance via secretion of a myriad of immunosuppressive and pro-resolving cytokines, including IL-10 and TGF-b. Here, we evaluated the dynamic regulation of inflammation and resolution in liver following chronic CCl_4-induced liver fibrosis. **Methods**: Wild-type C57BL/6J female mice were treated twice weekly with an injection of carbon tetrachloride (CCl_4) for five weeks; control mice received olive oil (OO) (*J Leuk Biology* 2015 97(1):161-9). Final doses were timed so that mice were euthanized 18, 24, 48, or 72 h post-injection so that dynamic responses to the final challenge with CCl_4 could be assessed. **Results**: CCl_4-treated mice displayed elevated indices of hepatocyte injury, including plasma alanine and aspartate aminotransferase (ALT, AST) activities; peak injury occurred 18 and 24hrs post-CCl_4 injection, which almost resolved by 72hrs. Markers of liver fibrosis were elevated in CCl_4-treated animals, with bridging deposition of collagen 1a1 (COL1A1) at 24hrs. Expression of pro-fibrotic genes alpha-smooth muscle actin (a-SMA) and COL1A1 were increased at early time points (18 and 24hrs), which was maintained at 72hrs. Similarly, CCl_4 treatment increased the expression of pro-inflammatory mediators TNFa, MCP-1, and IL-6 in liver, with peak elevations at 18 and 24hrs. FACS analysis of liver NPCs revealed infiltration of CD11c+ dendritic cells, Ly6G+CD11b+ neutrophils and CD11b+Ly6C^{hi} inflammatory scar-associated macrophages (SAM) at 24hrs following CCl_4 treatment. Interestingly, CD11b+Ly6C^{low} tissue restorative SAMs persisted throughout the recovery period. The mRNA expression of pro-resolution mediators TGF-b and IL-10 robustly increased at 18 and 24hr, decreased at 48hr, and moderately increased at 72hrs. Liver CD4+FoxP3+T_{regs} displayed a biphasic response after CCl_4 injection, with a modest increase at 18hrs, a decrease at 24hrs, a modest increase at 48hrs and a dramatic increase at 72hrs. The expression of FoxP3 mRNA in liver also followed this biphasic response. Interestingly, circulating CD4+FoxP3+T_{regs} increased at 18hrs, decreased at 24hrs, increased at 48hrs, and ultimately decreased again 72hrs post-CCl_4. **Conclusions**: These data demonstrate the dynamic response of the liver to the final challenge with CCl_4 after chronic CCl_4 exposure. Even after chronic injury, this additional insult led to leukocyte infiltration to the liver and induction of fibrogenic responses within 18-24hrs. By 48-72hrs after the final challenge, resolution of inflammation and extracellular matrix remodeling transpires. Here, we demonstrate that hepatic CD4+FoxP3+T_{regs} , major pro-resolution effector T cells, are also dynamically regulated, with increased recruitment to the liver during the wound healing/resolution phase of the response, likely serving to promote tissue repair following CCl_4-induced liver fibrosis. This work was supported by NIH/NIAAA R01AA011975 and R37AA011876 (LEN) and NIH/NIDDK 5T32DK007319 (RLM).

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Neutrophil Subsets and Activation Markers Are Sensitive Diagnostic Indicators of the Abdominal Aortic Aneurysm

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Objective

Neutrophils and monocytes are prominent cells in the intraluminal thrombus (ILT) associated with abdominal aortic aneurysm (AAA). Their recruitment and subsequent secretion of proteolytic enzymes may substantially contribute to the overall pathological role of the ILT in aortic wall weakening, thus increasing aneurysm growth and the risk of rupture. Recently, neutrophil subsets have been identified with distinct inflammatory activity. We hypothesised that the distribution of the quiescent (CD66b bright CD62L bright CD16 bright), activated (CD66b bright CD62L dim CD16 bright) and newly released (CD66b bright CD62L

bright CD16 dim) neutrophil populations as well as the level of neutrophil activation markers in plasma is altered in AAA patients compared to healthy controls. Comparably, the frequency of monocyte subsets (based on CD14 and CD16 surface expression) was investigated.

Methods

22 AAA patients with advanced disease (prior to surgical intervention) and 21 age, sex, BMI and smoker status-matched healthy individuals were assessed. Monocytes and neutrophils were stained in hirudinised, fixed whole blood with fluorescent antibodies and quantified by flow cytometry. Patient plasma was analysed for neutrophil elastase, NGAL and MPO by ELISA. SDF-1α, RANTES, MIP-1α, MIP-1β, MCP-1, IL-8, IP-10, GRO-α, and eotaxin were quantified by Procarta Multiplex Immunoassay.

Results

Among the secreted factors only MPO (mean+-SD 16.1+-11.2 vs. 7.3+-3.0 ng/ml, $p<0.001$) and MCP-1 (95.8+-29.4 vs. 62.7+-24.3 pg/ml, $p=0.002$) were significantly increased in AAA patients compared to healthy individuals. Correspondingly, a significant increase in the frequency of activated as well as newly released neutrophils was identified in AAA patient blood (2.95+-0.66 vs. 1.92+-0.36 and 3.20+-0.87 vs. 1.67+-0.39%, $p<0.001$). A significant correlation was identified between MPO level and the proportion of activated neutrophils ($r=0.407$, $p<0.001$). The distribution of monocyte subsets did not differ significantly between AAA patients and the control group.

Conclusion

An increased frequency of newly released and activated neutrophil subsets is significantly associated with AAA, as further reflected in a higher plasma level of MPO which is secreted by activated neutrophils. The potential of neutrophil subsets and activation markers to predict AAA progression is currently under investigation.

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Nitrogen Mustard-Induced Pulmonary Injury and Fibrosis Visualized by MRI and CT Imaging is Accompanied by an Accumulation of Foamy Macrophages in the Lung

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Nitrogen mustard (NM, mechlorethamine) is a cytotoxic alkylating agent known to induce extensive lung injury, which progresses to fibrosis. This is associated with a persistent macrophage dominant inflammatory response. Recent evidence suggests that lipid laden foamy macrophages play a role in fibrogenesis. In these studies we used high definition magnetic resonance imaging (MRI) and computed tomography (CT) to characterize the progression of lung injury and fibrosis induced by NM. We also determined if NM caused derangements in lung lipids resulting in the formation of foamy macrophages. Male Wistar rats were treated i.t. with 0.125 mg/kg NM or PBS control. Gradient (GRE) and fast spin (FSE) echo MRI, as well as CT scans were performed on each animal 1, 3, 7, 14, 21 and 28 d later. Image analysis and high definition 3D lung reconstruction showed that NM exposure resulted in a significant increase in injury volume within 1 d, which persisted up to 28d; this was accompanied by reduced volumes of air and increased consolidated lung tissue. NM exposure also resulted in phospholipid accumulation in bronchoalveolar lavage fluid (BAL). This was associated with a time-related accumulation of oxidized phospholipids in lung macrophages. By 28 d, these cells were enlarged, vacuolated, laden with neutral lipid, and clustered in areas of fibrosis. Liver-X-receptor (LXR) and Farnesoid-X-receptor (FXR) are transcription factors involved in lipid homeostasis. Three d post NM, LXR and its target genes, cholesterol efflux transporters ABCA1 and ABCG1, were decreased in lung macrophages; conversely, FXR was increased, along with scavenger receptor CD36. Treatment of alveolar macrophages with lipid enriched fractions of BAL collected from rats 3 d after NM resulted in upregulation of proinflammatory genes, NOS2, ApoE and PTGS2, while BAL collected from rats 28 d post NM induced the formation of foamy macrophages and upregulated anti-inflammatory IL-10, CD163, and CX3CR1 gene expression. These data demonstrate that MRI and CT imaging are effective tools to visualize the progression of NM-induced lung injury and fibrosis in rats. Moreover, altered lipid homeostasis induced by NM results in macrophage foam cell formation and differential cell activation, responses that likely contribute to fibrogenesis.

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Impact of Obesity on Formation of Neutrophil Extracellular Traps by Murine Neutrophils during Sepsis

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Neutrophils combat pathogens by phagocytosis, release of antimicrobial agents and proteases, and release of neutrophil extracellular traps (NETs) (Kolaczkowska & Kubes, Nat Rev Immunol. 2013;13:159). NETs are structures composed of DNA decorated with histones and granular proteins, including proteases such as neutrophil elastase (NE) and antimicrobials. NETs play both beneficial and disadvantageous role since they facilitate capturing of bacteria during infectious inflammation (Brinkmann et al. Science. 2004;303:1532) and bystander damage to host tissues due to their persistent presence in vasculature, respectively (Kolaczkowska et al. Nat Commun. 2015;6:6673). Both of these aspects of NET functioning have to be taken into consideration during inflammatory responses and their treatment. This is of importance especially during sepsis in which NETs are formed and present in blood vessels. Sepsis is a systemic, deleterious host response to infection with a high mortality rate. Surprisingly, however, obese patients have higher chances of surviving sepsis (e.g. Wacharasint et al. Crit Care 2013;17:R122). This phenomenon might be connected to differential immune responses in lean and obese individuals as it is well know that obesity is accompanied by low-grade chronic inflammation. Most importantly, obese individuals have elevated levels of NE and, as shown in obese mice, it contributes to inflammation-induced metabolic disease, namely, neutrophil NE mediates insulin resistance (Talukdar et al. Nat Med. 2012;18:1407). We hypothesized that NE present in higher amounts in obese individuals might at least partially originate from NETs, and that some NETs might be already present in adipose tissue and/or vasculature of obese individuals prior to sepsis. For this reason we studied NET formation by non-activated and liposaccharide (LPS)- or phorbol-12-myristate-13-acetate (PMA)-activated neutrophils from mice with diet-induced obesity (DIO) and age-matched lean mice. C57Bl/6J mice were maintained on high fat diet (DIO, 60% kcal from fat) or chow diet (lean mice) for at least 12 weeks. Increased release of NETs was detected by LPS-stimulated neutrophils from healthy DIO mice. However, when neutrophils where collected from LPS-challenged septic mice, the above effect was abolished. We further studied NET formation by neutrophils from DIO and lean mice upon incubation with different concentrations of glucose and again detected differential responses between the two groups. Our studies indicate that formation of NETs is altered is obese mice and this is at least partially connected to increased glucose levels present in obese individuals. Detailed evaluation of mechanisms responsible for the higher chances of surviving sepsis in obesity, in connection to NETs, might also help understand mechanisms of NET formation in relation to immunometabolism in general.

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Neutrophils Extracellular Traps (NETs) Formation in the Synovial Fluid of Heifers with Acute Ruminal Acidosis

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Neutrophil extracellular traps (NETs) were previously described as a defense mechanism against harmful stimuli in tissues, however also are involved in the pathogenesis of several joint disease including gout and rheumatoid arthritis in humans. NETs structures are composed by DNA strands, antimicrobial and cytotoxic peptides, such as myeloperoxidase, elastase neutrophilic, Matrix Metalloproteinase-9 (MMP-9) and histones. Aseptic polysynovitis is a pathological condition described in bovine with acute ruminal acidosis, a metabolic disease produced by high intake of fermentable carbohydrates. The acidosis is characterized by the presence of high levels of D-lactic acid in ruminal fluid. Until now the pathogenesis of aseptic polysynovitis in bovine with ruminal acidosis is unknown.

We assessed the synovial fluid of four heifers with acute ruminal acidosis induced by oligofructose overload (13 g/kg BW). pH of ruminal fluid was recorded with a portable pH meter after ruminocentesis. Arthrocenthesis of tarso-crural joint was performed to assess in the synovial fluid; total

protein, DNA, ATP, prostaglandin E2and IL-1β, using a microplate reader, MMP-9 by zymography, NETs formation was visualized by immunofluorescence microscopy using anti-citrunillated histone 3 antibody (Alexa 594) and Picogreen as DNA probe. Also a cytological analysis was done. The level of D-lactic acid in the synovial fluid was detected using HPLC, equipped with a column for chiral separation. Additionally, we assessed the role of D-lactic in the formation of NETs using blood neutrophils isolated from healthy bovines. All experiments were conducted in accordance with institutional review board-approved protocols and the National Guidelines on the Use of Experimental Animals of the Comision Nacional de Ciencia y Tecnologia de Chile. Ruminal fluid of animals with acute ruminal acidosis after oligofructose overload, showed a decrease of pH below 5.0. The synovial fluid appears cloudy and with abundant presence of neutrophils and high total protein, DNA and ATP level. The formation of NETs structures in the synovial fluid were confirmed by the visualization of DNA decorated with citrunillated histone 3. Additionally, MMP-9, prostaglandin E2and IL-1β were increased in the synovial fluid of heifer with acute ruminal acidosis. An increase of D-lactic acid was also detected in the synovial fluid. In vitro experiments revealed that D-lactic acid induced NETs formation with the presence of DNA, citrunillated histone H4 and CD11b. We demonstrated that Cl-amidine reduced the NET formation induced by D-lactic acid, suggesting a possible role of PAD4 in this response. We concluded that animals with acute ruminal acidosis develop an aseptic neutrophilic polysynovitis, characterized by the presence of NETs structures, D-lactic acid, ATP, prostaglandin E2 and IL-1β in the joint. Also, our results suggest that D-lactic acid could contribute to NETs formation in the initial steps of aseptic polysynovitis. Acknowledgments: This work was supported by FONDECYT 1151035, Beca Conicyt Doctorado Nacional, MECESUP AUS1203. Programa de Doctorado en Ciencias Veterinarias, UACh.

Assessment of Arl4c Expression in T-Cell Acute Lymphoblastic Leukemia and Acute Myeloid Leukemia Cell Lines

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Background: Arl4c is a small GTPase that belongs to the Arf family. Liver X-receptor (LXR) and retinoid X-receptor (RXR) agonists or cholesterol loading induces Arl4c expression. Arl4c is known to regulate cholesterol efflux and vesicular trafficking from early endosomes to recycling endosomes via interaction with microtubules. Wnt3a and EGF signaling synergistically induce Arl4c, which regulates cell migration and the actin cytoskeleton. As cancer cells often express high levels of Arl4c mRNA, we evaluated the expression of Arl4c in T-cell acute lymphoblastic leukemia (T-ALL) and myeloid leukemia (AML) cell lines.
Materials and methods: Arl4c monoclonal antibodies (mAb) were generated and characterized. Arl4c expression in T-ALL (MOLT-3, JJHAN, HSB2) and AML (PLB-985) cell lines was monitored using qPCR and Western blotting. PLB-985 cells were cultured with or without LXR (GW3965, T0901317) and RXR (LG268) agonists, either alone or in combination. Granulocytic differentiation was induced using db-cAMP, DMSO, or DMF.
Results: Arl4c mAb 21F12 was capable of detecting 2 ng of Arl4c, with no significant cross-reactivity to Arf proteins, Arl4a, and Arl4d. Higher levels of Arl4c protein were measured in MOLT-3 and HSB2 lymphoblastic T-cells compared to JJHAN cells. Higher Arl4c mRNA but lower Arl4c protein levels were detected in neutrophils compared to undifferentiated PLB-985 cells. Granulocytic differentiation of PLB-985 cells markedly enhanced Arl4c mRNA copy numbers. Differentiation with db-cAMP (3 days) increased Arl4c mRNA 500 to 1000-fold, while slightly decreasing Arl4c protein (15-40%). Combinations of LXR and RXR agonists during db-cAMP differentiation further decreased endogenous Arl4c protein.
Conclusions: Leukemia cell lines express Arl4c. Although granulocytic differentiation markedly increases Arl4c mRNA levels, those of Arl4c protein were decreased and down regulated by combinations of LXR and RXR agonists.

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Functional Role of Free Fatty Acid Receptor 4 (FFAR4/GPR120) Agonists in Bovine Neutrophils

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Omega-3 fatty acids are recognized ligands of the G-protein coupled receptor Free Fatty Acid Receptor 4 (FFAR4), and a role on inflammatory response has been suggested. Previously, we suggested the expression of FFAR4 in bovine neutrophils; however the role of FFAR4 agonists has not been demonstrated yet. The aim of this study was to determine the functional role of FFAR4 agonists in bovine neutrophils. First, we demonstrated the presence of FFAR4 in bovine neutrophils by RT-PCR, immunoblot and immunofluorescence. Then, the natural and synthetic FFAR4 agonist docosahexaenoic acid (DHA) and TUG891, respectively, were used to analyse the metalloproteinase-9 (MMP-9) granules release by zymography, and MAPK and Akt phosphorylation by immunoblot. We observed that DHA and TUG891, between 50 and 200 μM, significantly increased MMP-9 granules release. The analysis of protein phosphorylation showed that DHA (50 μM), between 15-30 min, induced the ERK1/2 and Akt phosphorylation. Similarly, TUG891 increased the phosphorylation of ERK1/2, p38 MAPK and Akt. In conclusion, our results showed that FFAR4 agonists increased granules release and phosphorylation of intracellular signaling pathways in bovine neutrophils, thus suggesting that bovine neutrophil function could be modulated in the presence of omega-3 fatty acids.

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Dual Immunometabolic Roles of the Melanocortin 5 Receptor in Obesity Induced Diabetes

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Type 2 diabetes mellitus (T2DM) is a serious global problem with obesity as a major risk factor. Since there is a connection between obesity and inflammation, inflammation can have a role in T2DM. The melanocortin 5 receptor (MC5r) has a role in lipolysis in adipocytes and in the induction of antigen-specific regulatory immunity, so had dual roles in both metabolism and inflammation. Ocular immune privilege exists to protect the eye from damaging and potentially blinding inflammation, and one aspect of ocular immune privilege is the induction of antigen-specific systemic regulatory immunity. In some cases, inflammation of the eye (uveitis) does occur and can be autoimmune in nature. Autoimmune uveitis is studied with a mouse model, experimental autoimmune uveitis (EAU). In mice, EAU spontaneously resolves and a consequence of the resolution is the induction of systemic regulatory immunity. This regulatory immunity requires MC5r expression on a $CD11b^{+}F4/80^{+}Ly6G^{+}Ly6C^{lo}$ ($Ly6G^{+}$) suppressor macrophage. We therefore asked if MC5r has a role in obesity induced hyperglycemia and if EAU-associated regulatory immunity can protect from obesity induced hyperglycemia. Body weight, blood glucose, and the macrophage composition in white adipose tissue (WAT) and brown adipose tissue (BAT) was compared between wild-type (WT) and $MC5r^{(-/-)}$ mice on a normal fat diet (NFD) or high fat diet (HFD), and compared with EAU mice. At 8 weeks on the HFD, $MC5r^{(-/-)}$ mice showed a significant increase in body weight compared to HFD WT mice. Despite the increase in weight, the HFD $MC5r^{(-/-)}$ mice showed significantly lower blood glucose compared to HFD WT mice. When immunized for EAU both HFD and NFD WT mice showed significantly lower blood glucose compared to HFD WT mice. However, $MC5r^{(-/-)}$ mice immunized for EAU showed significantly higher blood glucose on either NFD and HFD. The percentage of $Ly6G^{+}$ macrophages was 10 fold greater in BAT compared to WAT, and $Ly6G^{+}$ macrophages in BAT from HFD mice was reduced by half. This population showed a similar increased accumulation in BAT from mice at resolution of EAU on both NFD and HFD. These observations indicate that a deficiency in MC5r provides protection from HFD induced hyperglycemia. More interestingly, these observations suggest that as inflammation in the eye resolves systemic regulatory immunity emerges that is protective from HFD induced hyperglycemia and may be due to a MC5r-

dependent accumulation of Ly6G^{+} macrophages in BAT.

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Dendritic Cells: Holding the Balance of Immunity and Tissue Integrity

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Dendritic cells (DCs) can be subdivided in different subsets: conventional DC type-1 (cDC1), cDC2 and plasmacytoid DCs (pDCs). Each subset has specialized functions and is required for protection against selected pathogens. pDCs are major source of type-1 IFNs during viral infections. cDC1 are essential producers of IL-12 leading to IFN-g responses. cDC2 are a more heterogeneous subset; on one hand responsible for the production of IL-23, required for the induction of Th17 immunity, and on the other hand responsible for mediating Th2 immunity. This DC subset specialization is obtained and maintained through a complex network of transcription factors (TFs). We could establish the requirement for specific TF during DC development through the generation of KO mouse models. Further, we could correlate the expression of these TF with a specific function linked to a DC subset. In the absence of Irf8 or Batf3 cDC1 commitment is compromised, and their development abrogated. This results in impaired generation of Th1 and CTL immunity. Mice defective for either TF are therefore highly susceptible to intracellular pathogens such as parasites i.e *Toxoplasma gondii* or viral infections, i.e. *Herpes simplex virus*, and also show defective anti-tumor response. The other branch of cDCs depends on the TFs Irf4 and Klf4. In the absence of these TFs, Th17 and Th2 immune responses are defective. In particular, mice lacking Klf4 have impaired Th2 immunity against helminthes infection such as *Schistosoma mansoni* and are resistant to the development of house dust mite mediated asthma. Altered immunity in the absence of one of these TFs is however, not only rendering mice susceptible to specific pathogens, for which the TF is required, but also causing an overall unbalanced cytokine production and immunity. Batf3-deficient mice have impaired IL-12 production, which results in defective Th1 immunity, but also leads to an exacerbated Th2 immunity during helminthes infection such as *Schistosoma mansoni*. Increased Th2 polarization and cytokine secretion had major histo-pathological consequences with sever liver damage. However, following *H. Polygyrus* infection increased Th2 cytokines in Batf3 deficient mice, improved parasite burden, ensuing reduced worm and egg counts.

Collectively, the outcome of an immune response is highly dependent on the site of infection, the pathogen and the generation of the appropriate inflammatory microenvironment. A balanced cytokine production by DCs is instrumental to generate a pathogen-specific immune response. Altered DC development in the absence of one of the key TFs, will result in an improper innate cell activation as well as Th-cell polarization generating an unbalanced immune response. DCs are therefore not only professional antigen presenting cells, but also essential gatekeepers of innate immunity and tissue integrity.

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T Cells Undergoing Homeostatic Expansion Induce Acute Lymphoblastic Leukemia in IL-15-Deficient NOD.Scid Mice

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Non-obese diabetes mice with severe combined immunodeficiency (NOD/SCID) are widely used in leukemia research. We made a serendipitous finding that NOD/SCID.*Il15*$^{-/-}$ mice, but not NOD/SCID controls, became overtly ill following adoptive transfer of splenocytes from 8.3-NOD mice expressing an MHC class I-restricted, transgenic T cell antigen receptor (TCR). These NOD/SCID.*Il15*$^{-/-}$ recipients developed acute lymphoblastic leukemia that infiltrated the liver and kidney. This leukemogenic activity was restricted to donor CD4^{+} T cells and also occurred when non-TCR transgenic donor cells were used. The leukemic cells showed an immature T cell phenotype (CD4^{+}CD8^{int}TCR^{-}

CD5^{hi}B220^{-}), readily grew *in vitro*, showed multiple cytogenetic lesions and rapidly transferred disease to immune-competent NOD mice. Further investigation revealed that the leukemic cells did not arise from donor cells, but originated from recipient cells and expressed terminal deoxynucleotidyl transferase. In agreement, NOD/SCID mice lacking IL-15 or IL-15 receptor alpha developed spontaneous leukemia within 8 months of age with 100% penetrance. These findings indicate that IL-15 deficiency or impaired IL-15 signaling in a lymphopenic environment promotes leukemogenesis from T cell precursors during homeostatic expansion of CD4^{+} T cells. These findings highlight the importance of IL-15 signaling in conferring protection against potentially neoplastic T cell precursors from developing into fatal leukemia.

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Interleukin-31 and Thymic Stromal Lymphopoietin Expression and Clinical Relevance in Classical Hodgkin Lymphoma Patients

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Classical Hodgkin Lymphoma (cHL) is characterized by a low percentage of neoplastic Hodgkin/Reed-Stenberg (HRS) cells, that are embedded in a reactive microenvironment including CD4 T cells, B cells, macrophages, dendritic cells, eosinophils, fibroblasts, and basophils/mast cells. This inflammatory microenvironment provides essential signals for HRS cell survival. Interleukin 31 (IL-31) is related to the IL-6 family and secreted mainly by activated Th2 cells. Thymic Stromal Lymphopoietin (TSLP) is highly expressed in cutaneous and bronchial epithelial cells from atopic dermatitis and asthma patients. Both cytokines are mediators of chronic pruritus in various skin diseases. Pruritus is observed in about 30% of patients with cHL, more often in the nodular sclerosis type with mediastinal mass. Aim of this study was to investigate the possible role of IL-31 and TSLP in pruritus and the potential contribution of these cytokines to disease pathogenesis in patients with cHL.

Plasma samples from 109 HL patients were collected at diagnosis and tested for IL-31/TSLP secretion by ELISA. IL-31 was detected in 65 patients out of 109 tested and ranged from 5 to 7937 pg/ml with a median of 245 pg/ml; TSLP was detected in 52 out of 75 patients with a range from 9 to 4209 pg/ml, and a median of 171 pg/ml. IL-31/TSLP levels were found not to be associated with presence or degree of itching in our patient cohort. However, we identified a subgroup of patients showing elevated plasma levels of both IL-31 and TSLP who had an International Prognostic Score (IPS) >2, indicative of high risk disease (P=0.002 for IL-31, =0.03 for TSLP). Among the clinical parameters included in IPS, white blood cells count >15x10^{3}/ml was found to be significantly associated with high IL-31 and TSLP plasma levels (P=0.01, n=19/89 for IL-31; P=0.02, n=15/59 for TSLP). In addition, cHL patients with a positive interim PET-scan (indicative of insufficient response to chemotherapy) had higher IL-31/TSLP levels at diagnosis (P=0.01 for IL-31, =0.05 for TSLP) compared with patients with a negative interim PET-scan..

Then, we investigated by flow cytometry the expression of IL-31/TSLP and their heterodimeric receptors (IL-31R alpha/OSMR for IL-31 and TSLR/IL-7R alpha for TSLP) in lymph node biopsies form cHL patients. HRS cells were found to express intracytoplasmic and surface IL-31 and TSLP, as well as their receptors on the cell surface. Among the major immune cells infiltrating cHL lymph nodes, B cells and macrophages displayed surface and cytoplasmic expression of IL-31 and TSLP and expressed the corresponding receptors. CD4 T cells also expressed both cytokines (in the intracellular compartment only) and their surface receptors.

This study demonstrates for the first time that IL-31/TSLP are present in the plasma of HL patients but do not appear to be the mediators of itching. However, the cytokines are associated with HL clinical characteristics of prognostic relevance, suggesting a role of these two cytokines as potential biomarkers of chemoresistance and surrogate biomarkers of early-PET. Moreover, the expression of IL-31/TSLP and their receptors on both malignant and immune cells suggests that numerous paracrine and/or autocrine interactions involving these pairs of cytokines and receptors may take place in vivo.

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Functional Characterization of Meteorin-Like/IL-39, a Novel Cytokine Produced by Macrophages and Barrier Tissues

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Cytokines play a key role in nearly all biological processes including hematopoiesis, wound healing, inflammation, cell differentiation, lymphoid trafficking, proliferation and survival. The cytokine superfamily includes various cytokines, chemokines, interleukins, tumor necrosis and growth factor families. Thirty eight interleukins have been described. Recently, we reported the identification of a novel cytokine, Meteorin-like (Metrnl), for which we have proposed the name IL-39to reflect its association with the immune system. IL-39 is produced by macrophages in response to various stimulating agents including TNFα, IL-17α, IL-4 and IL-12 whereas TGFβ and IFNγ inhibit IL-39 production. Moreover, levels of IL-39 in circulation rise in response to inflammation induced by thioglycollate injection into the peritoneal cavity. To elucidate the function of IL-39, we have produced an *IL-39-/-* mouse. Although IL-39 deficient mice have comparable numbers of major immune cell populations in their immune organs, the effector functions of some of these cells are altered. For example, splenocytes isolated from *IL-39-/-*mice produce abnormal levels of several cytokines and chemokines following activation (with anti-CD3/anti-CD28) when compared to splenocytes isolated from their WT littermates. To elucidate the role of IL-39 in innate and/or adaptive immune responses, we tested the *IL-39-/-* mice in a model of acute *T.gondii* infection. We found that levels of IL-39 in serum and peritoneal cavity significantly increase in response to infection. Surprisingly, *IL-39* deficient mice were more resistant to the *T.gondii* infection as indicated by reduced pathogen load and increased survival. Analysis of various cytokines and chemokines in serum from infected WT and IL-39 deficient mice revealed that *IL-39-/-* mice had lower levels of several pro-inflammatory mediators including CXCL1 and CCL2. High levels of these chemokines have strong correlation with increased mortality suggesting that, with sublethal doses of *T.gondii* infection, mice die from sepsis rather than from the infection itself. Therefore, our results indicate that reduced inflammation levels in *IL-39-/-* mice protect them from immune-mediated tissue damage during *T.gondii* infection. Together, our studies indicate that IL-39 is a pro-inflammatory cytokine that plays important roles in both innate and/or adaptive immune responses.

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The Gut Microbiome is Essential for the Development of IL-33-Responsive ILC2s That Mediate Chronic Inflammation in Ileitis-Prone Mice

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It is well established that IL-33 and its receptor, ST2, are increased in several autoimmune and inflammatory disorders, including inflammatory bowel disease (IBD). In fact, IL-33 has been reported by several groups to be potently upregulated in patients with both ulcerative colitis and Crohn’s disease, the two main etiopathogenic forms of IBD, compared to healthy controls. However, uncovering its precise role utilizing (primarily acute, chemically-induced) models of colitis has produced ambiguous results in the literature, indicating both pathogenic and protective functions of IL-33. Using a relevant, spontaneous model of Crohn’s-like ileitis, *i.e.*, SAMP1/YitFc (SAMP) mouse strain, we recently reported that blockade of IL-33 signaling ameliorates chronic intestinal inflammation and that the gut microbiome is essential for the induction of IL-33 in these mice. Since IL-33 is also known to be important in the development of group 2 innate lymphoid cells (ILC2s), the aim of this study was to determine the

contribution of IL-33-responsive ILC2s to chronic intestinal inflammation in ileitis-prone SAMP mice. By flow cytometry, our results showed a dramatic increase in both the frequency and absolute numbers of ILC2s within the draining mesenteric lymph nodes (MLNs) ($P<0.05$ for both frequency and absolute numbers) as well as the ileal lamina propria (both $P<0.05$) of SAMP vs. AKR (parental control) mice, and increased with age as disease became more severe in 20- vs. 4-wk-old SAMP ($P<0.007$), which also demonstrated increased MLN-derived IL-5-expressing ILC2s ($P<0.05$). In fact, MLN-derived $ST2^+$ ILC2s potently expanded in normal AKR mice after exogenous IL-33 administration vs. vehicle-treated controls ($P<0.0001$). Interestingly, germ-free SAMP raised under gnotobiotic conditions exhibited a dramatic decrease in MLN-derived ILC2s compared to specific pathogen-free raised SAMP ($P<0.01$), which corresponded with a decrease in MLN-derived ILC2s in SAMP lacking the bacterial sensor for muramyl dipeptide (SAMPxNOD2$^{-/-}$ vs. SAMPxNOD2$^{+/+}$, $P<0.01$), suggesting that components of the gut microbiome may be necessary for ILC development and expansion during chronic gut inflammation. Together, our data indicate that the IL-33/ST2 axis may mechanistically contribute to chronic intestinal inflammation, such as that observed in IBD, through the development of pathogenic ILC2s.

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IL-22 and STAT3 Signaling Protect Intestinal Barrier Integrity despite Persisting Intestinal Inflammation following Alcohol and Burn Injury

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Burn injury has been shown to result in intestinal inflammation and leakiness, which can contribute to sepsis and multiple organ failure in burn-injured patients. Co-morbidities, such as ethanol intoxication at the time of trauma, negatively impact patient prognosis. We have previously shown that ethanol intoxication prior to burn results in gut barrier leakiness, inflammation, and bacterial overgrowth in the small intestine one day following injury. We have demonstrated that interleukin-22 (IL-22) administration helps maintain gut barrier integrity and prevents bacterial overgrowth following the combined injury. We also observed that IL-22 restores proliferation and reduces apoptosis in epithelial cells after ethanol and burn injury, which was partially dependent on signal transducer and activator of transcription factor 3 (STAT3). In this study, we wanted to elucidate whether IL-22 limits acute intestinal inflammation, and if STAT3 and downstream suppressor of cytokine signaling factor 3 (SOCS3) signaling is necessary for IL-22 mediated protection. To study this, male C57BL/6 or intestine epithelial cell specific VillinCre STAT3-/- knockout mice were gavaged with ~2.9g/kg ethanol or vehicle (water). When blood alcohol content was ~100mg/dL, mice were anesthetized and given a ~12.5% total body surface area scald burn by immersion in 85-90^{0}C water for 7 seconds. Mice in sham groups were immersed in 37^{0}C water for 7 seconds. Mice were immediately given 1mL normal saline resuscitation with or without 1mg/kg recombinant mouse IL-22. One day following injury total small intestine tissue, small intestine epithelial cells (IECs), and blood were collected. Small intestine tissue was analyzed for inflammatory cytokine expression (IL-6, IL-18, KC) by ELISA, small IEC SOCS3 gene expression was measured by qRT-PCR, and IL-22 levels in circulation were quantified by ELISA. We found increased levels of IL-22 in both sham IL-22 treated (70 pg/mL) and burn ethanol (38 pg/mL) animals compared to sham vehicle controls (undetectable). Burn ethanol mice receiving IL-22 treatment had significant increases in serum IL-22 (352 pg/mL) compared to all other groups. There was an increase in IL-6 (1.5-fold), IL-18 (3.75-fold), and KC (2.2-fold) in the small intestine following ethanol burn injury compared to shams, and a 6-fold increase in SOCS3 gene expression in small IECs. Burn ethanol animals treated with IL-22 displayed even higher SOCS3 gene expression (9-fold increase) in small IECs, but not reduced IL-6, IL-18, or KC levels. SOCS3 expression was completely diminished in small IECs of STAT3-/- mice, however, we observed similar increases in IL-6 (2.4-fold) and IL-18 (1.8-fold) in STAT3-/- burn ethanol injured animals compared to sham vehicle controls. Together along with our previous findings, these data suggest that IL-22 signaling does not directly

regulate gut inflammation, rather, IL-22 and STAT3 protect the intestinal barrier by promoting epithelial barrier regeneration following ethanol and burn injury.

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A Novel Role for IL-21 in Modulating T Cell Antigen Receptor Responsiveness towards Low Affinity Ligands in CD8+ T Lymphocytes

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IL-21 promotes the development of spontaneous and virus-induced autoimmune type-1 diabetes (T1D) by increasing the efficiency of $CD4^+$ T cell help. However, it is unclear whether IL-21 is also directly involved in the activation of autoreactive $CD8^+$ T cells, which play a key role in T1D pathogenesis. Here, we assessed the requirement of IL-21 in a virus-induced, $CD8^+$ T cell-mediated T1D model where disease occurs independently of CD4 help. In this model expressing the glycoprotein (GP) antigen of lymphocytic choriomeningitis virus (LCMV) under the rat insulin promoter (RIP-GP), LCMV infection activates $CD8^+$ T cells reactive to the GP-derived antigenic peptide GP33 that attack the islets and cause T1D. IL-21 deficiency in RIP-GP mice did not affect T1D induction by LCMV, indicating that IL-21 is dispensable for eliciting the pathogenic functions of autoreactive $CD8^+$ T cells. Previously we have shown that IL-21 enables autoreactive $CD8^+$ T cells to respond to weak TCR ligands and induce T1D in RIP-GP mice. Therefore, we assessed IL-21 requirement for T1D induction by variant LCMV expressing altered GP33 epitopes that are less efficient in inducing T1D in RIP-GP mice. Surprisingly, an LCMV variant expressing the altered peptide ligand induced T1D more efficiently in $Il21^{-/-}$RIP-GP mice than in control mice. Moreover, a LCMV variant expressing a very weak peptide mimic of the GP33 epitope activated diabetogenic T cells that caused disease in a lymphopenic environment. Using $Nur77^{GFP}$ reporter mice, we show that $CD8^+$ T cells from $Il21^{-/-}$ mice expressing the GP33-specific transgenic P14 TCR showed increased reactivity towards low affinity TCR ligands. Our findings suggest a novel role for endogenous IL-21 in restraining $CD8^+$ T cell reactivity towards low affinity TCR ligands.

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Role of PR3 in IL-1b Cleavage and Release in Human Neutrophils

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INTRODUCTION:
Interleukin 1b (IL-1b) is a key proinflammatory cytokine in innate immunity and host response to microbial invasion and tissue injury. IL-1b is first expressed as a biologically inactive precursor and the proIL-1b is processed into mature IL-1b by Caspase 1. It is known that not only Caspase 1 but also serine proteases such as proteinase 3 (PR3), neutrophil elastase and Cathepsin G cleave the proIL-1b in neutrophils. However, the roles of serine proteases, especially PR3, on IL-1b cleavage remain poorly understood. For example, there is no clear evidence suggesting mouse proIL-1b is cleaved by mouse PR3 even though previous studies have shown that human proIL-1b is cleaved by human PR3. Also, the exact PR3 cleavage site of IL-1b is unknown. In this study, we will analyze the role of PR3 on IL-1b cleavage both in human and mouse.
METHODS:
Recombinant human and mouse PR3 were incubated with recombinant human and mouse proIL-1b in vitro. The exact cleavage site of IL-1b was determined by ESI-TOF mass spectrometry. The PR3 cleaved mature IL-1b was expressed in E. coli and purified by Ni-NTA affinity column followed by SUMO protease cleavage. HeLa cells were incubated with the purified recombinant IL-1b and its biological activity was measured by quantifying the amount of IkB. The release of IL-1b was measured by ELISA using both human PR3 knockout HL-60 cells and mouse PR3 knockout neutrophils. The PR3 cleaved IL-1b specific antibody is generated.
RESULTS:
Human PR3, but not mouse PR3, generates a long form of mature IL-1b in vitro. The long mature IL-1b cleavage site is 9 amino acid upstream of Caspase 1 cleavage site. The IkB was rapidly degraded after treatment of the HeLa cells with the recombinant

long mature IL-1b, suggesting that the long mature IL-1b is biologically active. PR3 knockout human cells release significantly less IL-1b compared to wild-type cells, whereas there was no significant difference in IL-1b release between PR3 knockout and wild-type mouse neutrophils. The PR3 cleaved long mature IL-1b is specifically recognized by anti-long mature IL-1b, but not commercially available anti-mature IL-1b.
CONCLUSIONS:
PR3 cleaves proIL-1b in a species-specific manner. The long mature IL-1b may be a potential therapeutic target or biomarker for neutrophil-dominated inflammation in human.

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Aryl Hydrocarbon Receptor FICZ Protects, Intestinal Barrier by Upregulating Th17 Cell Responses in a Mouse Model of Alcohol and Burn Injury

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Aryl Hydrocarbon Receptor (AHR) is a ligand-dependent transcription factor and regulates Th17 cell differentiation and functions. IL-22 produced by Th17 cells plays a critical role in maintaining intestinal barrier function. In a previous study, we have shown that acute alcohol (ethanol) intoxication combined with burn injury suppresses T cell release of IL-17 and IL-22. This accompanied an increase in intestinal inflammatory cytokines, such as IL-6, KC, and IL-18, as well as an increase in intestinal permeability. In this study, we treated mice withan AHR agonist 6-Formylindolo (3,2-b) Carbazole (FICZ) to determine whether AHR activation modulates the Th17 cell response and protects gut barrier after ethanol and burn injury. To test this, male C57BL/6 mice were divided into four groups: sham vehicle, sham vehicle treated with FICZ, burn ethanol and burn ethanol treated with FICZ. In the ethanol and burn group, mice were gavaged with ethanol (~ 3.0 g/Kg BW) to achieve a blood ethanol level of ~100 mg/dL prior to receiving a ~12.5% total body surface area burn. In the FICZ treated group, mice were treated with FICZ (200 µg/Kg BW) by intraperitoneal injectionat time of injury. One day after injury, mice were sacrificed and spleen and small intestine were collected. Splenic T cells were isolated and cultured with plate-bound anti-CD3 and soluble anti-CD28 for 48 hours. Supernatants were collected for the measurement of IL-17 and IL-22 by ELISA, and cells were collected for determination of the AHR downstream molecule, CYP1A1, via mRNA expression by PCR. Lamina propria (LP) cells were also isolated from small intestine and stimulated with CD3 and CD28 for 16 hours to determine IL-17 and IL-22 by FACS. In addition, inflammatory cytokines were measured in small intestinal homogenates by ELISA. We observed significant decreases in IL-17 and IL-22 in both splenic T cells and LP cells as well as a significant decrease in CYP1A1 expression in splenic T cells harvested from mice receiving ethanol and burn injury compared to sham vehicle mice. Furthermore, significant increases in IL-6, KC, IL-18, and apoptosis in small intestine and a significant increase in intestinal permeability were observed in mice receiving ethanol and burn injury compared to sham vehicle mice. These data were similar to our previous findings. However, in this study we found that treatment of mice with FICZ prevented the decrease in IL-17 and IL-22, which was accompanied by a significant increase in CYP1A1 mRNA expression in T cells isolated from ethanol and burn mice. Treatment of mice with FICZ also prevented increases in IL-6, KC, IL-18, and apoptosis in the small intestine, as well as intestinal permeability following ethanol and burn injury. Together, these data suggest that the AHR agonist, FICZ, normalizes Th17 effector functions, prevents intestinal inflammation, and maintains intestine barrier after injury. (Support: R01 AA015731, R01 AA015731-08S1 and T32 AA013527)

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IL-33 Increases Cysteinyl-Leukotriene Receptor Expression in Human Peripheral Lymphocytes

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During allergic inflammation, interleukin-33 (IL-33) is released as an alarmin by damaged airway epithelial cells and fibroblasts. Following a proteolytic cleavage by inflammatory proteases, the

cytokine is recognized by its specific receptor ST2 expressed on leukocyte cells, contributing to cell activation and Th2 immune response. Cysteinyl-leukotrienes (CysLTs) are well characterized as contributing to the Th2 phenotype. CysLTs are lipid mediators derived from acid arachidonic metabolism and include leukotriene (LT) C4, D4 and E4. They have initially been described as slow-reacting substance of anaphylaxis for their strong broncho-constrictive activity, but they also target leukocyte populations and stimulate chemotaxis and cytokine production. The CysLTs act via, at least, two receptors, CysLT1 and CysLT2, which are expressed on the majority of leukocytes in different proportions.

In this study, we have explored the regulation of CysLT1 and CysLT2 expression by IL-33 in human peripheral lymphocytes isolated from healthy donors.

CysLT1 and CysLT2 mRNA expression levels were not affected by IL-33 treatment. However, cytometry analysis showed that IL-33 increased CysLT1 and CysLT2 protein levels, up to 120% and 60% respectively, in T and B lymphocytes, suggesting a translational or post-translational regulation of these receptors. In addition, calcium influx and chemotaxis in response to LTD4 were also increased in IL-33-stimulated T lymphocytes.

These results reveal a potential regulation of peripheral lymphocyte responses to cysteinyl-leukotrienes by IL-33, and reinforce the inflammatory capacity of this alarmin in allergic diseases.

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Alcohol Suppresses IL-22 Expression during UC Remission and Enhances Mortality to the Mouse Enteropathogen C. Rodentium

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Over 1.4 million Americans present with Inflammatory Bowel Disease (IBD), and ulcerative colitis (UC) accounts for half of those diagnoses. UC is a cyclical, life-long illness characterized by disease remission and active disease flares causing symptoms of abdominal pain, increased weight loss, intestinal inflammation, rectal bleeding, and dehydration. Alcohol consumption is known to be both pro-inflammatory and directly harmful to gut barrier function, and it was recently reported to induce flare periods in IBD patients. Our preliminary findings demonstrate increased weight loss, colon shortening, and large intestine inflammation in mice exposed to binge alcohol paradigm following DSS induced colitis. During UC remission, IL-22 expression is upregulated, acting as a hallmark of entrance into a UC remission period as it stimulates proliferation, mucous protection, and AMP secretion within the intestine. In this study, we examined the effect of alcohol on both IL-22 expression during UC remission and mortality from C. rodentium infection in DSS induced colitis.To accomplish this, male C57BL/6 were divided into two groups: DSS and sham. In DSS group, mice received 2% DSS ad libitum in their drinking water for 5 days to induce UC. Mice in sham/control group received water. On day 5, DSS and sham/control group mice were further subdivided into two subgroups: mice gavaged with alcohol (~3g/kg) or mice gavaged with water days 5, 6, and 7. This resulted in four overall experimental groups - sham vehicle, sham alcohol, DSS vehicle, and DSS alcohol. Three hours after the last gavage on day 7, mice were humanely euthanized and large intestines were harvested and processed for quantification of IL-22 and IL-17 protein levels by ELISA. A separate group of DSS vehicle and DSS alcohol mice were gavaged with 1 x105 CFUs of Citrobacter rodentium 3 hours after last gavage day 7. On day 11, mice were euthanized and large intestines were harvested and processed for quantification of IL-22, IL-6, and KC. Body weight and mortality were monitored over the 11 days. Protein levels of large intestine IL-22 were significantly decreased (6.9 fold) in DSS alcohol compared to DSS alone. In contrast, levels of the pro-inflammatory cytokine, IL-17, remained elevated in DSS alcohol mice compared to DSS vehicle. Upon gavage with C. rodentium, DSS alcohol mice experienced 25% mortality by day 9 and 50% mortality by day 11 compared to no mortality in DSS vehicle. This accompanied increases in weight loss in DSS alcohol plus C. rodentium mice compared to DSS vehicle C. rodentium treated mice. Furthermore, IL-22 levels in the large intestine trended toward a decrease in DSS alcohol receiving C. rodentium compared to DSS vehicle plus C. rodentium, while levels of IL-6 and KC were elevated. However, neither of these were

statistically significant. Along with our previous findings, these data suggest alcohol exacerbates an UC flare period by preventing upregulation of the IL-22 mediated repair response needed for entrance into remission. Additionally, alcohol further impairs DSS-colitis intestinal defense mechanisms against invading pathogens resulting in increased mortality, weight loss, and inflammation. (Support: R21 AA022324, T32AA013527)

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HIF-1α Inhibitor PX-478 Modulates Microrna Biogenesis and Inflammation in the Small Intestine following Alcohol and Burn Injury

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Alcohol intoxication at the time of burn injury is a major contributing factor in post burn pathogenesis. Numerous adverse effects after alcohol and burn injury are linked to gut barrier disruption. The gut is the largest bacterial reservoir within the body and a compromised barrier could result in bacterial translocation from the gut to extra-intestinal sites leading to systemic inflammatory response, sepsis and multiple organ failure. Earlier studies from our laboratory have shown that oxygen delivery is significantly reduced to the gut following alcohol and burn injury. Furthermore, we observed a significant increase in hypoxia inducible factor (HIF)-1α, a marker of hypoxia in small intestinal epithelial cells (IECs) after alcohol and burn injury. Hypoxia has been demonstrated to negatively affect microRNA biogenesis. MicroRNAs are major regulators of cellular homeostasis and play a critical role in gut inflammation and barrier maintenance. In a recent study, we observed a decrease in miR-150 expression in IECs following alcohol and burn injury. We further found that alcohol and burn injury reduced the expression of microRNA biogenesis components Drosha and Argonaute-2. To test whether altered microRNA biogenesis and reduced miR-150 following alcohol and burn injury are linked to increased HIF-1α, we treated a group of mice with HIF-1α inhibitor PX-478 immediately after injury. In short, male mice were gavaged with alcohol (~3 mg/kg) 4 hours prior to receiving a ~12.5% total body surface area full thickness burn injury. Mice were given 5 mg/kg of PX-478 via intraperitoneal injection immediately after burn injury. Mice were euthanized one day after the injury, small intestine was harvested and processed for isolation of epithelial cells. IECs were used for the measurement of HIF-1α, components of microRNA biogenesis (Drosha and Argonaute-2) and miR-150 expression. In addition, we measured IL-6 and IL-18 levels in the small intestine total tissue. As shown previously, alcohol and burn injury reduced Drosha (1.25 fold) and Argonaute-2 (1.5 fold) expression as well as miR-150 (2 fold) expression in IECs compared to sham vehicle. Treatment of mice with PX-478 at the time of injury normalized the expression of Drosha and miR-150 to sham values. PX-478 did not affect the expression of Argonaute-2 in IECs following alcohol and burn injury. The PX-478 restoration of miR-150 was accompanied by reduced inflammation as assessed by the measurement of IL-6 and IL-18 levels in the intestine following alcohol and burn injury. Together, these data suggest that the HIF-1α inhibitor PX-478 restores the expression of Drosha and miR-150 while reducing intestine inflammation after alcohol and burn injury. Supported by R01AA015731, R01AA015731-08S1, and T32AA013527-12.

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Macrophage Migration Inhibitory Factor is Hepatoprotective following Chronic-Binge Ethanol Feeding in Mice through Suppression of Neutrophil Accumulation.

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Background: Chronic alcohol abuse is a leading cause of preventable morbidity and mortality worldwide. Alcoholic liver disease (ALD) is characterized by a chronic progression from steatosis, inflammation, to fibrosis and cirrhosis. In addition, acute alcoholic hepatitis, characterized by massive neutrophilic infiltration, is a severe form of ALD with a high mortality rate. In order to better

understand the mechanisms underlying different stages of ALD, we make use of different animal models that model chronic progression (chronic ethanol feeding) or alcoholic hepatitis (Chronic-Binge/Gao-Binge). Macrophage Migration Inhibitory Factor (MIF) is a regulator of innate immunity with chemokine- and cytokine-like activities. MIF contributes to the progression of several diseases, including alcohol-induced liver injury in response to chronic ethanol feeding in mice. However, the role of MIF in models of alcoholic hepatitis is not known. Here, making use of MIF-/- mice, we have investigated the role of MIF in neutrophil recruitment in response to the Gao-binge model of alcoholic hepatitis Experimental Design: Wild-type C57BL6/J and MIF-/- female mice were fed a liquid diet of ethanol for 10 days (5% v/v) then acutely binged with ethanol (5 g/kg) on day 11 (Gao-binge model). Results: In wild-type mice, Chronic-Binge feeding resulted in liver damage characterized by increased ALT/AST and neutrophil accumulation. Chronic-binge ethanol exposure also resulted in a dynamic regulation of chemokines in circulation, including a robust increase in the C-X-C chemokine LIX, a potent chemotactic factor for neutrophils in mice, that preceded neutrophil appearance in the liver and injury. Interestingly, in contrast to the protection of MIF-/- mice from progression of chronic ethanol-induced liver injury, ALT/AST activities were exacerbated in MIF-/- mice in response to Chronic-Binge ethanol exposure compared to WT mice. MIF-/- mice had a more robust and rapid accumulation of neutrophils in the liver. Hepatic neutrophils from MIF-/- mice were also more activated, as measured by Ly6G+CD11b+ cells in FACS analysis. Peripheral chemokines in the plasma were largely blunted in MIF-/- mice, with the exception of LIX, which was present at higher concentrations in MIF-/- compared to WT mice. Interestingly, hepatic expression of LIX mRNA was actually suppressed following Chronic-Binge ethanol feeding in both WT and MIF-/- mice, suggesting an extrahepatic source of ethanol-induced LIX. Adipose tissue is an important source of cytokines and chemokines in response to ethanol. Indeed, expression of cytokines TNF and IL-1β, as well as C-X-C chemokines MIP2 and LIX mRNA was increased in adipose tissue following Chronic-Binge ethanol feeding in WT mice; this response was further exacerbated in MIF-/- mice. Conclusions: Chronic-Binge ethanol feeding induced marked chemokine synthesis in adipose tissue, contributing to neutrophil mobilization and recruitment into the liver in WT mice. MIF-/- mice showed greater chemokine expression and neutrophil recruitment, suggesting that MIF serves as a brake to neutrophil mobilization. These data suggest that, in addition to MIF's well-established chemokine activity, MIF also plays a robust upstream role in the regulation of chemokine expression in response to an acutely severe episode of alcoholic hepatitis in mice. Supported by R01-AA011876 and U01-AA020821 (L.E.N.) and F32-AA024955 (K.L.P.)

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Butyrate Protects Intestinal Immune Response during Acute on Chronic Ethanol Exposure

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Background: Excessive alcohol consumption is pathogenic contributing to increased morbidity and mortality. Following a physiologic insult, an immune response needs to be adequately mounted and resolved for proper organ function homeostasis. The immune response to EtOH exposure differs based on multiple factors including the duration of ethanol exposure. While chronic EtOH exposure is proinflammatory, acute EtOH can actually dampen immune responses. Butyrate, a fermentation byproduct of the gut microbiota, influences gut inflammation and immunity. Our prior work reveals butyrate protects against EtOH-induced intestinal and hepatic injury. This study aimed to determine butyrate's impact on innate immune responses in the intestine following acute binge-on-chronic EtOH exposure.

Methods: Female C57BL/6J age-matched mice were adapted to a control liquid diet for 5 days, then randomly assigned to a 5% v/v (27% total kcal) ethanol-containing diet or an isocalorically-substituted maltose dextrin containing diet for EtOH for 10 days. Ethanol-fed groups were allowed free access to the diet and control mice were pair-fed. On day 11, mice were orally gavaged a 5 g/kg dose of EtOH or isocaloric maltose. Mice were euthanized 9 hours post-binge. Mice were provided equimolar glycerol or tributyrin (5mM) as part of the liquid diet over the 10 days of EtOH exposure as well as added

into the EtOH or maltose gavage at a dose of 2.5 mM. Tributyrin is a structured lipid consisting of a glycerol backbone and 3 esterified butyrate molecules which are released following digestion by gastric and pancreatic amylase and lipase. Intestinal expression of markers of inflammation was evaluated. Additionally an in vitro co-culture model was developed using a transwell system. Caco2 (intestinal epithelial) and RAW 264.7 (macrophage) cells were seeded in the apical and basolateral chamber, respectively. Differentiated Caco2 cells were pretreated with sodium butyrate (5 mM) for 24 hours followed by EtOH (40 mM) for 3 hrs. Cell culture supernatant and cells were collected and processed for protein and mRNA expression of cytokines, a G-protein coupled receptor for butyrate and chemokine's via ELISA and real time PCR. All animal procedures were approved by Cleveland Clinic Institutional Animal Care and Use Committee.

Results: As predicted, in response to acute on chronic EtOH exposure, genes for immune mediators exhibited decreased expression in the proximal colon including IL12p40, IFNγ, IL1β, Ly6C, CD68 and NOD2. However, mice co-treated with tributyrin during EtOH exposure had comparable mRNA expression of these immune parameters as pair-fed control mice. Similarly, in response to EtOH exposure, supernatant from the apical co-culture system exhibited decreased protein expression of the chemokine IL-8 compared to control, butyrate and EtOH-butyrate treated cells. Interestingly, mRNA expression of a butyrate receptor (GPR109A) was depleted with EtOH treatment, in both in vivo and in vitro experimental models, and this depletion was mitigated with butyrate co-treatment

Conclusion: These findings show butyrate protects against the blunting of the immune response caused by acute binge ethanol exposure in the intestine and suggests that this response may be linked with a G-protein coupled receptor, GPR109A, for which butyrate is the main ligand.

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Regulation of Inflammation and Thermogenesis in Adipose Tissues by Pro-Inflammatory Cytokines

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During the past decade, obesity has become a global health problem and is associated with chronic diseases such as diabetes, hypertension and cardiovascular diseases, referred to as metabolic syndrome. Obesity and metabolic syndrome are associated with chronic inflammation. In this study we characterized the expression of chemokines and cytokines in subcutaneous and visceral adipose tissues obtained from obese patients undergoing bariatric surgery. Obese patients were classified into non-diabetic, pre-diabetic and diabetic groups. Lysates prepared from frozen tissues were analyzed for 42 analytes using commercial services. We observed that certain chemokines are expressed at higher levels in visceral adipose tissues when compared to subcutaneous adipose tissues. Furthermore we did detect significant expression of genes and cytokine signatures associated with T lymphocytes in the visceral adipose tissues from these patients. Our results show that the pattern of expression of chemokines and cytokines differ between the visceral and subcutaneous adipose tissues.

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Peroxisome Proliferator-Activated Receptor-Gamma (PPARγ) Plays a Critical Role in Pulmonary Immunity by Altering Macrophage Cell Surface Receptor Expression in a Mouse Model of *Pseudomonas aeruginosa* Infection

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Alveolar macrophages (AMs) play a pivotal role in regulating the pulmonary response to infection and maintaining tissue homeostasis. They initiate inflammatory responses, through pathogen phagocytosis and pro-inflammatory cytokine production, and then switch phenotype for inflammation resolution, through neutrophil efferocytosis and anti-inflammatory cytokine

release. PPARγ is a ligand-activated nuclear receptor involved in promoting resolution. Previous work in our laboratory showed that in comparison to wildtype mice, intratracheal *P. aeruginosa* infection caused impaired respiratory function and dramatically elevated levels of lung pro-inflammatory cytokines in macrophage-specific PPARγ knockout mice (Mac PPARγ KO). Histologically, lungs of Mac PPARγ KO mice also had increased cellularity, interstitial thickening, and alveolar collapse following intratracheal *P. aeruginosa* infection. Since AMs play a critical role in regulating lung inflammation following infection, we chose to examine AMs in this model. Wildtype (C57BL/6) or Mac PPARγ KO mice were given *P. aeruginosa* or PBS control (intratracheally, 40,000 CFU). 24 hours later, bronchoalveolar lavage cells were analyzed by flow cytometry for absolute numbers of AMs and cell surface receptor expression, as determined by mean fluorescence intensity (MFI). Markers for efferocytosis (CD11b and MARCO), antigen presentation (MHC Class II and Toll-like receptor-4 (TLR4)/MD-2), and macrophage phenotype (CD206) were assessed. When compared to vehicle-treated controls, CD11c+ CD11b-SiglecF+ AM were decreased by 64.7% following *P. aeruginosa* infection in wildtype mice and 96.9% in Mac PPARγ KO mice ($p<0.05$). Levels of CD11b were reduced by 26.4% and 50.14% in infected wildtype and Mac PPARγ KO mice, respectively ($p<0.05$). There was also a trend towards heightened MARCO expression, as levels were increased by 14.3% and 28.3% in infected wildtype and Mac PPARγ KO mice, respectively. No differences in TLR4/MD-2 were observed between groups. When comparing vehicle treated animals, Mac PPARγ KO mice had 38.8% higher expression of MHC Class II ($p<0.05$) than wildtype, which was not changed in the presence of infection. Lastly, we measured levels of the anti-inflammatory marker (M2), CD206. CD206 was reduced by 82% in AM from wildtype mice ($p<0.05$) and by 97.9 % in Mac PPARγ KO ($p<0.05$). These findings suggest that AM deficient in PPARγ upregulate cell surface markers important for efferocytosis and antigen presentation more robustly than wildtype AM do in response to *P. aeruginosa* infection and that the switch to a M2, anti-inflammatory phenotype may be impaired in Mac PPARγ KO AMs. Supported by R01AA012034 (EJK) and R01AG018859 (EJK).

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Microfluidics-Based Side View Flow Chamber Reveals Tether-To-Sling Transition in Rolling Neutrophils

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Neutrophils rolling at high shear stress (above 6 dyn/cm2) form tethers in the rear and slings in the front. Here, we developed a novel photo-lithographically fabricated, silicone(PDMS)-based side-view flow chamber to dynamically visualize tether and sling formation. Fluorescently membrane-labeled mouse neutrophils rolled on P-selectin substrate at 10 dyn/cm2. Most rolling cells formed 5 tethers that were 2-30 μm long. Breaking of a single tether caused a reproducible forward microjump of the cell, showing that the tether was load-bearing. About 15% of all tether-breaking events resulted in slings. The tether-to-sling transition was fast (<100 ms) with no visible material extending above the rolling cell, suggesting a very low bending modulus of the tether. The sling downstream of the rolling cell aligned according to the streamlines before landing on the flow chamber. These new observations explain how slings form from tethers and provide insight into their biomechanical properties.

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Morpho-Rheological Fingerprint of Neutrophils in fMLP Activated Blood and RSV Infections

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Background: Neutrophil activation induces changes in a number of biophysical characteristics including cell size, shape and mechanical properties. By a microfluidic technology named RealTime Deformability Cytometry (RTDC) we have advanced mechanical cell analysis from rates of 1 cell/min up to 3000 cells/s (Otto et al., Nature Methods, 2015). To enable label-free RTDC

identification of neutrophils in whole blood, we developed a cell distinction algorithm to allow novel morpho-rheological blood cell characterization. This morpho-rheological fingerprint of blood neutrophils was assessed in RSV infection and healthy volunteer neutrophils activated in-vitro in whole blood by the bacterial tri-peptide fMLP.
Method: Morpho-rheological neutrophil typing was assessed in five patients with RSV+ lower respiratory tract infections. All patients were recruited on the day of hospitalization with a core temperature > 38.5°C and the need for supplemental oxygen. The blood of 20 healthy donors was used as control. Blood samples were analyzed by RTDC within 30 minutes of venipuncture. For in-vitro fMLP activation, blood samples of five healthy donors were stimulated with 100nM fMLP. Samples were incubated at 37 °C, 450 rpm, analyzed at RT after 15, 30, 45 and 60 min and compared to unstimulated controls. In brief, RTDC blood analysis involved 50 µl blood diluted in 950 µl PBS methylcellulose at 15mPas and was carried out in 20 µm PDMD channels at a flow rate of 0.12 µl/s. Morpho-rheological phenotyping was processed via a series of in-house python scripts. Area and deformability were calculated for neutrophils with an area ratio of 1.0 to 1.05 (AR; reflecting concavity of shape).
Results: The cross sectional area of blood neutrophils of patients with RSV infections was increased in comparison to controls (p=0.0014). Additionally, blood neutrophils from RSV patients were more deformable in comparison to controls (p=0.0374). A change of AR was only detected in one patient with RSV infection, whereas the others did not differ from the control cohort (p=0.3624). fMLP activation of blood neutrophils caused an initial size reduction and the expected stiffening of cells (the ‘priming’ response). However, this was followed by an increase in cell size and an unexpected increase in deformability 30 min post stimulation (corresponding to the ‘de-priming’ phase); AR peaked at 15 min with consecutive decreases thereafter (all $p < 0.01$).
Discussion: Neutrophil activation can be detected label-free in whole blood of patients with RSV infection. The morpho-rheological fingerprint provides a novel view on mechanical neutrophil changes under ‘near to’ physiological conditions. Correlation to the in-vitro kinetics of fMLP activated purified neutrophis revealed that AR provides a sensitive way to detect even minimal shape change (priming). Additionally, AR realized the label-free distinction of neutrophil subsets during activation. Priming starts rapidly following fMLP stimulation, in which even neutrophils of AR < 1.05 decreased in size and were less deformable, and shape changed neutrophils (AR > 1.05) were detected. This was followed by a wave of de-priming characterized by increased neutrophil size and deformability as seen in clinical RSV infection. The mechanism and physiological meaning of this latter response is yet to be established.

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Long-Term Intensive Endurance Exercise Leads to an Innate Immune Response Characterised by Mobilisation of CD16+CD62L- Neutrophils

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Regular physical exercise is correlated with fewer upper respiratory tract infections (URTI), when compared to a sedentary lifestyle. In contrast, intensive endurance training is associated with an above average risk to URTI. Different studies have shown that peripheral blood neutrophil counts increase upon exercise and recover within 6-24 hours. This applies both for anaerobic as well as for aerobic exercise bouts. We have previously described the mobilisation of 3 neutrophil subsets in response to acute inflammation such as evoked by systemic LPS challenge in healthy volunteers. These cells can be identified by differential expression of FcgammaRIII (CD16) and of L-selectin (CD62L) (Pillay et al, 2012). The aim of this study was to test the hypothesis that these 3 subsets also arise upon long term endurance exercise.
To test this hypothesis we studied the leukocyte kinetics in the peripheral blood of thirty (11 female, 19 male) contestants of an 8-day intensive cycling tour (on average 160 km and 2500 altimeters per day). Next to this, we measured the expression of CD16, CD62L and an additional 8 markers on their peripheral blood neutrophils by flow cytometry. Blood was sampled on three mornings and three afternoons
During the second part of the tour overall neutrophil

numbers did not normalise overnight and an enhanced neutrophil count was found in the morning of day 8 (1.32 fold-increase; 95%CI 1.04-1.58 $p<0.05$). When analysing the 3 neutrophil subsets separately we found that "mature" CD16+CD62L+ neutrophils normalised to baseline overnight, whereas CD16+CD62L- neutrophils were significantly increased at the mornings of day 5 (1.66 fold-increase; 95% CI 1.10-2.22) to day 8 (2.65 fold-increase; 95% CI 1.9-3.4 $p<0.0001$) when compared to the morning of day 1. Also a significant increase in CD16-CD62L+ neutrophils was found in the morning of day 8 (2.78 fold-increase; 95% CI 1.33-4.23 $p<0.05$). Using FLow cytometric Orthogonal Orientation for Diagnosis (FLOOD), a computational analysis method for multidimensional flow cytometry data (Jansen, Hilvering et al 2016), we analysed our data in a response specific space. We identified a new CD62L- population within the total CD16+CD62L- population which has differential marker expression when compared to the classical CD16+CD62L- cells.
In conclusion, long-term intensive endurance exercise induces a systemic innate immune response characterised by a change in neutrophil phenotypes. This response does not recover to baseline overnight after 5-7 days of exercise. Multidimensional FLOOD analysis of peripheral blood neutrophils allows the analysis of multiple markers and the definition of a new neutrophil phenotype mobilised during extreme exercise.

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Neutrophil CARD9 Mediates Autoantibody-induced Autoimmune Diseases by Controlling Gene Expression Changes *in Vivo*

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Background: CARD9 is a CARMA-like intracellular protein that is highly expressed in myeloid cells. We previously presented that CARD9 plays an important role in the development of experimental arthritis and autoimmune blistering skin disease in mice, likely by linking the Syk tyrosine kinase to chemokine production in innate immune cells.Here, we investigated whether CARD9 expression in neutrophils is required for the *in vivo* pathogenesis.
Methods: Neutrophil-specific CARD9-deficiency was achieved by crossing MRP8 promoter-driven Cre recombinase transgenic (MRP8-Cre) animals with *Card9*flox/flox mice (MRP8-Cre *Card9*flox/flox animals). The efficacy and specificity of lineage-specific deletion was analyzed by Western-blot in neutrophil and macrophage cell lysates. Experimental arthritis was induced by a single intraperitoneal injection of K/BxN serum and was assessed by clinical scoring, ankle thickness measurements and by testing articular function. Epidermolysis bullosa acquisita was triggered by anti-collagen type VII antibodies. Global neutrophil gene expression changes were detected by Affymetrix Microarrays. CXCL2 levels in the synovium were measured by ELISA.
Results: In contrast to wild type animals, neutrophils of the MRP8-Cre *Card9*flox/flox mice did not express CARD9, while their macrophages had normal levels of the protein. Neutrophil-specific CARD9-deficiency caused a partial, but significant decrease in the clinical arthritis score, the ankle thickening and the articular dysfunction, which did not differ significantly from that seen in *Card9*–/– animals. A similar phenomenon was observed in connection with the autoimmune blistering skin disease as MRP8-Cre *Card9*flox/flox animals showed a substantial but incomplete reduction in inflammation, which was indistinguishable from the*Card9*–/– phenotype. Furthermore, CARD9-deficiency resulted in a 78 % reduction in immune complex-induced global gene expression changes in neutrophils *in vitro*. In line with this, synovial CXCL2 levels were significantly lower in mice with neutrophil-specific CARD9-deficiency compared to wild type animals.
Conclusions: CARD9 in neutrophils plays an important role in the development and progression of autoimmune arthritis and autoimmune blistering skin disease in mice, likely by controlling gene expression changes and chemokine release. These

are the first data in the literature showing a direct role for neutrophil gene expression changes *in vivo*. *This project was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.*

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Neutrophil Proteomics Reveals Pathways Involved in Preconditioning Prevention of Ischemia/Reperfusion Injury

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Intestinal ischemia and reperfusion injury often results into lung injury and multiple organ failure also observed after trauma and surgery. Ischemic preconditioning preceding ischemia and reperfusion was shown to attenuate this injury and has a potential therapeutic application; however the exact underlying mechanism is not clear. Neutrophils are key participants in the mechanism of ischemia and reperfusion injury while the ischemic preconditioning led to a decrease in neutrophil stimulation and activation. Proteomic analysis is widely used as an appropriate tool for studying complex systems. In order to evaluate the effect of IPC preceding a longer ischemia and reperfusion on the proteome of neutrophils we used Wistar rats divided in four experimental groups: Control, sham laparotomy, intestinal ischemia/reperfusion and ischemic preconditioning. Ischemia was performed by clamping the superior mesenteric artery for 45 min, reperfusion was allowed for 120 min. The preconditioning was obtained by a short (10 min) ischemia/reperfusion event preceding the longer ischemic event. At the end of the surgical procedure, central venous blood was collected. After neutrophil separation, proteins were extracted, trypsin digested and the resulting peptides were iTRAQ labelled followed by HILIC fractionation and nLC-MS/MS analysis. Database searches, normalization and statistical analysis of our proteomic analysis resulted in the identification of 2437 protein groups that were assigned to five different clusters (Fig.1) based on the relative abundance profiles among the experimental groups. Statistical analysis of the proteins within each cluster led to the identification of significantly up and downregulated proteins in IR and IPC. Cluster based analysis revealed that after intestinal ischemic preconditioning some KEGG pathways are regulated. The most relevant upregulated pathways were actin cytoskeleton, metabolism, phagocytosis, chemokine signaling, focal adhesion and leukocyte transendothelial migration whereas downregulation was detected in ribosome, spliceosome, RNA transport, protein processing in endoplasmic reticulum and proteasome. Furthermore, enzyme prediction analysis revealed the regulation of some important antioxidant enzymes and having their role in reactive oxygen species production. To our knowledge, this work describes the most comprehensive and detailed quantitative proteomic study of the neutrophil showing the beneficial role of ischemic preconditioning and its effects on the neutrophil proteome. This data will be helpful to understand the effect of underlying protective mechanisms modulating the role of PMNs after IPC and provide a trustworthy basis for future studies.

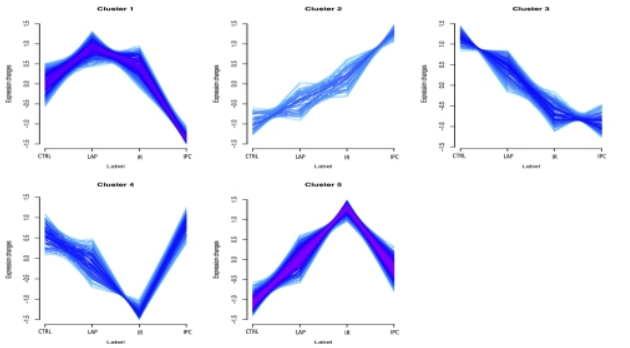

Figure 1: Expression profile of regulated proteins found in the four experimental conditions (quiescent control, laparotomy, ischemia / reperfusion and preconditioning). All identified proteins were grouped in 5 clusters.

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Direct One-Step Isolation of Untouched Human Neutrophils from Whole Blood without Lysis

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Neutrophils are the first line of defense against microbial infections, sensing pathogen-associated molecular patterns through a wide range of receptors. Once activated, neutrophils eliminate infection via phagocytosis of pathogens, releasing pro-inflammatory cytokines and antimicrobial peptides, and producing reactive oxygen species (ROS). Current procedures to enrich neutrophils from human whole blood involve at least two steps; one to remove red blood cells (RBC) and one to select neutrophils or to remove unwanted cells. For example, RBCs may be first depleted using ammonium chloride lysis or sedimentation, followed by fluorescence-activated cell sorting or magnetic cell separation. Alternatively, neutrophils may be separated from other leukocytes using density gradient centrifugation. However, in many cases mononuclear cells (MNC), eosinophils, and basophils may still contaminate the pelleted cells, and they are still mixed with RBCs which then need to be lysed. Finally, neutrophils may be lost or their viability compromised at every stage of a multi-step procedure, thus isolation in a single step from the starting sample is preferable.
We have developed a one-step immunomagnetic, column-free negative selection method to enrich neutrophils from whole blood which does NOT require RBC lysis, sedimentation or density gradient centrifugation. RBC, MNC, platelets, eosinophils and basophils were targeted for removal with antibody complexes which crosslinked them to magnetic particles. The sample was then placed in an EasySep™ magnet, and the labeled, unwanted cells were retained in the magnet while the neutrophils were simply poured off. The sample was diluted with buffer and the magnetic incubation and pour-off steps repeated once more, for a total protocol time of 25 minutes.
The purity of neutrophils isolated directly from whole blood was 97.3 ± 1.4%, with an average recovery of 0.6 – 2.9 x 10^6neutrophils / mL of blood (means ± SD, n=14). The isolated neutrophils were functional as shown by ROS production upon activation with PMA. This rapid method to isolate untouched neutrophils will facilitate studies of neutrophil function in inflammatory disease.

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Multiple Signals Coordinate Human-Neutrophil Migration during Swarming in a Novel Ex vivo Assays

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Neutrophils display a highly coordinated behavior against large microbial targets, a process known as swarming. Swarming is important for containing fungal infection but also key to the pathology of various inflammatory diseases, including rheumatoid arthritis or gout. Better understanding the molecular players that control the swarming of human neutrophils may help develop new treatments against infections and inflammatory diseases. However, despite some recent advances from the in vivo studies of swarming in mice, progress has been slow. Animal experiments are low throughput, have poor control over local microenvironment, and their relevance to human immune responses is untested. Here, we circumvent the limitations of current techniques by designing a novel ex vivo assay that enables us to control the size of swarming targets and space between arrays of targets (see figure). With this assay, we monitored the recruitment of neutrophils towards targets at highest resolution observation to date, we synchronized the starting time for thousands of swarms at once and analyzed the proteins and lipids in the supernatant, and verified the role of some of these mediators by targeted inhibition of receptors or neutralization of mediator. We uncovered a highly redundant network of chemotactic cytokines that coordinate neutrophil swarming, consisting of more than two dozen cytokines and lipid inflammation mediators that are differentially secreted by neutrophils between swarming vs. simple phagocytosis, and measured their temporal dynamics. Interestingly, some of the cytokines stimulate receptors present exclusively on other immune cells besides the neutrophils and also

non-immune cells, suggesting that the human neutrophils participating in swarms are at the center of a constellation of coordinated cellular responses of the innate and adaptive immune systems, blood vessels, adipose tissue, tissue fibroblasts etc. Overall, new knowledge regarding the nature and temporal dynamics of the cytokines coordinating swarming will advance our understanding of the physiologic and pathological processes associated with inflammation, during health and disease.

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Mapping of Glycoepitopes in Neutrophil Granules

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The time of protein synthesis during neutrophil differentiation is the main identified mechanism driving the targeting of proteins to granules. Unlike for the delivery of proteins to the endocytic pathway, no identified sugar residue participate in the sorting of proteins to neutrophils granules. Thus, granule subsets are characterized not only by a distinct protein content. Indeed, pauci-mannose residues have been identified by mass spectrometry on azurophilic proteins. Galectins bind to poly-N-acetyllactosaminyl backbones attached to proteins mobilized from specific granules. We also showed, by immunofluorescence, that the selectin ligands, CD15 and CD15S, localize to different intracellular compartments. Therefore, proteins carrying distinct sugars are targeted to distinct granules. These observations lead us to hypothesize that sugar residues might define other granule subsets than those identified by proteins and that they might also play a role in the genesis and structure of granules. Therefore, we started mapping glyocoepitopes on neutrophil granules using a combination of immunofluorescence, subcellular fractionation and glycomics. In an initial screen, we selected six lectins for their specific binding to myeloid cells and their ability to stain intracellular vesicles in neutrophils. The binding of two lectins to granules was strongly reduced in neutrophils lacking specific granules, due to a specific mutation in neutrophil differentiation. Interestingly, one of the two lectins co-localized partially with myeloperoxidase whereas none of the two lectins co-localized with CD15 or lactoferrin. These data indicate that two other types of sugar residue, distinct from CD15, characterize specific granules. One sugar type is present in specific granules and myeloperoxidase-positive azurophilic granules, thereby defining an alternative category of granules and supporting our initial hypothesis.

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Assessing the Production of Reactive Oxygen and Nitrogen Species by Human Neutrophils

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Neutrophils are the first immune cells to arrive at the site of inflammation, especially as a result of bacterial infections, where they recognize and phagocyte the microorganisms. One of the most important defense systems of neutrophils against the invaders corresponds to their ability to trigger a strong oxidative burst through the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). While oxidative burst is important for the elimination of invading microorganisms, the overproduction of reactive species or the impairment of endogenous antioxidant defenses may result in detrimental effects to the host. The nature and the extent of reactive species production by neutrophils in response to different stimuli is, consequently, a matter of extensive research, with scientific reports showing an enormous variability on the detection methodologies employed. This presentation attempts to provide a critical assessment of the most effective approaches to detect the reactive species formed during the neutrophils' oxidative burst. In that sense, some practical examples will be given arising from our experience in the modulation of human neutrophils' oxidative burst by bioactive compounds, as flavonoids. The detection mechanisms and performance, as well as advantages and limitations of the different methodologies, will be presented, focusing on the use of luminescent probes.

Acknowledgements

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Diversity of Neutrophil Subpopulations in Cancer

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Neutrophils, the predominant circulating leukocyte population, play a well-established role in host defense and are usually associated with inflammation and with counteracting infection. Neutrophils are largely viewed as a homogeneous population of terminally differentiated white blood cells lacking significant plasticity. Interest in these cells has increased during the last decade, because of the accumulating data that suggest important and significant roles for neutrophils in tumor biology. Controversy, however, surrounds their role as neutrophils were shown to provide both pro- and anti-tumor functions.

We hypothesized that the controversy regarding neutrophil function in cancer stem from the existence of distinct neutrophil subsets which may posses different and even conflicting functional properties. Along these lines, the ratio between these subpopulations and the potency of their activity should determine the overall contribution of neutrophils. We now show that two circulating neutrophils subpopulations may be detected in cancer based on their different densities. Using mouse models of cancer, and samples from breast and lung cancer, we identified a heterogeneous subset of low-density neutrophils (LDN) that appear and continuously accumulate with cancer progression. These LDN are phenotypically different from the normal high-density neutrophils (HDN) that are the predominant population in health. Morphological analyses show that, whereas HDN appear to be a homogenous population of mature, segmented neutrophils, LDN are heterogeneous and contain segmented as well as morphologically immature (banded and ring-shaped) neutrophils. Furthermore, LDN had higher forward scatter (FSC) than HDN with similar side scatter (SSC), suggesting that they are larger than HDN while maintaining similar granularity. LDN display a reduced inflammatory profile, impaired neutrophil function and immunosuppressive properties, characteristics that are in stark contrast to those of mature HDN. Importantly, we show that the mature subset of LDN is derived from HDN, in a TGFß dependent mechanism.

Our findings challenge the concept that mature neutrophils are a homogenous group of cells. HDN possess anti-tumor properties, whereas LDN have features associated with pro-tumor activity. During early tumor development, HDN are the predominant neutrophil subpopulation leading to an overall antitumor response. With tumor progression, LDN become dominant resulting in an overall neutrophil phenotype switch toward tumor-promoting functions. The functional differences between HDN and LDN together with the continuous increase in the proportion of LDN with tumor progression provide a solid explanation for the controversy that has surrounded neutrophil function in cancer.

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Neutrophils and Lymphocytes Express Vomeronasal Type-1 Receptor (VN1R) Genes

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Blood leukocytes express a variety of G protein-coupled receptors (GPCR) to distinguish non-self from self, and to orchestrate immune responses in adaptive and innate immunity alike. For example, some 40 GPCR are highly expressed in T-cells, B-cells, and polymorphonuclear granulocytes (neutrophils) [1, 2]. Recently, and in addition to the canonical immunologically relevant GPCR, we demonstrated the expression of some 80 chemosensory odorant, taste, and trace amine-associated GPCR in five different types of blood leukocytes [3, 4]. Here we show the expression in

neutrophils, T- and B-cells of yet another family of chemonsensory GPCR, comprising the four vomeronasal type-1 receptors (VN1R) 1, 2, 4, and 5. VN1R are receptors that are canonically expressed in a specialized chemosensory epithelium, the vomeronasal organ (VNO), enabling the detection of species-specific chemical cues pertaining to social interaction – humans, however, supposedly have no functional VNO. An ectopic expression of VN1R in blood leukocytes opens up new venues for research on the functional relevance of VN1R expression for our cellular immune system.

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Food Supplements Regulate the Expression of Chemosensory Receptor Genes in Neutrophils

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Neutrophils express a variety of G protein-coupled receptors (GPCR) to identify tissue damage, bacterial infection, and to orchestrate innate immune responses. For example, about 10 canonical, non-chemosensory GPCR are known to highly express in neutrophils [1]. There is, however, increasing evidence of an ectopic expression of both sweet and bitter taste receptors in a variety of non-chemosensory tissues and cells, including leukocytes. Recently, we demonstrated saccharin to be capable of activating chemotaxis in neutrophils via both sweet and bitter taste GPCR, which are functionally expressed in these and other leukocytes [2]. Here, we investigated the role of diet lemonade consumption-typical, postprandial saccharin, acesulfam-K and cyclamate concentrations on the regulation of sweet and bitter taste chemosensory receptor genes and genes of innate immunity in isolated PMN, in vitro and in a human intervention study. In summary, we suggest that artificial sweeteners beyond their role as dietary hedonic supplements are bioactives on our cellular immune system.

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Plasma Membrane Profiling of the Neutrophil by Amino-Oxybiotinylation and Tandem Mass Tag Mass Spectrometry

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Background

Following stimulation, a multitude of processes are instigated which require the neutrophil to modulate the expression of cell surface molecules. Comprehensive tracking of these changes by mass spectrometry against a background plasma membrane proteome has been limited by conventional cell and membrane purification techniques, which result in cell priming and organelle membrane contamination. In addition, the short neutrophil half-life ex-vivo precludes the use of stable isotype labelling by amino acids in cell culture (SILAC).

The use of amino-oxybiotin to label plasma membrane proteins prior to MS has been demonstrated in fibroblasts, resulting in high numbers of protein identifications with good membrane purity (Weekes 2010). Furthermore, it is now possible to multiplex this procedure, combining multiple culture conditions for comparison on a single MS run using Tandem Mass Tags (TMT) (Matheson 2015). This abstract describes the first

use of this technique in human neutrophils.

Methods

Pilot plasma membrane profiling was carried out on neutrophils isolated from 3 healthy volunteers. Plasma-Percoll gradient separated neutrophils were incubated with amino-oxybiotin, enriched with streptavidin, TMT labelled and subjected to high performance liquid chromatography MS. The resulting plasma membrane proteins were assigned corresponding gene ontology terms to determine subcellular localisation, and input cell numbers were increased to improve the number of proteins identified.

To validate the technique, neutrophils were incubated with the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) for up to 4 hours prior to biotinylation, and expression of the adhesion molecules CD11b and CD62L was compared to untreated neutrophils using both TMT MS and flow cytometry.

Results

Plasma membrane profiling of 2x10^7 neutrophils by amino-oxybiotinylation followed by MS resulted in identification of 263 unique proteins, with high enrichment for membrane proteins (92%) as judged by gene ontology annotation. Increasing the cell number to 1x10^8 neutrophils led to an increase in identified proteins to 819, of which 77% were membrane-associated. Biotinylation efficiency was high, with a 3-log increase in streptavidin binding in biotinyated neutrophils compared to unbiotinylated, and the protein set was comparable across replicates from 3 donors, with the most variable proteins being predominantly HLA molecules.

Plasma membrane profiling of GM-CSF-treated neutrophils over a 4 hour time course showed similar temporal changes across donors, and TMT MS temporal expression profiles of CD11b and CD62L correlated with that demonstrated by flow cytometry (r2=0.978 for CD11b, r2=0.594 for CD62L).

Conclusions

Here we describe an unbiased quantitative proteomic approach to characterizing the neutrophil plasma membrane by amino-oxybiotinylation and MS. The additional use of Tandem Mass Tags allowed comparison of multiple culture conditions on the same MS run, and we validated our findings by tracking cell surface molecule changes known to occur following stimulation with GM-CSF. This new proteomic approach has been described in other cell types but here is successfully adapted for the first time in a primary cell. It has the potential to allow investigation of how the neutrophil alters surface signalling in response to a wide range of stimuli and pathogens, using a candidate-free hypothesis, and ultimately to identify novel drug targets.

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A Genome-Scale Screen for New Players in Neutrophil Biology

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Genetic modification of neutrophils is frustrated by their short lifespan and sensitivity to manipulation. We circumvented these limitations by deriving conditionally immortalized murine myeloid progenitors that are receptive to gene knockout via CRISPR/Cas9. We expressed Cas9 endonuclease and the GeCKOv2 small guide RNA library in myeloid progenitors to achieve knockout of a single gene in each cell. The GeCKOv2 library targets more than 20,000 different genes. We performed proof-of-concept experiments using single cell PCR amplification and sequencing of the sgRNA barcode. The library-expressing myeloid progenitors were then differentiated into neutrophils to establish a range of functional assays. The tools developed in this study will allow us to identify novel genes that regulate neutrophil activation, recruitment and effector function.

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NADPH Oxidase Activation Regulates Apoptotic Cell Clearance and Antigenicity

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The phagocyte NADPH oxidase generates superoxide in response to multiple agonists. Superoxide and derivative reactive oxygen species (ROS) are important in microbial killing and many immunological processes including antigen

presentation, LC3-associated phagocytosis (LAP) and modulation of inflammatory responses. Chronic granulomatous disease (CGD) patients with inactivating NADPH oxidase subunit mutations are susceptible to recurrent microbial infections and chronic inflammatory conditions. Moreover, variant NADPH oxidase subunit alleles causing only partial loss of enzyme activity are also associated with increased susceptibility to autoimmune diseases. However specific molecular mechanisms by which NADPH oxidase and ROS prevent autoimmunity are poorly defined. Here, we demonstrate a mechanistic role of the NADPH oxidase in regulating apoptotic cell clearance by macrophages, dendritic cells, and prevention of autoimmune responses *in vivo*. Efficient clearance of apoptotic cells is essential to prevent secondary necrosis, inflammation and aberrant immune responses to apoptotic cell antigens. Our studies show that ingestion of apoptotic cells *in vitro* by wild type mouse macrophages and dendritic cells activated NADPH oxidase in a CD11b-TLR4-MyD88-dependent manner. Furthermore, apoptotic cell-derived ligands (MSU crystals, ATP) were strong activators of the oxidative burst, while complement deposition (iC3b) on apoptotic cells further augmented superoxide generation. After uptake, NADPH oxidase activity directly regulated the rate of proteolysis, and subsequent cross-presentation of apoptotic cell-derived peptides. Macrophages from CGD mice lacking NADPH oxidase activity due to inactivation of *Cybb* (encodes gp91^{phox}- the main catalytic subunit of NADPH oxidase), were delayed in digestion of ingested apoptotic cargo *in vitro* and strongly activated CD8 T cell responses that correlated with enhanced cross-presentation of model antigen - ovalbumin *in vitro* and *in vivo*. Long-term infusion of apoptotic cells in CGD mice resulted in anti-nuclear antibody generation and glomerulonephritis. Thus our studies identify receptors, ligands and molecular pathways leading to activation of NADPH oxidase following apoptotic cell ingestion. Furthermore, this study for the first time explains the direct role that NADPH oxidase-derived plays in the digestion of apoptotic cells, and is most likely to be the underlying mechanism leading to hyperinflammation and autoimmune complications observed in the of context NADPH oxidase deficiency.

Activated Protein C (APC) Binds Leukocytes and Signals to Inhibit Neutrophil Extracellular Trap Formation and Apoptosis; Possible Additional Mechanism for Anti-Inflammatory and Cytoprotective Effects of APC

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Background: At sites of microbial infections, activated neutrophils can expel their nuclear content to form microbicidal protein-exposing neutrophil extracellular traps (NETs). This process of NETosis provides a structural framework for pathogen clearance. The exact mechanism of NETosis, including its regulation, if any, is unclear. Activated protein C (APC) is a natural antithrombotic, anti-inflammatory and cytoprotective blood enzyme that inhibits the migration of neutrophils. We hypothesized that the anti-inflammatory function of APC is due in part to the inhibition of NETosis and cell death.

Aim: To determine the effect of APC on phorbol myristate acetate (PMA)-induced NETs formation and tumor necrosis factor-α (TNFα)-induced apoptosis.

Methods: Human neutrophils were purified from peripheral blood and allowed to adhere to a fibronectin-coated surface. Neutrophils were then pretreated with increasing concentrations (30-300 nM) of human APC prior to stimulation with PMA to induce NETosis. NETosis was quantified using the DNA-binding dye, Hoescht 33342, as well as neutrophil elastase and citrullinated histone. The area of DNA was quantified via fluorescence microscopy and MATLAB-based image analysis. For apoptosis studies, neutrophils were treated with increasing concentrations of APC prior to stimulation with TNFα in the presence or absence of cycloheximide (CHX) to induce apoptosis. Cells were stained with the apoptosis molecule Yo-Pro1 and Hoescht 33342 and quantified via fluorescence

microscopy as the percent of Yo-Pro1 cells over total cells.

Results: APC prevented NETosis induced by the protein kinase C activator, PMA. The ability of APC to prevent NETosis was reversed after pretreatment of APC with the protease inhibitor, Phe-Pro-Arg chloromethylketone (PPACK). Function blocking antibodies to endothelial protein C receptor (EPCR), protease activated receptor-3 (PAR-3) and Mac-1 and pharmacological inhibitors of the PDK and PLC signaling pathways prevented APC from inhibiting PMA-induced NETosis. Recombinant APC with several mutations in and near the protease domain failed to prevent the ability of PMA to induce NETosis. APC reduced the ability of TNFα to induce neutrophil apoptosis in a dose-dependent manner. Function blocking antibodies to EPCR, PAR-3 and Mac-1 abrogated the anti-apoptotic effect of APC.

Conclusions: Our data suggest that ex vivo, exogenous APC can inhibit PMA-induced NETosis and TNFα-induced apoptosis in a EPCR/Mac-1/PAR-3 dependent manner. Our data identify an additional possible link to the anti-inflammatory and antithrombotic activities of APC in immunothrombosis.

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Deficiency in IRAK-4 Activity Attenuates Manifestations of Murine Lupus

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IL-1R-associated kinase (IRAK) 4 initiates TLR signaling and plays an important role in host defense against infections. As an active kinase, IRAK4 elicits full spectra of MyD88-dependent responses, while kinase-inactive IRAK4 adapter induces a subset of cytokines and negative TLR regulators whose expression is not regulated by mRNA stability. While IRAK4 activity is critical for host resistance against *Streptococcus pneumonia*, its role in autoimmunity is incompletely understood. In this study, we determined the role of IRAK4 activity in systemic lupus erythematosus, using 4 month-old lupus-prone Bxsb male mice expressing the Y chromosome-linked autoimmunity accelerator (Yaa) and age-matched control Bxsb female mice without lupus manifestations. Compared to cells from control mice, splenic macrophages from Bxsb/Yaa mice had increased basal and TLR4/7-induced phosphorylation of IRAK1, ERK and p38 MAPKs, p65 NF-κB, and enhanced TNF-a and CCL5 gene expression. In contrast, lupus development led to decreased levels of Toll-interacting protein and IRAK-M in splenic macrophages, but did not affect IRAK4 or IRAK1 mRNA expression. Mice harboring kinase-inactive IRAK4 on the lupus-prone Yaa background had reduced glomerulonephritis, splenomegaly, and levels of serum anti-nuclear Abs compared to lupus-prone mice expressing kinase-sufficient IRAK4. A loss of IRAK4 kinase activity led to decreased numbers of splenic $F4/80^+$ macrophages, $CD11c^+$ dendritic cells, IFN-?$^+$ T-lymphocytes and $B220^+CD138^+$ B-lymphocytes and reduced TNF-a expression in macrophages. Thus, IRAK4 kinase activity positively regulates the development of lupus in mice and could represent a new target for therapeutic interventions in lupus via applications of peptide- or small molecule-based IRAK4 inhibitors.

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Cullin-5 Promotes TRAF6 Polyubiquitination and LPS Signaling

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TNF receptor-associated factor 6 (TRAF6) is an E3 ubiquitin ligase that integrates signals from multiple cell surface receptors for the activation of nuclear factor κB (NF-κB). However, the mechanism underlying lipopolysaccharide (LPS)-induced TRAF6 signaling remains largely unknown, nor is it clear how TRAF6 responds to various types of receptor signals. We report that cullin-5 (Cul-5), a cullin family scaffold protein that resides in the cytoplasm of unstimulated cells, forms a complex with TRAF6 and promotes TRAF6 polyubiquitination at Lys63 in LPS-stimulated macrophages. A direct interaction is established between the C-terminal domain of Cul-5 and the TRAF-C domain of TRAF6 that facilitates dimerization and polyubiquitination of TRAF6. Targeted deletion ofCul-5(cul-5+/-) abrogates LPS response and significantly improves the survival rate

of mice receiving systemic LPS challenge. Cul-5deficiency also attenuates acute lung injury resulting from intratracheal injection of LPS to thecul-5+/-mice. Macrophages isolated from the cul-5 hemizygous mice display significantly delayed phosphorylation of p65/RelA, ERK, JNK and p38 MAP kinases, with a marked decrease in NF-κB activation and significant reduction in inflammatory cytokine expression. The effect of Cul-5 to promote TRAF6 binding and polyubiquitination is abrogated by inhibition of NEDD8 conjugation, suggesting the involvement of neddylation in LPS signaling leading to TRAF6 polyubiquitination. Taken together, these findings identify Cul-5 as a novel link between the LPS-activated TLR4-MyD88 complex and TRAF6, an immune cell check-point that governsactivation of NF-κB.

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Toll-Like Receptor Trafficking and Cellular Responses are Determined by Common Single Nucleotide Polymorphisms

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Despite advances in personalized medicine for many disease processes, our therapies for sepsis remain algorithmic rather than patient specific, with morbidity and mortality still unacceptably high. However, it is well recognized that the host innate immune response has pronounced patient-specific variability and often determines the patient outcome. Neutrophil (PMN) activation, although required for pathogen killing, can be a mediator of significant host tissue damage during acute inflammatory events, via release of reactive oxygen species, granule exocytosis, and cytokine production. Bacterial ligands elicit priming or pre-activation of neutrophils through interaction with pattern-recognition receptors including Toll-like receptors. We recently demonstrated that stimulation of PMN with the synthetic TLR2/1 ligand Pam3CSK4 elicited a priming phenotype in approximately 50% of donors, with the remainder displaying minimal/no response. Genotyping studies revealed that PMN responsiveness to Pam3CSK4 was determined by a common SNP in TLR1 (1805 G>T). We subsequently analyzed a pediatric sepsis database and found that patients with this SNP had prolonged pediatric intensive care unit length of stay. Based on our finding of an alteration in the density of TLR1 on the cell surface in the setting of this SNP, we hypothesized that TLR1 biosynthesis and trafficking was impacted by this SNP. Moreover, we postulated that PMN responses to a "natural" bacterial ligand for TLR2/1 from *Mycobacterium tuberculosis* would be similarly affected by this SNP. Using confocal microscopy for quantitative colocalization analysis for TLR1 and TLR2 proteins, we found a Pearson's correlation coefficient of 0.9337 in a donor with the 1805T SNP. PMNs displayed colocalization of TLR1 with TLR2 both on the cell surface and in intracellular compartments. Immunoprecipitation studies demonstrated enhanced association of TLR1 with both TLR2 and with gp96, an ER chaperone protein, in PMNs from 1805T vs. 1805G donors. Unexpectedly, cell activation in 1805 G vs. T PMNs in response to purified lipoarabinomannan from *M. tuberculosis* (a TLR2/1 ligand) demonstrated a completely different phenotypic signature from that seen in response to Pam3CSK4. 1805T PMNs displayed no differences in priming of NADPH oxidase activity in response to fMLF as compared with 1805G PMNs, but there was significantly greater cytokine production by both monocytes and PMNs and enhanced phosphorylation of ERK 1/2. We conclude that leukocyte responses to TLR stimulation are significantly impacted by SNPs in these receptors and that may result from impaired trafficking within the cell. Moreover, cellular responses are highly ligand-specific with unique phenotypes that could be very relevant to disease pathogenesis. We speculate that these ligand-specific differences may result from modification of TLR2/1 responses by other pattern recognition receptors, including the inhibitory actions of TLR10.

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HHV-8 Replication in Subtypes of Myeloid Dendritic Cells is Regulated by Differential Expression of Virus Receptors

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Background: HHV-8 (KSHV) infection of myeloid dendritic cells (DC) is considered important in host control of the virus and prevention of Kaposi's sarcoma (KS). There is, however, limited

information of HHV-8-DC interaction. Monocyte-derived dendritic cells (MDDC) only support abortive replication of HHV-8 in vitro, with expression of viral lytic proteins but minimal virion production. HHV-8 infected MDDCs have impaired cytokine production and antigen processing, with a significant decrease in endocytosis and lower capacity to activate antigen-specific CD8+ T cells. Because DC are actually a family of functionally and regionally diverse antigen presenting cells (APC), we assessed Langerhans cells (LC) and interstitial/dermal DCs (IDDC) compared to MDDC to better define virus receptor usage and their ability to support virus replication.

Methods: The 3 DC populations were derived from neonatal cord blood and infected with BAC16, a recombinant virus expressing green fluorescent protein (GFP). The percentage of HHV-8-infected cells expressing GFP was enumerated by flow cytometry. To measure virus replication, viral DNA in cell pellets and cell cultures was quantitated by RT-PCR and the amount of infectious virus in cell culture supernatants was measured by a flow cytometry-based TCID50 assay. Virus receptor usage was determined by blocking infection of DC with mAbs directed against either DC-SIGN, Langerin or Ephrin type-A receptor 2 (EphA2).

Results: Phenotyping studies confirmed that LC express Langerin and EphA2 but not DC-SIGN, IDDC express DC-SIGN and low levels of EphA2 and MDDC express DC-SIGN but not Langerin or EphA2. Our data indicate that full cycle, HHV-8 lytic replication occurs in LC and IDDC as measured by at least one log10 increase in viral DNA copies and infectious virus over 72 hours of infection. Only abortive HHV-8 infection was detected in MDDC, in agreement with our previous results. Anti-DC-SIGN mAb blocked viral infection of both IDDC and MDDC by 75%. By contrast, HHV-8 appeared to use alternate receptors to infect LC, as blocking with mAb against either Langerin or EphA2 abrogated infection by 42% and 59%, respectively, and 83% when cells were treated with both mAbs prior to infection, with no detectable infectious virus production. Infected LC and DDC were poor MLR stimulators.

Discussion: We expand on our previous results by showing that HHV-8 utilizes DC-SIGN as a major receptor for infection of both DC and IDDC, but only fully replicates in IDDC. HHV-8 infection of LC is more complex in that the virus utilizes both Langerin and EphA2 as receptors. EphA2 is known to synergize with CIB1 (calcium and integrin binding protein 1) to facilitate HHV-8 entry and establish successful productive primary infection in endothelial cells. Ligand binding to EphA2 influence activation and maturation of DC by affecting integrin affinity and the cell cytoskeleton. Thus, HHV-8 binding to LC and DDC through EpHA2 receptor could render these cells less efficient in the ability to stimulate T cell responses. Taken together, our data show for the first time that HHV-8 differentially infects and replicates in several types of DC and supports a role of these cells in HHV-8 infection and pathogenesis.

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ICOS Signaling Contributes to Staphylococcus Aureus Pneumonia

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Staphylococcus aureus is a major cause of both community and healthcare acquired pneumonias. We have shown previously that $CD4^+$ T cells contribute to pneumonia through the production of proinflammatory cytokines and the CD28 co-receptor plays a role in this signaling. The ICOS pathway is part of the CD28 family of proteins and is a target for immune checkpoint therapy. To determine the contribution of ICOS to the pathogenesis of *S. aureus* pneumonia we intranasally infected WT and *Icos*$^{-/-}$ mice with *S. aureus* USA300, the current epidemic MRSA isolate. *Icos*$^{-/-}$ mice had improved survival in a mortality model and significant reductions in bacterial burden in the airway (65% reduction, $P<0.01$) and in lung tissue (92% reduction, $P<0.01$) in a non-lethal acute pneumonia model. We then examined the cytokine differences in bronchoalveolar lavage fluid between WT and *Icos*$^{-/-}$ mice. *Icos*$^{-/-}$ mice had major reductions in most cytokines analyzed including: IL-6, KC, TNF, GM-CSF, CCL2, Eotaxin (CCL11), IL-1β, IL-17, IL-5, IL-7 and IL-13. To better understand the mechanisms behind the improvement in the *Icos*$^{-/-}$ mice we examined the cellular response to infection by antibody staining and flow cytometry. Examination of recruitment to the airway identified significant reductions in two cell types, the $Ly6C^+$ inflammatory monocyte (82%, $P<0.05$; $CD45^+Ly6C^+Cd11b^+CD11c^-MHCII^-$) and

eosinophils (73%, $p<0.01$; $CD45^+Ly6C^-CD11b^+SiglecF^+CD11c^-$). It has also been recently shown that ICOS is expressed on group 2 innate lymphoid cells (ILC2) and we observed a 4-fold reduction in ILC2 cells in the *Icos*$^{-/-}$ mice. We are currently investigating the role of ICOS in other airway pathogens and delineating the respective roles of inflammatory monocytes, eosinophils and ILC2 cells in the pathogenesis of *S. aureus* pneumonia. Our results indicate that ICOS plays a significant role in orchestrating the innate immune response to *S. aureus* and could be potential immunomodulatory target to attenuate *S. aureus*-related immunopathology.

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Phagocytosis by Opsonic Receptors in Macrophages

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Our lab studies the activation of macrophages and cellular mechanisms behind the targeted destruction of pathogens by phagocytosis. We and others have shown that the type of receptor engaged on the macrophages influences the uptake of the phagocytic target into macrophages. We have done a comprehensive analysis of kinase and cytoskeletal requirements for Fcgamma and CR3-mediated phagocytosis when challenged with single or dual-opsonized particles. Scanning electron microscopy as well as fluorescent imaging of F-actin in macrophages has revealed that very different surface structures are induced by opsonic receptors and the timing of particle internalization varies accordingly. Interestingly, coating particles with both antibody and complement (to stimulate Fcgamma and CR3, respectively) has an additive effect on particle binding. The prominent membrane ruffles that capture complement-opsonized particles do not need Src activity but have a strong requirement for microtubules, while the opposite is observed during Fcgamma receptor engagement. The activation of the receptor also influences the maturation of the phagosome once inside. Phagosomes of complement-opsonized particles show delayed acidification and striking F-actin recruitment to the phagosome in waves or flashes. The presence of the F-actin flash correlates with deformation of the internalized particle, the contraction which is likely mediated by myosin IIA which was also present on the phagosome. Together this work indicates that opsonins direct a different fate for coated pathogens and CR3-mediated phagocytosis promotes mechanical over enzymatic destruction of target particles.

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Differential Activation of Formyl-Peptide Receptors by Staphylococcus Aureus and Consequences for Inflammation

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Formyl peptide receptors (FPR1-3) are crucial pattern recognition receptors governing leukocyte chemotaxis and cytokine release in response to microbe-associated molecular patterns (MAMPs). FPR1 senses formylated peptides produced by all kinds of bacteria, while FPR2 and 3 respond to certain endogenous peptides. In addition, we have recently demonstrated that phenol-soluble modulin (PSM) peptides from highly pathogenic *Staphylococcus aureus* are not only important cytolytic toxins but also highly efficient ligands for the human FPR2. Mouse neutrophils also respond to PSMs, but it has remained unclear, which of the mouse FPR paralogs senses staphylococcal PSMs.
To analyze the role of mouse FPRs, stably transfected RBL cells were generated, which either express mFpr1 or mFpr2. After stimulation with PSMs or culture filtrates of PSMs-secreting *S. aureus* strain USA300, we noticed strong calcium influx and degranulation in mFpr2-transfected cells, but no response in mFpr1-transfected cells and control cells. Moreover, by using HoxB8 neutrophils, a primary neutrophil cell line prepared from wild-type (WT) and mFpr2 knockout mice (Fpr2-/-), we observed strong calcium influx, chemotaxis, MIP2 release and CD11b upregulation

in wild-type HoxB8 but not in Fpr2-/- HoxB8 after stimulation with PSMs or culture filtrates of USA300.
These data indicate that the mouse Fpr2 is specifically activated by PSMs. Therefore, PSMs represent the first secreted MAMPs for the mouse Fpr2. Our data support the hypothesis that the mouse Fpr2 is the functional orthologue of the human FPR2 and that a mouse infection model may be a suitable model for analyzing the role of PSMs and FPRs during infection.

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LTD4-Induced Monocyte/Macrophage Migration: a Role for PTPε?

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Cysteinyl-leukotrienes (cys-LTs), which include leukotriene (LT) C4 (LTC_4), LTD_4 and LTE_4, have well-characterized physiopathological roles in the development of several inflammatory diseases, including asthma. Cys-LTs lead to the production of various inflammatory mediators, to the recruitment of multiple cellular subtypes and to an exacerbation of asthma symptoms. Thus, the function and signalling of $CysLT_1$ receptors ($CysLT_1R$), highly expressed on leukocytes, have been the focus of research in our laboratory. In our attempt to discover potential $CysLT_1R$-interacting proteins, we identified protein tyrosine phosphatase epsilon (PTPε). Interestingly, a polymorphism in the *PTPRε* gene, *PTPRε*_rs7081735A>G, has been associated with allergic asthma, potentially making its interaction with $CysLT_1R$ even more significant. Furthermore, as PTPε has been found to be involved in the formation and function of podosomes in osteoclasts, through Src activation, we were interested in its role in monocyte migration, as well as, in LTD_4-induced $CysLT_1R$ signalling in polarized monocyte-derived macrophages (M1 and M2).
There are two major protein isoforms of PTPε, the receptor-like (RPTPε) and the cytoplasmic (cyt-) PTPε. In most cells, only one of the isoforms is expressed, however in human monocytes both are present, although the cyt-PTPε predominates. In this regard, we demonstrate a positive modulation of cyt-PTPε expression following stimulation by LTD_4, IL-4 and IFNγ of monocytes. However, stimulation by IL-4 and IFNγ, when polarizing monocyte-derived macrophages, resulted in a decreased expression of cyt-PTPε among M1 and an increase of RPTPε in M2. Activation of Src is also different in M1 and M2 macrophages subtypes, after stimulation with LTD_4, as a decrease in the phosphorylation of the Src inhibitory residue (Y530) was observed in M1 and an increase in M2. The opposite tendency was observed at the ROCKII inhibitor residue (Y722) where M1 had an increase in phosphorylation of the residue and M2, a decrease.
Using Boyden chambers for directional migration experiments, our results demonstrate a correlation between PTPε expression, ROCK activity and monocyte chemotaxis. Indeed, monocytes treated with siRNA against PTPε (siPTPε) or with a ROCK inhibitor, Y27632, display a significantly reduced LTD_4-induced chemotaxis. However, the combination of siPTPε and Y27632, results in a restoration of migration.
Our results demonstrate a possible interaction of PTPε and ROCK in LTD_4-induced monocytes chemotaxis. In addition, the differential modulation of cyt-PTPε and RPTPε expression in polarized macrophages M1 and M2 may result in differential $CysLT_1R$ signalling and/or migration.

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The Role of IL-15 Trans-Presentation in Antigen Cross-Presentation

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Interleukin-15 (IL-15) is a pro-inflammatory cytokine that is required for the survival and activation of memory $CD8^+$ T cells, NK cells, innate lymphoid cells, macrophages and dendritic cells. IL-15 is implicated in the pathogenesis of various autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and autoimmune type 1 diabetes (T1D). IL-15 receptor (IL-15R) consists of a specific a chain, the b chain that is shared with IL-2R and the common g chain.

IL-15 is unique in the manner in which it binds and signals through its receptor subunits. IL-15 that is complexed with IL-15Ra binds to the bg receptor complex present on the responding cell to mediate its biological effects, through a process referred to as trans-presentation. The trans-presented IL-15 is essential to mediate the biological effects on T lymphocytes and NK cells. We show that in vitro, neither IL-15 nor IL-15Ra is required for cross-presentation of bacterial antigens. However IL-15, but not IL-15Ra is required for cross-presentation of antigens derived from intracellular pathogens in vivo. Our findings provide insight into the complexities of IL-15 signaling in the initiation and maintenance of $CD8^+$ T cell-mediated immune responses.

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Stabilin-1 is a Functional Biomarker for Pro-Fibrotic Alternative Macrophages Predicting Pathological Heart Remodelling in Patients with Heart Failure during the Left Ventricular Assist Device Therapy

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Alternatively activated macrophages (M2) control immunological tolerance and healing processes. Stabilin-1 expressed on M2 and acts as a multifunctional scavenger/sorting receptor that mediates clearance of unwanted-self components and delivery into secretory pathways of soluble mediators, including chitinase-like proteins. Stabilin-levels can be also elevated in chronic inflammatory conditions. Chronic inflammation in myocardium results in development of caridomyopathy and heart failure (HF). Advanced HF is a life-threatening disorder affecting 6-10% of people above 65 y.o. Less than 50% of HF patients survive 5 year after the first symptoms are identified. Advanced HF is treated with mechanical circulatory support LVAD (left ventricular assist device). The outcome of LVAD therapy differs significantly between patients with over 50% mortality after LVAD explantation and successful myocardial recovery in others. Identifying the immunopathological mechanisms and biomarkers of patients' reaction to LVAD are urgently needed. We used stablin-1 as a bimarker to idensitfy M2 subsets in healing heart. Heart sections were obtained directly before LVAD installation and out the explained hearts at the moment of donor heart transplantation. Analysis of macrophages phenotypes in paraffin sections of 21 patients with HF was performed using IHC, IF/confocal microscopy. Three types of macrophage subtypes have been identified: CD68+stabilin-1; CD68+Stabilin-1+; CD68-stabilin-1+. In 10 patients the percentage of CD68-Stabilin1+ was decreased or not changed during LVAD therapy. In 8 out of these 10 cases ejection fraction (EF) of the heart was improved after LVAD therapy. In 11 cases the percentage of CD68-Stab1+ was increased after LVAD therapy. In 10 out of these 11 cases EF was not improved. Pro-fibrotic cytokine TGFbeta strongly activated functionally active stabilin-1 in human macrophages in vitro. We concluded that CD68-Stabilin-1+ macrophages represent new subtype of pro-fibrotic M2 in healing heart, and dynamic accumulation of CD68-Stabilin-1+ macrophages is predictive for the pathological heart remodelling during LVAD therapy.

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Loss of MEKK2 and MEKK3 Results in Increased ROS and is Protective in LPS-Induced Lung Injury

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Introduction:

Patients with chronic granulomatous disorder (CGD) have defective NOX2 and impaired release of ROS. These patients not only suffer from recurrent infections but also develop sterile inflammatory lesions. This latter observation suggests that ROS have anti-inflammatory potential.

MEKK3 is highly expressed in neutrophils while, MEKK2,its homolog is expressed at lower level. However, the two homologs share over 95% sequence identity in their kinase domains suggesting

that MEKK2 can compensate for the loss of MEKK3.

Hypothesis:

MEKK2 and MEKK3 regulate NOX2 in neutrophils.

Methods:

1. Generation of double knock out (DKO) mice with loss of MEKK3 in myeloid cells and global loss of MEKK2.
2. Generation of bone marrow (BM) chimeric mice with transplantation of DKO BM into wild type (WT) mice and WT BM into WT mice.
3. Development of LPS-induced lung injury via intranasal delivery of 50 ul of LPS (1mg/ml).
4. Expression of MEKK3 , kinase domain mutant MEKK3 (KD), PB1 domain deficient MEKK3 (delta PB1 MEEK3) in neutrophils and cos-7 cells.
5. Expression of NOX2 components and p47phox mutant S208E (47phoxSE) incos-7 cells.
6. In vitro kinase assay.
7. GST pulldown assay.

Results:

1. DKO neutrophils release significantlymore ROS compared to WT neutrophils.
2. DKO mice have significantly less permeability to FITC albumin as compared to WT mice in an LPS- induced model of lung injury.
3. This permeability change is sensitive to anti-oxidant treatment (BHA) suggesting that the protective effect is being mediated by ROS.
4. Overexpressed MEKK3GFP inhibited ROS release in WT and DKO neutrophils suggesting that MEKK3 regulates NOX2 negatively.
5. MEKK3 mediated NOX2 inhibition is dependent upon its kinase activity.
6. MEKK3 phosphorylates p47phox on S208.
7. MEKK3 mediated phosphorylation of p47phox on S208 disengages p47phox from p22phox.

Conclusion:

Increased ROS can be protective under certain circumstances, and the regulation of NOX2 by MEKK3 opens a new direction for the development of novel anti inflammatory therapies.

Distinct Role for MyD88 Signaling in Myeloid Cells and Stromal Cells in an Imiquimod-Induced Mouse Model of Psoriasis

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Psoriasis is a chronic skin disease associated with deregulated interplays between immune cells and keratinocytes. The contribution of T cells and DCs to psoriasis pathogenesis is well established, whereas the role of innate myeloid cells is not well understood. Aim of this study was to investigate the contribution of MyD88 signaling in neutrophils and monocytes/macrophages during psoriatic inflammation.

Materials & Methods. We induced imiquimod-induced psoriasis-like skin model in conditional knockout mice lacking MyD88 in monocytes/macrophages and neutrophils (*Myd88$^{fl/fl}$ LySM-Cre* mice) or in the total hematopoietic compartment (*Myd88$^{fl/fl}$ Vav-Cre* mice). Total MyD88 knockout mice (*MyD88 $^{-/-}$*), *Myd88$^{fl/fl}$* and C57BL/6 mice were also included as controls. The development of psoriasis was induced by topical application of Aldara™ (5% IMQ cream) on the shaved back for 6 consecutive days. *Results. Myd88$^{fl/fl}$ LySM-Cre* mice manifested a significant reduction in the activation and recruitment of innate immune cells and gd T cells in the draining lymph nodes and in the skin. This correlates with a reduction of the expression of several inflammatory mediators in these tissues, including a specific proliferative and inflammatory skin markers, however keratinocyte proliferation and epidermal acanthosis were not significantly reduced in *Myd88$^{fl/fl}$ LySM-Cre* mice. Interestingly, we observed that significant levels of skin inflammation were still present, at similar levels, also in both *Myd88$^{fl/fl}$ Vav-Cre* and *MyD88 $^{-/-}$* mice, probably due to the reported TLR7-indipendent signaling activation induced by IMQ in keratinocytes. *Conclusions*. These data demonstrate that MyD88

signaling in innate myeloid cells plays an important role on disease propagation and exacerbation in the IMQ-induced model of psoriasis. However, MyD88 signaling is dispensable for the initiation of keratinocyte-mediated inflammatory responses.

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Responses of Donkey Monocytes to Toll-Like Receptor Agonists

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As opposed to other animal species, stimulation of equine monocytes with lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR4) leads to induction of pro-inflammatory gene expression profiles showing engagement of only the MyD88 pathway. In contrast, activation of TLR2 or TLR3 shows the expected engagement of the pro-inflammatory MyD88 and the anti-inflammatory TRIF pathways in these cells. These unique responses of equine monocytes to LPS, coupled with the low EC50 of LPS in these cells, may be responsible for the finding that endotoxemia is associated with the leading causes of death in horses. In contrast colic events in the university donkey herd are typically not associated to clinical endotoxemia as seen in the equine population. In addition there appear to be no reports on the response of donkey monocytes to Toll-like receipt agonists in the literature. To determine whether closely related animal species have similar responses to TLR agonists we have examined the responses of isolated donkey peripheral blood monocytes to the respective TLR4, TLR3 and TLR2 specific agonists LPS, Poly I:C and PAM3CSK4

Donkey monocytes were isolated from peripheral blood using density gradient centrifugation over Histopaque gradients and adherence of the monocytes to plastic. In the first series of experiments the EC50 of E. coli O55:B5 LPS and PAM3CSK4 was determined by stimulating the monocytes (>95% purity) with a range of agonist concentrations and measuring secreted TNF-α by ELISA assays. In the second series of experiments donkey monocytes were stimulated with diluent only (control), LPS, PAM3CSK4, or poly I:C for fixed periods of time (0, 1, 4, 8, 12 and 20 hrs). RNA was isolated from these cells and induced expression of the TNF-α, IL-6, IL-1β, IFN-β, RANTES (CCL-5), IL-10 and IP-10 genes was determined by SybrGreen RT-qPCR assays, with 18S rRNA used as housekeeping control.

Our preliminary data shows that donkey monocytes are highly sensitive to LPS (EC50 = ~27 pg/ml) and are less sensitive to the TLR2 agonist PAM3CSK4 (EC50 = ~8 ng/ml), similar to what is observed in equine monocytes. LPS and PAM3CSK4 induced higher expression of TNF-α, IL-6, IL-1β than poly I:C , with weaker expression of IL-10 only at early time intervals. Poly I:C induced higher expression of IL-10 (late time intervals), RANTES and IFN-β. These findings are similar to those observed in equine monocytes indicating that LPS activation of these cells leads to a largely pro-inflammatory gene expression profile.

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Differential Role of the Transcriptional and Non-Transcriptional Functions of IRF-3 in High Fat Diet-Induced Liver Injury

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Background and Aims. Metabolic diseases, like non-alcoholic fatty liver disease (NAFLD), are characterized by hepatic steatosis and increased expression of inflammatory cytokines and chemokines. These responses in turn contribute to hepatocyte injury characterized by endoplasmic reticulum (ER) stress and mitochondrial dysfunction resulting in hepatocyte injury. While the pathogenesis of NAFLD is complex and not completely understood, activation of Toll-like receptor (TLRs), through increased exposure to fatty acids, as well as gut derived microbial products, contribute to disease progression. Interferon regulatory factor 3 (IRF3) is a master regulator of host response to viral infection, but is also activated via the TLR4-MyD88-independent pathway. Upon activation, IRF3 is phosphorylated and translocates

to the nucleus where it initiates a specific transcriptional program. Recently, two non-transcriptional functions for IRF3 have been identified: 1) a novel pro-apoptotic function termed RIG-I-induced IRF3-mediated pathway of apoptosis (RIPA) and 2) an interaction with the p65 subunit of NFκB that prevents p65 translocation to the nucleus. While IRF3 has been implicated in the progression of high fat diet-induced steatosis and insulin resistance, the contribution of the transcriptional vs non-transcriptional functions of IRF3 are not well understood. Here we made use of IRF3-deficient mice (IRF3 KO), as well as a novel knock-in of a mutated IRF3 that cannot translocate to the nucleus and only retains the non-transcriptional functions of IRF3 (nt-IRF3 KI).
Methods: Wild-type C57BL/6, IRF3-KO and nt-IRF3 KI mice were allowed free access to a high fat diet or chow-fed control diets for 12 weeks. Measures of liver injury were assessed.
Results: High fat diet (HFD) increased plasma ALT and AST activities indicators for hepatic injury and expression of inflammatory cytokines and chemokines in liver of wild-type mice. These responses were exacerbated in IRF3 KO mice, but completely ameliorated in nt-IRF3 KI mice. In contrast, hepatic triglycerides were elevated after HFD in wild-type mice, with a modestly reduced steatosis in IRF3 KO mice and further reduced in nt-IRF3 KI mice. Fibrosis, assessed by expression of collagen 1α mRNA and Sirius red staining, was increased in wild-type mice after HFD; the fibrotic response was even higher in IRF3 KO mice, but nt-IRF3 KI mice were protected from HFD-induced fibrosis.
Conclusion: IRF3 has previously been implicated in the development of HFD-induced steatosis and insulin resistance. Here we have identified a differential contribution of the transcriptional vs non-transcriptional actions of IRF3, finding that the expression of only the nt-IRF3 can protect from HFD-induced inflammation and fibrosis in the liver.

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Activity of Cell Surface 90kDa Heat Shock Protein (HSP90) is Necessary for Cytokine Production but Not for Target Engulfment following Pattern Recognition by Macrophages

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Professional phagocytes are equipped with complex mechanisms of pattern recognition, allowing them not only to identify different pathogens (PAMPs), but also to recognize the self-derived molecular patterns of dying, damaged or stressed cells (DAMPs and ACAMPs). A term: engulfment synapse, has been proposed to depict the complex nature of the phagocyte-target contact site which comprises hundreds of proteins that play a key role in deciphering the target surface and in defining host response. HSP90, a stress-induced chaperone protein, has been demonstrated as a regular component of LPS-signalling complexes on macrophages but its role has not been elucidated. As LPS is a prototypical PAMPs it is tempting to speculate that HSP90 also assists recognition of different molecular patterns by professional phagocytes.
In this study, we detected the cell surface HSP90 on human monocyte-derived macrophages by staining with a cell-impermeable specific inhibitor. Confocal analysis of live hMDMs revealed that the HSP90-inhibitor complexes were rapidly clustered on the macrophages surface and, when incubation was prolonged, recycled from cell surface through endosomal compartment. This finding, supported by detection of N-terminal domain of surface HSP90 by specific mAbs suggests that N-terminal (ATP-ase) domain of HSP90 is exposed to and accessible from the outside of the cell. To study the role of surface HSP90 on phagocytic cells we used two different classes of molecular patterns: pathogen- or apoptotic cell-associated. We have shown that blocking the cell-surface HSP90 pool leads to the dramatic decrease of TNF production by primary human monocytes and hMDMs exposed to wide range of soluble (TLRs-specific ligands) and particulate (bacteria *S. aureus* and *P. gingivalis*) PAMPs. Surprisingly, in human macrophages the functional cell-surface HSP90 was not necessary for the engulfment of either apoptotic neutrophils or bacteria cells. The presented data suggest that the surface HSP90 is a ‘signaling complex chaperone’ which activity is essential for cytokine response but not for target engulfment following pattern recognition by macrophages.

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Silencing Endothelial Cell Receptor, Tie1, Reduces Inflammation and Vascular Permeability following Hemorrhage Priming for the Development of Acute Respiratory Distress Syndrome in Mice

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Loss of endothelial cell barrier function plays a significant role in the development of acute respiratory distress syndrome (ARDS). Angiopoietins 1 & 2 mediate endothelial cell (EC) activation through competitive binding to their shared receptor, Tie2, expressed on ECs. Ang-1 binding induces Tie2 phosphorylation and signaling for downstream anti-inflammatory and anti-apoptotic protein synthesis and vessel barrier integrity. Ang-2/Tie2 binding, alternatively promotes increased pro-inflammatory signaling (increased ICAM-1 expression) and decreased barrier function (lung leak). Ang-2 is significantly elevated in plasma from patients with ARDS and in our murine model of hemorrhagic shock priming for the development of indirect (i)ARDS followed a subsequent septic challenge. We have demonstrated that EC interaction with hemorrhage-primed neutrophils contributes to EC activation and Ang-2 release, and that depletion of peripheral blood neutrophils prior to hemorrhage/sepsis increases Tie2 phosphorylation. Recent publications as well as our preliminary data suggest that the orphan receptor, Tie1, may also play a role in modulating EC activation by forming a complex with Tie2. This complex inhibits Ang-1/Tie2 interactions promoting an activated/pro-inflammatory EC phenotype. To better understand the significance of Tie1/Tie2 interaction in mediating EC activation leading to loss of barrier function and the development of ARDS in our model, we delivered liposomal-encapsulated short interference (si)RNA, targeting Tie1, via tail vein injection 1 hour post hemorrhage. Mice were made septic [cecal ligation and puncture (CLP)] 24 hour following hemorrhage and euthanized 24 hours after CLP. Targeted siRNA treatment decreased Tie1 expression in lung tissue homogenates by 65% +/- 7% compared to mice that received non-targeting/scrambled siRNA (control). Importantly, while silencing Tie1 did not alter lung tissue Ang-2/Ang-1 ratio, we found inflammatory cytokines, vessel permeability and lung injury to be decreased in Tie1 siRNA treated mice. These data suggest that Tie1 contributes to EC activation by inhibiting Ang-1/Tie2 interaction, potentially leading to EC dysfunction and the development of iARDS. (Funded by NIH GM103652)

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Role of CK2 in Neutrophil Signaling and Functional Responses

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Casien kinase 2 (CK2) is a highly conserved, ubiquitously expressed, and pleiotropic Ser/Thr kinase, endowed with constitutive activity, that can be modulated by phosphorylation. The CK2 holoenzyme consists of two regulatory (beta) and two catalytic (alpha or alpha') subunits, the latter of which can also function independently of the tetramer. CK2 has hundreds of substrates participating in the regulation of numerous cellular processes including oncogenesis, cell cycle progression, proliferation, apoptosis, transcription, inflammation, and immune responses. Neutrophils and their products profoundly influence innate and adaptive immunity, notably through the generation of inflammatory cytokines. Several signaling cascades are known to control neutrophil responses, including the TAK1, IKK/NF-kB, p38 MAPK, MEK/ERK, and PI3K/Akt pathways. Whether CK2 affects neutrophil functional responses or signaling, however, remains unknown.

In this study, we report that neutrophils constitutively express CK2 subunits. Following cell stimulation by TNFalpha or LPS, CK2alpha becomes rapidly phosphorylated. Similarly, the phosphorylation of several phospho-(Ser/Thr) CK2 substrates is observed in resting neutrophils, and increases following TNF or LPS stimulation. Inhibitors of MAPKs, PI3K, or TAK1 failed to affect the pattern of phospho-(Ser/Thr) CK2 substrates. This suggests that these various kinases do not act upstream of CK2. Conversely, neutrophil pretreatment with a CK2 inhibitor (CX-4945) led to a dose-dependent inhibition of phospho-(Ser/Thr)

CK2 substrates. The kinase, Akt, appeared to represent one such substrate, as its phosphorylation was attenuated by CX-4945 in activated neutrophils. By contrast, the phosphorylation of other signaling intermediates, including p38 MAPK, JNK, or IKK was unaffected by CK2 inhibition. We next investigated whether CK2 might affect neutrophil functional responses. The inducible gene expression and secretion of several inflammatory cytokines were profoundly impaired following CK2 inhibition. Similarly, the delayed apoptosis observed in response to various neutrophil stimuli was mostly reversed by CK2 inhibition. Our data unveil a role for CK2 in controlling the Akt cascade and downstream functional responses in primary human neutrophils, making it a promising target for therapeutic intervention in view of the foremost role of neutrophils in several chronic inflammatory conditions.

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A Novel Role for JNK in Neutrophils: JNK Uniquely Regulates ROS Production and NADPH-Oxidase-Dependent Netosis

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Introduction & Objectives: JNK is a stress-activated kinase that regulates cell survival and death under different conditions. However, the roles of JNK in neutrophils are not clearly established. We considered that JNK plays an important role in neutrophil Extracellular Trap (NET) formation or NETosis. NETs are decondensed DNA decorated with antimicrobial peptides that are capable of trapping bacteria. The molecular pathway of NETosis is not fully understood. Although the participation of mitogen-activated protein kinases (MAPKs) ERK and p38 in NETosis has been studied in considerable detail, the role of c-Jun N-terminal Kinase (JNK), another MAPK, in NETosis is not clearly established.

Methods: NETosis was activated in human neutrophils by a diacylglycerol mimic phorbol 12-myristate 13-acetate (PMA), gram-negative bacteria cell wall component lipopolysaccharide (LPS), and gram-negative bacteria such as E.coli and Pseudomonas aeruginosa (PA). JNK specific inhibitor SP-600125 was used for inhibiting the JNK pathway. NETosis kinetics was measured by the Sytox-Green assay and ROS production was measured by the DHR123 assay. Immunoblots and confocal microscopy was used for determining the pattern of NET inhibition, JNK activation, and apoptosis.

Results: Our immunoblot results suggest that JNK is activated in response to LPS, but not PMA or media control. Further, JNK inhibition by SP-600125 significantly reduces LPS-induced ROS production while PMA-induced ROS production was unchanged. This shows that JNK is essential for LPS-mediated ROS production. In addition, JNK inhibition suppresses LPS-induced NETosis but does not affect PMA-induced NETosis. Our microscopy images show that JNK inhibition suppresses LPS-mediated NETosis such that the cells resemble the media control. However, JNK inhibition did not inhibit PMA-mediated NETosis such that no difference was observed compared to the PMA group. Additionally, our cleaved caspase-3 immunoblots and confocal images indicate that JNK suppression does not induce apoptosis in neutrophils but prolongs the lifespan of neutrophils in the LPS group. To further confirm the biological significance of LPS-induced NETosis, we tested the effects of JNK inhibition in E. coli- and PA-induced NETosis. We found that pretreating neutrophils with SP-600125 significantly suppresses E. coli and PA-induced NETosis.

Conclusion: We show that JNK is essential in LPS-, E. coli- and PA-mediated, but not PMA-mediated NETosis. JNK is involved in ROS production in LPS-mediated NETosis pathway such that its inhibition reduces LPS-induced ROS production and subsequent NETosis. Additionally, JNK inhibition does not lead to apoptosis but increases the life span of the LPS treated neutrophils.

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Neutrophils and Influenza-Specific CD8 T Cells in the Flu-Infected Airways

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Influenza virus infects the epithelial cells that line the respiratory tract. Therefore, cytotoxic $CD8^+$ T cells must traffic to the mucosal epithelium to mediate elimination of infected cells. Neutrophils are key players that help organs initiate and maintain immune reactions and shape the overall immune response by signaling to DCs, monocytes, and T cells. Under most inflammatory conditions, neutrophils are the first cell type that crosses the blood vessel endothelium into the tissue, often preceding a subsequent wave of effector T cells. Although neutrophil-mediated recruitment of T cells into infected sites has been documented in both bacterial and viral infections and in chronic inflammatory diseases, the molecular mechanisms that link neutrophil and T cell migration remain unknown.

The chemokine receptor family is known to be the most potent and subset-selective, tissue-specific homing receptors for T cell. Therefore, it is widely assumed that the differential expression of the chemokines and their receptors is responsible for the distinct migratory properties and distribution patterns of different subsets of specialized T cells. However, although this idea has been verified experimentally in some settings, multiple chemokine receptors expressed on the effector T cells and the redundancy in their signaling pathways suggest the presence of a more complex mechanism that can confer the specificity and selectivity of T cell recruitment. Furthermore, less is known about how chemokines released from newly recruited leukocytes act together with the local chemokines produced within the inflamed tissue. To address this, we performed intravital multi-photon microscopy (IV-MPM) imaging of the influenza-infected mouse trachea and explored how neutrophil-derived chemokines cooperates with the tissue-specific inflammatory cues to finely control the recruitment of $CD8^+$ T cells to the influenza-infected trachea. Here, we show that the early recruitment of neutrophils into influenza-infected trachea is essential for $CD8^+$ T cell-mediated immune protection. Especially, the relative motility of virus-specific $CD8^+$ T cells in the trachea was determined by their localization to the epithelium, which was governed by the presence of neutrophils during early infection. Both *in vitro* and *in vivo* imaging showed that migrating neutrophils leave behind long-lasting trails from their elongated uropods that are prominently enriched in the chemokine CXCL12. We observed that CXCL12 derived from the epithelial cells remained close to the epithelium, while CXCL12 derived from neutrophils was the main source of CXCL12 in the tissue interstitium during infection. Experiments with granulocyte-specific CXCL12 conditional knock-out mice and a CXCR4 antagonist revealed that CXCL12 derived from neutrophil trails is critical for virus-specific $CD8^+$ T cell recruitment and anti-viral effector functions.

The data presented here demonstrate that migrating neutrophils leave behind chemoattractant-containing trails, resulting in the local accumulation of neutrophil-derived chemoattractant signals in inflamed tissues. As chemokines are small, diffusible molecules, perhaps these trails serve to package the chemoattractant so that it can be preserved and survive severe mechanical perturbation during inflammation, lest it be present only transiently or immediately diffuse away from the site.

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Role of Neutrophils at the Site of Unhealing *Leishmania mexicana* Induced Lesion

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The protozoan *Leishmania mexicana* parasites cause chronic non-healing cutaneous lesions in humans and mice with poor parasite control. Promastigote forms of the parasites are injected by sand flies and transform into intracellular amastigote stage forms in the vertebrate hosts. We have previously shown that within hours of infection, *L. mexicana* promastigotes induce the local recruitment of neutrophils that sequester the parasites, shaping the microenvironment, eventually favoring the development of a chronic lesion. Here, we investigated interactions between neutrophils and the intracellular amastigote form of the parasite. We first show that amastigotes are 2-3 times less internalized

by neutrophils than promastigotes *in vitro*, however opsonization with immune serum led to comparable internalization. In line with these data, injection of mice with amastigote forms of *L. mexicana* only poorly recruits neutrophils. This is in contrast to what is observed following injection of the infective promastigotes. Of note, intralesional neutrophils represent 6-10% of the local cells during chronic infection. Strikingly,these neutrophils are heavily infected with over 70% of them harboring two or more intact amastigotes, as determined by the use of fluorescently labelled parasites and ImageStream analysis. Recent reports reveal increased lifespan of tissue neutrophils upon inflammation, suggesting that neutrophils could not only harbor live parasites but that parasites may replicate within these cells. To explore this, we have generated transgenic *L. mexicana* parasites expressing the photoswitchable kikume protein, allowing visualization of intracellular parasite replication. New data will be presented which focus on a better understanding of the role of neutrophils at the site of unhealing parasite infection.

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TNFα Drives the Migration and Crawling of Neutrophils into Afferent Lymphatic Vessels during Antigen Challenge In vivo.

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Neutrophils are now viewed as key effectors of both innate and adaptive immunities in many physiological and pathological conditions. Whilst the trafficking of neutrophils through blood vessels has been extensively studied, the mechanism that control their entry into the lymphatic system is poorly understood. For this purpose, we have developed a murine model of cremasteric inflammation to visualise by intravital confocal microscopy the migration of neutrophils into tissue-associated afferent lymphatic vessels in vivo. In the present study, we report that neutrophils migrate rapidly into the lymphatic vessels of inflamed cremaster muscles of WT animals upon antigen challenge in a CCR7-dependent manner. Interestingly, neutrophil intravasation into lymphatics (but not extravasation through blood vessels) is inhibited (~70%) in mice genetically deficient in both receptors for TNFα. Furthermore, the directional crawling of neutrophils onto the lumen of lymphatic endothelial cells is altered in mice pre-treated with an anti-TNFα blocking antibody, a response associated with a reduction in ICAM-1 expression by lymphatic endothelial cells in WT animals. Collectively, our results demonstrate the critical role of TNFα in promoting neutrophil recruitment into the lymphatic vasculature in vivo during the inflammatory response of antigen challenge.

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Effects of Antibody Opsonization on Early Yersinia Pestis-Host Cell Interactions in the Skin and Draining Lymph Node in a Mouse Model of Bubonic Plague

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Plague is a zoonosis caused by the bacterial pathogen *Yersinia pestis*. Bubonic plague, the most prevalent form in humans, results when *Y. pestis* is deposited in the skin via the bite of an infected flea. Bacteria subsequently traffic from the dermis to the draining lymph node (dLN) where they replicate to large numbers forming a severely enlarged lymph node called a bubo. The bacteria can escape from the bubo and spread systemically, which if left untreated, is highly fatal. Though several plague vaccine candidates are currently at various stages of development, no licensed vaccine is available in the United States. Polyclonal and several monoclonal antibodies (Ab) can provide complete protection against plague in animal models. The mechanisms involved in this antibody-mediated immunity (AMI) to *Y. pestis* remain poorly understood. Here we examine the effect of opsonizing Ab on *Y. pestis* interactions with macrophages and neutrophils *in vitro* and *in vivo*. We generated mouse anti-*Y. pestis* immune serum by vaccinating C57Bl/6 mice with an attenuated pCD1+ strain of *Y. pestis* and confirmed the presence of high Ab titers by western blot and immunofluorescence assays. Immune serum-opsonized *Y. pestis* showed modest but significant increases in phagocytosis by mouse macrophages and neutrophils *in vitro* compared to naïve serum

controls. Ab opsonization had little effect on intracellular survival of bacteria within these cells or on neutrophil oxidative burst. Previous intravital microscopy studies showed a rapid and robust neutrophil response to *Y. pestis* in the dermis of naïve mice. We used this system to determine if Ab opsonization alters this response. We observed increased association of immune serum-opsonized *Y. pestis* with neutrophils in the dermis of LysGFP mice compared to naïve serum-treated controls. Similar results were obtained with passively and actively immunized mice. We also imaged the popliteal LNs after i.d. injection of bacteria in the footpad and observed increased *Y. pestis*-neutrophil interactions and increased neutrophil clustering in response to Ab-opsonized bacteria. Thus, despite only having a modest effect in *in vitro* assays, opsonizing Ab has a dramatic *in vivo* effect on *Y. pestis*-neutrophil interactions in the dermis and dLN very early after infection. Additionally, anti-*Y. pestis* Ab may alter neutrophil recruitment and localization within infected dLNs. These data shed new light on the importance of neutrophils in AMI to *Y. pestis* and may provide a new correlate of protection for evaluation of plague vaccine candidates.

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Fc Receptors on Neutrophils: Two Sides of the Same Coin?

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Neutrophils express Fc receptors, which recognize the antibodies IgA (FcαRI) or IgG (Fcγ receptors). We previously demonstrated that neutrophil migration is only induced after IgA activation, which may play a role in IgA-autoantibody mediated autoimmune diseases. The aim of this study is to investigate in depth the differences between IgA- versus IgG-induced neutrophil effector functions and to elucidate the specific signaling pathways of FcαRI in neutrophils, responsible for IgA-induced neutrophil migration and activation.

Methods

The quantitative number of Fc receptors expressed was determined with a Qifikit. Furthermore, neutrophils were stimulated with complexed serum IgA or IgG to determine the differences in phagocytic index, lactoferrin release, induction of reactive oxygen species (ROS), NETosis, cytokine release, metabolite release, calcium flux and induction of signaling.

Results

Neutrophils express a higher number of Fcγ receptors (FcγRII and FcγRIII) compared to FcαRI. No differences were observed in phagocytosis of IgA- versus IgG-coated beads, but triggering the Fc alpha receptor with IgA led to enhanced release of cytokines, chemokines and pro-inflammatory metabolites. Additionally, IgA stimulation resulted in a higher induction of ROS, NETosis and calcium flux. Furthermore, crosslinking the FcαRI led to a stronger and more sustained phosphorylation of different signaling molecules.

Conclusion

Activating neutrophils with IgA leads to increased pro-inflammatory effector functions and enhanced signaling, which could not be explained by a higher expression of FcαRI on neutrophils. This suggests that the signaling routes after IgA and IgG triggering are different, which is against the current dogma which dictates that both FcαRI and Fcγ receptors use similar signaling pathways via immunoreceptor tyrosine-based associated motifs (ITAMs). The delineation of the exact signaling pathways of FcαRI and Fcγ receptors in neutrophils is the subject of further studies, as specifically targeting IgA signaling pathways may represent a novel therapeutic strategy to prevent tissue damage in IgA-mediated autoimmune diseases.

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Neutrophil Dysfunction in the Pre-Hospital Setting following Traumatic Injury: Results from the "Golden Hour" Study

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Providing immediate frontline protection against rapidly dividing bacteria, fungi and yeast, neutrophils are critical effector cells of the innate immune system. In the acute phase (4-12 hours) following major traumatic injury, significant alterations have been described in neutrophil phenotype and function, changes that may contribute to the increased susceptibility of trauma patients to nosocomial infection. However, no study to date has examined neutrophil behaviour in the immediate aftermath of major injury. Thus, it remains to be established how quickly following a traumatic insult neutrophil biology is altered. Here, by analysing blood samples acquired in the *pre-hospital* setting from 75 adult trauma patients (mean time to blood sample 43.69±1.37 minutes post injury) we have performed for the first time a detailed assessment of the composition, function and phenotype of the circulating neutrophil pool in the ultra-acute phase of major trauma.

We observed within minutes of injury a significant neutrophilia, which was accompanied by a marked elevation in the percentage and number of circulating immature granulocytes (IGs), suggesting enhanced bone marrow haemopoietic activity in the immediate post injury phase. Relative to a cohort of age and sex-matched healthy controls, freshly isolated neutrophils from patients exhibited significantly reduced surface expression of CD62L, CD88, CXCR1 and CXCR2 as well as increased expression of CD11b, phenotypical changes that are indicative of neutrophil activation. Furthermore, multi-colour flow cytometry revealed a significant trauma-induced increase in the frequency and absolute number of circulating $CD16^{BRIGHT}$ $CD62L^{DIM}$ suppressive neutrophils. In e*x vivo* functional analysis, neutrophils from trauma patients exhibited impaired phagocytosis of the gram negative bacteria *Escherichia Coli* and were refractory to stimulation with the bacterial peptide formyl-methionine-leucine-phenylalanine as evidenced by significantly reduced up-regulation of CD11b and shedding of L-selectin. The phagocytic activity of neutrophils in *pre-hospital* samples was negatively associated with the absolute number of circulating IGs and $CD16^{BRIGHT}$ $CD62L^{DIM}$ neutrophils. As a readout of neutrophil function *in vivo*, plasma samples from patients were screened for citrullinated histone H3 (CitH3). A marker of neutrophil extracellular trap (NET) formation, we detected CitH3 in *pre-hospital* samples, but not those acquired 4-12 hours or 48-72 hours post injury, thereby demonstrating that NET formation is a feature of the ultra-early immune response to sterile trauma.

Taken together, we have shown for the first time that neutrophil dysfunction is evident within minutes of major traumatic injury and attribute this phenomenon to a trauma-induced creation of a heterogeneous neutrophil pool, consisting of IGs, activated neutrophils and $CD16^{BRIGHT}$ $CD62L^{DIM}$ suppressive neutrophils.

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The Type of Monosaccharides Influences the Metabolic and Functional Responses of Neutrophils Activated with Pro-Inflammatory Cytokines

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Pro-inflammatory cytokines IL-6, TNF and IL-1β are involved in chronic inflammatory diseases. They modulate the responses of immune cells like polymorphonuclear neutrophils (PMNs), but their impact on their bioenergetics is not well defined. Using the extracellular flux analyzer, we observed a quick and robust glycolytic response in TNF-activated PMNs compared to IL-1β- or IL-6-activated cells. TNF also induced rapid oxygen consumption, an effect enhanced with IL-1β. IL-6 or IL-1β alone had no effect on PMNs' oxygen consumption. At the molecular level, we observed that TNF- and IL-1β-activated PMNs had enhanced gene transcripts coding for pro-inflammatory cytokines and for the metabolic adaptor *HIF-1A*. The expression of the glucose transporter *GLUT-3* and the amino acid transporters *CD98* and *ASCT2*

transcripts were only modulated by TNF. Pre-incubation of PMNs with a competitive inhibitor of glucose revealed that the cytokine-activated oxidative burst depended on glycolysis. Since monosaccharides regulate this process, we examined the effect of sugars on TNF and IL-1β-activated PMNs. The cytokine-induced oxidative burst occurred in the presence of sugar-free medium, or in media containing glucose and galactose. This effect was completely inhibited in the presence of mannose, even when glucose and galactose were added. Moreover, the induction of pro-inflammatory cytokine transcripts in response to TNF and IL-1β was significantly increased in PMNs when mannose was used compared to glucose-, galactose- or sugar-free medium. These results indicate that pro-inflammatory cytokines exert different effects on PMNs' energy metabolism and that the type of monosaccharides can influence their cytokine expression and functional responses.

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PHD2 as a Regulator of Myeloid Inflammatory Response

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Hypoxia-inducible factors (HIF) are master regulators of the cellular response to oxygen deprivation and coordinate a transcriptional program that ensures optimal functional, metabolic, and vascular adaptation to oxygen levels. Regulation of these transcription factors relies on the oxygen-dependent enzymes prolyl-hydroxylase domain (PHD1-3) proteins. Although several studies have shown how the hypoxia pathway controls innate and adaptive immunity response in different inflammatory disorders, the role of these oxygen-sensors during inflammation is still largely unknown.

Using an antibody-induced arthritis model, we observed that loss of PHD2 in hematopoietic cells (Vav:cre-PHD2f/f) leads to an augmented inflammatory response, revealed by an increase in paw thickness in conditional deficient mice. Therefore, we hypothesize that PHD2 (main regulator of the HIFα proteins) in hematopoietic cells might have a protective role during the onset of inflammatory arthritis. Indeed, the observed differences in swelling directly correlate with neutrophil/macrophage accumulation in the synovial cavity of these mice. These findings are also supported by data obtained in a model of PMA-induced ear inflammation. Currently, our research is focused on the potential cross-talk between the involved PHD2-deficient myeloid populations and its effect on chemotaxis in both in vivo models.

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Secondary Necrotic Neutrophils Release Interleukin-16C and MIF from Stores in the Cytosol

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Background: Neutrophils harbor a number of preformed effector proteins that allow for immediate antimicrobial functions without the need for time-consuming de novo synthesis. Evidence indicates that neutrophils also contain preformed cytokines, including IL-1ra, CXCL8. The present study aimed to identify additional preformed cytokines in primary human neutrophils and investigate the conditions of their release/secretion.

Materials and methods: In search for additional preformed cytokines, a cytokine array analysis was performed with lysates from human primary neutrophils. Confocal immunofluorescence microscopy as well as western blot analysis of subcellular fractions was carried out to investigate the subcellular localization of cytokines. Neutrophil apoptosis was induced by overnight incubation and by UV irradiation.

Results: IL-16 and macrophage migration inhibitory factor (MIF) were identified as preformed cytokines in human primary neutrophils. Both IL-16 and MIF are unconventional cytokines because they lack a signal sequence. IL-16 and MIF were found to be stored in the cytosol rather than in the granules of human neutrophils, which implies an unconventional secretion mechanism for both cytokines. IL-16 is synthesized and stored as a precursor (pre-IL-16). We present evidence that the processing of pre-IL-16 to the biologically active IL-16C is mediated by caspase-3 and occurs during both spontaneous and UV-induced apoptosis of human neutrophils. Although IL-16 processing occurs during apoptosis, IL-16C and MIF release was observed only during secondary necrosis of neutrophils. Screening a panel of microbial substances and pro-inflammatory

cytokines did not identify any stimulus that induced the release of IL-16C and MIF independent of secondary necrosis.
Conclusions: The data presented here suggest that IL-16 and MIF are neutrophil-derived inflammatory mediators released under conditions of insufficient clearance of apoptotic neutrophils, as typically occurs at sites of infection and autoimmunity.

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Morpho-Functional Changes of Human Neutrophils Treated with Dopaminergic Agents: Relevance for Multiple Sclerosis

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Polymorphonuclear leukocytes (PMN) are innate immune cells that during inflammatory or infective conditions leave the bloodstream and enter the tissues, promoting repair or contributing to the amplification of chronic damage depending on local and systemic factors.
Multiple sclerosis (MS) is the main autoimmune disease of the central nervous system (CNS), and emerging evidence suggest novel roles for PMN in this disease. In MS patients PMN show an activated profile and, in response to pro-inflammatory stimuli, they migrate into the CNS through the compromised blood-brain-barrier, producing proteases and reactive oxygen species (ROS) thus resulting in increased inflammation and disease progression. Dysregulation of dopaminergic pathways in the immune system play a key role in multiple sclerosis (MS), however few information exists on dopaminergic regulation of PMN function in healthy conditions and during MS. Our aim was to investigate the morpho-functional changes induced by dopaminergic agents in PMN from healthy controls (HC) and from MS patients (PTS). Cell migration was measured by optic microscopy and quantified as the difference (Δ) between resting values and values induced by treatments. ROS produced by PMN were assessed by use of the redox-sensitive dye C-DCDHF-DA coupled to spectrofluorimetry. Cell morphology was assessed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). D1-like (D1 and D5) and D2-like (D2, D3 and D4) dopaminergic receptor (DR) expression was analyzed by flow cytometry.
In PMN from HC, migration induced by fMLP 0.1 μM was (mean±SEM) 9.4±1.9 μm. DA 1 μM reduced migration to 26.2±18.0% of fMLP-induced values (n = 5, P<0.01 vs fMLP alone). The effect was mimicked by the D1-like DR agonist SKF-38393 0.1 μM (22.3±6.4% of fMLP-induced values, n = 5, P<0.01 vs fMLP alone) and reverted by the D1-like DR antagonist SCH-23390 1 μM (22.7±13.9%, n = 3, P>0.05 vs fMLP alone, and P<0.05 vs fMLP+DA). The D2-like DR agonist pramipexole 1 μM did not affect migration (not shown). ROS generation induced by fMLP was 302.8±124.1 arbitrary units. DA 1 μM reduced fMLP-induced ROS generation to 63.0±18.0% of fMLP-induced values (n = 11, P<0.01 vs fMLP alone). The effect was mimicked by SKF-38393 0.1 μM (56.7±44.3%, n = 13, P<0.01 vs fMLP alone) but not by pramipexole 1 μM (not shown). DA 1 μM prevented fMLP-induced morphological changes of PMN and was antagonized by SCH-23390 1 μM. Flow cytometric assay of DR showed that PMN were 79-80% and 48-63% positive for D1-like and D2-like DR in HS (n = 9) and 65-72% and 37-63% positive in PTS (n = 7). Functional experiments on PMN from PTS are ongoing.
DA exerts inhibitory effects in human PMN, which might be beneficial in MS. Dopaminergic modulation of PMN function must be however assessed also in cells from PTS.

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Zebrafish as Animal Model to Study Intestinal Inflammation

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Inflammatory Bowel Disease (IBD) is an immunological mediated disorder of the gastrointestinal tract, characterized by uncontrolled inflammation resulting from inappropriate and persistent activation of the mucosal immune system. Although it is widely accepted that IBD results from

a deregulated mucosal immune response to environmental factors in genetically susceptible hosts, the precise cause of the disease has not yet been fully elucidated. The hallmark of active IBD is an aberrant mucosal infiltration by innate immune cells (primarily neutrophils) and adaptive immune cells.

In our laboratory, we developed a novel approach using the zebrafish model to study the intestinal inflammation triggered by the intake of a soybean-meal based diet (50SBM). It is well accepted that soybean is a potent allergen and that produce intestinal inflammation in fish. By the use of Tg(bacmpx:gfp)^{i114} zebrafish larvae line, that labels specifically neutrophils fluorescently, we evaluated the presence of this granulocyte in the intestine, as a marker of an inflammatory process. Likewise, we also determined the effect of the soybean meal on the intestinal barrier integrity by immunofluorescence on intestinal section. Also, qPCR assays were performed to analyse the transcription level of mucosal immunity markers. To determine if the susceptibility to pathogen was increased in larvae with intestinal inflammation, we developed a challenge assay with the enteropathogen *Edwardsiella tarda*.

Our results indicate that after as early as 4 day of feeding, larvae fed with 50SBM showed an increase in the number of neutrophils present in the intestine compared to control ones. Also, experimental larvae were more susceptible to infection. At the moment, we are working on the epithelial integrity assays.

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Human Neutrophils Phagocytose Mycobacteria through Interactions between Laccer and α1,2-Monomannose Side Branches of LAM

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Microorganisms express highly conserved pathogen-associated molecular patterns (PAMPs). These molecules are able to bind to a diverse array of pattern-recognition receptors (PRRs), such as αMβ2-integrin, expressed on the surfaces of neutrophils and macrophages. The binding of PAMPs to PRRs on phagocytes initiates pathogen-specific innate immune responses to eliminate the invading microorganisms. To evade host immune systems, pathogenic mycobacteria express effector molecules, including lipoarabinomannan (LAM), on their cell surface. ManLAM has been associated with several PRRs and binding molecules on phagocytes, including αMβ2-integrin, macrophage mannose receptor, and DC-SIGN. Moreover, *Mycobacteria* have been shown to target lipid rafts (membrane microdomains) to enter into host cells. However, little is known about the molecular mechanisms by which LAM associates with lipid rafts. Previously, we found that a neutral GSL, lactosylceramide (LacCer, CDw17), forms lipid rafts on cell membranes. Moreover, we showed that LacCer-enriched lipid rafts mediate the phagocytosis of non-opsonized mycobacteria by human neutrophils. Here, we investigated the binding specificity of mycobacterial LAM to LacCer and LAM-induced phagocytosis.

We first examined the binding specificity of LAM to LacCer using surface plasmon resonance (SPR). SPR analysis showed that LacCer liposomes bind specifically to *Mycobacterium tuberculosis*-derived mannose-capped LAM (ManLAM). In contrast, ganglioside GM3 liposomes failed to bind to ManLAM. We also tested whether LAM binds to glycosphingolipids-coated plastic plates, finding that both ManLAM and non-pathogenic *M. smegmatis*-derived phosphatidylinositol-capped LAM (PILAM) bind specifically to LacCer- but not GM1-coated plastic plates, In contrast, PILAM from an *M. smegmatis* α1,2-mannosyltransferase deletion mutant (*ΔMSMEG_4247*), which lacks α1,2-monomannose side branches of the LAM mannan core, did not bind to LacCer. Next, we examined the phagocytosis of LAM-coated polystyrene beads by human neutrophils. Neutrophils phagocytosed *M. tuberculosis*-derived ManLAM- and *M. smegmatis*-derived PILAM-coated beads, with the phagocytic indices of these ManLAM- and PILAM-coated beads being similar. Moreover, as observed in the binding experiments, neutrophils did not phagocytose beads coated with PILAM derived from *ΔMSMEG_4247*. Taken together, these findings suggest that, regardless of the pathogenicity of *Mycobacteria*, α(1→2)-monomannose side branches of LAM mannan core are essential for LacCer

binding and for neutrophil phagocytosis of *Mycobacteria* in humans.

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Cytoskeleton Re-Organization during NET Formation

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Neutrophils are most prominent cells in blood circulation and their anti-microbial defence mechanism has been defined by their ability to phagocytise microbes as well as forming Neutrophil Extracellular Trap (NET). For the last decade efforts has been done to elucidate the molecular mechanism of NET formation. In this study we demonstrate that physiological activation of neutrophils generate reactive oxygen species (ROS), specifically hydrogen peroxide (H_2O_2), which then negatively regulate actin polymerization by inducing actin glutathionylation. In absence of NADPH oxidase activity, CGD patients' neutrophils have defect in actin reorganization, and NET formation. Exogenous source of H_2O_2 however, restores actin glutathionylation, and ability of neutrophils to trap and kill bacteria. Consistent with above observations, Grx1-deficient mouse neutrophils upon activation accumulated high level of actin glutathionylation, which attenuated actin polymerization, and impaired NET formation despite presence of high level of ROS.

Taken together these data support our hypothesis that cytoskeleton rearrangements are essential for physiological functions of neutrophils such as NET formation. Pre-requisite for F-actin recycling is ROS activity; which is reversibly regulated by Grx1 enzyme activity. These findings enlighten our understanding of regulated molecular mechanism involved in NET formation.

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Organization and Immunological Functions of Lactosylceramide-Enriched Lipid Rafts

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Neutrophils play a central role in the innate immune system, and express several specific pattern recognition receptors (PRRs) on their cell surface that recognize pathogen- associated molecular patterns (PAMPs) on microorganisms. Microorganisms possess binding avidities to several types of glycosphingolipids (GSLs), suggesting that GSLs are involved in host-pathogen interactions. GSLs cluster to form lipid rafts with several transducer molecules on plasma membranes, which have been implicated in a number of important membrane events. A neutral GSL, lactosylceramide (LacCer, CDwl7) has been shown to bind specifically to several types of pathogenic microorganisms, including *Escherichia coli, Bacillus dysenteriae, Candida albicans* and *Mycobacteria*. LacCer is highly expressed on human neutrophils, and forms lipid rafts coupled with Src family kinase Lyn and trimeric G proteins. LacCer-enriched lipid rafts directly mediate neutrophil chemotaxis, phagocytosis and superoxide generation. Moreover, LacCer-enriched lipid rafts serves as signal transduction platforms for $\alpha_M\beta_2$ integrin-mediated phagocytosis.

Here we introduce how LacCer organizes the lipid rafts with several transducer molecules and mediate signaling. Two different anti-LacCer monoclonal antibodies T5A7 and Huly-ml3 showed the different binding behaviors and specificities. Although Huly-ml3, but not T5A7, immunoprecipitated LacCer-enriched domains, the biological activity of T5A7 on neutrophil functions was much higher than that of Huly-ml3. T5A7 bound to human and mouse neutrophils, while Huly-ml3 only bound to human neutrophils. SPR analysis revealed that the binding affinity of Huly-ml3 but not T5A7 to LacCer depended on the amount of LacCer in the domains. The ultra-high resolution STED microscope observation revealed that Huly-ml3 and T5A7 bound to distinct LacCer domains/clusters. The binding experiments revealed that T5A7 recognizes the phosphatidylcholine (PC)-enhanced three-dimensional structure of LacCer clusters. In contrast, PC did not affect the binding of Huly-m13 to LacCer cluster. It seems, therefore, that Huly-ml3 binds to the core region of lactose clusters in LacCer-enriched domains, while T5A7 binds to dispersed LacCer clusters in the phase boundary regions of the

lipid rafts. Immune-electron microscopic observations revealed that Lyn molecules were located at the phase boundary regions of the rafts. Photo labeling experiments revealed that LacCer directly associated with Lyn and Gαi proteins through the direct connection between C24 fatty acid chains of LacCer and palmitic chains of these signaling molecules. A Src family kinase Hck was not associated with LacCer-enriched lipid rafts in resting neutrophils. During phagocytosis, Hck was associated with LacCer-enriched lipid rafts on the membrane of non-pathogenic but not pathogenic mycobacterial phagosomes. *M. tuberculosis*-derived lipoarabinomannan (ManLAM) directly bound to LacCer clusters. ManLAM inhibited the association of LacCer-enriched lipid rafts with Hck on phagosomal membranes, and prevented phagosome maturation. Thus, ManLAM may interfere the formation of LacCer clusters to make direct association of LacCer and Hck. Taken together, it seems that reorganization of LacCer-enriched lipid raft with signal transducer molecules is essential for immunological functions of neutrophils.

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Optic Atrophy 1 (OPA1) Controls Energy Metabolism and Contributes to Antibacterial Functions of Neutrophils

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Neutrophil extracellular traps (NETs) are implicated in killing extracellular pathogens. Our laboratory demonstrated that activated neutrophils form extracellular DNA traps, whereby the DNA originates from mitochondria.

Content exchange between mitochondria, mitochondrial shape control, and mitochondrial communication with the cytosol are processes that require mitochondrial fusion and fission. OPA1 in inner membrane, and MFN1 and MFN2 in outer membrane of mitochondria are three proteins, which orchestrate fusion of mitochondria in mammalian cells. OPA1, a large GTPase protein, is required for fusion of phospholipid bilayer and tightness of cristae junction in mitochondria. Pathogenic OPA1 mutations cause autosomal dominant optic atrophy (ADOA), a condition characterized by the preferential loss of retinal ganglion cells and subsequent progressive optic nerve degeneration in ADOA patients.

We studied the role of OPA1 in mtDNA release, NET formation and antibacterial activity of neutrophils. We generated mice lacking Opa1 gene specifically in myeloid population, by cross breeding Opa1flox/flox with Lyz2Cre/Cre mice. These mice have Opa1 knockout restricted to myeloid lineage (Opa1Δneut). In addition, we used isolated neutrophils from blood of autosomal dominant optic atrophy (ADOA) patients.

Freshly isolated neutrophils from Opa1Δ mice are unable to form functional NETs while the neutrophils from genetically matched mice (Lyz2Cre/Cre) retain this ability. NETs are known to contribute to antibacterial defence mechanisms. Intranasal inoculation with the nonmucoid Pseudomonas aeruginosa revealed failure of Opa1Δ mice to clear the bacteria from lung despite increased neutrophil infiltration in their lung and intact phagocytic activity compare to control (Lyz2Cre/Cre) mice. We also confirmed the absence of NETs formation by staining of extracellular DNA fibres associated with MPO and infiltrated neutrophils in lung sections of infected Opa1Δmice. Moreover, inability of NETs formation and disruption of microtubule was also observed in ADOA patient's neutrophils. We conclude that, the difference in antibacterial killing ability is mainly due to the fact that Opa1Δneut are not able to form NETs.

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Activation of Neutrophils by Immunoglobulin a (IgA) Exacerbates Pathogenesis of Inflammatory Bowel Disease (IBD)

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Background

Mucosal lesions in IBD, and particularly ulcerative colitis, are characterised by massive neutrophil

infiltration and aggregation. Earlier we showed that binding of IgA, the prominent antibody in the gut mucosa, to its receptor FcalphaRI on neutrophils initiates chemotaxis of these cells. As such, we hypothesize that abnormal activation of neutrophils by mucosal IgA might explain enhanced neutrophil infiltration in IBD, which results in undesirable tissue damage.

Materials and methods

Fresh blood and snapfrozen colon biopsies of IBD patients were stained for different immunological markers and analysed with FACS or immunofluorescence, respectively. Neutrophils were activated by IgA and co-cultured with CaCo2 epithelial cells. A humanised mouse model, expressing both human IgA and FcalphaRI, was used to study the role of IgA/FcalphaRI interactions in pathogenesis in a DSS-induced colitis model.

Results

Phagocytosis of IgA opsonised particles led to activation of neutrophils, leading to extensive reactive oxygen production and release of Neutrophil Extracelluar Traps (NETs), which consisted of aggregated DNA strands covered by reactive enzymes like myeloperoxidase (MPO) and neutrophil Elastase (NE). Culturing 'NETosing' neutrophils with epithelial cells led to enhanced apoptosis and cell death of epithelial cells. In human IBD biopsies enhanced neutrophil infiltration correlated with presence of NETs positive for MPO and NE. FcalphaRI on neutrophils furthermore, resulted in enhanced DSS induced colitis in IgA/FcalphaRI mice, compared to mice that only expressed human IgA.

Conclusions

Aberrant activation of neutrophils in the gut mucosa via IgA/Fcalpha interactions may lead to extensive NETs release, resulting in epithelial cell death and tissue damage. Inhibiting migration and activation of neutrophils by restraining IgA/FcalphaRI interactions can be a valuable new approach to dampen the disease burden.

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Refractory Neutrophils in the Closed Eye Are Associated with Ocular Surface Dysregulation

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Background: The normal, healthy, closed eye is remarkably different than the open eye, and is characterized by many pro-inflammatory cytokines, complement activation products, matrix metalloproteinases, and has a characteristic accumulation of hundreds of thousands of neutrophils–500,000 on average. Previous work has demonstrated that these neutrophils have a refractory phenotype, whereby they do not upregulate surface membrane receptors in response to inflammatory stimuli. However, these neutrophils have an activated phenotype as compared to blood-isolated neutrophils with increased expression of CD66b, CD11b, and CD54. The closed eye leukocyte milieu also contains a small percentage (2%) of T cells. Preliminary investigations have shown that roughly 20% of all CD4+ T cells in the closed eye are Th17 cells. Dry eye disease is a complex multifactorial disease of the ocular surface, which results in symptoms of pain, irritation, and discomfort and may ultimately lead to a significant reduction in quality of life. Dry eye disease is commonly associated with inflammation and Th17 influx, and in severe cases, neutrophil extracellular traps have also shown to be present. The purpose of this pilot investigation was to determine if additional neutrophil hyperactivity or increased T cell infiltration could be observed as part of the closed eye leukocyte population in dry eye disease. Methods: Six normals and six dry eye subjects were recruited and were trained to wash the ocular surface with phosphate buffered saline for at-home self-collection of tear film leukocytes following a full night of sleep. Cells were isolated and counted, and were incubated with fluorescently-labeled antibodies against CD45, CD14, CD15, CD16, CD11b, and CD66b to identify neutrophils. Antibodies against CD45, CD3, CD4, CD8, CD196, and CD161 were used to identify T cells. A Becton Dickinson (BD; San Jose, CA) proprietary fixable viability stain was used to exclude dead cells from analysis. A BD LSR II flow cytometer was used for all analyses. Results: Neutrophils isolated from dry eye subjects demonstrated increased expression of CD11b, CD66b, and CD16 as compared to normals, indicating a hyperactive neutrophil phenotype. Similarly, there were more CD4+ T cells and more Th17 cells (as identified as being CD196+CD161+) in the closed eye leukocyte populations as compared to normals. Combined, the results demonstrate increased inflammation in the dry eye closed eye

compared to the normal closed eye. Conclusions: In a small cohort, the closed eye leukocyte population in dry eye disease appeared to have increased presence of Th17 cells and an associated neutrophil hyperactivity. The normal, homeostatic, closed eye is in an inflammatory state, and this investigation suggests that local inflammatory markers may be upregulated in dry eye disease. This allows for novel biomarkers of study and may offer a potential site for intervention. The authors thank Dr. Karen Ersland for her assistance with flow cytometry.

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Deficiency of NADPH Oxidase NCF2 Synergizes with Other Lupus-Predisposing Genes to Accelerate Lupus in NZM.2328 Mice

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Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by extensive immune dysregulation and loss of tolerance to self nucleic acids, inducing chronic inflammation in multiple organs. Variants in multiple genes involved in immune regulation can influence the development of lupus. Recent genetic studies revealed that missense variants in Neutrophil Cytosolic Factor 2 (NCF2) are associated with SLE. NCF2 is an essential subunit of the leukocyte NADPH oxidase that generates superoxide and derivative reactive oxygen species (ROS), which have both antimicrobial and immunomodulatory functions. Human SLE shows substantial variability in clinical, genetic, disease status and medications, limiting the mechanistic studies on the impact of NCF2 mutations in human SLE leukocyte samples. Thus, to investigate the role of NCF2 in SLE, we generated NCF2 null mice on the C57BL/6 background and backcrossed the mutation into the New Zealand Mixed (NZM).2328 spontaneous lupus model. NZM.2328 mice are an inbred strain derived from NZB x NZW mice, and females develop SLE with lupus nephritis by 7 – 8 months of age. NZM.NCF2-/- knockout mice lacked leukocyte NADPH oxidase activity whereas this was reduced in NZM.NCF2+/- haploinsufficient mice. Interestingly, we found that both female NZM.NCF2-/- and NZM.NCF2+/- mice showed accelerated onset of lupus nephritis. Proteinuria and renal pathologies including glomerular hypercellularity, glomerular crescents, mesangial matrix deposition and interstitial inflammation, were comparable in 4 month old NZM.NCF2-/-, ~5.5 month old NZM.NCF2+/- and ~7.5 months old wild type NZM females. Correspondingly, survival was shortened in NCF2 null mice (4~6 months), followed by the haploinsufficient strain (6~10 months), as compared to NZM.NCF2+/+ mice (8~12 months). We evaluated splenic immune cell populations and found that the appearance of hyperactive B cells including GC-B cells and antibody producing plasma cells, and T cells including activated T and CD69+ T cells was significantly accelerated in NZM.NCF2-/- mice. Moreover, increased type I IFN activity is spotlighted as a mediator in autoimmune disorders such as SLE. We demonstrated that a type I IFN signature gene expression was significantly increased in NZM.NCF2-/- mice even in the pre-disease state (7 wks age old females). Finally, neutrophil extracellular traps (NETs) have been associated with SLE pathogenesis. Given the hypothesized role of NETosis as a potential mediator to induce type I IFN signaling and inflammation and/or a role as autoantigens in SLE, we measured NET formation. As expected, NCF2-null PMNs did not release NETs following phorbol myristate acetate stimulation. However, lupus serum stimulated NETosis by NCF2-null neutrophils to a similar extent as wild type NZM neutrophils, as studied in 7 wk old mice. In conclusion, deficiency of NCF2 and NADPH oxidase activity synergizes with other lupus-predisposing genes in NZM mice to accelerate immune dysregulation and the emergence of lupus. This study complements the genetic studies in human SLE that identified NCF2 variants as linked to SLE, and establishes a new experimental system to dissect mechanisms for the role of NADPH oxidase-derived ROS in regulating autoimmunity.

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Neutrophil Activation in Systemic Anaphylaxis: Results from the Multicentric NASA Study

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Background

Anaphylaxis is a severe systemic allergic reaction that can be life-threatening. Evidence of the classical anaphylaxis pathway, which involves IgE and IgE receptors, can be detected in most (85%) but not all cases. An alternative pathway involving IgG and neutrohil IgG receptors (FcγRs) has recently been suggested by animal models. We hypothesized that such a mechanism may also exist in humans and studied this possibility in a multicentric prospective cohort of patients suspected of perioperative anaphylaxis to neuromuscular blocking agents (NMBA).

Materials and Methods

Consecutive patients suspected of perioperative anaphylaxis (n=86 cases) were recruited and paired with 86 control patients. Blood samples were collected for cases and controls promptly after anesthesia induction. For cases, an extensive allergological evaluation was performed 6-8 weeks after the reaction. Circulating elastase, neutrophil extracellular traps (NETs), tryptase, histamine, and IgG and IgE anti-NMBA were measured by ELISA. FcγR expression on the major blood cell populations was analyzed by flow cytometry.

Results

We found higher circulating NETs and elastase levels during an anaphylactic reaction as compared to controls. IgG anti-NMBA were found in both cases and controls, however for cases the IgG titer was associated with anaphylaxis severity. Finally, we show a significant decrease of neutrophil FcγR expression, pointing towards their engagement by immune complexes. This decrease correlated significantly with NET release and with the severity of the anaphylactic reaction. Together, our results strongly suggest an activation of neutrophils by NBMA-IgG complexes during anaphylaxis.

Conclusion

We provide for the first time evidence for of an IgG-dependent neutrophil activation pathway during anaphylaxis in human. This additional mechanism opens potential applications in anaphylaxis diagnostics and treatment.

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Neutrophils and Monocytes Induce a Synergistic Increase of Blister Formation in a Human Skin Cryosection Model of Bullous Pemphigoid

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Background: Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease characterized by tissue bound and circulating autoantibodies to the hemidesmosomal antigens BP180 (BPAG2, type XVII collagen) and BP230 (BPAG1). Although neutrophils have been described to mediate blister formation, the contribution of other immune cells to neutrophil-dependent tissue damage in BP is unknown.

Objective: To investigate whether neutrophils collaborate with other immune cells in the induction of BP autoantibody-dependent blister formation and the mechanisms involved.

Methods: In an *ex vivo* skin model, cryosections of normal skin were incubated with purified human neutrophils and peripheral blood mononuclear cells (PBMCs) in the presence or absence of BP autoantibodies. Reactive oxygen species (ROS) and granule protein release was inhibited by using chemical inhibitors of NADPH oxidase or serine proteases, myeloperoxidase or MMP-9 respectively. The dermal-epidermal separation (DES) was assessed by light microscopy and quantified by Fiji

software.
Results: Neutrophils synergised with monocytes to induce enhanced DES in a BP autoantibody-dependent manner. This synergistic effect on DES was inhibited by the NADPH-oxidase inhibitor diphenyleneiodonium (DPI). Blockage of MMP-9 resulted in decreased DES
Conclusion: Our study revealed a partnership between neutrophils and monocytes, resulting in increased MMP-9 release, leading to an enhancement of BPS-dependent DES in a human BP model. More studies are needed to elucidate the role of an immunological synapse between neutrophils and monocytes, and the molecular pathways and mechanisms involved, in their synergistic induction of skin blister formation in BP.

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Do NETs Matter? Establishing a Role for Neutrophil Extracellular Traps in Patho-Biology of Indirect Acute Lung Injury

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Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) represent clinical syndromes of acute respiratory failure, with an incidence rate between 80 and 60 per 100,000 per year, respectively. Indirect ALI (iALI) is caused by a non pulmonary inflammatory process as a result from insults such as non-pulmonary sepsis, hypotensive shock, etc. Neutrophils are thought to have a significant role in mediating ALI, with the development of iALI being characterized by dysregulation and recruitment of activated neutrophils to the lung. Recently a novel mechanism of microbial killing by neutrophils was identified through the formation of neutrophil extracellular traps (NETs). NETs are comprised of large webs of decondensed chromatin coated with granule proteins that are released from activated neutrophils into the extracellular space which is regulated by the enzyme PAD4 through mediating chromatin decondensation via citrullination of target histones. Components of NETs including neutrophil derived circulating free DNA as well as circulating histones has been implicated in ALI. However, it is unknown if there is any pathological significance of NET formation in ALI caused by non-pulmonary insult. Firstly, utilizing blood neutrophils obtained from WT and PAD4-/- mice we determined that the only neutrophil functional deficit in the PAD4-/- mice was the inability to make NETS; since neutrophils from both groups were comparable in ex vivo motility, ROS production, and phagocytosis killing. To examine the role of NETs in iALI we subjected PAD4-/- mice and WT mice to a “2 hit” model of traumatic shock (fixed-pressure hemorrhage; Hem) followed by septic (CLP) insult (Hem/CLP). Mice were hemorrhaged, resuscitated, 24 hours post-Hem mice were then subjected to CLP, and 24 h later lung tissue, blood, and bronchial lavage fluid (BAL) samples were collected. To evaluate PMN targeting to the lung we looked at MIP-2, KC, and MPO levels in lung tissue after Hem/CLP. PAD4-/- mice displayed an increase in MIP-2 levels compared to WT mice while KC levels were comparably elevated in both WT and PAD4-/-. However, there was a marked decrease in MPO in PAD4-/- lung tissue. To the extent that these changes were associated with local lung as opposed to systemic cytokine response to iALI we noted that while, there were no differences in BAL IL-10 or TNF-α levels between the two groups, PAD4-/- mice displayed a marked decrease in IL-6 levels in BAL after Hem/CLP. PAD4-/- appears to have no significant effect on indices of lung injury, as increased vascular permeability was noted in the lungs of both negative control and experimental mice. Conclusion: These data taken together suggest that NET formation appears to contribute to neutrophil influx as well as the pro-inflammatory response within the lung, in response to pathological processes regulating iALI here

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Neutrophils Contribute to Pathology but not Neovascularization following Ocular Herpes Simplex Virus Type 1 Infection

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Neutrophils - one of the “first responders” to ocular herpes simplex virus type 1 (HSV-1) infection - traffic to the cornea within the first 24 hr following infection. Previous studies indicated depletion of Gr-1+ ‘neutrophils’ exacerbated virus replication and spread. In addition, earlier studies reported

neutrophils contributed to HSV-1-induced neovascularization of the normally avascular cornea through the production of pro-angiogenic factors and enzymes. We proposed to follow-up these reports by other groups and more closely evaluate the role of neutrophils in ocular pathology in response to acute corneal HSV-1 infection. Initially using IFNAR1-deficient (CD118-/-) and CCL2-deficient mice along with neutrophil-specific depleting antibody—Ly6G (1A8), we found inflammatory monocytes (F4/80+Gr-1+) but not neutrophils (F4/80– Gr-1+) controlled HSV-1 infection within the first 48-72 hr post ocular infection - as measured by flow cytometry and viral plaque assay. These results were consistent with our findings that showed an increase in infectious virus recovered in the cornea of mast cell-deficient KitW-sh mice, which was associated with a specific increase in neutrophil infiltration but not that of other myeloid populations. Enrichment of neutrophils from the corneas of infected mast cell-deficient KitW-sh mice using immunomagnetic separation showed they possessed an elevation in the HSV-1 lytic gene, *thymidine kinase*, in comparison to enriched neutrophils from wild type mice which we interpreted to suggest the cells were possibly a source of virus replication. Indeed, upon transfer of neutrophils from the cornea of infected mice into naive CD118-/- mice resulted in significant mortality reinforcing the notion that neutrophils were a source of HSV-1 replication and likely contributed to virus dissemination. We further investigated the role of neutrophils in corneal pathology by assessment of corneal neovascularization following HSV-1 infection of mice. Depletion of neutrophils as well as other leukocyte populations in the cornea using anti-Gr-1 (RB6-8C5) antibody had no significant consequence on the genesis of corneal blood or lymphatic vessels in response to HSV-1 infection as measured by confocal microscopy. In contrast, the intraperitoneal administration of the potent anti-inflammatory steroid, dexamethasone, as a single bolus following ocular clearance of virus dramatically reduced neovascularization without compromising the number of leukocytes, including neutrophils, residing in the cornea. In summary, our results show neutrophils are a detriment to the host in response to ocular HSV-1 infection as they serve as an additional source for virus replication and do not contribute to viral surveillance during acute infection of mice. However, in contrast to previously published data, our results suggest neutrophils do not play a significant role in the neovascularization of the mouse cornea following HSV-1 infection.

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Tumor-Associated Neutrophils from a Bird's Eye View

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Tumor-associated neutrophils (TAN) were recently identified as the most important prognostic leucocyte subset associated with an adverse outcome over a broad range of tumor diseases. However, the underlying pathophysiological mechanisms still remain unclear and a dichotomy of pro- versus antitumoral functions is described in the literature. Insight into TAN function is yet restricted to classical histology or in vitro models. In this project we combine the advances of intravital two-photon imaging with a novel transgenic mouse model with red fluorescent neutrophils (Catchup^{IVM}) to longitudinally explore the interaction of TAN and tumors in the living mouse. Additionally the establishment of a tumor clearing protocol allows for three-dimensional reconstruction of whole tumor samples. With this approach we were able to monitor neutrophil infiltration in newly established tumors and characterize the migratory behavior of TAN in the tumor environment. Ultimately, this project will provide novel insight into the complex biology of TAN leading to innovative approaches for neutrophil targeting in the tumor host.
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Hypoxia Upregulates PI3Kinase-Dependent Neutrophil Degranulation and Neutrophil-Mediated Tissue Injury

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Introduction:
Neutrophils are the first line of defence against invading pathogens. However, damage to host tissue from persistent neutrophilic inflammation has been implicated in the pathogenesis of many diseases, including chronic obstructive pulmonary disease (COPD). Infected and inflamed tissues can be profoundly hypoxic; hypoxia promotes a destructive neutrophil phenotype with prolonged neutrophil survival and augmented degranulation of histotoxic proteases. Hence, hypoxic neutrophils have the potential for enhanced tissue damage.
Methods:
Neutrophils from healthy volunteers were isolated by centrifugation over discontinuous plasma-Percoll® gradients. Neutrophils were re-suspended in IMDM under normoxia (21% O_2) or hypoxia (0.8% O_2; Ruskinn SCI-tive dual hypoxia workstation) for 4 hours, and subsequently treated with granulocyte-macrophage colony-stimulating factor (GM-CSF), platelet activating factor (PAF) or tumour necrosis factor-α (TNFα), followed by formylated peptide (fMLP). Neutrophil elastase (NE) activity was measured by Enzchek® assay. Western blotting for total and phosphorylated Akt was performed on cell lysates. Neutrophil extracellular trap (NET) production was assessed by incubation of neutrophils with SYTOX Green and measurement of extracellular DNA by fluorescence absorbance. Neutrophil supernatants were incubated with primary human pulmonary artery endothelial cells (HPAEC, Lonza) for 4, 24 or 48 hours. HPAEC death and detachment were measured by MTT assay and confocal microscopy; HPAEC activation was assessed by flow cytometric analysis of ICAM-1, VCAM-1 and E-selectin. TCA-precipitated neutrophil supernatants were separated by SDS polyacrylamide gel electrophoresis (PAGE) and silver stained. S100A8, S100A9 and A8/A9 heterodimer content of neutrophil supernatants was assessed by ELISA.
Results:
Following exposure to hypoxia, neutrophils treated with GM-CSF or PAF, and fMLP displayed significantly greater NE release. TNFα treatment did not increase NE release under hypoxia. Hypoxia also augmented resting and cytokine-stimulated Akt phosphorylation; PI3Kinase-γ inhibition abrogated Akt phosphorylation and prevented the hypoxic uplift of NE release. There was no increase NET release in response to hypoxia alone or in combination with GM-CSF/fMLP. Hypoxic neutrophil supernatants induced extensive HPAEC detachment and death, which was prevented by co-incubation with the anti-protease alpha-1 antitrypsin. Neither normoxic nor hypoxic neutrophil supernatants changed HPAEC expression of adhesion molecules ICAM-1, VCAM-1 or E-selectin. Silver stained protein bands from precipitated neutrophil supernatants separated by SDS-PAGE, identified by mass spectrometry, suggested a hypoxic increase in damage associated molecular pattern (DAMP) proteins S100A8 and S100A9 (cytoplasmic rather than granule-associated). However, when this was interrogated by ELISA, there was no significant difference between the amount of S100A8, S100A9 or A8/A9 heterodimer in normoxic versus hypoxic supernatants.
Conclusion:
Hypoxia augments neutrophil azurophilic granule exocytosis in a priming agonist- and PI3Kinase-γ-dependent manner but NET production is not increased. Hypoxic neutrophil supernatants have enhanced capacity to damage endothelial cells *in vitro* but do not affect endothelial adhesion molecule expression. Endothelial cell damage is likely due to increased release of neutrophil elastase and can be prevented by the addition of alpha-1 antitrypsin. The contribution of S100A8 and S100A9 proteins to this damage is currently unclear. Hence, hypoxia promotes a destructive neutrophil phenotype with enhanced capacity to damage pulmonary endothelial cells *in vitro*, with relevance to chronic inflammatory diseases such as COPD.

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Rhesus Monkey Neutrophils are Hyper Reactive as Compared to Human and Marmoset Monkey Neutrophils; the Rhesus Monkey Experimental Autoimmune Encephalomyelitis (EAE) as a Model for Acute Disseminated Encephalomyelitis (ADEM).

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The rhesus monkey experimental autoimmune encephalomyelitis (EAE) model is characterized by an early onset after disease induction and rapid deterioration. Attempts to alter the disease course in this species have been largely unsuccessful. EAE models in the other non-human primates, cynomolgous and marmoset monkeys, have a less acute and destructive disease course.
In rhesus monkeys, EAE onset is preceded by a peripheral neutrophilia and lesions are characterized by perivenular lesions with a prominent influx of macrophages and neutrophils. This is in contrast to the lesions in MS patients and the EAE model in marmosets in particular, which are characterized by T-cell infiltrates and macrophages. The pathology and clinical presentation observed in the rhesus monkey EAE model is much more reminiscent of human acute disseminated encephalomyelitis (ADEM), or even its most severe variants acute haemorrhagic
leucoencephalitis/leukoencephalomyelitis
(AHL/AHEM), two forms of acquired demyelinating syndromes mostly observed in children that have been linked to viral infections and vaccination. The involvement of neutrophils in the rhesus monkey EAE model with the subsequent differences in pathology and in contrast to EAE in marmosets or cynomolgous monkeys points to a particular role of neutrophils in this species. The prominent role of neutrophils in rhesus monkeys has also been observed in other disease models, such as collagen-induced arthritis (CIA).
Characterization of rhesus monkey neutrophils in vitro indicates that they have a higher tendency to produce reactive oxygen species (ROS) as compared to humans or marmosets.
The EAE and CIA models in rhesus monkeys have a clear dependency on autoantibody induction, which correlates with ADEM and the childhood version of arthritis, juvenile idiopathic arthritis (JIA). Similar to what has been described for systemic lupus erythematosus (SLE), we are currently studying the role of Fc receptor mediated activation of neutrophils by disease specific autoimmune antibodies.
The rhesus monkey EAE model presents a unique opportunity to study neutrophil mediated inflammation and may be useful for evaluation of treatments aimed at the modulation of neutrophils in general, and more specifically for ADEM or AHL.

Functional and Metabolic Reprogramming Drives the Development of a Pathogenic Subset of Neutrophils in Inflammatory Airway Diseases

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Airway inflammation in Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD), and severe asthma is in part characterized by the massive recruitment of neutrophils (PMNs) into the lumen of the airways. In CF in particular, neutrophil elastase (NE) that is released from the primary granules of PMNs is a strong predictor of lung function and survival. However, it is still not clear why and how long PMNs are able to survive in the airways. **Methods:** Blood and sputum were collected from CF, COPD, severe asthma, and HC patients, and analyzed for viability, degranulation, and other functional changes. In a transepithelial migration assay, airway fluid from patients with CF, COPD, and severe asthma were used to recruit PMNs and analyzed for survival, degranulation, metabolic activity, and bacterial killing capacity. **Results:** From these analyses we observed that (i) CF, COPD, and severe asthma airway fluid induces rapid migration and survival of neutrophils, (ii) live PMNs release their primary granules and (iii) migration towards CF airway fluid metabolic reprograms PMNs to become increasingly glycolytic while at the same time decreasing their ability to kill *P. aeruginosa*. Finally, (iv) In our model system we can utilize inhibitors of neutrophil migration (LTA4H inhibitor) that significantly reduces the chronic influx of PMNs. **Conclusions:** These data taken together suggest a primary role for the inflammatory airway microenvironment in inducing increased survival, phenotypic and metabolic reprogramming of PMN and identifies a pathogenic subset of neutrophils that can targeted therapeutically in various inflammatory airway diseases.

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Galangin as Modulator of NADPH Oxidase and Myeloperoxidase Activity in Peripheral Blood Neutrophils from Patients with Rheumatoid Arthritis

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Introduction and Objective: Rheumatoid arthritis (RA) is a chronic disease characterized by synovial tissue inflammation, where neutrophils are the most abundant cells. These cells are important players in tissue damage in RA, which involves the excessive production of reactive oxygen species (ROS) triggered by immune complexes (IC) via Fcg and complement receptors (FcgR and CR, respectively). NADPH oxidase (NADPHox) and myeloperoxidase (MPO) are the key enzymes that generate ROS in activated neutrophils. In this sense, modulation of neutrophil ROS generation can help to maintain the tissue homeostasis in RA. Flavonols are natural compounds with promising anti-inflammatory and immunomodulating activity that have emerged as selective modulators of the effector functions of neutrophils. In this study, we examined whether galangin modulates the neutrophil NADPH oxidase and MPO activities in methotrexate-treated RA patients with active disease who do not respond very effectively to this drug therapy. **Methods:** Peripheral blood was collected from healthy volunteers and RA patients, and neutrophils were isolated by the gelatin method. The total ROS production was measured by the luminol-enhanced chemiluminescence assay. The NADPH oxidase activity was assessed by the lucigenin-enhanced chemiluminescence assay, which detects the reaction product superoxide anion. The MPO activity was measured by a colorimetric assay using 3,3′,5,5′-tetramethylbenzidine, which detects the end-product taurine chloramine formed by the reaction of HOCl with taurine. **Results:** The levels of total ROS, superoxide anion, and taurine chloramine production in RA patients' neutrophils were significantly higher than those detected in healthy subjects' neutrophils ($p < 0.001$). Galangin equally suppressed the production of all the aforementioned oxidant species in RA patients' and healthy subjects' neutrophils: it inhibited nearly 60% of total ROS production at 2.5 µM, 30% of MPO activity / taurine chloramine production at 20 µM, and ~50% of NADPH oxidase activity / superoxide anion production at 5 µM. **Conclusion:** Galangin acts as an effective inhibitor of IC-stimulated neutrophil ROS generation by modulating the activities of NADPH oxidase and MPO, in RA patients who are resistant to methotrexate therapy. The use of this flavonol to modulate neutrophil functions can be a promising therapeutic strategy to control inflammation in RA patients. Support: FAPESP, CNPq.

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Neutrophils' Influx and Propensity to Form NETs is Regulated by SLPI in Experimental Model of Psoriasis

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Introduction

Psoriasis is a chronic autoinflammatory skin condition. As skin infiltrating neutrophils that often form epidermal microabscesses are a hallmark of psoriasis, it is likely that these cells play a role in the disease. Neutrophils have been implicated in variety of autoimmune-like conditions, largely due to their ability to form neutrophil extracellular traps (NETs). These structures can serve as a source of autoantigens as well as stimulate production of type I interferon (IFNI) in plasmacytoid dendritic cells (pDC). The production of IFNI by pDCs is considered pivotal for the development of psoriasis, indicating that NETs may play a role at early stages of the disease. Neutrophil elastase (NE) – enzyme crucial for NET formation (NETosis) – is inhibited by secretory leukocyte protease inhibitor (SLPI). Together, these data suggest that SLPI can impact the severity of this disease possibly by regulating NETosis.

Methods

To investigate if/how neutrophils and SLPI contribute to psoriasis we took advantage of experimental model that closely resembles human disease. The model is based on multiple applications of imiquimod (TLR7-ligand)-containing cream

(Aldara) on murine skin. C57Bl/6xSvj129 SLPI knock-out and wild type mice were treated twice a day for up to 6 days and analyzed daily for psoriasis-like changes by macroscopic and microscopic observation. Next SLPI+/+ and -/- animals were injected intracuteneously with SLPI in PBS or PBS only and subsequently treated with Aldara for 2 days. Samples of treated skin were analyzed by histology and immunofluorescence.
Results
SLPI deficient mice exhibited increase in scaling and expanded skin lesions which are indicative of more severe psoriasis. These changes were accompanied by enhanced skin inflammation measured by the amount of infiltrating leucocytes in lesions. These included Ly6G+ granulocytes that were predominant immune cells observed at early stages of skin alteration. Interestingly, neutrophils from the early influx showed increased propensity for NET-formation in SLPI KO mice as compared to control mice. The ectopic delivery of recombinant SLPI alleviated skin inflammation and significantly reduced frequency of neutrophils producing NETs in SLPI -/- animals.
Conclusions
The differences in abundance and function of neutrophils and corresponding severity of psoriatic symptoms are dependent on the presence of SLPI which suggests that regulation of NETosis may play a role in the development of psoriasis. Together, these findings establish a protective role for SLPI during the pathogenesis of experimental psoriasis.
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Different Functions of Neutrophil-Derived Microvesicles and Trails

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Neutrophils release different types of extracellular vesicles with diverse biological activities. Neutrophil-derived microvesicles induces aggregation of bacteria and, hence, arrest the growth of bacteria. Neutrophils also deposit chemokine-containing extracellular vesicles known as trail, which can guide the migration of virus-specific CD8+ T cells. Although these neutrophil-derived extracellular vesicles have diverse functions ranging from the immune modulation to antimicrobial activity, their specific characterization has not been fully understood. Here, we studied the differences in compositions and functions between neutrophil-derived microvesicles and trails. We investigated the effects of different stimulants on microvesicles and trails formation. Chemoattractants, inflammatory cytokines, and bacteria induced the generation of microvesicles and trails from neutrophils. Microvesicles and trails showed similar patterns of surface marker expression including phosphatidylserine and MCP-1. Both mircovesicles and trails have direct bactericidal activity and induced chemotaxis of monocytes. However, microvesicle and trails have different effects on the phenotype polarization of macrophages. Additionally, neutrophil-derived extracellular vesicles were also detected in the serum of healthy donors, and their number was significantly increased in the serum of septic patients. Together, our study suggests the important insights into the understanding the neutrophil-derived microvesicles.

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Targeting Specific Phosphoinositide 3-Kinase Isoforms Reduces Damaging Neutrophil Functions without Impairing Bacterial Phagocytosis in Chronic Obstructive Pulmonary Disease

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INTRODUCTION: Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory lung condition, with systemic manifestations, and is the fourth leading cause of death worldwide. Neutrophils are centrally implicated in COPD pathogenesis and their

numbers, and products, correlate with clinical decline. Inaccurate neutrophil migration, increased degranulation and excess superoxide release have all previously been described and may contribute to disease development and lung tissue damage. Less is known about phagocytosis. There are currently no disease-modifying treatments available for patients with COPD and generic anti-inflammatory preparations do not prevent COPD progression. There is great interest in modifying disease-specific targets to improve outcomes and phosphoinositide 3 kinase (PI3K) has been identified as a potential target. Delta and Gamma isoforms of class I PI3K are of particular interest, as these are central to accurate migration. We wished to assess if a PI3Kdelta selective inhibitor (PI3Kdi), PI3Kgamma selective inhibitor (PI3Kgi) or SHIP1 activator (SHIP1a) could improve neutrophil migration in COPD without inhibiting other cell functions important for bacterial clearance. **METHODS**: Peripheral blood neutrophils were isolated from 70 COPD patients and 70 age matched controls (aged 39 – 80 years). Functional assays were also conducted with PI3Kgi, PI3Kdi, SHIPa or vehicle control (DMSO). Neutrophil migration was assessed using an Insall chamber towards CXCL8, CXCL1, LTB4 and fMLP. Surface expression of CD63 (a measure of primary granule release) and phagocytosis of opsonised and non-opsonised pHrodo-conjugated *Staphylococcus aureus*, or alexa-fluor488 labelled *Streptococcus pneumoniae* and *Haemophilus influnzae* was assessed by flow cytometry. Extracellular reactive oxygen species (ROS) production was studied using an isoluminol assay in basal conditions and following stimulation with fMLP.
RESULTS: Neutrophil migration in COPD remained faster but less accurate than healthy controls across all age groups. COPD neutrophils demonstrated systemic cell activation (evidenced by increased basal cell polarization), and increased degranulation (evidenced by increased CD63 expression). This cell phenotype could not be replicated by exposing "healthy" neutrophils to COPD plasma. Phagocytosis of all bacterial species was preserved in COPD but total extracellular reactive oxygen species production was increased. The PI3K delta and gamma isoform selective inhibitors and SHIP1-activator restored neutrophil migratory accuracy and reduced total extracellular reactive oxygen species production. At the same concentration, these compounds did not impair opsonised or non-opsonised phagocytosis of *Staphylococcus aureus*.
CONCLUSIONS: COPD neutrophils display a functional phenotype favouring host damage that may contribute to disease pathogenesis. The PI3Kgi/PI3di/SHIP1a restore these functions to "healthy" levels, without impairing bacterial phagocytosis. Their use in COPD may reduce the potential for neutrophil-related host damage while maintaining bacterial clearance. This further supports their development as potential therapies.

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Peptidomimetics as novel formyl peptide receptor 2 (FPR2) modulators in human and mouse neutrophils

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Formyl peptide receptor 2 (FPR2) is a chemoattractant receptor belonging to the family of G-protein coupled receptors (GPCRs). FPR2 is abundantly expressed in neutrophils and have a major regulatory role in the inflammatory processes involving these cells, indicating that modulators of FPR2 have therapeutic potential. However, most molecules that interact with the receptor are peptides that display low bioavailability and low affinity for the mouse counterpart (Fpr2), which render them inadequate for animal studies. Peptidomimetics are peptide-like molecules that have unnatural residues incorporated in their backbones, thus making them proteolytically stable and attractive as novel modulators for both FPR2 and Fpr2. Isolated human peripheral blood neutrophils and bone marrow-derived mouse neutrophils were used in this study**.** A library of lipidated α-peptide/β-peptoid hybrid peptidomimetics was screened for the ability to: (i) activate/inhibit the NADPH-oxidase in human and mouse neutrophils, (ii) induce an intracellular calcium transient in HL-60 cells transfected to overexpress FPR2 or FPR1. Through the screening process, peptidomimetics that activates NADPH-oxidase activity in both human and mouse

neutrophils were identified. The lipid headgroup was found to be critical for the agonistic activity. The activities of the compounds were sensitive to FPR2 antagonists and the receptor specificity was confirmed in the cells overexpressing either of the two FPRs.
In conclusion, a novel class of lipidated α-peptide/β-peptoid hybrid peptidomimetics was found to activate both human and mouse neutrophils. The preferred receptor was human FPR2 and the mouse orthologue Fpr2. This novel class of peptidomimetics is expected be an excellent tool in mouse models of human diseases as they enable experiments that will increase our understanding of the role of FPR2 in the complex processes of inflammation, and they might constitute the basis for the development of therapeutic drugs that specifically target FPR2.

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Pepducins Designed to Target Their Cognate G-Protein Coupled Receptors FPR1, $P2Y_2R$ And, CXCR4 Allosterically Modulate the Function of FPR2

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Background: Pepducins are a class of lipopeptides with a short peptide sequence identical to one of the intracellular domains of a G-protein coupled receptor (GPCR) that act as allosteric GPCR activators or inhibitors. Their activities are transduced following interaction with a receptor distinct from the orthosteric binding site used by conventional ligands. The physio-chemical properties of a pepducin (i.e. fatty acid chain, charge and hydrophobicity) suggest a "flipping" of the molecule through the cell membrane and an interaction directly with the cytosolic parts of the cognate receptor. Human neutrophils express many GPCRs important in immune regulation including $P2Y_2R$, the receptor for the "danger signal" ATP, the chemoattractant receptors CXCR4, as well as the pattern recognition receptor FPR1 and the closely related receptor FPR2. Using the pepducin approach, we have earlier shown that pepducins with amino acid sequences derived from the third intracellular loop of FPR2 specifically activate this receptor. In this study, we have determined the effects (rise in intracellular Ca^{2+} and NADPH-oxidase activity) of pepducins generated from some other GPCRs expressed in neutrophils.
Results: A pepducin with an amino acid sequence derived from the third intracellular loop of FPR2 (F2Pal) specifically activates this receptor and the signaling cascade initiated was similar to the one triggered by a classical FPR2 agonist. A pepducin with a peptide sequence identical to the third intracellular loop of FPR1 (F1Pal), however, inhibited neutrophil function and unexpectedly the inhibition was found to be selective for FPR2 but not FPR1. Also pepducins with amino acid sequences corresponding to the second and third intracellular loops of $P2Y_2R$ activated neutrophils. But the receptor desensitization profile and the effects of receptor specific antagonists suggest the involvement of FPR2 rather than $P2Y_2R$. Similarly to FPR1 and $P2Y_2R$ pepducins, a pepducin derived from the first intracellular loop of CXCR4 (ATI-2341) targeted FPR2 to activate neutrophils.
Conclusions: In summary, our data demonstrate that pepducins designed to target FPR1, $P2Y_2R$, and CXCR4 constitute a novel class of modulators for FPR2. These findings are in contrast to the generally accepted dogma for the mode of action of pepducins and raise the possibilities of FPR2 being a pattern recognition receptor for lipopeptide structures, as well as a possible "off-target" receptor for pepducins designed to allosterically modulate other receptors.

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An Emerging Oral Pathogen, Peptoanaerobacter Stomatis, Promotes Degranulation and Primes the Respiratory Burst Response in Human Neutrophils.

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Peptoanaerobacter stomatis is a newly appreciated species associated with chronic inflammatory periodontal disease. However, little is known about this organism's potential virulence and its interaction with the host immune system. Therefore, to better understand the role of *P. stomatis* in periodontitis, we studied its interactions with human neutrophils.

P. stomatis strain CM2 was used to challenge neutrophils at a multiplicity of infection of 10. Bacterial internalization was examined by imaging flow cytometry and bacterial viability was assessed by colony-forming unit assays. Phagocytosis-stimulated respiratory burst response was measured by flow cytometry, and superoxide release by the reduction of ferricytochrome *c*. Exocytosis of secretory vesicles, specific granules and azurophil granules was determined using flow cytometry to measure the expression of CD35, CD66b and CD63, respectively. Exocytosis of gelatinase granules was determined by ELISA for gelatinase (MMP9) release. After 30 min of incubation, only 25% of the *P. stomatis* associated with neutrophils in suspension were internalized. Approximately 43% of the bacterial inoculum was killed within a 30 min interaction with neutrophils. *P. stomatis* induced a robust intracellular respiratory burst response compared to *S. aureus*. Minimal superoxide release was observed by the bacterial challenge after 5-30 min, however *P. stomatis* significantly increased fMLF-stimulated superoxide release to a similar extent as cells primed with TNFα. *P. stomatis* induced significant exocytosis of secretory vesicles, gelatinase granules, specific granules, and azurophil granules. These results suggest that although neutrophils have a low phagocytic efficiency for *P. stomatis*; robust intracellular ROS production nonetheless occurs, and exocytosis of the four neutrophil granules is induced. The release of reactive oxygen intermediates and matrix metalloproteinases such as MMP9 from neutrophil granules could contribute to tissue damage in periodontal disease.

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Autophagy Primes Neutrophils for Neutrophil Extracellular Trap Formation during Sepsis

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Excessive or dysregulated functions of neutrophils are considered responsible for the pathogenesis of sepsis. To understand the function of neutrophil during sepsis, we hypothesized that neutrophils from septic patients are either primed or easily stimulated, and autophagy is responsible for the activation of neutrophils. To investigate this, we isolated neutrophils from community acquired pneumonia-induced septic patients, and investigated the functions of neutrophils. Neutrophils were isolated from septic patients who have admitted to the intensive care units, and the morphology, the expression of surface phenotypic markers, the generation of reactive oxygen species (ROS), neutrophil extracellular traps (NETs) formation, granule release, and autophagy were examined. Neutrophils isolated from septic patients showed increased vacuolization with decreased mean lobe counts and showed several changes in phenotypic markers. Further, sepsis neutrophils have increased NETs formation and degranulation markers in response to stimulation. We also found that autophagy is responsible for the priming effect of sepsis neutrophils. Moreover, sepsis neutrophils from non-survivor showed impairment in autophagy with decreased NETs formation. In murine sepsis model, the enhancement of neutrophil autophagy improved survival via increased NETs formation. Together, our study suggests an important insights into role of autophagy in neutrophils during sepsis.

Cross-Talk of Dendritic Cells with Neutrophils that have been Activated with Immunoglobulin A

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Introduction
Immunoglobulin A (IgA) is the most prevalent antibody at mucosal sites and a potent stimulus of neutrophils (PMN) via the IgA Fc receptor (FcαRI). In patients with ulcerative colitis, mucosal infiltration of PMNs as well as interaction with dendritic cells (DCs) is seen. We hypothesize that mucosal pathology and immune responses are influenced by FcαRI induced PMN activation. The aim of this study is to investigate crosstalk of IgA activated PMN with DCs.
Methods
Retinoic acid stimulated DCs (RA-DCs) and monocyte-derived DCs (MoDCs) were derived from monocytes after being cultured for 6 days in the presence of IL-4 and GM-CSF, with or without retinoic acid (RA) respectively. Aldehyde dehydrogenase activity was measured using the ALDEFLUOR kit. Fresh isolated PMNs phagocytosed IgA coated latex beads (IgA PMN) after which they were co-cultured with DCs for 16h. Subsequent, maturation marker expression on DCs was assessed. Cell-cell interactions were analyzed with live cell microscopy and cytokine production in supernatants of co-cultures was measured.
Results
RA-DCs differed in morphology, expressed more CD103, and had higher aldehyde dehydrogenase activity compared to MoDCs. RA-DCs expressed less CD1c, CD80, CD83, CD86 and HLA-DR, and more CD141 and CD103 after co-culture with IgA PMN compared to MoDCs. Cell interactions and bead transfer between IgA PMN and DCs were seen in live cell microscopy. IL-12 was produced by MoDCs after co-culture with IgA PMN, but not by RA-DCs.
Conclusions
Both MoDCs and RA-DCs interacted with IgA PMN, and bead transfer from PMNs to DCs was observed. However, after co-culture with IgA PMN, RA-DCs did not produce IL-12, suggesting induction of immune tolerance.

Neutrophil Expression of B7-H6 and HLA – Implications on Interactions between NK Cells and Neutrophils in Inflammation

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As neutrophils contain toxic content and degrading enzymes, they constitute a potential danger to the surrounding tissue. A silent neutrophil death, i.e., apoptosis, is therefore of importance for termination of an inflammatory process. We have previously demonstrated that Natural Killer (NK) cells can induce apoptosis in healthy neutrophils via the activating NK cell receptor NKp46 and in a Fas-dependent manner.
NK cell cytotoxicity is regulated by array of activating and inhibitory receptors. The major inhibitory receptors are the killer immunoglobulin-like receptors (KIRs), which bind to corresponding HLA ligands, while the activating receptors include the natural cytotoxicity receptors (NCRs). B7-H6 has been identified as a ligand to the activating NCR NKp30, and it was recently reported that neutrophils display B7-H6 on their surface after pro-inflammatory stimulation and that B7-H6 exists in a soluble form in inflammatory conditions.
In this study we investigated how the human neutrophil surface expression of B7-H6 and HLA was regulated during inflammatory conditions and how the relative expression of these ligands affected the outcome of NK cell interactions with neutrophils.
We found B7-H6 to be expressed already on the surface of resting, circulating neutrophils. Furthermore, an additional pool of pre-formed B7-H6 was localized in easily mobilized neutrophil granules. When we monitored the expression of B7-H6 on the surface of neutrophils over time in response to inflammatory stimuli, we found the expression of B7-H6 to be highly dynamic. Further, our *in vitro* studies demonstrated that the surface

expression of HLA was rapidly decreased upon neutrophil activation.
These *in vitro* observations were confirmed in a human *in vivo* skin chamber model where transmigrated neutrophils demonstrated decreased levels of HLA on their cell surface. Also the surface expression of B7-H6 was altered and we could detect soluble B7-H6 in exudates of the skin chambers. In an *ex vivo* cytotoxicity assay, we observed that the transmigrated neutrophils from the skin chambers were significantly more sensitive to NK cell-mediated cytotoxicity, supporting our hypothesis that altered expression of NK cell receptor ligands on inflammatory neutrophils determines their susceptibility to NK cell-induced death.
Collectively, our data shed light on the complex regulation of interactions between NK cells and neutrophils during an inflammatory response and suggest a role for NK cells in the resolution phase of inflammation.

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Identification of Granulocytic Myeloid-Derived Suppressor Cells (G-MDSCs) in the Peripheral Blood of Hodgkin and Non-Hodgkin Lymphoma Patients

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Purpose: Human granulocytic myeloid-derived suppressor cells (G-MDSCs) have been described as low-density immunosuppressive CD66b+CD33dimHLA-DR- granulocytes that co-purify with mononuclear cells after density gradient centrifugation of blood from cancer patients. The role of G-MDSCs in Hodgkin (HL) and non-Hodgkin lymphoma (NHL) remains unclear. **Methods**: The frequency and immunophenotype of CD66b+CD33dimHLA-DR- cells were analyzed in PBMCs from HL and NHL patients (n=124) and healthy donors (n=48). The immunosuppressive functions of these cells were tested in vitro. Correlations between CD66b+CD33dimHLA-DR- cells and patient clinicopathological features and outcome, were evaluated. **Results**: CD66b+CD33dimHLA-DR- cells were increased in PBMCs from HL and NHL patients as compared to healthy donors. Their frequency remained significantly higher even considering HL (n=31), indolent (n=31) and aggressive (n=62) NHL patients separately. CD66b+CD33dimHLA-DR- cells in patient PBMCs were mostly composed of mature CD11b+CD16+ low-density neutrophils in an activated status as compared to conventionally isolated autologous or healthy donor neutrophils. The in vitro depletion of CD66b+ cells from patient PBMCs restored the proliferation of autologous T cells. Higher frequencies of CD66b+CD33dimHLA-DR- G-MDSCs correlated with unfavorable prognostic index scores and a significantly shorter freedom from disease progression. **Discussion**: Novel areas of research for lymphoma patients include improving the efficacy of adoptive cellular therapies, modulating T regulatory cells, developing novel lymphoma vaccine and enhancing tumor-specific innate immune response. In this context, our study demonstrate that in HL and B-cell NHL patients a population of functionally defined G-MDSCs mainly composed of mature activated LDNs is present and its generation seems to be more related to the biological aggressiveness of the disease than to its size. **Conclusions**: Our findings disclose a previously unknown G-MDSC-mediated mechanism of immune-escape in lymphomas, therefore anticipating possible targets for therapeutic interventions. Further studies are awaited in order to establish whether CD66b+CD33dimHLA-DR- G-MDSCs could be inhibited or reprogrammed for therapeutic purposes.
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Role of Neutrophils in an Imiquimod-Induced Mouse Model of Psoriasis

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Psoriasis is a chronic immune-mediated skin disease associated with deregulated interplays between immune cells and keratinocytes. Despite the fact that a prominent skin infiltration of neutrophils is a distinctive hallmark feature of psoriatic inflammation, the role of neutrophils in psoriasis pathogenesis remains unclear. Aim of this study was to investigate the specific contribution of neutrophils during psoriatic inflammation.

Methods. Psoriasis was induced by topical application of Aldara™, 5% Imiquimod (IMQ) cream in B6 mice treated or not with anti-LY6G (1A8) antibody to deplete neutrophils. Disease development was evaluated by flow cytometry and gene expression analysis of draining lymph nodes and skin biopsies, as well as by histological evaluation of skin inflammation.

Results: Epidermal thickening, together with the expression of several inflammatory cytokines (i.e. IL-22, IL-17, CXCL1 and IL-23) and psoriatic associated genes (i.e. Lipocalin 2 and S100A7), was significantly increased in the skin of neutrophil depleted mice in response to IMQ treatment. Interestingly, IMQ-treated neutrophil depleted mice manifested also a significantly increased recruitment of activated IL-17-producing γδT cells in the draining lymph nodes. In line with the latter observation, we demonstrated that neutrophils inhibited γδT cell proliferation and IL-17 production *in vitro*.

*Conclusion:*Overall, these data demonstrate that neutrophils may negatively contribute to disease propagation and exacerbation in the IMQ-induced mouse model of psoriasis by impairing γδT cell effector functions.

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Is There a Role for Neutrophils in the Adjuvanticity of Alum?

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Insoluble aluminum salts (alum) are still the most widely used adjuvants in humans. The actual mechanism of the adjuvanticity of alum is still not fully understood. In vivo studies are limited to animals and in the human system only its effect on monocytes and dendritic cells has been assessed. Recently, it has been shown in mice that host-derived DNA, which could not be assigned to a single cell type, is involved in the adjuvant effect of alum. Neutrophils are the most abundant circulating white blood cells in humans, the first cell type to enter sites of tissue damage or inflammation and can produce so-called NETs, i.e. “neutrophil extra-cellular traps” upon different stimuli (e.g. bacteria, fungi, immune complexes, C3a). These NETs consist of decondensed nuclear chromatin (DNA, histones) or mitochondrial DNA forming web-like filaments and are decorated with antimicrobial granular material such as myeloperoxidase, elastase or LL37, which are expelled during an induced death process called “NETosis”. In addition to their obvious antimicrobial activity, components of NETs are meanwhile also discussed as host derived danger signals (DAMPs) in the initiation of innate immune responses and may play an important role for the adjuvant effect of alum vaccines.
The aim of this study is to investigate, whether alum induces NETosis in human neutrophils and hereby may influence the initiation of immune responses to alum-containing vaccines.
First, freshly isolated human neutrophils were stimulated with alum to induce a possible NET-response and PMA or ionomycin were used as positive controls. Strong NET-formation was induced in neutrophils by all three stimuli when visualized by co-staining of extracellular DNA and different granular proteins using fluorescence/confocal microscopy. In addition, the enzymatic activity of neutrophil elastase in culture supernatants indicated alum-induced NETosis. Regarding the mode of action, the production of cellular reactive oxygen species (ROS) were

analysed by flow cytometry or plate reader assays. Prominent cytosolic (c)ROS production was only observed with PMA, as expected, whereas alum and ionomycin induced significant production of mitochondrial (m)ROS. Using a plate-reader method to quantify DNA-release over time, for alum and ionomycin a similar time course (namely much faster than with PMA) was observed and application of different ROS-inhibitors or EDTA revealed a significant dependence on mROS and extracellular Ca++ for both stimuli. Inhibition of NET-release with cytochalasin D indicated phagocytosis as initial step for alum-induced NETosis, which in aqueous solution is present in colloid particles, as also evaluated by microscopy. Similarily, amine- or carbonate-modified latex beads (3 µm) were phagocytosed by neutrophils and induced NETs, whereas uncharged beads did not. First co-cultures of NET-forming neutrophils and monocyte-derived dendritic cells (mdDC) showed a decrease in LPS- or alum-induced upregulation of CD83 and co-stimulatory molecules of these mdDC.
In summary, alum is capable of potently inducing NETosis in human neutrophils. This response is dependent on phagocytosis (most probably facilitated by its charged nature), mROS production and extracellular Ca++. While Alum directly activates mdDC, co-cultured neutrophils producing NETs may rather inhibit mdDC activation.

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Signaling Pathways Mediating the Human Neutrophil Response to Fungal ß-Glucan

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Neutrophils are the critical immune cell necessary for eliminating invasive fungal infections from the tissue and recognize fungi *via* CR3, a β_2 subfamily integrin involved in phagocytosis. CR3 is a unique receptor with two spatially distinct binding domains, the I-domain and the lectin-like site. The I-domain binds number of ligands including iC3b, ICAM, fibrinogen, and fibronectin (Fn). The lectin-like site binds polysaccharides, including β-glucan, a pathogen associated molecular pattern found within the cell wall of fungi. Co-ligation of CR3 with Fn at the I-domain and β-glucan at the lectin-like domain is possible due to spatially distinct locations and generates unique cellular responses not seen with ligation of either domain separately. In the setting of immobilized β-glucan and Fn, CR3 exhibits immune responses not seen when exposed to either substrate alone correlating to *in vivo* fungal infection. Recent work in our lab demonstrates in response to Fn + β-glucan, as well as *Candida* hyphae in the context of Fn, neutrophils form homotypic aggregates and rapid NET release.
In this study, we further characterized the chemotactic signals underlying neutrophil homotypic aggregation. Moreover, while the key surface receptors have been identified, less is known about the intracellular signaling pathways downstream of these receptors in the context of neutrophil aggregation. We additionally interrogated the role of a variety of signaling proteins that were identified *via* mass-spectrometry as having differential tyrosine phosphorylation in neutrophils clustered on surfaces coated with Fn and β-glucan, including extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase kinase 1 (MEK1), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), spleen tyrosine kinase (Syk), glycogen synthase kinase 3 β (GSK3β), and protein kinase C (PKC). The long term goal is to determine differential stimulation of intracellular signaling pathways as a function of CR3 domain occupancy. Understanding the signaling mechanisms underlying chemoataxis, aggregation, and NETosis in the neutrophil anti-fungal response may provide therapeutic targets which may benefit to candidiasis patients in the future by reducing harmful neutrophil behaviors and/or stimulating behaviors crucial to fungal clearance.

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STAT Factors Control the Expression of Certain Genes in Human Neutrophils, Yet without Much Impact on Functional Responses

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Inflammation and immunity are crucially impacted by cytokines and chemokines produced by

neutrophils. We have previously shown that this response is tightly regulated by transcription factors NF-κB, C/EBP and CREB in response to physiological stimuli. The STAT family of transcription factors is also thought to control cytokine production in neutrophils, as they do in other leucocytes. Supporting this idea is the fact that several STAT proteins are phosphorylated on both tyrosine residues in response to neutrophil stimuli such as IFNγ, GM-CSF, or G-CSF, that are known to induce responses such as cytokine production, delayed apoptosis, and expression of surface receptors. However, a direct link between STAT activation and these functional responses remains to be demonstrated.

Using pharmacological inhibitors selective for STAT1/3 or for STAT3/5, and DNA microarray analysis, we identified numerous genes that are controlled by STATs in response to IFNγ, GM-CSF, or G-CSF. Among them were genes encoding cytokines or chemokines (IL-1b, CXCL1, CXCL10), surface receptors (CD64, PD-L1, TLR5, IL-18R), and SOCS proteins (CIS, SOCS1, SOCS3). From a more functional standpoint, cytokine/chemokine secretion was largely unaffected by STAT inhibitors, or by siRNAs directed against STAT1 or STAT3. Likewise, the increase in CD64 membrane expression induced by IFNγ or G-CSF was not impaired in presence of STAT inhibitors or siRNAs. Finally, neutrophil apoptosis is strongly delayed by IFNγ, G-CSF, or GM-CSF, but STAT inhibition only hindered the survival effect of IFNγ, while that of G-CSF or GM-CSF was unaffected. Together, these results indicate that STAT proteins play but a marginal role in neutrophil responses, despite controlling the expression of several genes involved in said responses. In the particular case of CIS/SOCS, while their inducible gene expression was found to be under STAT control, the corresponding proteins were not detected, in keeping with other studies. Thus, the actual outcome of STAT-controlled SOCS expression is difficult to assess. Studies are in progress to clarify the issue. In summary, while STAT factors definitely govern the expression of several target genes in neutrophils, this seems to have little or no effect on functional responses in these cells.

The Role of Hemorrhage-Primed Neutrophils in Altering Endothelial Cell Tie1/Tie2 Expression in the Development of Indirect Acute Respiratory Distress Syndrome

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Severe hemorrhage/hemorrhagic shock(Hem) has been shown to be causal for the development of extra-pulmonary/indirect (i)ARDS and is associated with severe endothelial cell (EC) injury. EC growth factors, Angiopoietin (Ang)-1 & 2, maintain vascular homeostasis via tightly regulated competitive interaction for the tyrosine kinase receptor, Tie2, expressed on ECs. Ang-1/Tie2 binding leads to Tie2 phosphorylation (pTie2) and signaling for downstream anti-inflammatory and pro-survival phenotype, whereas Ang-2, stored pre-formed and released for activated ECs, can act as an apparent antagonist. We have shown Ang-2 significantly elevated in our murine acute respiratory distress syndrome (ARDS) model of hemorrhage (priming) followed by secondary septic challenge, and additionally, that neutrophil (PMN) interaction with resident pulmonary vascular ECs contributes significantly to Ang-2 release and mediates Ang-2-associated pulmonary EC dysfunction central to the development of indirect ARDS. The orphan receptor, Tie1, has recently been described as contributing additional level of regulation to Ang/Tie2 signialing. While neither Ang-1 nor Ang-2 bind Tie1, Tie1 complexing with Tie2, has been shown to inhibit Ang-1/Tie2 interactions, thus promoting an activated, pro-inflammatory EC phenotype. However, Whether Hem alters Tie1/Tie2 expression and interactions leading to the primed/activated EC phenotype, and further, how the Hem primed PMN contribute to this altered expression, is not known. To begin to investigate this, we tested the hypothesis that Tie1/Tie2 expression is altered following Hem, and this change is a result of EC interaction with activated (shock primed) PMN.

A murine model of hemorrhagic shock–induced priming for the development of iARDS after subsequent septic challenge was used in this study. To assess the contribution of Hem-primed PMN to

changes in Tie1/Tie2 expression, mice were depleted of peripheral blood PMN via intra-peritoneal (i.p.) injection 500 ug of rat anti-mouse PMN antibody, anti-Gr1(clone RB6–8C5, rat IgG2b)/mouse, 48 hours before Hem. Mice were euthanized at 6 and 24 hours following Hem. Tie1, Tie2 and phosphorylated Tie2 expression in lung homogenates were assessed using commercial ELISA kits and Western blot. Our findings show that 1) p-Tie2 is decreased at 6h, but is restored by 24h after hemorrhagic shock; 2) Tie1 expression is elevated at both 6h and 24h after hemorrhagic shock; 3) Depletion of neutrophil significantly increases p-Tie2 and decreases Tie1 expression. These findings support our hypothesis and suggest 1) that Tie1 plays a role in mediating EC dysfunction potentially by prolonging EC activation by decreasing Tie2 availability for Ang-1 binding and, 2) that Hem-primed PMN contributes to the altered Tie2/Tie1 expression. (Funded by NIH.5P20GM103652 [JLN] &R35GM118097[AA])

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TNFα Promotes CCR7-Dependent Migration of Neutrophils and Their Subsequent Crawling into the Lymphatic Vessels in Vivo

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Neutrophils through their diverse immune functions play an essential role in innate immunity against invading pathogens and during inflammatory processes. Interestingly, these leukocytes have been shown recently to be capable to shape the adaptive immune responses following immunisation and during pathogen infections; however, the mechanism of neutrophil trafficking to the lymphatic system is still unclear. In the present study, we have used the murine model of cremasteric inflammation to decipher some of the molecular mechanisms of neutrophil trafficking into the tissue-associated lymphatic vessels. Here we show that *in vivo* stimulation of the tissues with TNFα induces the migration of neutrophils into cremasteric lymphatic capillaries in a time-dependent manner. This response is strictly CCR7-dependent as the neutrophil trafficking into lymphatic vessels is suppressed in CCR7KO animals (~97% inhibition as compared to WT mice). Furthermore, using intravital confocal microscopy, TNFα also promotes the intraluminal crawling of all intravasated neutrophils along the lymphatic endothelium. Additionally, TNFα-induced inflammation results in ICAM-1 upregulation on lymphatic vessels. Taken together, our results demonstrate for the first time a critical role for TNFα in priming neutrophils to migrate into the lymphatic vessels *in vivo*, through the upregulation of ICAM-1 and CCR7 signalling.

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Deficiency in Toll-Interacting Protein (Tollip) Skews Inflamed yet Incompetent Innate Leukocytes in Vivo during DSS-Induced Septic Colitis

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Functionally compromised neutrophils contribute to adverse clinical outcomes in patients with severe inflammation and injury such as colitis and sepsis. However, the ontogeny of dysfunctional neutrophil during septic colitis remain poorly understood. We herein report that the dysfunctional neutrophil may be derived by the suppression of Toll-interacting-protein (Tollip). We found that Tollip deficient mice were more susceptible to DSS induced acute colon injury. Tollip deficient mice displayed serious rectal bleeding, quicker weight loss, higher mortality, and shorter colon length significantly as compared to wild type mice. Further studies showed that Tollip deficient neutrophils had compromised migratory capacity, as well as reduced potential to generate bacterial-killing neutrophil extra-cellular trap (NET), and compromised bacterial killing activity. Together, our data reveal a novel mechanism in Tollip alteration that underlies the inflamed and incompetent polarization of neutrophils leading to severe outcomes of colitis.

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Toxoplasma gondii down Regulates Il-1ß Production by Human Neutrophils in the Presence of Lipopolysaccharide

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During acute infection with the intracellular parasite, *Toxoplasma gondii*, monocytes and neutrophils are among the first immune cells recruited to the site of infection. These cells can be infected by *T. gondii*, but, how neutrophils, in particular, respond to this pathogen is poorly understood. Since IL-1β is a key regulator of innate immunity, we investigated the production of this cytokine during *T. gondii* infection. Human peripheral blood monocytes or neutrophils were infected with *T. gondii* (MOI 2) and after 16 hours, the secretion of IL-1β was measured by ELISA. Unlike monocytes, which produce IL-1β in response to *T. gondii* infection, the infection of neutrophils did not induce IL-1β release. Interestingly, in neutrophils, *T. gondii* infection also inhibited LPS-induced IL-1β by 62%. This inhibition was observed when the stimulation with LPS and the infection were simultaneous or when LPS was added 3 hours before infection. To test if IL-1β inhibition required active parasite invasion, neutrophils were cultured with parasites that were heat killed, treated with DMSO (vehicle control) or pretreated with mycalolide B (5 µM), an irreversible actin polymerization inhibitor, which prevents parasite invasion. Heat killed and mycalolide B-treated parasites did not limit IL-1β production, indicating that this effect required active parasite invasion. Since LPS activates NF-κB, which is involved in transcriptional activation of pro-IL-1β and the inflammasome sensor NLRP3, a possible effect of *T. gondii* on the transcription of these two genes was investigated by qPCR. *T. gondii* reduced the transcription of IL-1β by 95% and NLRP3 by 75% in the presence of LPS. Furthermore, since LPS typically activates NF-κB through the canonical pathway, we investigated if *T. gondii* inhibition of IL-1β and NLRP3 transcription was related to an effect on NF-κB activation. In *T. gondii*-infected neutrophils stimulated with LPS, phosphorylation of p65 was reduced by 60%, compared to LPS treatment alone, indicating that the parasite prevents NF-κB activation. Finally, we investigated the effect of *T. gondii* on caspase-1, a key mediator of proteolytic processing of IL-1β. *T. gondii* inhibited LPS-induced caspase-1 cleavage by 83%. Together, these results show that *T. gondii* limits the production of LPS-induced IL-1β in human neutrophils and that this effect is associated with the down regulation of IL-1β and NLRP3 at the transcript level and a reduction in NF-κB activation. Moreover, *T. gondii* inhibited the cleavage and activation of the inflammasome protease caspase-1. Collectively, these results describe a new strategy of immune evasion in which *T. gondii* down regulates the immune response of human neutrophils, potentially facilitating the survival and spread of this parasite during acute infection.

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Long Noncoding RNA MEG3-4 Tissue Specifically Regulates IL-1β during Pulmonary Bacterial Infection by Repressing MicoRNA-138

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Long noncoding RNAs (lncRNAs) modulate various biological processes; however, their function in host immunity response against infection remains elusive. Here, we identify an intergenic lncRNA MEG3 (linc-MEG3) as a tissue specific regulator for pulmonary immunity during Gram-negative bacterial infection. Among the 10 transcripts of linc-MEG3, transcripts 1 and 4 are main regulators as being discovered the most downregulated in mouse lungs after bacterial infection, but with insignificant alterations in other organs. Overexpression of linc-MEG3-4 in mice led to exacerbated inflammatory response, severe lung injury, systemic dissemination and ultimately mouse death. Alveolar epithelial cells and alveolar macrophages (AM) were the major cell types that are targeted by linc-MEG3-4. Due to its 3' sequences complementary to microRNA-138 (miR-138), linc-MEG3-4 competitively binds miR-138 and thereby freeing its target IL-1b mRNA to intensify inflammatory responses. Our results characterize linc-MEG3 as a novel pulmonary inflammatory regulator of bacterial infection through a miR-138/IL-1b/NF-kB pathway, indicating that lincRNAs may be critical for coordination of complex immunity and inflammation in response to infection.

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USP18 is Required for HIV-1 Infection in a Human iPSC-Derived Macrophage Infection Model

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The type I interferon [T1-IFN] pathway plays a dual role in viral defense by inducing expression of restriction factors to limit viral replication, while simultaneously increasing expression of host factors involved in antigen processing and presentation. Although the innate antiviral response can potently restrict virus replication in nearly all cell types, in some antigen presenting cells like macrophages, it is important for viruses to replicate sufficiently that antigen is present for priming cell-mediated adaptive immunity. For HIV-1, however, this defensive strategy is not effective for eradicating the virus. Recruitment of CD4+ T cells actually provides additional target cells for the virus and macrophages themselves become persistent virus producing cells. CD4+ T cells are highly susceptible to the cytopathic effects of HIV-1 while macrophages are relatively resistant to virus-induced cell death pathways. To understand the mechanisms of survival of HIV-1-infected macrophages, we performed whole transcriptome profiling of HIV-1-infected cells and found that ubiquitin specific proteinase 18 [USP18] was highly upregulated in infected cells. USP18 is a negative regulator of T1-IFN signaling through the Jak/STAT pathway. We hypothesized that USP18 expression inhibits the antiviral response normally initiated through the T1-IFN signaling pathway allowing for macrophage survival and HIV-1 replication. Indeed knockout of USP18 in iPSC-derived macrophages results in marked reduction of HIV-1 replication. USP18 deficiency also makes the cells hypersensitive to stimulation with T1-IFNs and other pattern recognition receptor ligands. Together our data suggest that USP18 expression is essential for HIV-1 replication and persistence in macrophages.

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Signaling Pathways in Neutrophils are Regulated by Surgical Trauma - a Proteomics Study

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Neutrophils are important players in the development of the systemic inflammatory response after trauma. To understand in more detail the neutrophil activation at early stages after trauma, in this study neutrophils were isolated from rats divided in two groups: control and surgical trauma. Rats from the surgical trauma group were submitted to laparotomy, eviscerated and kept hydrated for 120min, after what the abdominal wall was closed and blood was collected. The control group was anesthetized and observed for the same period. Isolated neutrophils were lysed and the extracted proteins were analyzed using nano liquid chromatography coupled to tandem mass spectrometry. We identified a total of 2924 rat neutrophil proteins in our analysis, of which 393 were found differentially regulated between control and surgical trauma groups. Functional pathways analysis revealed transcription initiation and protein biosynthesis to be enriched containing several of the 190 proteins up-regulated in surgical trauma. On the other hand, among the 203 proteins down-regulated in surgical trauma we found enrichment for proteins of the immune response, proteasome degradation and actin cytoskeleton (Fig.1). Moreover, we performed enzyme prediction analysis, that revealed regulated enzymes involved in neutrophil apoptosis, directional migration and chemotaxis. Our

observations were confirmed by in silico protein-protein interaction analysis. Collectively, our results reveal that neutrophils drastically regulate their biochemical pathways after the early stages of surgical trauma, showing lower activity. This implies higher susceptibility of the trauma patients to infection and bystander tissues damage.

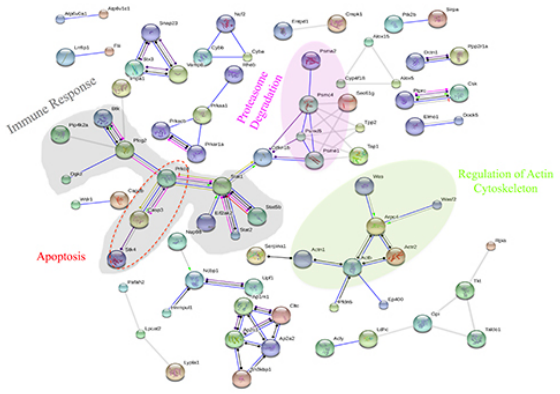

Figure 1: Protein interaction diagram showing the proteinsdownregulatedin surgical trauma, highlighting proteins involved in significantly enriched pathways.

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Early Tyrosine Phosphorylation and Comparative Proteomics Provide New Insights into Adenosine A_{2A} Receptor Activities in Human Neutrophils

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Adenosine, through activation of the adenosine A_{2A} ($A_{2A}R$) receptor, elevates intracellular levels of cyclic AMP and in this way acts as a physiological inhibitor of inflammatory neutrophil functions. In the present study, we looked into the impact of $A_{2A}R$ engagement on early phosphorylation events. Neutrophils were stimulated for 2 min at 37˚C with well-characterized, pro-inflammatory agonists, in absence or presence of the $A_{2A}R$ agonist, CGS 21680. As assessed by immunoblotting, several protein bands were tyrosine phosphorylated; CGS 21680 markedly decreased tyrosine phosphorylation levels of four regions (37-45 kDa, 50-55 kDa, 60 kDa and 70 kDa) and key-signaling protein kinases, p38 MAPK, Erk-1/2, PI3K/Akt, Hck and Syk were targets of $A_{2A}R$ activities, while Lyn, SHIP-1 or PTEN phosphorylation levels were spared. Prostaglandin E_2, or a mixture of the compounds RO 20-1724 (inhibitor of phosphodiesterase IV) and forskolin (activator of adenylate cyclase) largely mimicked the effect of CGS 21680. We used LC-MS/MS for the global survey of the neutrophil phosphoproteome, mainly occurring on serine and threonine residues. Stimulation of the cells solicited the MAPK, PIP3/Akt, Rho GTPases, NF-kB, cytokine signaling, inflammasomes and mTOR pathways. Activated $A_{2A}R$ positively impacted proteins involved in functions of the Golgi, nitric oxide, and sumoylation of proteins. In contrast, ERK/MAPK targets, the AP-1 family of transcription factors and DNA replication processes, were down-regulated. In resting cells, $A_{2A}R$ engagement down-regulated phosphorylation events linked with receptor signaling pathways, transcription and glucose uptake processes. Results unveal a number of intracellular signaling pathways targeted by the $A_{2A}R$, several of which might be key in the regulation of neutrophils functions during inflammation.Proteomics data are available via ProteomeXchange with identifier PXD004172.

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Identifying Novel Immune Modulating Factors in a Genome-Wide S. aureus Screen in Human Neutrophils

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Rationale:

Staphylococcus aureus is a highly adaptive and widespread bacterial pathogen causing numerous clinical problems due to antibiotic resistance. Recent human-pathogen interaction studies reveal *S. aureus* uses multiple evasion mechanisms to survive or even replicate within neutrophils, the primary cellular defence against this pathogen. In addition, *S. aureus* induces rapid and profound neutrophil necrosis, which further disables the immune response. Our study aims to identify novel immune modulators

through the screening of a genome-wide *S. aureus* mutant library in neutrophil cell death assay.

Methodology:

The 1,952 strain mutant library was constructed by transposon insertion in the clinically relevant community acquired methicillin-resistant *S. aureus* (USA300) background. Individual *S. aureus* strains were co-incubated with primary human neutrophils isolated from healthy volunteers, at an MOI of 10 for 3 hours before ToPro-3 staining and assessment of cell loss via flow cytometry.

Findings:

Wild type USA300 caused profound neutrophil cell loss by 3 hours (≥75%). Quantification of ToPro-3 negativity and viable cell counts identified 34 mutant strains that showed attenuated neutrophil cell death. A number of internal controls with known pro-death functions including *lukAB, lukGH, agrA* and *saeS* were among the identified attenuated strains. Four gene mutations not previously associated with cell death: *purB, lspA, clpP* and *pfo* were also among the most highly-attenuated strains and have been further validated by transduction strategies, and current studies to verify them by gene complementation are underway. These findings may identify novel mechanisms of *S. aureus* induced neutrophil cell death, which may aid in the design of future antibiotic-independent therapeutic strategies to restore failures of innate immunity.

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Genome-Wide Localization of PU.1 and H3K4me1 in Human Neutrophils and Monocytes

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Neutrophils and monocytes are the two most represented population of phagocytes in the human bloodstream. Neutrophils are integrated in the activation, regulation and effector mechanisms of the innate and adaptive immune systems. Beyond their well-known role in acute inflammatory responses and resistance to extracellular pathogens, neutrophils also function as major players of diverse pathologies via the release of *de novo* formed mediators. However, the mechanisms that in neutrophils control transcription of mediators are still very poorly studied, mainly because neutrophils are seen as short-lived effectors cells. Multiple knockout models have demonstrated the importance of the transcription factor (TF) PU.1 in the differentiation of myeloid cells. Indeed, this pioneer TF, that acts as a master transcriptional regulator, initiates nucleosome remodelling followed by histone modification depositions at large numbers of genomic regions. These changes in the chromatin landscape serve as beacons for the recruitment of additional factors, which ultimately drive both cell-specific gene expression and signal-dependent responses.

Herein, by performing chromatin immunoprecipitation (ChIP) assays followed by high-throughput sequencing in freshly isolated human neutrophils and monocytes, we globally mapped the genomic distribution of the enhancer mark histone H3 lysine 4 monomethylation (H3K4me1) and the master regulator PU.1. We found that the numbers of PU.1-bound genomic sites were 63461 in neutrophils and 49041 in monocytes, whereas the H3K4me1 peaks were 54269 in neutrophils and 63661 in monocytes. While most of the PU.1 peaks that are localized in promoter-proximal regions showed similar occupancy levels in both neutrophils and monocytes, cell-specific PU.1 recruitment was predominantly located at enhancer-distal regions in both types of phagocytes. Analysis of the genomic distribution of H3K4me1 within a definite region of approximately 3000 bp from the centre of enhancer-distal PU.1 peaks showed a typical bimodal distribution. In addition, we found that PU.1 recruitment at cell-specific enhancer sites strictly correlates with distal cell-specific H3K4me1 also in neutrophils. Moreover, by performing microarray gene expression profiling, we identified either common or cell-specific transcripts in freshly isolated neutrophils and monocytes. Finally, by integrating the transcriptomic and epigenetic signatures, we observed a stringent correlation between cell-specific transcript expression and H3K4me1 and PU.1 deposition at specific enhancer regions of the related genes. Altogether, these findings suggest that histone modifications and PU.1 recruitment at cell-specific genomic regions are likely responsible of the differences observed at the transcriptional level between neutrophils and monocytes.

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Analysis of NCF1 in Patients with P47phox Deficient Chronic Granulomatous Disease and Normal Subjects by Droplet Digital PCR

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Mutations in gene *NCF1* (encoding protein $p47^{phox}$ of the NADPH oxidase) result in an autosomal recessive form of chronic granulomatous disease (CGD), a rare genetic disease with impaired phagocyte production of reactive oxygen species and recurrent infections, granulomatous complications, and premature death. Identification of the specific genetic defect in patients with $p47^{phox}$ CGD is complicated by the presence of two highly conserved (>98%) pseudogenes. The *NCF1* gene has a GTGT at the start of exon 2 while the pseudogenes (*NCF1B* and *NCF1C*) delete one GT (ΔGT). Unequal crossing-over events between the wild-type gene and the pseudogenes have been suggested to account for the majority of mutations in $p47^{phox}$ deficient CGD. Due to the sequence identity between the wild type gene and pseudogenes, standard Sanger sequencing has proven to be inadequate to assign a specific genetic mutation in these patients. Using droplet digital polymerase chain reaction (ddPCR), we developed an assay to differentiate between ΔGT and other mutations in *NCF1*. Using the ratio of intact, GTGT alleles to total number of *NCF1* alleles, this assay can accurately determine carrier status of relatives within the $p47^{phox}$CGD families. Unexpectedly, analysis of normal subjects revealed that a significant proportion (14%) exhibited >2 alleles containing the GTGT, while 17 subjects exhibited 3 alleles and 4 subjects (of 151 normal subjects tested) exhibited 4 alleles. Despite increased numbers of GTGT alleles, neither $p47^{phox}$ protein expression nor the production of reactive oxygen species was increased in these normal subjects. Funded by NCI Contract No. HHSN261200800001E

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Neutrophil and Monocyte Survival in Vivo and in Vitro Depends on an Additive Cytoprotective Shield by Serpinb1a and Serpinb6a against Granule Proteases

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Background: Serine protease inhibitors, serpins, are highly conserved proteins regulating many physiological processes. While most serpins are secreted into the plasma, the clade B serpins control proteases intracellularly in the cytoplasm. We have previously shown that Serpinb1a is essential in regulating a cell intrinsic cathepsin G-dependent neutrophil death in steady state and in infection studies leading to impaired bacterial clearance. Mice lacking Serpinb1a were previously shown to have 50% less neutrophils in the bone marrow. SerpinB6, a related clade B serpin expressed in myeloid cells, also inhibits cathepsin G. Yet, lack of SerpinB6 leads to a recessive inherited stimulus-induced deafness in humans and mice but the role of SerpinB6 in monocytes and neutrophils remains unknown.

Results: First, we generated double knock-out mice by crossing *Serpinb1a*$^{-/-}$ mice with mice lacking Serpinb6a, the mouse homolog of SerpinB6. *Serpinb1a.Serpinb6a*$^{-/-}$mice showed a more severe neutropenia in the bone marrow compared to the *Serpinb1a*$^{-/-}$ mice. Strikingly, *Serpinb1a.Serpinb6a*$^{-/-}$ mice also presented reduced blood neutrophil as well as blood and bone marrow monocyte percentages, which were not observed in *Serpinb1a*$^{-/-}$ mice. Accordingly, *Serpinb1a.Serpinb6a*$^{-/-}$ neutrophils and monocytes had a considerably higher kinetic of cell death induced by granule permeabilization in vitro. Caspase inhibition did not rescue neutrophil death in cells lacking serpins. In contrast, cathepsin G deletion fully rescued granule permeabilization-induced cell death in neutrophils, but not in monocytes.

Conclusion: Our results demonstrate that Serpinb1a and Serpinb6a cumulatively provide a cytoprotective shield in neutrophils and monocytes in steady state and after granule leakage by inhibiting the serine protease cathepsin G, which induces death independently of caspases.

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Neutrophil Differentiation and Functions after Modulation of DDR Pathway

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The main function of genes involved in DNA damage pathway is regulation of cellular response to genotoxic stress. Modulation of expression and/or activity of DNA damage response (DDR) genes in leukocytes and their progenitors affects not only DDR but also other functions of neutrophils such as differentiation, proliferation and pro-inflammatory functions. Deregulation of expression and activity of wild type p53-induced phosphatase in mouse and human neutrophils led to their stabilization in *in vitro* culture, promoted neutrophil differentiation and proliferation and changed the repertoire of secreted cytokines. Using genetic deletion or chemical inhibition in deregulated neutrophils, we manipulated p38MAPK, tp53 and Stats pathways to determine dependency of observed neutrophil phenotypes from indicated pathways. The results obtained in *in vitro* experiments were confirmed by manipulation with indicated above pathways in *in vivo* mouse models of several human inflammatory and oncological pathologies.
The novel strategies in treatment of human diseases based on our findings were proposed.
The work was supported by Grant #14-15-00636, Russian Scientific Fund

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Inflammation-Resolving Lipid Mediator, Resolvin D2, Promotes Revascularization and Tissue Regeneration during Limb Ischemia

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Tissue injury arising from trauma, surgery or ischemia elicits a prompt inflammatory response. A primary goal of the inflammatory response is to eliminate exogenous pathogens and to promote the clearance of dead cells. Timely resolution of the inflammatory phase enables the transition from inflammation to tissue repair, whereas chronic unresolved inflammation perturbs tissue repair. Resolvins are lipid mediators generated by leukocytes (neutrophils and macrophages) during the resolution phase of inflammation. Here, we show that resolvins promote the transition from inflammation to tissue repair and enhance tissue revascularization following ischemia. In mice undergoing hind limb ischemia (HLI) induced by femoral artery ligation, we identified resolvin D2 (RvD2) in bone marrow and skeletal muscle by mass spectrometry. We also identified RvD2 in skeletal muscle biopsies from humans with peripheral artery disease. Monocytes were recruited to skeletal muscle during HLI and isolated monocytes produced RvD2 in a lipoxygenase-dependent manner. Using laser Doppler perfusion imaging, we found that exogenous RvD2 enhanced perfusion recovery in HLI and micro-computed tomography (microCT) of limb vasculature revealed greater volume, with evidence of tortuous arterioles indicative of arteriogenesis. Unlike other treatment strategies for therapeutic revascularization that exacerbate inflammation, RvD2 did not increase vascular permeability, but reduced neutrophil accumulation and the plasma levels of TNF-a and GM-CSF, while promoting a tissue reparative monocyte/macrophage phenotype. In mice treated with RvD2, histopathological analysis of skeletal muscle of ischemic limbs showed more regenerating myocytes with centrally located nuclei and mice deficient in RvD2 receptor, Gpr18, had an endogenous defect in perfusion recovery following HLI. Thus, RvD2 stimulates arteriogenic revascularization during HLI suggesting that resolvins may be a novel class of mediators that both resolve inflammation and promote arteriogenesis.

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Protein Kinase Inhibitor Compound Screen Reveals ErbB Family Kinases as Regulators of Neutrophil Apoptosis in the Context of Inflammatory Diseases

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Rational and Hypothesis:
Persistent neutrophilic inflammation is observed in a number of inflammatory conditions, including chronic obstructive pulmonary disease (COPD). Prolonged neutrophil survival in the lung is known to contribute to COPD pathophysiology. Uncovering the mechanisms underpinning pro-survival phenotypes may allow development of new and effective therapies to treat the cause of inflammation in COPD. Protein kinase cascades play pivotal roles in diverse cellular functions including cell proliferation and apoptosis, and kinase inhibitors are used as pharmacologic drugs in multiple pathologies. Here, we screened a protein kinase inhibitor library to identify therapeutically targetable novel neutrophil apoptosis pathway(s).
Methods:
Neutrophils were isolated from peripheral blood of healthy subjects and COPD patients by Percoll gradient centrifugation. A protein kinase inhibitor library consisting of 298 compounds that have been fully profiled against 224 human protein kinases, was screened in neutrophil apoptosis assays. Neutrophil apoptosis was assessed by light microscopy and flow cytometry. Inhibitors were tested in mpx:GFP zebrafish tail injury model of inflammation by counting GFP positive neutrophils at the site of inflammation. LPS nebulized C57BL/6 mice were injected intraperitoneally with the kinase inhibitor Tyrphostin AG825, and bronchoalveolar lavage (BAL) was performed at 48 hours.
Results:
Screening and validation experiments identified 12 compounds that robustly increased neutrophil apoptosis ≥2 fold and have greatest specificity for their kinase targets. Epidermal Growth Factor Receptor family (ErbB) was identified as a frequent target of the identified compounds. Inhibition of ErbB1 and ErbB2 with selective small molecule inhibitors, Erbstatin analog and Tyrphostin AG825 respectively, accelerated spontaneous apoptosis in neutrophils from healthy subjects and COPD patients, and also reversed GMCSF-, LPS-, hypoxia- and cAMP-mediated neutrophil survival. Tyrphostin AG825 was also able to reduce GMCSF induced AKT-phosphorylation, suggesting that ErbB2-mediated neutrophil survival signals may be transduced via the PI3-AKT pathway. In an in vivo zebrafish tail transection model, Tyrphostin AG825 significantly reduced neutrophils at the site of inflammation at 4 and 8 hours post injury. Tyrphostin AG825 also significantly increased BAL neutrophil apoptosis and alveolar macrophage efferocytosis of apoptotic neutrophils in an acute lung injury mouse model.
Conclusion:
We are hopeful that our findings may identify clinically targetable protein kinase dependent pathways that will allow the development of novel therapeutic strategies for chronic inflammatory diseases.

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Atypical Molecular Pattern Exposed by Peripheral Blood Neutrophils Treated with Sphingolipid Analog Drug FTY720

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Recently we have described an atypical cell death induced in human neutrophils by sphingolipid analog drug FTY720 (fingolimod). The neutrophil death is characterised by rapid translocation of HSP27 to the cell surface and externalization of phosphatidylserine (PS) and annexin I (Skrzeczynska-Moncznik et all. 2015, J Leuc Biol). In continuation we studied the engulfment of the FTY720-treated neutrophils by macrophages. The FTY720-treated neutrophils were extensively phagocytized by monocyte-derived macrophages

although the exposure of PS or annexin I did not correlate with the clearance. Interestingly, inhibitors of HSP90 (geldanamycin, 17-DMAG, radicicol) reduced the externalization of PS, moreover some features of FTY720-induced neutrophil death were regulated by albumin or sphingolipids. Paradoxically, the decrease of the established 'eat me' signals was accompanied by a substantial increase in the neutrophils clearance. The engulfment of the FTY720-treated neutrophils by macrophages was mediated by scavenger receptors and triggered cytokine production. Cumulatively, our data strongly argue that the clearance of FTY720-treated neutrophils by macrophages is not related to the typical changes associated with apoptosis, especially the PS redistribution to the cell surface. In conclusion, we postulate the existence of a new molecular pattern (DAMP) on neutrophils which is independent of physiological apoptosis, regulated by sphingolipids and HSP90, results in rapid neutrophil clearance, and thus may importantly contribute to the neutrophil turnover.

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The Dual Role of the Endogenous Homeostatic Factor Del-1 in the Modulation of Acute Self-Limited Inflammation

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Acute inflammation is initiated by neutrophil and monocyte recruitment to the inflamed site, followed by release of pro-inflammatory mediators. After their acute proinflammatory actions, recruited neutrophils undergo apoptosis and are phagocytosed by macrophages, a process that is mediated by the recognition of phosphatidylserine (PS) on apoptotic neutrophils by “eat-me” signals and specific macrophage receptors. This process is called “efferocytosis”. Macrophage engulfment of the apoptotic material leads to the induction of a downstream signaling in which efferocytic macrophages acquire a resolving phenotype by releasing TGFb1 or IL-10 and by further upregulation of phagocytic receptors and bridging molecules. Resolving macrophages are central to resolution of inflammation (RoI), which promotes restoration of tissue homeostasis.
We have previously described Developmental endothelial locus-1 (Del-1) as an endogenous negative regulator of leukocyte adhesion by blocking the interaction between the integrin LFA-1 and ICAM-1. Here we show that Del-1 also acts as an eat-me signal thereby enhancing the efferocytic ability of macrophages in the context of RoI. This action is facilitated by a high affinity binding of the discoidin I-like domains of Del-1 to PS on apoptotic cells, and the interaction of Del-1 with the phagocytic receptor alphaVbeta3-integrin on macrophages, which is mediated by the RGD site in the second EGF-repeat of Del-1. The increased efferocytosis in the presence of Del-1 was associated with the induction of the resolving macrophage phenotype, as demonstrated by upregulation of phagocytic receptors, bridging molecules, the nuclear receptor LXRa and TGFb1. These Del-1 effects were dependent on the alphaVbeta3-integrin, as beta3-integrin deficient macrophages did not display the Del-1-mediated upregulation of LXRa and TGFb1. Blockade of LXRa decreased efferocytosis-dependent upregulation of TGFb1 expression. The presence of the apoptotic neutrophils rather than solely PS was required to mediate the Del-1-mediated macrophage re-programming, as phagocytosis of PS-coated beads in the presence of Del-1 was not sufficient to induce a resolving phenotype in macrophages, while inhibition of the degradation of phagocytosed apoptotic material resulted in decreased LXRa and TGFb1 expression. In vivo, monosodium urate (MSU) crystal-induced peritonitis resulted in increased accumulation of neutrophils and monocytes/macrophages due to Del-1 deficiency, consistent with the effect of Del-1 to block LFA-1-dependent neutrophil and Mac-1-dependent monocyte recruitment, respectively. Conversely but consistently, mice with endothelial over-expression of Del-1 displayed significantly decreased leukocyte numbers 8h after MSU injection. Besides this acute

anti-inflammatory effect of Del-1, Del-1 contributed to RoI in vivo, as Del-1-deficient mice displayed enhanced numbers of apoptotic neutrophils and impaired induction of the resolving macrophage phenotype. Local administration of Del-1 resulted in enhanced efferocytosis of apoptotic neutrophils in MSU-induced peritonitis.
Taken together, our data provide evidence that Del-1 exerts a dual immunomodulatory role by both blocking early inflammatory cell recruitment and by promoting macrophage efferocytic capacity, thus identifying new mechanisms that can be targeted to modulate RoI.

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Flavonoids Can Mediated Inflammation Resolution through Inhibition of Human Neutrophils' Oxidative Burst and Leukotriene B4 Production

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The first reports of the clinical features of inflammation date from 3000BC and this complex process and their modulation have been object of study for many years. Inflammation is a carefully orchestrated response of the organism, involving the recruitment of inflammatory cells, namely neutrophils. Neutrophils are the major body's first line defence, but they are also found in some chronic inflammatory diseases. They are responsible for the production of various inflammatory mediators, such as reactive species (RS) by the rapid consumption of oxygen, prostaglandins, leukotrienes, cytokines, among others. Although inflammatory response is generally self-limited, the balance between the pro- and anti-inflammatory signals may become dislocated, leading to deleterious widespread inflammatory responses. Therefore, the need for efficient drugs with low side effects has become a hot topic in the scientific research field. Flavonoids have been studied as possible alternatives for the anti-inflammatory therapy, as they already demonstrated antioxidant effects. In this sense, the main aim of this work was to study the potential of flavonoids as modulators of human neutrophils' oxidative burst and as inhibitors of leukotriene B_4 (LTB_4) production. As it is a comprehensive and systematic study, a series of 24 structurally related flavonoids (fig. 1) was studied and the structure-relationship was stablished. For this purpose fresh human neutrophils were daily isolated. The RS produced were detected through the use of chemiluminescent and fluorescent probes and the LTB_4 produced was detected by applying an ELISA kit. The obtained results showed that the catechol group in B-ring is important for the activities studied. Indeed, 3',4'-dihydroxyflavone, 5,3',4'-trihydroxyflavone, 7,3',4'-trihydroxyflavone, and luteolin prove to have the essential structural features to influence the inflammatory mediators studied, constituting promising alternatives for the resolution of inflammatory process.

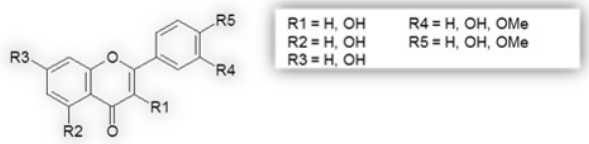

Fig. 1 - Substitution patterns of the studied flavonoids.

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Coagulation Induced by C3aR-Dependent NETosis Drives Protumorigenic Neutrophils during Small Intestinal Tumorigenesis

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Cancer patients show hypercoagulation and neutrophila but a link has up to now been unclear. We set out to study hereditary colorectal cancer (CRC) in the $APC^{Min/+}$ model. Similar to familial adenomatous polyposis (FAP) in humans in this model CRC development is driven by the truncation at codon 850 of the apc gene. Subsequently $APC^{Min/+}$ mice develop mainly in the small intestine (ileum) and sporadically in the large intestine (colon) polyps. Tumorigenesis is associated with the manifestation of hypercoagulation and neutrophil accumulation. All these effects on the haematopoietic system were found to be driven by haematopoietic extrinsic factors. During tumoroginesis neutrophils showing a low-density (LDN) phenotype associated with a functional pro-tumorigenic N2 profile emerged that were pre-set to spontaneous NETosis and were directly imprinted by blood clots. Finally, tumorigenesis results in increased circulating lipopolysaccharide induced activation of the complement cascade and we demonstrate the existence of a feedback loop between neutrophils and complement. We confirm these results in a cohort of patients with small bowel cancer. In our model enhanced thrombus formation induces a protumorigenic phenotype in neutrophils which in addition to spontaneous NETosis, further strengthens hypercoagulation. This study expands our view on the mechanisms underlying cancer-associated hypercoagulation, which could be further exploited as a therapeutic intervention.

FURTHER READING: **Guglietta S**, Chiavelli A, Zagato E, Krieg C, Gandini S, Ravenda PS, Bazolli B, Lu B, Penna G, Rescigno M. Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. **Nat Commun. 2016** Mar 21;7:11037. PubMed PMID: 26996437.

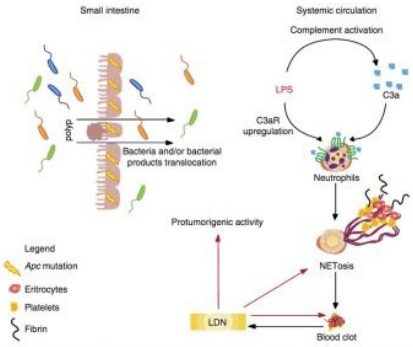

Figure 9 | Neutrophils and coagulation during small intestinal tumorigenesis. The Apc mutation induces growth of polyps in the small intestine of $APC^{Min/+}$ mice and results in defects in gut permeability, ultimately leading to bacterial and/or bacterial products translocation in the systemic circulation. LPS induces upregulation of C3aR on neutrophils (HDNs and LDNs), thereby enhancing the interaction with C3a produced via alternative complement activation. These events result in NET induction, which form a scaffold for the recruitment of components of the coagulation cascade. By interacting with the circulating neutrophils, the formed clot induces LDNs, which can further fuel coagulation in a positive feedback loop by undergoing NETosis and can exert protumorigenic functions.

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Myeloperoxidase in the Regulation of Polymorphonuclear Neutrophil Cell Death

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Polymorphonuclear neutrophils (PMNs) play a key role in host defense. However, their massive accumulation at the site of injury can initiate chronic inflammatory processes, thus the clearance of PMN mediated by regulated cell death is key process. Myeloperoxidase (MPO), highly abundant enzyme in PMN granules, primarily connected with PMN defense machinery is suggested to be involved in PMN regulated cell death. Nevertheless, mechanisms how MPO affects PMN cell death remain incompletely characterized. Interestingly, MPO deficient PMNs revealed significantly decreased rate of cell death characterized by phosphatidylserine surface expression in response to activation of oxidative

burst by12-myristate 13-acetate (PMA). An inhibitor of MPO activity 4-ABAH (50 μM) showed only limited effect on PMA induced cell death compared to MPO deficiency. Interestingly, PMA stimulated PMNs do not present activation of other markers characteristic for apoptotic cell death including activation of caspase 3 and 8 and DNA fragmentation. In contrast, markers characterizing autophagy such as cleavage of LC3 protein and increased expression of p62 were observed in PMA stimulated PMNs.
The important role of MPO in the regulation of the course of inflammation, independent of its putative microbicidal functions, can be potentially linked to MPO ability to modulate the life span of PMN accumulated at the site of inflammation.

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Mice Expressing Human Proteinase 3 Show Sustained Neutrophil Infiltration and a Defect in the Resolution of Inflammation

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Proteinase 3 (PR3) is serine protease expressed exclusively in neutrophils, monocytes and macrophages. This protein has a number of well-characterized pro-inflammatory activities including cleaving extracellular protein matrix, activating chemokines as well as controlling cell survival and proliferation. To better understand the function of PR3 in vivo, a transgenic mouse was generated expressing human PR3 (hPR3Tg). hPR3 mRNA was detected in myeloid cells isolated from the bone marrow of transgenic mice. During zymosan-induced peritonitis, hPR3Tg mice displayed an increased accumulation of neutrophils compared to WT controls 24 hours after the induction of inflammation and this difference increased by 48h. There was no difference in the recruitment of macrophages, B or T lymphocytes at any time point examined. Mice were also subjected to cecum ligation and puncture, a model used to mimic human sepsis. In these experiments hPR3Tg mice displayed decreased survival where 50% of WT mice survived for 7 days post-surgery while 100% of hPR3Tg mice died prior to day 5. This decreased survival was also associated with increased neutrophil infiltration. In vitro, neutrophils from hPR3Tg mice displayed enhanced survival during spontaneous apoptosis compare to WT controls, as well as decreased apoptosis induced by TNF-α treatment and this may in part explain the increased neutrophil numbers observed during the later stages of inflammation. Taken together, our data suggests that hPR3 plays a proinflammatory role in vivo and increased levels of this protein during inflammation affects neutrophil accumulation and survival.

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Polarization of Innate Neutrophils Due to Tollip Deficiency Modulates Colonic Tumorigenesis

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Differential programming of innate neutrophils may bear critical relevance in host defense as well as tumorigenesis. However, molecular mechanisms responsible for neutrophil polarization are still not well understood. Here, we aim to identify and characterize the neutrophil phenotypes in chronic colitis (CC) mouse model. The study focuses at clarifying the molecular and physiological signature of neutrophils in mice subjected to chronic colitis with AOM and DSS challenges. We identify that Toll-interacting- protein (Tollip) may play a key role in modulating the long-term polarization and function of neutrophils as well as anti-tumor defense. We observed that Tollip deficient mice had reduced generation of colon polyps when subjected to prolonged challenges with AOM and DSS. At the cellular level, Tollip deficient mice challenged with AOM-DSS had distinct neutrophil activation profiles as compared to wild type mice, including the differential expression of cell surface co-stimulatory molecules, migratory behaviors, as well as cytokine expression profiles. Based on the in vivo and in vitro studies, we conclude that Tollip may play a key role during the polarization of innate neutrophils involved in the modulation of chronic colitis tumor environment. Further studies with regard to the molecular mechanisms may enable a better

understanding of neutrophil modulation in colon tumorigenesis.

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Cystic Fibrosis is a New Type of Leukocyte Adhesion Deficiency Disease

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RATIONALE Cystic fibrosis (CF) is a genetic disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Persistent lung inflammation, characterized by increasing polymorphonuclear leukocyte recruitment, is a major cause of the decline in respiratory function in patients with CF and is a leading cause of morbidity and mortality. CFTR is expressed in various cell types, including leukocytes, but its involvement in the regulation of leukocyte recruitment in inflammation is unknown. OBJECTIVES We evaluated whether CF leukocytes might present alterations in integrin activation, cell adhesion and migration controlled by chemoattractants, key processes governing innate and acquired immune responses. METHODS
In this study we used integrin affinity assays, ex vivo adhesion and chemotaxis assays, flow cytometry, immunofluorescence staining, in vivo analysis and rho small GTPase signaling activity assays. MEASUREMENTS AND MAIN RESULTS We found that chemoattractant-induced activations of β1 and β2 integrins and of chemotaxis are defective in mononuclear cells isolated from patients with CF. In contrast polymorphonuclear leukocyte adhesion and chemotaxis were normal. The functionality of β1 and β2 integrins was restored by treating CF monocytes with the CFTR-correcting drugs VRT325 and VX809. Moreover, treatment of healthy monocytes with the CFTR inhibitor CFTR(inh)-172 blocked integrin activation by chemoattractants. In a murine model of lung inflammation, we found that integrin-independent migration of CF monocytes into the lung parenchyma was normal, whereas, in contrast, integrin-dependent transmigration into the alveolar space was completely impaired. Finally, signal transduction analysis showed that, in CF monocytes, chemoattractant-triggered activation of RhoA and CDC42 rho small GTPases (controlling integrin activation and chemotaxis, respectively) was strongly deficient. CONCLUSIONS
Altogether, these data highlight the critical regulatory role of CFTR in integrin activation by chemoattractants in monocytes and identify CF as a new, cell type-selective, leukocyte adhesion deficiency disease (LAD-IV), providing new insights into CF pathogenesis.

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Human Blood Monocytes are Able to Form Extracellular Traps

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Background
Neutrophil extracellular traps (NETs) are extracellular DNA filaments formed during neutrophil activation, a process called netosis and originally associated with neutrophil antibacterial properties. Several lines of evidence now suggest a major role for netosis in thrombosis, auto-immune diseases and cancer. A similar mechanism, called etosis, has been reported in other immune cells such as eosinophils. We investigated if such a mechanism could exist in human monocytes.
Materiel and Methods
Magnetically sorted CD14+ monocytes and monocyte-derived dendritic cells were stimulated with phorbol-12-myristate-13-acetate, calcium ionophore (A23187), platelet-activating factor and zymosan A. Extracellular traps release was quantified by Sytox Green fluorescence. NETs were visualized by immunofluorescence with antibodies against myeloperoxidase, lactoferrin, citrullinated histones, elastase, and tissue factor. DNA-

myeloperoxidase complexes were measured by ELISA and histone citrullination was assessed by western blot.

Results

We demonstrate that human blood monocytes are capable of extracellular trap (ET) release in response to several chemical and biological stimuli. In contrast, monocyte-derived dendritic cells are not capable of etosis but rather undergo necrotic cell death upon stimulation. By microscopy, we show that monocyte ETs display a morphology analogous to NETs, and are associated with myeloperoxidase, lactoferrin, citrullinated histones and elastase. Monocyte etosis depends on oxidative burst via NADPH oxidase activation but not on myeloperoxidase activity, in contrast to neutrophils. Finally, we provide evidence of tissue factor on monocyte ETs, a feature that could be relevant to monocyte thrombogenic properties.

Conclusion

We demonstrate that upon ex vivo stimulation, human blood monocytes can release extracellular traps bearing several active proteins. This new cellular mechanism is likely to improve our understanding of monocytes' role in multiple pathological contexts such as inflammatory disorders, infection or thrombosis.

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Arthritogenic Citrullinated Peptides Present in Neutrophil Extracellular Traps are Internalized by Synovial Fibroblasts through a RAGE-TLR9 Axis and Activate Adaptive Immunity in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder characterized by inflammation and destruction of the synovial joints and by the development of humoral and cellular autoimmunity to citrullinated proteins. Autoimmunity to citrullinated antigens appears years before the onset of clinical symptoms of RA and is highly specific for this disease. Previous studies implicate the formation of neutrophil extracellular traps (NETs) as a source of citrullinated autoantigens and as a mechanism promoting the activation of RA synovial fibroblasts (FLS), cells with crucial roles in joint damage. We investigated the molecular mechanism by which NETs promote a proinflammatory phenotype in FLS, and whether these interactions play a role in the generation of adaptive immune responses to citrullinated autoantigens. Autoantibodies recognizing citrullinated antigens(ACPAs), isolated from RA patients, canenhance NETosis and recognize multiple citrullinated peptides present in NETs. Proteomic analysis of NETs demonstrates that myeloperoxidase (MPO) and neutrophil elastase, among other proteins, are citrullinated. Autoantibodies against citrullinated MPO are present in synovial fluid from RA patients. Furthermore, NETs containing these citrullinated peptides are internalized by FLS through a RAGE-TLR-9 pathway. NETs' internalizationis required for the upregulation of proinflammatory cytokine synthesis by FLS. Interleukin-17B (IL-17B), externalized in NETs, induces upregulation of major histocompatibility complex class II (MHCII) in FLS. Once internalized, NET-peptides are loaded into MHCII intracellularly and trafficked to the FLS plasma membrane. Arthritogenic NET peptidesare then presented by FLS to Ag-specific T cells and induce their activation. Humanized HLA-DRB1*0401 transgenic mice immunized with mouse FLS loaded with NETs

develop ACPAs and antigen-specific T cell responses. Epitope mapping arrays demonstrate the enhanced presence of autoantibodies against citrullinated forms of relevant autoantigens implicated in RA pathogenesis in those animals immunized with FLS-NETs. These results suggest that NETs are a source of arthritogenic peptides in the synovium and implicate FLS as mediators in RA pathogenesis, through the internalization and presentation ofcitrullinated peptides to the adaptive immune system leading to ACPA formation and T cell activation.

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PI3K Activity in the Tumor Microenvironment Promotes Mammary Tumor Growth and Metastasis and Inhibits Anti-Tumor Immunity

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Identification of targeted therapies that also activate anti-tumor immunity could offer significant hope for cancer patients. In this report, therapeutic effects of the pan-PI3K inhibitor, BKM120, on growth and metastasis of mammary tumor allografts in mice, were observed to be associated with increased infiltration of IFN-γ and TNFα expressing immune cells into tumors. Moreover, in a humanized mouse model using patient derived xenografts growing in immune compromised mice engrafted with the patient's hematopoietic cells, BKM120 also inhibited tumor growth and enhanced anti-tumor immune responses. Surprisingly, similar effects occurred when $PI3K\gamma^{-/-}$ mice were implanted with $PI3K^{WT}$ MMTV-PyMT tumor, without any exogenous inhibitor treatment. Effects of host PI3Kγ loss on tumor growth were partially reversed by CD8+T depletion. Altogether our data indicate a key mechanism for PI3K inhibition of tumor growth and metastasis involves enhancement of anti-tumor immunity, particularly CD8+T effector cells, in addition to direct effects on tumor cells.

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Ifn-γ and IL-17A Establish the Balance between P. aeruginosa Clearance and Inflammatory Potential during Infection of Human Macrophage-Neutrophil Co-Cultures

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Neutrophils are essential for protection against extracellular bacteria but neutrophil-dominated inflammation can also compromise organ function through tissue damage. Optimal anti-bacterial immunity should harness the microbicidal activity of neutrophils while minimising their potential for causing injury. Neutrophils do not act in isolation and during inflammation, they respond to cues provided by other cells such as macrophages, which influence their activation and life span.In vivomodels have dominated the study of anti-bacterial immunity and there is a need of human models for the dissection of macrophage-neutrophil communication during bacterial infection. This work describes the development of a human macrophage-neutrophil infection assay and its use to model Th1 (IFN-γ)- and Th17 (IL-17A)-driven microbicidal activity and inflammatory potential upon infection. Results show that IFN-γ and IL-17A have opposite effects on the killing ability of macrophages and neutrophils co-cultures; bacterial killing was reduced by IFN-γ and promoted by IL-17A. In addition macrophages and neutrophils specifically collaborated for the production of IL-1β and IL-1α and IFN-γ-treated co-cultures generated significantly less IL-1β and IL-1α compared to those treated with IL-17A. This effect was not observed with other cytokines such as TNF-α, IL-6, MIP-1α and MCP-1. Thus phagocyte co-cultures provide a suitable model of human anti-microbial immunity and unveil an unappreciated collaboration between macrophages and neutrophils in the promotion of IL-1-mediated inflammation which is quenched in the presence of IFN-γ.

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Advances on the Biology of 6-Sulfo LacNac (slan)/M-Dc8+ Cells

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A population of human myeloid cells, sharing some functional characteristics with classical myeloid dendritic cells (DCs), have been recently identified by using specific mAbs (M-DC8) recognizing the 6-Sulfo LacNac (slan) carbohydrate modification of PSGL-1, whose acronym gave thus origin to their name (namely, "slanDCs"). However, on a two-dimensional flow cytometry dot plot of CD14 and CD16 expression in peripheral blood mononuclear cells (PBMCs), slanDCs/M-DC8$^+$ myeloid cells overlap, in part, with the CD14^{dim}CD16$^+$ monocytes, suggesting that they might actually represent a subset of the so-called non-classical monocytes. Functionally, blood slanDCs/M-DC8$^+$ cells have been described as potent pro-inflammatory cells, based on their capacity to produce large amount of TNFα and IL-12p70 upon stimulation with TLR ligands. In addition, blood slanDCs/M-DC8$^+$ cells are known to promote proliferation, cytotoxicity and IFNγ production by natural killer (NK) cells, as well as strong antigen-specific T-cell responses. Furthermore, it is well established that slanDCs/M-DC8$^+$ cells locate in peripheral tissues, especially under inflammatory conditions, and selectively in carcinoma-draining lymph nodes, where they marginate metastatic cells. However, even though blood slanDC/M-DC8$^+$ cell function and phenotype have been broadly delineated, an extensive comparison between blood and tissue slanDCs/M-DC8$^+$ cells, as well as between tissue slanDCs/M-DC8$^+$ cells and other tissue DC/macrophage populations, has never been performed. For such a purpose, we recently made an extensive phenotypic and functional characterization of slanDCs/M-DC8$^+$ cells in human tonsils. We found that tonsil slanDCs/M-DC8$^+$ cells represent a unique population of dendritic cells, displaying a surface marker repertoire distinct from their circulating counterpart and from other tonsil DC and macrophage subsets described to date. Functionally, tonsil slanDCs/M-DC8$^+$ cells exhibit an efficient antigen presentation capacity and a constitutive secretion of TNFα. Notably, such DC phenotype and function were substantially reproduced by culturing blood slanDCs/M-DC8$^+$ cells in tonsil-derived medium, supporting the hypothesis of a full DC differentiation program occurring within tonsils. Our data uncover novel information on plasticity by blood slanDCs/M-DC8$^+$ cells and their ultimate commitment within tissue microenvironments.

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IFNα Enhances the Production of IL-6 by Human Neutrophils Activated via TLR8

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PURPOSE

Recently, we reported that human neutrophils produce biologically active IL-6 when incubated with agonists activating TLR8[1], a receptor recognizing viral single strand RNA. Herein, we investigated the effect of IFNα, a cytokine known to modulate the early innate immune responses toward viral and bacterial infections, on the production of IL-6 by TLR8-activated neutrophils.

METHODS

Human neutrophils isolated from healthy donors or systemic lupus erythematosus (SLE) patients by negative selection using immunomagnetic beads (99.7 ± 0.2 % purity), were incubated for up to 20 h with or without 5 μM R848 (a TLR8 agonist), in the presence or the absence of 1000 U/ml IFNα. mRNA expression and cytokine production were then measured by, respectively, RT-qPCR and ELISA, while C/EBPβ transcription factor recruitment at the IL-6 genomic locus was investigated by chromatin immunoprecipitation (ChIP) assays.

RESULTS

In this study[2], we demonstrate that IFNα potently enhances the production of IL-6 in neutrophils incubated with R848. Such an effect is not caused by an IFNα-dependent induction of TLR7, another receptor for R848, but, rather, by an increased release of TNFα, which in turn amplifies IL-6 expression. Endogenous TNFα, in fact, was shown to promote an augmented synthesis of the IkBζ co-activator and an enhanced recruitment of C/EBPβ to the IL-6 promoter. Moreover, our data uncover that neutrophils from active SLE patients, displaying an IFN-induced gene expression signature, produce

increased amounts of both IL-6 and TNFα in response to R848 as compared to healthy donors.

DISCUSSION

Altogether, data clarify the molecular bases of the IFNα-dependent enhancement of IL-6 production in TLR8-activated neutrophils. More in general, we show that TLR8 ligands, IFNα and TNFα, three players often coexisting in many diseases of viral or autoimmune origin, promote a strong production of IL-6 in human neutrophils, placing the same neutrophils among potential targets for immunotherapeutic interventions.

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Emperipolesis is a Novel Cell-In-Cell Phenomenon that Mediates Transfer of Neutrophil Membrane to Megakaryocytes and Platelets

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BACKGROUND: Emperipolesis (EP) is a poorly-understood phenomenon wherein bone marrow megakaryocytes (MKs) actively enclose intact neutrophils. Common even in healthy individuals, EP increases in frequency in myeloproliferative states and in states of accelerated platelet production. However, the mechanism and significance of EP are unknown.

METHODS: We assessed the frequency of EP in healthy mice and in animals subjected to systemic LPS and to induced thrombocytopenia. A novel model of EP was developed through incubation of cultured bone marrow MKs together with neutrophils, and characterized using immunofluorescence microscopy, electron microscopy and live-cell spinning disk imaging. The mechanism of EP was interrogated using specific inhibitors as well as in mice with informative genetic deletions. The impact of EP was interrogated through engraftment of MKs co-cultured with neutrophils back into recipient mice.

RESULTS: EP is evident in approximately 5% of MKs in bone marrow, increasing 2-3-fold with LPS and peripheral platelet depletion. Imaging discloses sequential phases, beginning with uptake into a distinct vacuole followed by release of the neutrophil directly into the MK cytoplasm (Figure 1). Uptake is mediated in part through neutrophil beta2 integrins and requires active cytoskeletal rearrangement by both neutrophils and MKs. Within the cytoplasm, neutrophils develop direct membrane contiguity with the MK's platelet-producing demarcation membrane system (DMS), resulting in transfer of neutrophil proteins to the MK surface and to platelets produced by MKs engrafted into live mice. MK cell contents are reciprocally transferred to neutrophils prior to their release from MKs alive and intact. Compared with MKs cultured alone, MKs cultured together with neutrophils demonstrate more efficient platelet generation in vivo, suggesting that one effect of EP is to enhance the ability of MKs to respond to thrombocytopenic stress.

CONCLUSIONS: Emperipolesis is a highly novel cell-in-cell interaction that is mediated in part by neutrophil beta2 integrins and results in the reciprocal exchange of cell contents between megakaryocytes and neutrophils, potentially enhancing production of platelets. Additional consequences of emperipolesis for MKs and neutrophils remain to be defined.

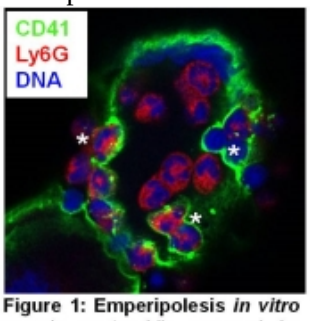

Figure 1: Emperipolesis *in vitro* reveals neutrophils encased by CD41+ MK membrane (i.e. in vacuoles (★)) or free in cytoplasm

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Type I Interferon-Mediated Polarization of Tumor-Associated Neutrophils Depends on CSF3R Signaling Pathway

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Neutrophils play strategic role during inflammatory responses of the host. Importantly, inflammation has long been associated with enhanced susceptibility for cancer thus neutrophils, as a crucial component of this process, play essential role in tumorigenesis and remain an independent prognostic marker in a broad variety of neoplasias.

It is known that neutrophils are influenced by the tumor microenvironment and differentiate into anti-tumor (N1) or pro-tumor (N2) cells. The most plausible explanation for the different neutrophil phenotypes in tumor is alteration of the local cytokine milieu. Notably, we could recently demonstrate that type I interferons polarize neutrophils into N1 phenotype. In mice that lack endogenous type I IFNs elevated numbers of N2 neutrophils could be observed, accumulating in tumors and pre-metastatic lungs. Such neutrophils efficiently support tumor growth by up-regulating pro-angiogenic molecules e.g. VEGF and MMP9. Moreover, these cells display prolonged survival, secrete higher amounts of neutrophil-attracting chemokines and support metastatic processes by up-regulation of pro-metastatic proteins, like Bv8, MMP9, S100A8 and S100A9. Importantly, these neutrophils show also impaired tumor cell killing due to repressed ROS production and less effective NETs release. Treatment with rmIFN-b reverses these effects, leading to anti-tumor N1 polarization. Importantly, neutrophils isolated from melanoma patients undergoing type I IFN therapy show augmented N1 anti-tumor characteristics, suggesting similar regulating mechanisms in human.

Here, we evaluated the molecular mechanisms involved in interferon-mediated alterations of neutrophil polarization. We observed that in the absence of type I interferons the expression level of G-CSF is markedly elevated in neutrophils from different anatomical compartments. This could be reversed by rmIFN-β treatment. Importantly, we could show that G-CSF induces through its receptor CSF3R the synthesis of Nicotinamide phosphoribosyltransferase (NAMPT), which is an unique enzyme-cytokine molecule. It is a rate-limiting enzyme, converting nicotinamide (NA) into NAD+ that in turn activates NAD+-dependent protein deacetylases sirtuins (SIRTs). Moreover, extracellularly, NAMPT exhibits also the cytokine-like functions that lead to upregulation of pStat3. Extracellular NAMPT levels are increased in various inflammatory conditions, e.g. tumors. That makes this pleiotropic molecule an important player in tumor/host cross-talk. NAMPT serves as an inhibitor of neutrophil apoptosis and as neutrophil chemoattractant. It is a potent pro-inflammatory factor upregulating ROS release and pro-angiogenic factor (smooth muscle maturation). NAMPT is strongly overexpressed in many cancers and its elevated levels are correlated with tumorigenicity. Analysis of CSF3R downstream signaling in neutrophils from interferon deficient mice revealed highly elevated expression of NAMPT, several Sirtuins and pStat3, on gene and protein expression level. This was in line with enhanced tumorigenesis in these mice. Notably, treatment of such animals with Nampt or Sirtuin inhibitors leads to strongly inhibited tumor growth and altered neutrophil differentiation towards N1 phenotype.

Based on these observations, we identified a new mechanism of interferon-mediated polarization of TANs that involves NAMPT inhibition. Importantly, both – enzymatic and cytokine-like activity of NAMPT seems to be influenced by interferons. Since TANs represent a highly potent therapeutic target, these data highlight the therapeutic potential of interferons as NAMPT inhibitors, suggesting optimization of their clinical use as potent anti-tumor agent.

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Tumor Associated Neutrophils (TAN) Impair Cytotoxic T-Cells Anti-Tumor Effect by Inducting CD8+ Cells Apoptosis and Inhibiting Proliferation

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The tumor microenvironment includes a complex network of immune cells comprising macrophages, MDSCs, neutrophils, T-cells and more. The

contribution of Tumor Associated Neutrophils (TAN) to tumor progression has been a matter of debate as both pro- and anti-tumor functions have been reported. In recent years, there has been a growing interest in the relationship and co-regulation between tumor-infiltrating myeloid cells and cytotoxic T cells (CTLs). Infiltrating T lymphocytes have been attributed an anti-tumor function since the presence of activated CD8+T cells both within the tumor and in the peritumoral stroma provided a positive prognosis for patients. In our current work, we evaluated the effects of TAN on CD8+ cytotoxic T-cells (CTLs) survival, proliferation and anti-tumor effect. Using a modified Winn assay, we first demonstrated *in vivo* that while CD8+ T-cells have the ability to slow down tumor initial growth, TANs revert this anti-tumor effect of CD8+ CTLs. We found that neutrophils isolated from murine tumors of lung cancer and mesothelioma, induce significant apoptosis of CD8+ CTLs. This effect was found to be contact-dependent, and mediated by iNOS. Furthermore, this effect was markedly reduced in TNFα knock-out mice, suggesting that TNFα has an essential role in this process. Although TAN were found to have a mild positive effect on the activation of CD8+ T-cells, the use of activation markers such as CD69 and intracellular cytokines IFN-γ showed that TAN primarily promote the apoptosis of non-activated CD8+ T-cells. Using the CFSE method, we also demonstrated that TAN inhibit the proliferation of CD8+ CTLs.

Our findings demonstrate the importance of TAN in the regulation of the immune reaction to cancer, adding another layer on the understanding of the role and effects of neutrophils in cancer.

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Differential Mobilization of Circulating Neutrophil Subpopulations in Breast Cancer Metastasis

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Breast cancer is the most common cancer among Canadian women over the age of 20, representing 26% of all cancer cases in Canadian women. Metastatic breast cancer is the most advanced stage (stage IV) of the disease and it is largely incurable. Breast cancer cells display preferences for specific metastatic sites including the bone, lung, and liver. The liver represents a prominent site for breast cancer metastasis, with 50-70% of women with metastatic breast cancer developing hepatic metastases.

The steps involved in the metastatic cascade rely on reciprocal interactions between cancer cells and their microenvironment. Distinct immune infiltrates can either impair the metastatic process or conversely, assist in the seeding, colonization and growth of disseminated cancer cells. Within distal organs, immune cells and their mediators are known to facilitate metastasis formation. However, the contribution of tumor-induced systemic inflammation to metastasis and the mechanisms regulating systemic inflammation are not well characterized. Using lung and liver-metastatic variants of 4T1 breast cancer cells model, we have revealed that there are increased recruitment of myeloid-derived/granulocytic (Gr-1+) and neutrophils (NE+) in the lungs and livers of mice bearing lung and liver metastasis respectively. However, based on the Gr-1+ depletion studies, it was observed that infiltrating myeloid-derived/granulocytic cells, including neutrophils, were essential for the formation of liver metastases but not for lung metastases. Intriguingly, we have found that in peripheral blood, lung and liver metastases have the ability to mobilize differently the two distinct populations of high-density (HDNs) and low density neutrophils (LDNs) based on a density gradient centrifugation. In the peripheral blood of mice bearing liver metastases, there is a dramatic increase in the mobilization of LDNs compared to mice bearing lung metastases. While HDNs are characterized as being more of an anti-tumor “N1” neutrophil with increased phagocytic activity, production of reactive oxygen species, and N1 associated genes (IFN-B and iNOS), LDNs function as a pro-tumorigenic “N2” neutrophil with increased expression of N2 associated genes (Arg1 and MMP9). These two different neutrophil populations are functionally important in liver metastases where HDNs have the ability to suppress metastases while LDNs cannot. Thus, we believe

that liver metastatic breast cancer cells rely on interactions with neutrophils within the liver microenvironment for colonization and growth. Our results demonstrate the importance of investigating the role played by these two neutrophil subpopulations which may represent a new potential therapeutic strategy to inhibit metastatic diseases.

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Characterization of Neutrophil Sub-Populations in Steady State and during Cancer Inflammation Using High Dimensional Data Analysis

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Experimental and clinical evidences of the past years revealed an unexpected plasticity of neutrophils in several pathologic conditions including cancer. Similarly to macrophages, it has been proposed that neutrophils show a pro-tumorigenic (N2) or an anti-tumorigenic (N1) phenotype. In addition, it has been suggested that neutrophils are of key importance in the metastatic process. Whether and how many neutrophil sub-populations could potentially exist in cancer is still a matter of debate. Here we used a mass cytometry based high dimensional staining panel to analyze blood neutrophils in healthy donors. Mass cytometry uses metal-tagged instead of fluorochrome-tagged antibodies thus eliminating spectral overlap and in combination with barcoding enables the measurement of more than 30 molecular species on a single cell in multiple parallel samples. Due to the complexity of the data set and in order to reduce analytical bias we use advanced bioinformatics in order to identify key immune populations. Using this approach we were able to identify multiple neutrophil populations in the peripheral blood of healthy donors and now you want to explore their role and changes during cancer inflammation.

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Elucidating the Mechanism of Neutrophil Apoptosis Inhibition by Francisella Tularensis

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Francisella tularensis is a Gram-negative, facultative intracellular pathogen and the etiologic agent of the zoonotic disease tularemia. Inhalation of as few as ten organisms can result in severe, and potentially lethal, pneumonic disease. The pathogenicity of *F. tularensis* is dependent on its ability to modulate host immune responses, and to survive and replicate within several different types of cells, including neutrophils. It is well established that defects in neutrophil turnover are defining features of an abnormal and ineffective immune response that exacerbates inflammation and results in host tissue destruction. Notably, published data indicate that neutrophils contribute to the pathology of tularemia, as demonstrated by the fact that blocking neutrophil migration into infected tissues decreases bacterial burden and tissue damage, and enhances host survival. Previous data from our laboratory demonstrated that *F. tularensis* prevents NADPH oxidase assembly and activation, escapes the phagosomes, and replicates in the neutrophil cytosol. Furthermore, we have demonstrated that *F. tularensis* inhibits spontaneous neutrophil apoptosis and significantly extends neutrophil lifespan. At the molecular level, *F. tularensis* impairs activation and activity of the intrinsic and extrinsic caspases in addition to modulating various anti- and pro-apoptotic proteins; however, the underlying molecular mechanisms are incompletely defined and bacterial factors required for this aspect of virulence are unknown. We now show that one or more factors secreted by *F. tularensis* can extend neutrophil lifespan, albeit to a lesser extent than direct infection. Additional studies have identified a role for bacterial lipoproteins (BLP) in delaying neutrophil apoptosis, whereas two major *Francisella* virulence factors, lipopolysaccharide and capsule, are neither necessary nor sufficient for this delay. Preliminary data suggest BLP act via a Toll-like receptor (TLR) 2/1-dependent manner; this mechanism is influenced by a TLR1 single nucleotide polymorphism that affects TLR1

trafficking to the plasma membrane. Our data also indicate that phosphoinositide-3-kinase activity is essential for *F. tularensis*-mediated neutrophil apoptosis inhibition, and whether this response is mechanistically linked to TLR2/1 is under investigation. Finally, we show that a related organism, *F. novicida*, also delays neutrophil apoptosis by mechanisms similar yet distinct from *F. tularensis*. Taken together, our data are significant as they provide fundamental insight into the mechanisms of pathogen-mediated perturbation of neutrophil lifespan during infection and may contribute to the identification of targets for therapeutic intervention or vaccines.

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Graphene Oxide (GO) Triggers Neutrophil Extracellular Trap (NET) Formation in Human Neutrophils in a Size- Dependent Manner

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Neutrophils, as the first-line defense of the innate immune system, are capable of detecting and promptly eliminating a wide range of foreign intruders in order to maintain organismal homeostasis. Neutrophils can thus engulf and digest microbes intracellularly or they may release neutrophil extracellular traps (NETs) to capture and destroy microbes extracellularly. In this study, we investigated whether neutrophils are also capable of 'sensing' and responding to graphene oxide (GO). GO is a synthetic, carbon-based material envisioned for a range of different applications including in medicine. To this end, we isolated primary human peripheral blood neutrophils and exposed them to GO displaying average lateral dimensions of 80-100 nm (small) or 10 µm (large). First, GO flakes were confirmed to be endotoxin-free. Both small and large flakes decreased cell viability (about 30% loss of cell viability at 3 h versus control, as assessed on ATP measurements). Using transmission electron microscopy, we noted that GO flakes were aligned with the cell membrane, possibly leading to membrane stripping, which could be linked to the loss of cell viability. Furthermore, we observed GO-triggered NET formation, as evidenced by scanning electron microscopy and confocal microscopy. Using an enzymatic assay for neutrophil elastase (NE), as an indirect readout for NET release, we showed that GO triggers NET formation in a size-dependent manner, with the large flakes inducing stronger NET release. NETs were, in turn, able to degrade GO. Hence, using confocal Raman analysis, we observed degradation of GO in purified NETs, and this was blocked in the presence of a selective inhibitor of myeloperoxidase. Additionally, using confocal microscopy, we observed that small and large GO flakes completely disrupted lipid rafts in a manner comparable to methyl-β-cyclodextrin. Overall, our work has provided new insights regarding the interaction of neutrophils with GO, a 2-D carbon-based material.

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Neisseria gonorrhoeae Co-Opts Complement Receptor 3 (CR3) for Silent Entry into Human Neutrophils

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Neisseria gonorrhoeae (Gc) is a human-specific, gram-negative bacterium that causes the sexually-transmitted infection gonorrhea. Symptomatic gonorrhea is characterized by the influx of neutrophils to sites of infection. Notably, Gc can be cultured from neutrophil-rich secretions. The ability of Gc to survive after exposure to human neutrophils is influenced by bacterial expression of phase-variable opacity-associated (Opa) outer membrane proteins. Using adherent, chemokine-treated primary human neutrophils, we found that Opa- Gc delays

phagosome fusion with neutrophil primary granules to prevent exposure to granule degradative components and consequent bacterial killing (Johnson and Criss, *Cellular Microbiology* 2013). In addition, Opa- bacteria do not promote NADPH oxidase activation and do not induce oxidative burst in neutrophils. In contrast, Opa+ Gc that engage neutrophil CEACAMs induces a potent oxidative burst in neutrophils and is phagocytosed into mature phagolysosomes that are bactericidal. These findings suggest that the route of bacterial phagocytosis influences the extent of neutrophil activation and thus Gc survival. However, the mechanism by which neutrophils phagocytose unopsonized, Opa- Gc is unknown. In primary cervical epithelial cells, Opa- Gc are internalized via a non-opsonic interaction between pili and porin on the Gc surface and epithelial Complement Receptor 3 (CR3; CD11b/CD18; αMβ2integrin; Mac-1) (Edwards et al, *Cellular Microbiology* 2002). In the current study, we tested the hypothesis that Opa- Gc uses CR3 for phagocytosis by primary human neutrophils. We found a dramatic decrease in the binding and internalization of Opa- Gc by neutrophils following treatment with blocking antibodies against either CD11b or CD18. Conversely, expression of CR3 enhanced uptake of Opa- Gc by HL-60 cells, and was dependent on the I-domain of CD11b. We found that IL-8 primed, adherent neutrophils express more total and activated CR3 on their surface than suspension neutrophils. Treating unprimed neutrophils in suspension with phorbol ester increased total and activated CR3 on the neutrophil surface and was sufficient to increase Gc association with the cells. These findings indicate that activated CR3 is required for Opa- Gc uptake and may in part explain the discrepancy between our results and those in the field regarding phagocytosis of Opa- Gc. We found no evidence that human neutrophils released C3 to opsonize Gc and generate the iC3b ligand for CR3. Interestingly, unlike in the epithelial cell model, the CR3-mediated association of Opa- Gc with neutrophils was independent of pili or pilin glycosylation. However, pili were found to enhance contact and Gc proximity to the neutrophil surface, in a CR3-independent manner. We conclude that unopsonized, Opa- Gc uses activated CR3 as the predominant route of entry into primary human neutrophils. Our findings help explain why unopsonized, Opa- Gc phenocopy complement-opsonized Gc in their interactions with neutrophils (*e.g.* lack of oxidative burst, delayed phagosome maturation, actin-dependent phagocytosis). We posit that Opa- bacteria co-opt CR3 to "silently" infect neutrophils and avoid cellular activation, to establish a safe niche that promotes bacterial survival.

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Epithelial Cells Directly Contribute to Attraction and Activation of Neutrophils during Infection with the Intracellular Bacterium Chlamydia trachomatis

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Genital infection with the obligate intracellular bacterium *Chlamydia trachomatis* can cause inflammation of genital tract and pelvis with the potential consequence of tubal scarring and female infertility. The mechanisms that lead to genital tract tissue damage are still unclear but previous studies suggest a contribution from neutrophil granulocytes. Although infected epithelial cells may secrete various mediators it is not clear how these cells are recognized by and stimulate neutrophils. We here rebuild this situation *in vitro* with the goal of understanding the factors involved on the sides of *C. trachomatis*, infected epithelial cell and responding neutrophil. Mouse neutrophils were generated from progenitors 'conditionally immortalized' with regulable Hoxb8. Epithelial cells were infected with *C. trachomatis*; to test for the role of the chlamydial cryptic plasmid known to contribute to tissue inflammation we also used a plasmidless bacterial strain. Supernatants of infected cells were collected and coincubated with neutrophils.
In transwell migration assays, neutrophils showed considerably higher migration towards supernatants of *Chlamydia* infected HeLa cells than to those of uninfected cells. When wt neutrophils were incubated in supernatants of infected HeLa cells, neutrophil apoptosis was significantly reduced. This activity was neutrophil MyD88/TRIF independent and was absent from supernatants of HeLa cells infected with the plasmidless strain. Surprisingly, supernatants from both strains induced similar levels of TNF in wt but not $MyD88^{-/-}TRIF^{-/-}$ neutrophils. These findings suggest that neutrophil-activation by

infected epithelial cells occurs both TLR-dependently and –independently and at least partly depends on the chlamydial plasmid. We are currently investigating the role of the cryptic chlamydial plasmid with particular focus on the virulence factor Pgp3 using a Pgp3-deficient plasmid-reconstituted chlamydial strain. Pgp3 had been shown by others to play a role in pathogenicity and tissue damage formation during chlamydial infection in mice and thus is a promising candidate. Our system illustrates that the interaction of infected epithelial cells with neutrophils alone may entertain inflammation and will permit more in-depth study of immune response and tissue damage during chlamydial infection.

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Release of Neutrophil Extracellular Traps (NETs) and Production of Reactive Oxygen Species (ROS) in Response to Acinetobacter Baumanii Isolated from Septic Patients.

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Neutrophil extracellular traps (NETs) constitute an important part of innate immunity. These threads of DNA in complex with bactericidal enzymes, which are primarily accumulated within cytoplasmatic granules of neutrophils, are actively released from cells to immobilize and entrap invading pathogens. Several studies indicated that NETs are released during sepsis, as confirmed by plasma freeDNA and citrullinated histones presence in patients blood. Acinetobacter baumanii is a Gram-negative bacteria that can cause opportunistic infections, including sepsis in immunocomprised patients.

The aim of the study was to assess the ability of Acinetobacter baumanii isolated from blood of septic child to induce NETs and production of ROS from healthy neutrophils.

Neutrophils were isolated from 8 buffy coats of healthy blood donors using density gradient centrigugation and sedimentation in 1% polyvinyl alcohol. Bacteria were isolated from peripheral blood after culturing for 24 hours in BacT Alert PF media, kept at -80ºC and thawed directly before experiments. Bacteria were allowed to grow on Columbia agar with 5% sheep blood, and were then suspended in 0.9% NaCl in OD600 of 0.5. Neutrophils were stimulated with 80 µl of bacteria suspension or phorbol 12-myristate 13-acetate (PMA, 100 nM). NETs were quantified as amount of extracellular DNA bound to Sytox Green and measured with a fluorometer and visualized by immunofluorescent microscopy with Sytox Orange and antibody anti-MPO conjugated with FITC. Intracellular production of reactive oxygen species (ROS) was analyzed by flow cytometry after dihydrorhodamine 123 (DHR) staining. Extracellular release of ROS was measured by chemiluminescence assay using luminol as a substrate and PMA, bacteria or medium alone as stimulators. Degradation of NETs was evaluated by fluorescent microscopy after adding bacteria to NETs already released by PMA stimulation, after 1,2, 3 and 4 hours of coincubation of NETs and bacteria at 37ºC and 5% CO2.

Acinetobacter baumani stimulated NETs release which was confirmed in qualitative and quantitative experiments. Amount of NETs released from cells measured as fluorescence of Sytox Green bound to extracellular DNA was 29428±8 713RFU in unstimulated samples and 35878±11809 RFU in bacteria stimulated samples, p=0.047. Intracellular ROS production was slightly induced: DHR fluorescence was 15.3±3.97 mcf for live cells and 23.2±4.7 mcf for cell incubated with bacteria, p=0.03; however fluorescence for PMA stimulated samples was 123.3±31.7 mcf. A. baumanii did not caused release ROS to extracellular matrix, as found in chemiluminescent assay (lack of increase in luminol fluorescence comparing with non stimulated cells, p=0.94). A. baumani did not degrade NETs.

We found that Acinetobacter baumanii isolated from patients with sepsis stimulates innate immunity by induction of NETs release and intracellular ROS production. No NETs degradation proves lack of nucleases expression in the bacteria.

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The Role of Neutrophils in Mice during Chlamydia Infection

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The sexually transmitted Chlamydia trachomatis genital infection is prevalent worldwide. This obligate intracellular bacterium can cause severe tissue damage such as hydrosalpinx formation and subsequent infertility in women. It is still not fully understood how the immune reaction causes this pathology. Neutrophil granulocytes are candidates because of their ability to induce tissue damage in other infections. We studied infiltration of immune cells and tissue damage in a model of genital infection with Chlamydia muridarum in mice. We analyzed lymphoid and myeloid subpopulations in the genital tract after various time points by flow cytometry. To analyze the effect of neutrophils on chlamydial infection and inflammation we used conditional Mcl-1 knock out (Mcl-1 ko) mice deficient in mature neutrophils.
As expected, the chlamydial burden in the genital tract increased in the first week after infection and bacteria were cleared 3 weeks later. There was no difference in chlamydial burden during the course of infection between wild type (wt) and Mcl-1 ko mice. In the first two weeks, neutrophils, monocytic cells and T cells infiltrated the genital tract of wild type mice. We could not detect mature neutrophils in Mcl-1 ko mice but identified a population of γδ T cells recruited to the genital tract that appeared only in Mcl-1 ko mice. Compared to the wt mice, the number of monocytic and NK cells was reduced in the Mcl-1 ko mice. Preliminary data indicate that all of the infected mice developed hydrosalpinx but the dilatation of the hydrosalpinx seemed to be less prominent in the Mcl-1 ko mice. Our findings suggest that neutrophils had an impact on the recruitment of other infiltrating immune cells and may have an influence on genital tract tissue damage. Other immune cell populations may contribute to these events.

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Cytotoxic PSMα Peptides from S. aureus Induce Rapid Cytotoxicity and NET Formation

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Community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) are MRSA strains that have emerged over the last few decades and are both antibiotic resistant and have enhanced virulence and fitness. Among the secreted virulence factors are phenol-soluble modulin (PSM) peptides. A group of PSM peptides of the alpha type are capable of activating human neutrophils, e.g. to produce reactive oxygen species, but they also possess cytotoxic properties to neutrophils at higher concentrations.

We here corroborated that PSMα peptides were cytotoxic to neutrophils in a concentration dependent manner and found that cell death was associated with the formation of structures indistinguishable from neutrophil extracellular traps (NETs). These PSMα-induced NETs contained DNA, histones, and granule proteins. As for the function of the PSMα-induced NETs we found that both bacteria (*S. aureus*) and fungi (*Candida albicans*) were captured in NETs, however with prolonged incubation *S. aureus* could degrade the NET structures, which is in accordance with their ability to secrete DNase. *C. albicans* on the other hand could not degrade the NET structures and the PSMα-induced NETs could inhibit the growth of *C. albicans* at comparable levels to NETs induced by the classic inducer phorbol myristate acetate (PMA).

In contrast to NET formation induced by PMA, PSMα-induced NETs formed very rapidly (within minutes) and the process was independent of ROS production and MPO activity. The nuclear structure of neutrophils treated with PSMα was rapidly disintegrated suggesting that the peptide-induced

nuclear destabilization could result in NET formation.

To establish whether the effect of PSMα peptides was specific for neutrophils we studied the effects on non-myeloid cells treated with PSMα. A melanoma cell line was also sensitive to the cytotoxicity of PSMα peptides, but this death was not associated with expulsion of DNA extracellularly.

Our data reveal an unusual cytotoxic effect of PSMα peptides that lead to the formation of NETs and show that rapid NET formation may be triggered through membrane disturbance by the cytotoxic peptides.

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Genetic Susceptibility to Hypersensitivity Pneumonitis

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Hypersensitivity Pneumonitis (HP) is a T cell-mediated interstitial lung disease that develops following repeated exposure to a wide variety of inhaled environmental antigens. The disease is characterized by alveolitis, noncaseating granulomas and, in some patients, develops into a chronic form which is associated with fibrosis and emphysema. Not all individuals exposed to HP causative antigens develop disease suggesting that genetic factors play a role in susceptibility. The host co-factor(s) that play a role in determining whether an individual is susceptible to disease are unknown. We used the innovative systems genetics tool, the BXD panel of recombinant inbred mice with the *Saccharopolyspora rectivirgula* (SR) model of HP to identify genetic loci and candidate genes associated with disease development. We exposed the parental strains, C57Bl/6 and DBA/2, and eight BXD strains to SR for 3 weeks and analyzed the lungs 18h after the last exposure. Bronchoalveolar lavage (BAL) was performed and cells recovered from the BAL fluid were analyzed by flow cytometry to determine the frequency of infiltrating immune cells. The results reveal wide variability in the cellular composition of the BALF; with some BXD strains exhibiting a neutrophilic alveolitis whereas other BXD strains exhibited a more lymphocytic alveolitis. For example, BALF recovered from mice of the BXD45 strain was 68% neutrophils and 7% T lymphocytes whereas; BALF recovered from mice of the BXD73 strain was 92% neutrophils and 2% T lymphocytes. In addition, there was considerable variability in activated $CD4^+$ T cells; BALF from mice of the BXD24 strain had 33% $CD4^+/CD69^+$ T cells and from the BXD74 strain had 72% $CD4^+/CD69^+$ T cells. To examine the extent of inflammation in the lung tissue H&E staining was performed. The results demonstrate differences in the lung pathology; the C57Bl/6 mice exhibited typical foci of inflammation whereas mice from the BXD45 strain exhibited a limited diffuse infiltration into the lungs. These data support the contention that genetic differences critically influence SR responsiveness given that environmental factors were held constant.

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Neutrophil Plasticity in Thyroid Cancer

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Background: Neutrophil function has long been viewed limited to the acute phase of inflammation and resistance against pathogens. The role(s) of neutrophil in tumor initiation and progression remain poorly understood. Neutrophils are found within inflammatory cells infiltrating the tumors and recent studies placed them as key effector cells in the orchestration of the immune and inflammatory responses. However, the association between neutrophil infiltration, clinicopathological features and outcome in cancer patients remain to be clarified. Thyroid cancer (TC) is the most frequent type of cancer of the endocrine system, accounting for 70% of deaths due to endocrine cancers. No studies are so far available investigating the role of neutrophils in TC.

Objective: The objective of this study was to

investigate the role of tumor-infiltrating neutrophils in TC. **Method:** Highly purified human neutrophils (>99%) from healthy donors were stimulated, in *vitro,* with conditioned media derived from thyroid cancer cell lines (TC-CM). Neutrophil biology and functions (e.g. chemotaxis, activation, plasticity, survival, gene expression and protein release of neutrophils) were evaluated. **Results:** We found that TC cell lines produced soluble factors able to promote neutrophil chemotaxis and significantly improved neutrophil survival. In addition, TC-CM induced neutrophil morphological changes and activation (CD11b and CD66b up-regulation, CD62L shedding). Finally, TC CM induced the production of reactive oxygen species (ROS), the expression of pro-inflammatory and angiogenic stimuli (CXCL8/IL-8, VEGF-A) and the release of matrix metalloproteinase-9 (MMP-9). **Conclusions:** TC cell lines produce soluble factors able to 'educate' neutrophils towards an activated functional state. Experiments are in progress to understand the role of these "tumor-educated neutrophils" in modifying TC behavior. These data will advance our understanding on the molecular and cellular mechanism of the innate immune system in cancer.

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Octa-Branched Peptide (MAP) Vaccination against Emmprin/CD147 Inhibits Tumor Growth and Metastasis by Immune Modulating the Microenvironment

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The Extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) is a multifunctional protein that mediates tumor cell-macrophage interactions, leading to induction of MMPs and VEGF. EMMPRIN is weakly expressed on immune cells and normal glandular epithelial cells, but overexpressed in tumor cells of high grade and stage and metastasis, in correlation to poor prognosis. We previously identified a novel epitope in EMMPRIN that is responsible for both MMP-9 and VEGF induction, and developed a polyclonal antibody that efficiently inhibited tumor growth and metastases *in vivo.* Here, we used this epitope, synthesized as an octa-branched multiple antigenic peptide (MAP), to vaccinate mice implanted with subcutaneous syngeneic colon (CT26), prostate (TRAMP-C2) or renal (RENCA) cell line carcinomas. Vaccination inhibited, and in some of the cases regressed, tumor growth in a dose-dependent manner (94%, 71% and 72%, respectively, $p<0.01$), and demonstrated immune memory that prevented tumor recurrence ($p<0.001$). When tumor cells were administered through the tail vein to generate lung metastases, vaccination effectively reduced the number of metastatic foci (by 15- and 23-folds, $p<0.001$), and increased mice survival ($p<0.01$) relative to the scrambled control peptide. In all experiments, no significant adverse responses were observed. Mechanistically, these effects were achieved by significantly immune-modulating the tumor microenvironment (TME): vaccination induced production of EMMPRIN-specific antibodies, increased $CD8^+$ T cells infiltration, enhanced their cytotoxicity, and alleviated immune suppression by decreasing TGFbeta and elevating TNFalpha and IL-1beta concentrations. Additionally, vaccination reduced angiogenesis and cell proliferation, but enhanced apoptosis. Thus, our results establish EMMPRIN as an attractive target, and show that peptide vaccination could be considered an efficient immune modulating approach for the treatment and prevention of some types of cancer.

LB01

The Role of Scavenger Receptor a (CD204) in Apoptosis of Septic Neutrophils

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In the United States ~750,000 patients/year develop sepsis syndrome with an overall mortality rate of 28.6% (~215,000 deaths/year). Those patients that survive the initial event may ultimately succumb to widespread organ dysfunction due to prolonged immune dysfunction and infection. Scavenger receptor A (**SRA**, aka CD204) is a multi-functional receptor which binds endogenous ligands and pathogen associated molecular patterns. We have shown that SRA plays a pivotal role in mediating the pathogenesis of sepsis. Specifically, SRA deficient mice exhibit improved survival and decreased bacterial burdens in cecal ligation and puncture (CLP) induced sepsis. SRA has previously been considered a macrophage specific receptor; however, we have discovered that neutrophils express SRA in response to sepsis. Neutrophils play an integral role in host defense by killing pathogens either after internalization or by releasing degradative enzymes, reactive oxygen species, and neutrophil extracellular traps into the extracellular milieu. However, neutrophils can also inflict significant collateral damage to the tissues. One way in which this collateral damage is limited in the normal immune response is by apoptosis of activated neutrophils. In fact, it has been shown that delayed neutrophil apoptosis is associated with increased inflammation and tissue damage. Our data have revealed high levels of SRA expression on tissue infiltrating neutrophils during CLP sepsis in WT mice. In contrast there is decreased inflammation in the lungs of SRA deficient mice, though neutrophil infiltration was similar to that of WT mice. It is possible that the decreased inflammation found in SRA deficient septic mice could be the result of a higher rate of neutrophil apoptosis compared to that seen in WT septic mice. To determine if this is the case bone marrow neutrophils were harvested 72 h after CLP. Cells were cultured with media alone or with ultra-pure LPS (100 ng/ml) for 16 h. Untreated cells were considered primed, while LPS treated cells were considered primed and activated. Cells were fixed with ethanol and stained with a propidium iodide/RNase solution, and apoptosis was determined by measuring the sub-G0 population by flow cytometry. We found that in untreated cells the percentage of apoptotic WT cells was lower than the percentage of apoptotic SRA deficient cells. When treated with LPS, the percentage of apoptotic cells increased in both groups; however, the percentage of apoptotic cells was greater in the WT cells. We can conclude that apoptosis of primed neutrophils is increased in the absence of SRA while apoptosis of activated neutrophils is increased in the presence of SRA. Thus, the decreased inflammation seen in septic SRA deficient mice is unlikely to be attributable to increased neutrophil apoptosis.

LB02

Source and Mechanisms of Recruitment of a New Subset of Neutrophils, the Pro-Angiogenic Neutrophils

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Background: We have recently described that hypoxic tissues induce the recruitment of a distinct population of neutrophils ($CD49d^{+}$ $VEGFR1^{high}$ $CXCR4^{high}$ and $MMP9^{high}$), called pro-angiogenic neutrophils, crucial for restoring the oxygen supply of the tissue by inducing the development of new and functional blood vessels. This study aims to characterise the origin of the pro-angiogenic neutrophils and the signals driving their recruitment into the hypoxic tissue.

Materials and methods: The study is performed in a mouse model of ischemic hind limb following ligation of the femoral artery. Different organs (muscles, spleen, lung and blood) are dissociated and cell populations are analysed by flow cytometry 3 hours following ischemia induction. Different pharmacological inhibitors are used to identify the molecular signals inducing the mobilisation of pro-angiogenic neutrophils visualised by histological staining and intravital confocal microscopy.

Results: Pro-angiogenic neutrophils are stored in two different pools in lung and spleen where they represent respectively 10 and 15% of the total neutrophil population of these organs. Following induction of muscle ischemia, the pro-angiogenic neutrophils are recruited into the hypoxic muscle and increase in numbers more than 200%. These neutrophils are mobilised into circulation mainly

from the spleen. Moreover, the inhibition of the adrenergic receptors impairs the release of the pro-angiogenic neutrophils from the spleen. Furthermore, the inhibition of the chemokine receptor CXCR4, of the vascular endothelial growth factor receptor 1 (VEGFR1) or of the integrin CD49d blocks the intramuscular recruitment of the pro-angiogenic neutrophils.

Conclusions: The newly described pro-angiogenic neutrophil are recruited into hypoxic tissues from the spleen by a multistep process. The recruitment is driven first by the release from the spleen after the activation of the receptors of the catecholamines and then by the extravasation into the muscle involving the receptors CXCR4 and VEGFR1 and the integrin CD49d.

LB03

Distinct Modulating Effect and Receptor Preference on the Formyl Peptide Receptors Expressed in Mouse Neutrophils for Ligands with Known Specificities for the Receptors Expressed in Human Cells

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Background: The neutrophil expressed formyl peptide receptors (FPR1 and FPR2 in human; and Fpr1 and Fpr2 in mouse) are pattern recognition receptor belonging to the family of G-protein coupled receptors (GPCRs). The activities of the FPRs, that play pivotal roles in host defense and inflammatory reactivity, are regulated by a large number of conventional peptide agonist/antagonists, small molecules, reversed agonists, and allosteric modulators which display binding preference for FPR1 or FPR2, or both (dual ligands). It is well known that the prototype high affinity FPR1 agonist fMLF is a very low affinity agonist for Fpr1, but very little is known about the activity induced in mouse neutrophils by other FPR ligands. In this study, we have determined recognition profiles for the mouse Fprs of a number of ligands with known specificities for FPR1/FPR2.

Results: The very potent FPR1 antagonist cyclosporine H and the most potent FPR2 inhibitor described, a rhodamine linked PIP2 binding peptide derived from gelsolin were without effects on the neutrophil activity induced in mouse cells by formylated agonistic peptides specific for Fpr1 or Fpr2, respectively.

We have earlier shown that a pepducin derived from the third intracellular domain of FPR2 activates neutrophils through FPR2, whereas the corresponding pepducin derived from FPR1 inhibits human neutrophil activity. The inhibitory effect was not mediated through FPR1 as expected. Instead the closely related FPR2 was hijacked by the pepducin. This FPR2 inhibiting pepducin activates mouse neutrophils and it was a dual ligand that targeted both Fpr1 and Fpr2.

Conclusions: Our basic pharmacological characterization of a number of human FPR ligands in mouse neutrophils reveals that the modulating effect and receptor preference by certain ligands differ significantly across species. Prominent examples are, i) that the most potent FPR antagonists have no effects on the activities triggered by the corresponding mouse receptors and, ii) the FPR1 derived pepducin that inhibits FPR2 is a dual agonist for both Fpr1 and Fpr2.

LB04

Immune Cell Response to Bacterial Infection

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Sepsis arises when the body's response to an infection damages its own tissues and organs; defined as life threatening organ dysfunction caused by a dysregulated host response to infection. The body's entire system is engulfed by a deleterious inflammatory response as opposed to local inflammation resulting from a local infection. This can lead to septic shock and death if not recognised early and treated promptly. Eicosanoids, including prostaglandins and leukotrienes, are a family of lipids that play key roles in inflammation including helping leukocytes fight infection. Cells of the innate immune system are major contributors of local eicosanoids. It is known that varying bacterial components result in a different profile of lipids and cytokines, and by characterising this lipid signature it may be possible to define a biomarker fingerprint predictive for sepsis. This study will address the hypothesis that the profile of the eicosanoid and

cytokine response varies depending on the nature of the bacterial pathogen. The experimental aim is to identify signatures of eicosanoids and cytokines that may represent useful prognostic indicators of bacterial infection.
The spectrum of the human innate immune response is influenced by a number of factors including host genetic background and physiology. A standardised human monocyte cell line (THP-1) was employed to characterise the eicosanoid and cytokine response of different bacterial agonists. A culture of THP-1 monocyte cells was differentiated into monocyte-derived macrophages and production of eicosanoids and cytokines was analysed. Eicosanoids were identified using a combination of high performance liquid chromatography and multiple-reaction monitoring mass spectrometry. A DuoSet ELISA was used to identify the production of inflammatory cytokines. Primary cell responses to bacterial components were analysed using neutrophils isolated from healthy human donors. Isolated neutrophils were activated under various conditions and lipid extracts were characterised as described above.
Initial THP-1 cell optimisation found that monocyte-derived macrophages differentiated from THP-1 cells with 0.025nM, 2nM, 25nM and 100nM phorbol 12-myristate 13-acetate allowed no generation of the eicosanoid 15-hydroxyicosatetraenoic acid when stimulated with calcium ionophore. THP-1 cells differentiated for 24-hours with 0.025nM PMA produced the inflammatory cytokine IL-1β following treatment with calcium ionophore, and TNF-α following treatment with lipopolysaccharide from Escherichia coli. Generation of the eicosanoid 5-hydroxyicosatetraenoic acid by isolated human neutrophils was detected following exposure to human toll-like receptor agonists (InvivoGen). Once the ability to detect agonist specific eicosanoid and cytokine profiles in THP-1 cells has been established primary immune effector cells will be used for further experimental validation.
In conclusion, preliminary results indicate that isolated human neutrophils display suitable physiological characteristics and substantiate that THP-1-derived cells are indeed macrophages. In future work these approaches will be utilised to characterise the innate immune responses to bacterial infection with a view to identifying pathogen-specific profiles. Bacteria-specific bio-signatures will underpin the development of rapid diagnostics, allowing clinicians to administer the correct antibiotics for sepsis patients at the earliest opportunity.

LB05

A Mouse Model of Neonatal Staphylococcus Epidermidis Infection

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Background and objective: Neonatal sepsis is a serious problem in neonatal intensive care units, causing increased hospitalization costs and prolonged hospitalization stays. The coagulase-negative staphylococci, *Staphylococcus epidermidis*, have emerged as the predominant pathogen of late-onset neonatal sepsis in premature infants, accounting for up to 78% of neonatal late-onset sepsis. Our laboratory previously determined that intravenous injection of *S. epidermidis* in postnatal day 1 mice induces systemic cytokine production and affects brain development. As neonatal mice have an immature immune system and deficiencies in leukocyte populations, we tested the response of older neonatal mice to the bacteria, so as to determine the vulnerability window for infection-induced brain injury in neonates.
Methods: *S. epidermidis* grown to mid-log phase was administered intraperitoneally into postnatal day 4 mice. Animals were monitored over time for body weight and temperature changes and long-term survival. Bacterial colony-forming units from the peripheral blood, liver and spleen was determined by spot plating. Cytokine production was determined by multiplex cytokine bead array analysis. Circulating

cell-free DNA was detected using a Quant-iT PicoGreen dsDNA Assay Kit. Immuno-histochemistry of brain sections was performed to determine the effects of the infection and inflammation in the brain.
Results: Injection of *S. epidermidis* led to bacteremia within two hours in a dose-dependent manner. There was a dose-dependent decrease in body weight gain after the infection. Pro-inflammatory cytokines were significantly up-regulated in the peripheral blood of infected animals by 2 to 24 hours post infection and tapered down by 48 hours, in association with the clearance of the bacteria from the blood. Neutrophil and monocyte chemotactic cytokines (CXCL1 and MCP-1), granulocyte-colony stimulating factor, and caspase-3 activity was also elevated in the brain by post infection. An elevated level of cell-free DNA was observed in postnatal day 9 but not day 4 mice after *S. epidermidis* infection. No difference in the gray and white matter volume in the brain was detected 10 days after the infection.
Conclusions: Although intraperitoneal injection of *S. epidermidis* into postnatal day 4 animals led to a similar infection pattern and response compared to intravenous infection of 1-day old animals, no gross morphological injury in the brain was detected. These data give support to the hypothesis that the response to infection, and the vulnerability of the immature brain to infection and inflammation is age dependent. Delineating the difference between neonatal mice of different ages will provide insight into factors leading to brain injury upon infection and inflammation, and allow us to develop therapeutic treatments for infants affected by these conditions.

LB06

Immune Complex Induced Apoptosis is Controlled by Non-Canonical PI3K – Cdc42 – Pak – Erk Signaling Promotes in Human but not Mouse Neutrophils

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Neutrophils are peripheral blood leukocytes that represent the first line of defense against bacterial and fungal infections and are also key to generating inflammatory response. Immune complexes are important mediators of many neutrophil effector functions; immune complexes are critical in driving range of chronic autoinflammatory disorders that rely on neutrophilic inflammation. However, insoluble immune complexes are also known to induce neutrophil apoptosis, an essential process in the resolution of inflammation.
Here we present a novel, non-canonical signaling pathway, FcgR – PI3Kb/d – Cdc42 – Pak – Mek – Erk, that promotes immune-complex induced apoptosis in human neutrophils. This pathway represents an example of Ras and Raf-independent Erk activation, where instead, an alternative small GTPase (Cdc42) and an alternative MAP3K (Pak) are involved. The non-canonical pathway is moreover a rare example of PI3K driven activation of Cdc42 rather than Rac.
Mechanistically, we show that the non-canonical signaling pathway drives apoptosis of human neutrophils by regulating the ratio of the Bcl2 family proteins Mcl1 and Bax1. We conclude that our novel, non-canonical signaling pathway may be important for the resolution of inflammation in chronic inflammatory diseases that rely on immune-complexes driven neutrophil activation.
In addition, we demonstrate that other effector functions induced by insoluble immune complexes (ROS generation, selectin shedding, cytokine release) are also PI3Kb/d dependent but employ separate pathways downstream of PI3K.
Given that neutrophils are terminally differentiated cells that are not amenable to transfection or transduction, we analysed mouse neutrophils with a view to making use of available knock-out models to enable studying the novel pathway genetically. Although insoluble immune complexes drive PI3K and Erk dependent apoptosis of mouse neutrophils, the two species use separate pathways. Control experiments carried out with fMLP stimulated neutrophils demonstrated that fMLP stimulated Erk activation is also controlled by separate pathways in human and mouse neutrophils. Our work therefore emphasizes that results obtained with mouse neutrophils need to be interpreted with caution, as they may or may not be conserved in human.

LB07

Neutrophil Extracellular Trap Formation among Neutrophil Subpopulations.

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Neutrophils have traditionally been considered to contribute to the non-specific first line of immune defence during acute inflammation, and as such disregarded as rudimentary leukocytes. However, as their contributions to tumour homeostasis, chronic inflammation and adaptive immunity were discovered, neutrophils have been more recently acknowledged for offering a diverse set of specialized functions. We recently identified a pro-angiogenic circulating neutrophil subset (CD49d+ VEGFR1high CXCR4high) that was specifically recruited by VEGF-A to sites of hypoxia, where they were fundamental for angiogenesis and restitution of blood flow at least partly due to their high expression levels of MMP-9. The current project investigates the ability of classic as well as proangiogenic neutrophils to form neutrophil extracellular traps (NETs), and if this is altered in persons with cancer. In addition, the ability of ascites fluid from cancer patients to induce NET formation is studied in the different neutrophil subsets. Interestingly, our results indicate that when isolated from cancer patients, the proportion of pro-angiogenic neutrophils positive for NETs was higher than that of the pro-inflammatory subset. Further, ascites fluid from patients with cancer strongly induced basal neutrophil NET formation.

LB08

Neutrophil Chromatin Undergoes Dramatic Stimulation-Specific Topological Changes during Extracellular Trap Formation

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Upon encountering activating stimuli neutrophils undergo a unique form of lytic cell death termed NETosis. During NETosis the neutrophil nucleus undergoes a catastrophic loss of organization, as the integrity of the nuclear envelope is compromised and chromatin is extruded into the extracellular space. The resulting cytotoxic granule-laced neutrophil extracellular trap (NET) confines and kills pathogens. In contrast to NETs, chromatin within intact nuclei is highly organized in three-dimensional space. Here we utilized genome-wide chromosome conformation capture to map the organization of neutrophil chromatin during NETosis. We find that the lobed morphology of the neutrophil nucleus enforces a unique organization of chromatin compared to that of non-lobed nuclei. We further identify distinct changes in chromatin organization that are dependent on the NET-inducing stimulus. Together with epigenetic and transcriptional profiling, we offer the first molecular-resolution view of neutrophil activation and NET formation.

LB09

Role of the Atypical Chemokine Receptor CCRL2 in Intestinal Inflammation

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Intestinal bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, is a chronic inflammatory immune-mediated disease at the intersection of complex interactions between genetics, environment and gut microbiota. The main pathological feature of IBD involves a massive infiltration of immune cells, in particular neutrophils into the intestinal tissue. However, the beneficial versus deleterious role of neutrophils during this pathological condition is still a matter of debate. Chemokine and their receptors play an important role in the recruitment of leucocytes in the intestinal mucosa of IBD patients (1-2). CCRL2/ACKR5 is an orphan chemokine receptor which apparently does not activate any chemokine conventional signaling. For this reason, it has been included in the subset of "atypical chemokine receptors" (ACKRs; 3), which are also characterized by ligand scavenging functions that dampen local inflammation. This receptor is rapidly induced in many activated cells, including macrophages, neutrophils, and dendritic cells (4) and in preliminary experiments is upregulated in the mucosa of Crohn's patients. The aim of our work was to define the role of CCRL2 during an experimental model of inflammation-induced colitis (dextran sodium

sulphate-DSS model) by using CCRL2 deficient mice. CCRL2 KO mice treated with 2.5% DSS for 7 days show exacerbated phenotype, including higher body loss, increased bleeding and shorter colon length compared to WT animals. The pathogenic mechanism underlying the observed phenotype in CCRL2 KO is under investigation. The understanding of the role of CCRL2 in the recruitment of immune cells at colonic mucosa level might provide new insights in the treatment of pathological intestinal inflammation.

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LB10

Gene Expression of Neurotransmitter Receptors and Immune-Related Proteins in the Nucleus Accumbens during Endotoxin Tolerance

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The nucleus accumbens (NAc) is the brain area involved in the reward system and the development of addictive behavior. We previously reported that systemic disorders, such as HIV and bacterial infections, may trigger neuro-inflammation leading to increased use of addictive substances. We also found that, during endotoxin tolerance, lipopolysaccride (LPS)-induced expression of TNF-alpha, IL-1beta, and IL-6 still occurs in the brain even when these three cytokines are no longer induced in the periphery. In the present study, we measured the gene expression of the dopamine (DA) receptors, GABA-A receptors, serotonin receptors (Htr), glutamate receptors (Gr), glutamate receptor ionotropic NMDA (Grin), cholinergic (Ch) receptors, and immune-related genes in the NAc of male F344 rats (7-8 wks) treated with LPS (progressively from 250 μg/kg, 500 μg/kg, 1 mg/kg, 2 mg/kg, 4 mg/kg, 8 mg/kg, 16 mg/kg to 32 mg/kg) using real-time PCR assay. Among the 68 genes examined, we found increased gene expression for lL-1alpha, IL-1beta, as well as the Types 1 and 2 IL-1 receptors. There was decreased gene expression for key neurotransmitter receptors, including dopamine receptors D1A, D2, and D4, GABA-A receptors a2, a3, and a4, serotonin receptor 3a, glutamate receptor m5, glutamate receptor ionotropic NMDA 1, 2a, 2b, and 2c, and the cholinergic receptor nicotinic beta 4 subunit. In addition, we found decreased expression of immune-related IL-5, IL-12a, IL-16, and solute carrier family 6 (Slc6), members 2 and 4. We then used the Library of Integrated Network-Based Cellular Signatures (LINCS), a biological network-based strategy that utilizes gene profile signatures to suggest probability outcomes in terms of drug mimics and effects, to further examine the altered gene profile during endotoxin tolerance. LINCS analysis revealed that the top three compounds having similar effects as those seen in the endotoxin tolerant state are RG108, an inhibitor of DNA methyltransferase; roscovitine, a potent and selective cyclin dependent kinase (CDK) inhibitor for Cdc2, CDK2, and CDK5; and selumetinib, a highly selective mitogen-activated protein kinase 1 (MEK1) inhibitor. Our LINCS analysis data suggests that, during endotoxin tolerance, expression of DNA methyltransferase, CDK, and MEK1 in the NAc may be inhibited.

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LB11

Neutrophils are Recruited to the Lung in a CCR1 and Complement C5 Dependent Manner Independent from Complement C3 and Type I IFN Receptor during Infection with Modified Vaccinia Virus Ankara

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Innate immune responses influence the outcome of

vaccination. Modified Vaccinia virus Ankara (MVA), which is used as a versatile vector for antigen delivery, has been proven effective in preclinical and clinical studies. To understand why vaccination with non-replicating MVA induces immunity without need for an adjuvant, we study MVA-induced cytokine expression in macrophages and leukocyte recruitment to the site of inoculation. We and others have shown that MVA, but not other vaccinia virus strains, induces robust innate immune responses including recruitment of leukocytes to the lung within hours (Lehmann et al. 2009 J. Virol., Lehmann et al. 2015 Virol. J.). During the early stages of MVA infection neutrophils represent the largest cell population of the infiltrating leukocytes, which are recruited by a mechanism involving chemokine (C-C motif) receptor 1 (CCR1) (Price et al. 2014 J. Virol.) but not type I interferon receptor (Lehmann et al. 2016 J. Leukoc. Biol.). Vaccinia virus activates both the classical and alternative complement pathways and complement is known to play an important role in poxvirus immunity. Activation of the terminal C5 component leads to the release of the small peptide fragment C5a, which is a potent immune activator and chemoattractant, particularly for neutrophils. Therefore, the role of complement component C5 in MVA-triggered recruitment of leukocytes was investigated in C5 deficient mice as well as in wild type mice treated with an antibody that blocks C5. Neutrophil recruitment to the lungs of C5 deficient mice and anti-C5 treated wild type mice was dramatically reduced as compared to untreated control mice at 12 h post infection. Additionally, using C3 deficient mice, we found that C5 functions independently of the central complement component C3 to support neutrophil recruitment during the inflammatory response to MVA (Price et al. 2015 J. Immunol.). We conclude that CCR1 and complement component C5 play non-redundant roles during the process of neutrophil recruitment to the site of viral infection.

LB12

Monocytic MKP-1 is a Sensor of the Metabolic Environment and Regulates the Functional and Phenotypic Fate of Monocyte-Derived Macrophages in Atherosclerosis

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Diabetes promotes the S-glutathionylation, inactivation and subsequent degradation of mitogen activated protein kinase phosphatase 1 (MKP-1) in blood monocytes, and hematopoietic MKP-1-deficiency in atherosclerosis-prone mice accelerates atherosclerotic lesion formation, but the underlying mechanisms were not known. To determine the mechanisms through which MKP-1 deficiency in monocytes and macrophages promotes atherogenesis. Transplantation of MKP-1-deficient bone marrow into LDL-R-/- (MKP-1LeuKO) mice accelerated high fat diet (HFD)-induced atherosclerotic lesion formation. After 12 weeks of HFD feeding, MKP-1LeuKO mice showed increases in lesion size in both the aortic root (1.2-fold) and the aorta (1.6-fold), despite reduced plasma cholesterol levels. Macrophage content was increased in lesions of MKP-1LeuKO mice compared to mice that received wildtype bone marrow. After only 6 weeks on a HFD, the in vivo chemotactic activity of monocytes was already significantly increased in MKP-1LeuKO mice. MKP-1 deficiency in monocytes and macrophages promotes and accelerates atherosclerotic lesion formation by hyper-sensitizing monocytes to chemokine-induced recruitment, predisposing macrophages to M1 polarization, decreased autophagy and oxysterol-induced cell death whereas overexpression of MKP-1 protects macrophages against metabolic stress-induced dysfunction. MKP-1 serves as master-regulator of macrophage phenotype and function and its dysregulation by metabolic stress may be a major contributor to atherogenesis and the progression of atherosclerotic plaques.

LB13

QUANTIM- Quantification of Innate Immune Function in Whole Blood Infection Assays

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Little progress has been made in recent years towards bringing immunomodulatory therapies for sepsis into clinical use. A major reason for this is the heterogeneity of sepsis as a clinical syndrome, resulting in highly diverse pathological conditions and showing variable kinetics within patient populations. Resultingly, classification of sepsis patients in terms of their immune status is important and necessary for future studies (e.g. immunomodulatory therapy approaches). With QUANTIM we seek to address this using a human whole blood model of infection combined with biomathematical modeling to quantify the global status of the innate immune response to infection (Hünniger *et al*, PLOSCompBiol 2014).

In preliminary work, human peripheral blood was collected from healthy volunteers and infected with a broad range of relevant pathogens. The resulting data provides time-resolved data on cell activation and allows a comparative analysis of regulatory networks governing inflammation and pathogen elimination. Before the whole blood model is ready to be examined with as heterogeneous of a population as septic patients, it needs to be validated with a more homogeneous population. For this purpose, cardiac surgery with extracorporeal circulation is ideal, as it provides an inflammatory stimulus that is both time-defined and relatively homogeneous. With the ability to investigate the blood of the same patient at defined time points before and after extracorporeal circulation, inter-individual differences and effects of inflammation can be clearly distinguished.

For this study, blood was taken from patients before cardiac surgery (pre-operative) immediately after surgery (post-operative) and 1 day after admission to intensive care. Whole blood infection was performed with two model pathogens, representing bacterial (*Staphylococcus aureus*) and fungal (*Candida albicans*) sepsis for a 4h time course, followed by FACS-based analyses of cell activation and pathogen association with immune cells.

Preliminary analysis showed a strong post-operative increase in neutrophil cell counts. Interestingly, the increase in cell number results in a faster association of *C. albicans* and *S.aureus*, but does not correlate with a stronger activation of neutrophils. In ongoing experiments, differences in pathogen elimination before and after extracorporeal circulation will be quantified and analyzed using biomathematical modeling. Additionally, cytokine levels will be assessed and compared to known cytokine profiles in terms of immune activation.

In conclusion, with QUANTIM we provide novel methods for individualized quantification of alterations in immune effector functions by using an *ex vivo* whole blood model of infection combined with advanced biomathematical quantification.

LB14

Recycling and Scavenging Properties of CCRL2

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CCRL2 (C-C chemokine receptor-like 2) is a non-signalling seven-transmembrane domain receptor related to the atypical chemokine receptors (ACKRs) family. CCRL2 is rapidly expressed by many activated leukocytes, including neutrophils[1]. At present, the best-characterized function of CCRL2 is its ability to act at the endothelial surfaces as a presenting molecule for chemerin[2], [3]. The aim of this study was to explore the biology of CCRL2, focusing on the common ACKRs features, that include constitutive internalization, recycling, ligand scavenging and transcytosis[3], [4].

To this goal and to avoid the steric hindrance of antibodies, HeLa cells were transfected with a construct carrying the Acyl Carrier Protein (ACP) tag at the N-terminus of CCRL2. Receptors were then labeled using a phosphopantetheine transferase that binds a fluorophore to the ACP tag. Cell analysis by fluorescence microscopy revealed

constitutive internalization and recycling of CCRL2. Inside the cell, CCRL2 co-localized with EEA1-positive and Rab5-positive vesicles and with recycling compartments mainly characterized by Rab11-positive vesicles. Apparently, CCRL2 internalization was not regulated by the presence of chemerin or CCL19, a chemokine also suggested binding this receptor. However, the expression of CCRL2 conferred to transfected cells the ability to scavenge chemerin, but not CCL19, from the cell supernatant.

To further investigate the mechanisms involved in CCRL2 internalization, receptors carrying different degree of truncations at the C-terminus were expressed in HeLa cells. FACS analysis of transfected cells showed reduced membrane expression of all the truncated forms, as described for other ACKRs, such as ACKR2[5]. Nevertheless, preliminary experiments highlighted a lower rate of internalization of the truncated receptor forms suggesting the involvement of CCRL2 C-terminus in the internalization process.

Future experiments will be performed to better characterize the mechanisms of CCRL2 internalization. Also, it will be interesting to confirm the scavenging ability, to date observed in transfected cells, in a more physiological setting.

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LB15

Leukaemia-Associated Mutations of C/Ebpa Inhibit Neutrophil Differentiation

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CCAAT enhancer binding protein alpha (C/EBPα), a master regulator of granulopoiesis, is known to be involved in cell proliferation and differentiation. C/EBPα has been reported to be mutated in 9 % of acute myeloid leukaemia (AML) cases. We here use an inducible Hoxb8 system to generate and differentiate mouse neutrophil progenitors to investigate the capacity of leukaemia-associated mutations to affect differentiation and survival of neutrophil granulocytes. Several C/EBPα mutations were expressed in these neutrophil progenitors, including an internal tandem duplication (K313 duplication) in the leucine zipper region, the BRM2 mutation (which carries two amino acid substitutions in the basic region) and the p30 N-terminal truncation mutant, which is generated by using an alternative start codon.

Expression of p30 C/EBPα impaired neutrophil differentiation and had a negative effect on survival during differentiation. Expression of C/EBPα carrying the K313 duplication exerted a strong differentiation-retarding effect while the BRM2 mutant only led to mild differentiation defects. The lack of differentiation was associated with maintained high expression levels of anti-apoptotic Bcl-2 and Mcl-1 and low levels of pro-apoptotic Bim.

The K313 mutation has been reported to have low transcriptional activity in other cellular systems. However reporter assays showed strong C/EBPα activity in neutrophil progenitors expressing the K313 mutant. We show that C/EBPα expression is upregulated during differentiation and subsequently downregulated at later stages of maturity, but interestingly, expression of the K313 mutant as well as the BRM2 mutant was much higher than wild type despite similar levels of transcription. These expression levels remained high during differentiation. These results suggest that the K313 and BRM2 mutations may contribute to AML by blocking the downregulation of C/EBPα at later stages of differentiation keeping the cells in an incompletely differentiated state.

LB16

Platelets Enhance Antifungal Activity of Human Neutrophils during Candida Infection

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Besides their crucial importance in blood hemostasis, platelets have been identified as effector

cells with diverse immune functions that participate in the host defense against pathogens. Using an *ex vivo* human whole blood infection model we have already shown that innate immune activation triggered by the two most prevalent *Candida spp*., *Candida albicans* and *Candida glabrata*, differs considerably. However, if platelets contribute to these differences needs to be elucidated. To compare platelet activation triggered by *C. albicans* and *C. glabrata*, we infected human whole blood and quantified platelet effector mechanisms after confrontation with the two species at different time points. Both species induced increase in surface exposure of activation marker CD62P on platelets associated with immune cells over time. However, infection with *C. glabrata* resulted in a less pronounced response than *C. albicans*. In line with this, secretion of platelet effector proteins, soluble P-Selectin (sCD62P), PDGF-BB and PF4, was more induced by *C.albicans.* Whereas platelets interact with various leukocytes during whole blood infection, no association to the pathogens has been observed. To get further insight in the interaction of platelets with the two *Candida spp.* we performed confrontation assays with purified platelets. Using autologous plasma with heat-inactivated complement during confrontation, a time-dependent increase in platelet-*C. albicans* interaction could be observed. Binding of platelets to *C.glabrata* was markedly lower than compared to *C. albicans*. Beside direct interaction with pathogens, immune functions of stimulated platelets are mediated by crosstalk with immune cells. Therefore, we investigated the contribution of platelets on *Candida*-induced activation of neutrophils, which have been shown to play a predominant role during *C. albicans* infection. When confrontation of primary neutrophils and *C.albicans* was performed in the presence of platelets, neutrophil activation detected by increased surface levels of CD66b and down-regulation of CD16 was markedly higher than induced by *C. albicans* alone. This synergistic effect seems to be independent of the complement. In contrast, neutrophil activation in response to *C. glabrata* was markedly lower than compared to *C. albicans* and only slightly enhanced by the presence of platelets.

Taken together, our data demonstrate a strong activation of platelets by *C. albicans* that synergistically influence neutrophil activation induced by *C. albicans*.

LB17

Swimming Motility Mediates NET Formation Stimulated by Flagellated Bacteria

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Pseudomonas aeruginosa is an opportunistic pathogen causing severe infections often characterized by robust neutrophilic infiltration. Neutrophils provide the first line of defense against P. aeruginosa. Aside from their phagocytic defense, neutrophils also release neutrophil extracellular traps (NETs) to kill bacteria. Although NET formation is an important antimicrobial process, the details of its mechanism are largely unknown. The identity of the main components of P. aeruginosa responsible for triggering NET formation in neutrophils is unclear. In this study, our focus was to identify the main bacterial component mediating NET formation and to gain insight into the underlying mechanism. We found that bacterial flagellum is the primary component of P. aeruginosa responsible for inducing NET extrusion as flagellum-deficient P. aeruginosa does not trigger NETs. Surprisingly, purified P. aeruginosa flagellin, the monomeric component of the flagellum, does not stimulate NET formation in human neutrophils. P. aeruginosa-induced NET formation is independent of the flagellum-sensing receptors TLR5 and NLRC4 in both human and mouse neutrophils. Flagellum-expressing but flagellar motor-deficient bacterial strains failed to induce NET release. On the contrary, when flagellum- and flagellar motor-deficient P. aeruginosa mutants were centrifuged on neutrophils, their abilities to trigger NET formation were entirely restored. Thus, we found that swimming motility, but not flagellum expression, mediates NET release induced by flagellated P. aeruginosa. The motAB operon of P. aeruginosa encoding essential flagellar motor genes proved to be critical. Complementation experiments showed that reintroduction of the motAB genes into motAB-deficient P. aeruginosa strains also restored their impaired ability to trigger NET formation. Phagocytosis of P. aeruginosa and

superoxide production by neutrophils were also essential for flagellum to mediate NET production. This indicates that flagellated bacteria are first phagocytosed prior to release NET formation. Taken together, our study presents here for the first time flagellum as the main organelle of planktonic bacteria responsible for mediating NET release. Furthermore, swimming motility propelled by the flagellum, rather than binding of the bacteria to flagellum-sensing receptors on host cells, is required for P. aeruginosa to induce NETs.

LB18

Simultaneous Dual Proteome Analysis towards Understanding Neutrophil Interaction with Aspergillus Fumigatus

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Systemic infections in immunocompromised hosts caused by the opportunistic filamentous fungus *Aspergillus fumigatus* are a significant burden for the hospital community due to limited therapeutic options and poor understanding of efficient immune mediated clearance in healthy individuals. Neutrophils are indispensable in executing the elimination of fungal spores and hyphal elements by various intra-and-extracellular means: degranulation, phagocytosis, formation of reactive oxygen (ROS) -and–nitrogen species (RNI) as well as neutrophil extracellular trap (NET) formation. We demonstrated that NETs occur both *in vitro* and *in vivo* but exhibit only fungistatic effects. Dual transcriptome analysis resulted in high coverage of the fungal transcriptome and low coverage of the human transcriptome. Stress-response proteomics revealed that neither ROS, nor RNI mediate the direct killing of *A. fumigatus*, since the fungus expressed an arsenal of fungal ROS/RNI detoxifying systems (catalase, superoxide dismutase, flavohaemoproteins) switched by the transcription factors (TFs) *Afyap1*, *AfSkn7*. These factors, were surprisingly dispensable for fungal virulence in infection mouse models.
Thus, a key paradigm- how *A. fumigatus* hyphae activate neutrophils and how they ultimately kill the fungus, needs be resolved. We therefore conducted a simultaneous dual proteome study of the activation of human neutrophils by *A. fumigatus* CEA10 hyphae without prior separation of the two cell types at a time point of NET formation (3.5h). We developed a protocol for parallel extraction and enrichment of both NETs covering hyphae and secreted proteins during NET formation. Besides a lysis-based approach for the cellular fraction consisting of hyphae entrapped in NETs, secreted proteins were enriched by C4 solid-phase extraction of the supernatant fraction. Using a multiplexed isobaric labelling approach and TiO2 phosphopeptide enrichment combined with nLC-MS/MS analysis, we were able to identify and quantify 273 differentially regulated proteins of 856 proteins in total on the fungal side and 298 differentially regulated proteins of 1950 proteins in total on the host side compared to NET controls induced with PMA and hyphae grown alone. We showed that neutrophil activities induce higher expression of fungal signal transduction proteins from the GPCR-cAMP and CalA axis as well as Ste-20 tyrosine kinase compared to hyphae grown alone as control. Neutrophil stress caused repression of fungal proteins responsible for siderophore–and-ergosterol biosynthesis and of the 60S ribosomal translation. Fungal Afyap1 dependent and independent heat-shock and ROS response was overall repressed.
Antifungal NETs contained in higher abundance the calprotectin complex, lactotransferrin and PTX3 versus control NETs and alternative and core histones with changed stoichiometric ratios were detected. We observed that neutrophil activation by the fungus and by PMA has distinct signatures by detecting unique 275 interactome-specific proteins and 50 PMA-specific proteins. The results of the phosphopeptide enrichment were implemented in a model of the signal transduction cascade of NET formation.

LB19

Endogenously Produced Tnfa Contributes to the Expression of CXCL10 in IFN?3- Activated Plasmacytoid Dendritic Cells

Giulia Finotti, Nicola Tamassia, Federica Calzetti, Marco A. Cassatella, *University of Verona*

The interplay between plasmacytoid dendritic cells (pDCs) and members of the IFNλfamily (IFNλ1, IFNλ2 and IFNλ3) is becoming increasingly

relevant, particularly at the light of their key role in induction of the antiviral state during HCV infection. Previous data showed that pDCs respond to IFNλ1 (1). Hovewer, the immunomodulatory activities of IFNλ3 on pDCs are poorly defined.
We report (2) that pDCs incubated with IFNλ3: prolong their survival; alter their expression pattern of surface HLA-DRα, CD123, CD86, and CD303; are induced to express typical ISG mRNAs, including IFIT1, ISG15, and CXCL10; and time dependently produce IFNα, CXCL10 and even modest quantities of TNFα. Nevertheless, endogenously produced TNFαwas found to be essential for driving the expression of CXCL10 in IFNλ3-treated pDCs.
Our data report that human pDCs respond to IFNλ3. Moreover, we demonstrate that IFNλ3 induces the production of biologically active TNFα. In fact, antibodies neutralizing the IFNαR orendogenoTNFαconfirmed that the production of CXCL10 is scarcely dependent on endogenous IFNαbut rather driven by TNFα, regardless of the CXCL10 amounts in the cell supernatant.
Our study suggest that IFNλ3, by triggering the production of IFNα, CXCL10 and TNFαmay impact on the polarization of Th cells as well as the recruitment/activation of $CXCR3^{+}$cells in HCV patients.

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LB20

The Deactivator Effect Elicited by Paracoccidioides Brasiliensis in Neutrophils Can Be Reversed with Low Level Laser Therapy Applied "In Vivo"

Eva Burger[1], Ana Cristina L. Pereira[1], Julianne C. Grisolia[1], Giulia Maria C. Bani[1], Felipe F. Sperandio[1], Liana Maria C. Verinaud[1,2], Zoilo P. Camargo[1,3], Ana Carolina S.C. Mendes[1], *[1]Federal University of Alfenas (UNIFAL-MG), Alfenas, MG, Brazil ; [2]State University of Campinas (UNICAMP), Campinas, SP, Brazil ; [3]Federal University of São Paulo (UNIFESP), São Paulo, SP, Brazil*

Introduction: Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the thermodimorphic fungus *Paracoccidioides brasiliensis* (*Pb*). Polymorphonuclear neutrophils (PMN) participate in an active way of the innate immunity and directing the acquired immune response towards an effective response. PMN of paracoccidioidomycosis patients are less effective than those of healthy individuals, suggesting that *Pb* exerts some deactivating effect on this cell population. In that way, we aimed to develop a treatment capable of stimulating PMN that migrate to the site of injury through low-level laser therapy (LLLT). **Methodology:** We employed LLLT (50 mW of power; wavelength of 780 nm; energy density of 37.5 J/cm2; 30 seconds per point) applied *in vivo* on alternate days at two points on each hind paw of mice with Zymosan (Z) in subcutaneous air pouches. Unirradiated animals were used as controls. PMN that migrated to the inoculation site provided, 10 days later, a highly pure population of cells which were subsequently cultured in the presence of either a virulent (Pb18) or an avirulent (Pb265) isolate. Experiments to assess the viability of PMN after culture, mitochondrial activity and reactive oxygen species (ROS) production by these cells were performed. **Results:** PMN were more deactivated in the presence of Pb18 than those maintained in culture with Pb265. LLLT treatment of PMN had the ability to keep them more active even when exposed to Pb18. This virulent isolate increased the mitochondrial activity of PMN compared with the avirulent isolate. Treatment with LLLT increased mitochondrial activity of PMN exposed to either Pb isolate when compared to controls. PMN co-cultured with Pb265 produced more ROS than those maintained in culture with Pb18, which demonstrates that the virulent Pb isolate determines more marked deactivation of PMN. The *in vivo* exposure of immature PMN in the bone-marrow to LLLT was able to activate these cells , significantly increasing their ROS production when co-cultured with the virulent Pb isolate. Curiously, PMN exposed to Pb265 when treated with LLLT decreased their ability to release ROS. **Conclusion:** *Paracoccidioides brasiliensis* exerts an inactivating effect on neutrophils, which depends on the virulence of the fungal isolate. *In vivo* LLLT treatment reverts this effect, rendering PMN more active metabolically against the virulent isolate, probably due to increased production of ROS. **Acknowledgements:** The authors were recipient of the following Grants:

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LB21

Neutrophils are Protective in Cancerogenesis by Altering Tumor Microenvironment and Controlling Intestinal Microbiota

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The view of neutrophil as a cell involved only in the early phases of inflammation has recently been challenged and neutrophils are now considered key players in the orchestration of the immune response. Though several studies relied on antibody-based neutrophil depletion to determine their contribution to tumor development, rigorous in vivo genetic evidence explaining the neutrophil role in cancerogenesis is missing.
We investigated this issue using key preclinical models of chemically-induced cancer (3-MCA induced sarcoma and AOM/DSS induced colorectal cancer, CRC) and taking advantage of a genetic model of neutrophil deficiency (i.e. c*sf3r*-/- mice). Neutrophil deficiency was associated with increased susceptibility to sarcoma and CRC, and tumor microenvironment displayed protumoral features (e.g. increased frequency of M2 macrophages and reduced IFNg concentration). In addition, in WT mice increased neutrophil infiltrate significantly correlated with reduced proliferation rate of tumor cells and adoptive transfer of naïve neutrophils reduced tumor growth in c*sf3r*$^{-/-}$ mice. Importantly, the increased susceptibility to CRC in c*sf3r*$^{-/-}$ mice was dependent on intestinal microflora, and was abolished in cohousing experiments. In addition, the dysbiosis displayed byc*sf3r*$^{-/-}$mice was also responsible for the increased susceptibility to acute colitis, which has a causal link with intestinal cancer development.
Collectively, our data support that genetic deficiency of neutrophils affects the anti-tumor response and is associated with increased susceptibility to chemically-induced cancerogenesis.
Until recently neutrophil function was mostly related to acute inflammation and defense against pathogens. We (and others) have challenged this dogma and demonstrated that neutrophils represent an essential component in the control of tumor onset and development.

LB22

The Differential Metabolic Responses of Non-Reprogrammed Vs Reprogrammed Human Neutrophils Involved in Chronic Inflammation

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Neutrophil plasticity is characterized by the ability of some neutrophils to be reprogrammed into other cell types. Reprogramming of neutrophils into dendritic-like cells (DC) is obtained by culturing blood neutrophils with diverse combinations of GM-CSF, IFN-γ, IL-4 and TNF. Importantly, neutrophils with DC characteristics are present in rheumatoid synovial fluids in which GM-CSF, TNF and IL-4 are present. Human neutrophilic skin inflammation is locally regulated by GM-CSF, TNF, IFN-γ, IL-4 and IL-5. GM-CSF, TNF and IL-4 are also factors responsible for the presence of neutrophils in lungs of a mouse model of asthma. These pathological conditions lend support to study these reprogrammed neutrophils in vitro. We showed that normal human blood neutrophils cultured with GM-CSF, IL-4 and TNF lead to long-lived (LL) neutrophils with expression of DC markers and modified functions (i.e. elevated production of superoxide anions and leukotrienes in response to second signals, and decreased degranulation). Compared to adequate controls, do these LL neutrophils exhibit changes in their bioenergetics? Using the extracellular flux analyzer, we observed that LL neutrophils maintain their glucose metabolism over time while only a transient increase in glucose metabolism was observed in control neutrophils. Moreover, upon stimulation, LL neutrophils increase their glucose metabolism to the level of glycolysis of freshly purified neutrophils. The increase in glycolysis was associated with an increase in oxygen consumption, while adequate control neutrophils were unresponsive to stimulation in terms of their

bioenergetics. These results suggest that reprogramming of neutrophils leads to neutrophils with major changes of their energy metabolism in parallel to their modified functions.

LB23

An Investigation into the Pathways Involved in Neutrophil Extracellular Trap Induction

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To date many of the investigations into the mechanism of neutrophil extracellular trap (NET) formation have relied on the use of the ligand PMA. We aimed to examine if the ligands A23187 (a calcium ionophore), nigericin (a potassium ionophore), group B streptococcus (GBS, a gram positive bacteria) and Candida albicans (a fungus) utilise a similar pathway as PMA to induce NET formation. To carry out this study healthy neutrophils were examined for NET production in response the chosen ligands in the presence of inhibitors of proteins known to be central to PMA induced NET formation (ex: PKC, neutrophil elastase, myeloperoxidase and reactive oxygen species, ROS). Neutrophils from patients with mutations in the pathways involved in NET formation, such as chronic granulomatous disease patients (CGD, patients with a mutation in proteins of the NADPH oxidase complex resulting in a lack of ROS generation by granulocytes) or MPO-deficient patients were also use to verify the pathways required for NET formation formed by the ligands of interest.

Our findings reveal that PMA and the physiological ligands Candida albicans and GBS make use of a related pathway for NET induction whereas the ionophores require very few of these signalling molecules for NET induction. This demonstrates that NET induction does not occur through one signalling pathway but is a widely varied method of host cell defence against pathogen attack.

LB24

Dynamics of Ezrin Location at the Plasma Membrane: Relevance to Neutrophil Spreading

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Background:

The spreading of neutrophils onto a surface involves a massive change in cell shape. As ezrin forms crucial crosslinks between the plasma membrane and cortical F-actin, in neutrophils and other myeloid cells, it is thought to maintain the structure of cell surface microridges which act as a reservoir of plasma membrane for spreading. It has been suggested that the effect of elevating cytosolic Ca^{2+} concentration, and thereby activation of the Ca^{2+}-activated cysteine protease calpain, would be to break the ezrin link and permit cell spreading. In this work, we have expressed fluorescent constructs of ezrin to investigate the dynamic relationship between elevatedCa^{2+} concentration in the microdomain of ezrin and its release from the cell periphery.

Materials and methods:

Blood was taken from healthy donors and human neutrophils were isolated by dextran sedimentation. Immunocytochemistry was performed using FITC-conjugated secondary antibodies. RAW 267.4 cells were transfected with plasmids encoding ezrin-mEmerald, mCherry-ezrin, ezrin-mCherry, GFPi-ezrin and ezrin-CEPIA3 using the Cell Line Nucleofector™ Device (Lonza). Ca^{2+} influx wasachieved using a high Ca^{2+} cocktail with thapsigargin and ionomycin.

Results:

Immunocytochemical detection showed that endogenous ezrin was exclusively at the cell periphery in human neutrophils. Ezrin constructs with available N-FERM domain (e.g. ezrin-mEmerald, ezrin-mCherry and GFPi-ezrin) were all located at the cell membrane in transfected myeloid cells, whereas ezrin constructs with a fluor linked to this domain (as with mCherry-ezrin) remained cytosolic. This suggests a dominant role of N-FERM binding in ezrin localisation to the cell periphery, compared to C-ERMAD domain binding to F-actin. Elevation of cytosolic Ca^{2+} concentration resulted in a rapid loss of ezrin from the cell cortex, and a simultaneous dramatic increase in cell size. By attaching a low affinity Ca^{2+}-sensing fluor to ezrin (ezrin-CEPIA3), it was found that in the membrane protrusions, or ezrin-containing microdomains, Ca^{2+} concentration was elevated to approximately 60 μM before release of ezrin and cell expansion.

Conclusions:
It was concluded that elevation of cytosolic Ca^{2+} concentration caused ezrin to be lost from the cell periphery. This may result in the reduction of membrane surface microridges, and contribute to the observed increase in cell size. For the first time, Ca^{2+} concentration has been measured locally in the microridges of myeloid cell membranes. Ca^{2+} concentration in the microridges of myeloid cells increased upon cell stimulation, to a sufficiently high concentration to activate calpain (K_d 30 µM). The dynamics of ezrin localisation and response to Ca^{2+} concentration at the cell periphery can be modelled into the mechanism of neutrophil spreading upon extravasation, where localised elevated cytosolic Ca^{2+} levels activate local calpain to cleave ezrin and liberate plasma membrane for neutrophil spreading.

LB25

Deciphering the Recognition Process of the Human Pathogenic Fungus Aspergillus Fumigatus by the Immune System with Live-Cell Imaging and Analysis of Fungal Mutant Strains
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Invasive aspergillosis caused by the fungus *Aspergillus fumigatus* (*Af*) is a life-threatening infection of immunocompromised hosts with high mortality rates and annually increasing incidences [1]. The small-sized conidia of *Af* are inhaled and easily reach the lung alveoli. Here, they encounter the first line of defense, mainly neutrophilic granulocytes and macrophages [2]. Neutrophils are fast-moving cells that are able to quickly invade infected tissue. We studied their motility by live-cell imaging. Neutrophils were coincubated with GFP-labeled spores (conidia) and images were taken every 10 seconds. A bioinformatic algorithm tracked the movement of every single neutrophil, which enabled us to perform an in-depth analysis of the interaction. More sophisticated parameters were applied to determine reactions of neutrophils like touching events, phagocytic efficiency, the number of phagocytosed spores per cell or the average neutrophil granulocyte speed over time. By contrast, macrophages are slowly moving cells whose main role is the phagocytosis on the spot [3]. The surface composition of the conidia plays a decisive role in their recognition. Our further interest was to investigate the importance of GPI-anchored proteins on fungal spores. GPI (glycosylphosphatidylinositol)-anchors fix proteins to the conidia surface and are highly abundant in fungi. By proteome analysis of the conidial surface 8 GPI-anchored proteins were identified. Knock-out mutants of the encoding genes were generated. Then, the phagocytosis rate in MHS-macrophages was determined microscopically. The images were analysed by an algorithm. As a result, for 4 fungal mutants significantly reduced phagocytosis was calculated. From these, we chose 3 mutants for further analysis. The respective genes code for a ß-1,3-glucanosyltransferase, a β-1,3-endoglucanase and a glutaminase. The mutant conidia were coincubated with neutrophils and the oxidative burst was measured. All 3 mutants initiated a stronger oxidative burst than wild-type conidia. Thus it is likely that the analysed GPI-anchored proteins affect the recognition of conidia in macrophages and neutrophils.
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LB26

EFFECT of ADMINISTRATION of ANTI-INFLAMMATORY DRUG CELECOXIB SIMULTANEOUSLY with LOW POWER LASER THERAPY in MURINE NEUTROPHILS
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Introduction: Celecoxib (CX) is a nonsteroidal anti-inflammatory drug, extensively employed in patients' therapy. Previously, we studied the effect of administration by the oral route of this drug in mice infected ip with *P. brasiliensis* (Pb), and observed that the treatment increased the expression of GM-CSF, responsible for the bone marrow

maturation of polymorphonuclear neutrophils (PMN). We also studied the effect of low level laser therapy (LLLT) applied to the bone marrow of animals infected with Pb or inoculated with Zymosan (Z) in subcutaneous air pouches, and reported that this therapy was able to activate PMN that subsequently migrated to the site of the inoculum. In the present study we evaluate the overall effect of LLLT application on bone-marrow immature neutrophils followed by administration of CX locally, at the site of subcutaneous air pouches in a sterile inflammation model (Z inoculation) in mice. **Methodology:** The treatments employed were: A) laser radiation, employing LLLT (50 mW of power; wavelength of 780 nm; energy density of 37.5 J/cm2; 30 seconds per point) at two points on each hind paw of mice on alternate days in mice previously inoculated with Z in subcutaneous air pouches, B) local treatment at the site of the air pouch with CX, in the last three days prior to collection of cells (at day +8), C) a sum of both treatments and D) Untreated mice. Cells were collected and the number of total and viable PMN, mitochondrial activity, reactive oxygen species (ROS) and proteins production were determined. **Results:** The administration of CX significantly decreased the influx of cells at the inoculation site, while treatment with LLLT managed to increase it. Submitted to both, CX and LLLT, the number of cells was similar to that of controls, as these two treatments had opposite effects. None of the treatments affected cell viability. CX treatment was able to strikingly increase mitochondrial activity, when compared to both, the LLLT-treated group, the CX+LLLT treated group and the non-treated controls. The group of CX-LLLT treated mice also had significantly increased the metabolic activity as compared to the LLLT-treated group. Regarding the release of ROS and production of proteins there were no difference between treatments. When analyzing GM-CSF-production, CX caused a remarkable increase as compared with all groups (non-treated, laser-treated and even LLLT+CX treated group). LLLT treatment increased the expression this cytokine when compared to control, and the combined treatment (LLLT+CX treated group) has antagonistic effect on this production. This cytokine is known to stimulate the maturation of neutrophils in the bone marrow, which may have caused the increased mitochondrial activity of the CX group.

Conclusions: Our results suggest that administration of CX increases mitochondrial activity by PMN resulting, eventually in increased GM-CSF production. Laser therapy acts favoring these cellular processes, but with less efficiency than the anti-inflammatory drug Celecoxib alone.

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LB27

The Effects of High-Dose Statins on Neutrophil Functions in Healthy Elders and during Respiratory Infections and Sepsis; in Vitro Studies and a Randomised, Placebo Controlled Cross-Over Clinical Trial.

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Background

Life expectancy is increasing leading to unprecedented global health resource use. Pneumonia a leading infectious cause of death in the elderly with mortality rates unchanged over the last decade(1). Sepsis is a clinical syndrome associated with systemic immune dysregulation produced by invasive microorganisms. Severe sepsis is seen in 48% of pneumonia patients and is more common (with worse outcomes) in the elderly(2). Innate immunosenescence (the decline in innate immunity with age) is thought to contribute to these poorer outcomes(3).Neutrophils are key effector cells during bacterial infections, and there is evidence of sub-optimal neutrophil responses in the elderly. There is great interest in developing therapeutic strategies to enhance immune responses. HMG CoA Reductase Inhibitors (“statins”) are used to reduce cardiovascular risk. Observational studies of patients taking statins suggest improved survival during infections, *in vitro* studies describe enhanced neutrophil function, yet interventional trials of statins during severe sepsis have not shown benefit. However, clinical trials have recruited younger

patients with severe sepsis for an acute intervention, observational studies included older patients and *in vitro* studies used pharmacologically irrelevant concentrations. To clarify the potential utility of statins for bacterial infections we assessed *in vitro* and *in vivo* affects of pharmacologically relevant concentrations on neutrophil functions in relation to donor age, infection severity and sepsis.

Methods

Migratory dynamics (with simvastatin or control) were assessed for neutrophils from healthy subjects, patients with lower respiratory tract infections, pneumonia or pneumonia-associated sepsis. Prior statin use and outcomes in pneumonia were assessed retrospectively from hospital records in an observational study of 2068 patients. Simvastatin effects on neutrophil functions were assessed in a randomised, double-blinded cross-over clinical trial in healthy elders.

Results

In health, increasing age was associated with reduced neutrophil migratory accuracy (R^2=-0.48, p<0.0001). Infection progressively worsened neutrophil functions in the elderly, in accordance with insult severity (chemotaxis: mean difference to aged healthy; Lower Respiratory Tract Infection (LRTI) -0.7μm/min, p=0.04; Pneumonia -1.1μm/min, p=0.02; Pneumonia with Sepsis -1.6μm/min, p=0.01).

Pharmacologically relevant doses of simvastatin rescued the inaccurate neutrophil migration seen with age in a dose dependent manner. Simvastatin (1uM) also restored "old" neutrophil migratory accuracy during LRTI and pneumonia, but not in patients with sepsis and only at high dose. An observational pneumonia study confirmed improved survival with prior statin use, demonstrating a dose response with better outcomes associated with higher doses.

2 weeks of 80mg simvastatin improved neutrophil chemotaxis in cells isolated from healthy elderly subjects in response to interleukin-8 (IL8) (p=0.04) and fMLP (p=0.006) compared to placebo, without impeding other crucial neutrophils functions. Simvastatin effects appear to be mediated through normalisation of cell adherence.

Conclusion

Age is associated with a reduction in neutrophil migration accuracy, which worsens during infections in accordance with the severity of the infectious insult. Simvastatin improves neutrophil function in the elderly in health and during infections, but not at low dose nor during sepsis. This supports pre-emptive statin treatment in at risk populations but not as an acute intervention.

LB28

Secretion of the Phosphorylated Form of the Alarmin S100A9 from Neutrophils is Essential for the Pro-Inflammatory Functions of Extracellular S100A8/A9, Véronique Schenten, Fabrice Tolle, Sabrina Bréchard, Sébastien Plançon, Eric J. Tschirhart, *University of Luxembourg, Life Sciences Research Unit- Calcium Signalling and Inflammation Laboratory*

S100A8 and S100A9 are members of the S100 family of cytoplasmic EF-hand Ca^{2+}-binding proteins and are abundantly expressed, mostly under heterodimeric form, in the cytosol of neutrophils. In addition to various intracellular roles, S100A8/S100A9 can be secreted in the extracellular environment and be considered as alarmins modifying the inflammatory response through the interaction with pattern recognition receptors such as TLR4 or RAGE. High concentrations of S100A8/A9 are found at local sites of inflammation or in the serum of patients with inflammatory diseases and are thus used as inflammation biomarkers. Although S100A8/A9 secreted by neutrophils are of importance in the pathophysiology of many inflammatory diseases, the mechanisms by which these proteins are released remain unclear. The intracellular activity of S100A8/A9 was shown to be regulated by S100A9 phosphorylation among other, however the phosphorylation state of S100A9 in the extracellular environment and the importance of this putative phosphorylation on the extracellular activity of S100A8/A9 has not yet been extensively studied. Therefore, we focused our work on one hand on the phosphorylation state of the secreted S100A9 and on the other hand on the effects of the unphosphorylated and the phosphorylated complex on the pro-inflammatory function of neutrophils. First of all, we characterized the secretion of S100A8/A9 under different stimulatory conditions and investigated the phosphorylation state of secreted S100A9. Our results on neutrophil-like differentiated HL-60 cells (dHL-60) and purified human neutrophils show a time-dependent secretion of S100A8/A9 when induced by PMA and

importantly, S100A9 was found in a phosphorylated form in these supernatants. Then we studied the influence of S100A8/A9 or S100A8/PhosphoS100A9 stimulation on pro-inflammatory cytokine expression and secretion in dHL-60 cells. In this aim, time course experiments with unphosphorylated or phosphorylated S100A8/A9 were performed and the expression and secretion levels of IL1α, IL1ß, IL6, TNFα, CCL2, CCL3, CCL4 and CXCL8 were measured by real-time PCR and cytometry bead array respectively. Our results clearly show that only the phosphorylated form of the complex induces pro-inflammatory cytokine expression and secretion. Finally, we were able to show that S100A8/PhosphoS100A9 is inducing cytokine secretion through TLR4 signaling. As a conclusion, we were able to show for the first time that S100A8/A9 is released under a phosphorylated form from both dHL60 cells and purified human neutrophils and that this phosphorylation is essential for the pro-inflammatory activity of the S100 complex on neutrophil-like dHL-60 cells.

LB29

Occurrence and Significance of Tumor-Associated Neutrophils in Patients with Colorectal Cancer

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Purpose: Colorectal cancer (CRC) is a major public health problem, representing the fourth cause of cancer death worldwide. Prediction of outcome in patients with CRC remains difficult because of the complexity and heterogeneity of this pathology [1]. Inflammation, including soluble and cellular effectors is an essential component of the tumor microenvironment [2]. Tumor-associated neutrophils (TAN) are a component of the inflammatory microenvironment of tumors but the significance of TAN in CRC has been the subject of conflicting reports [3]. The present study was designed to set up a reliable methodology to assess TAN infiltration in CRC and to evaluate their clinical significance. **Methods:** CD66b and myeloperoxidase (MPO) were assessed as candidate neutrophil markers in CRC using immunohistochemistry. CRC patients (n=271) (stageI-IV) were investigated retrospectively by computer-assisted imaging on whole tumor sections. **Results:** CD66b was found to be a reliable marker to identify TAN in CRC tissues, whereas MPO also identified a subset of $CD68^+$ cells. Higher TAN density in stage I-IV patients was associated with better prognosis. Importantly, an interaction was observed between clinical stage, TAN density and 5-FU-based chemotherapy, and, in stage III patients, TAN density had a dual clinical significance depending on the use of chemotherapy. **Discussion:** TAN are an important component of the immune cell infiltrate of CRC which can be reliably quantified by CD66b staining. Unexpectedly, higher TAN density was associated with response to chemotherapy.

Conclusions: Assessment of TAN infiltration may help identify patients likely to benefit from 5-FU-based chemotherapy.

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LB30

Neutrophil Infiltration Enhances the Favorable Prognostic Significance of CD8+ T Cell Infiltration in Colorectal Cancer

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OBJECTIVE: Tumor infiltration by different T lymphocyte subsets was repeatedly reported to be associated with favorable prognosis in colorectal cancer (CRC). Still debated is the role of innate immune system. We investigated clinical relevance, phenotypes and functional features of CRC infiltrating CD66b+ neutrophils and their crosstalk with CD8+ T cells.
DESIGN: CD66b and CD8 immunohistochemical evaluation was performed on a tissue microarray including >650 evaluable CRC samples. Phenotypic profiles of tissue infiltrating and peripheral blood CD66b+ cells were evaluated by flow cytometry. CD66b+/ CD8+ cells crosstalk was investigated by in vitro experiments.
RESULTS: CD66b+ cell infiltration in CRC is significantly associated with increased survival. Interestingly, neutrophils frequently co-localize with CD8+ T cells in CRC tissues. Functional studies indicate that although neutrophils are devoid of direct antitumor potential, co-culture with peripheral blood and tumor associated neutrophils (TANs) enhances CD8+ T cell activation, proliferation and cytokine release induced by stimulation with suboptimal concentrations of anti-CD3 monoclonal antibody (mAb). Moreover, under optimal activation conditions, CD8+ cells initially stimulated in the presence of CD66b+ cells show decreased expression of PD-1 "exhaustion" marker and are significantly less susceptible to apoptosis induced by T- cell receptor triggered re-stimulation. Importantly, combined tumor infiltration by CD66b+ and CD8+ T lymphocytes is associated with significantly better prognosis, as compared to CD8+ T cell infiltration alone.
CONCLUSIONS: Neutrophils enhance the responsiveness of CD8+ T cells to TCR triggering. Accordingly, infiltration by neutrophils enhances the prognostic significance of CRC infiltration by CD8+ T cells, suggesting that they might modulate antitumor immunity.

LB31

Mechanobiology of dendritic cell migration in confined environments

Pablo Vargas, *Institut Curie*

Dendritic cell (DC) migration in peripheral tissues serves two main functions: antigen sampling by immature DCs, and chemokine-guided migration towards lymphatic vessels (LVs) upon maturation. These migratory events determine the efficiency of the adaptive immune response.
In this seminar, I will discuss about the cellular rearrangements of the actin cytoskeleton needed to optimize migration of DCs in tissues. I will show that migration of immature DCs depends on two major actin pools: (1) a RhoA-mDia1-dependent actin pool located at their back, which facilitates forward locomotion and (2) a Cdc42-Arp2/3-dependent actin pool present at their front, which limits migration but promotes antigen capture. Following LPS-induced maturation, Arp2/3-dependent actin enrichment at the cell front is drastically reduced. Consequently, mature DCs switch to a faster and more persistent locomotion mode that facilitates chemotactic migration to LVs and lymph nodes. I will further discuss on how these cytoskeleton rearrangements are used by DCs to move in irregular landscapes facilitating the migration of leukocytes in the complex geometry of tissues.

LB32

Ultra High Throughput Purification and Concentration of Leukocytes and Progenitor Cells from Peripheral Blood and Bone Marrow

Kyle Smith[1], Thomas Barber[1], Mehmet Toner[2], Ron Tompkins[2], Ravi Kapur[1,2] ,*[1] MicroMedicine, Boston, MA , [2] Massachusetts General Hospital, Boston, MA*

While tremendous advances have been made in extracting valuable information from specific cellular components in blood (and other bodily fluids), the bulk processing methods used to isolate these subpopulations have remained crude and stagnant for decades, thereby compromising precious patient samples and hindering downstream clinical assays. MicroMedicine's innovative LeukoChip™ cell isolation platform addresses this issue by enabling enrichment with commensurate efficiency and elegance to the available backend cell and molecular interrogation techniques. The core of the platform is a high-precision, injection-molded microfluidic disc that enables ultra high-throughput purification and concentration of leukocytes from

peripheral blood or bone marrow.

The LeukoChip™ cell isolation platform achieves this high level of performance by utilizing inertial focusing to sort and concentrate cells. In the first stage, the sample stream enters the microfluidic channel flowing alongside a stream of buffer solution. Target cells flowing near the elongated island structures in the channel experience a fluidic lift force that induces them to gradually migrate laterally from the sample stream into the buffer stream, leaving behind the contaminating cells. The target cells then proceed to the second stage of the device to a series of curving channels in which the fluidic lift force induces them to migrate towards the center of channel. This creates a cell-free stream of fluid at the edge of the curving channel that is siphoned into an adjacent channel. After a number of iterations, the volume in which the target cells are suspended is significantly reduced. The platform processes blood at a rate of 200 mL/hour with each cell spending just a fraction of a second in the chip.

The LeukoChip™ platform maintains cells in their endogenous state, with no contact with physical objects or antibodies, and with minimal change in homeostasis. Minimal manipulation enables recovery of target cells (rare or abundant) with high yield, high purity, and high functionality. Preserving the functionality of the cells, and integrity of the protein, DNA, and RNA sub-components, enables ex-vivo, and in-vivo therapeutic and diagnostic applications. Specific examples include: (1) Retention, enrichment, and concentration of abnormal cells (and their phenotype, genotype, proteomics, and metabolomics) from blood or bone marrow, enables diagnosis of disease using traditional tools of cytopathology, and recent advances in next generation sequencing of RNA/DNA. (2) Retention of cellular motility, and cell signaling enables isolated autologous radioisotope tagged neutrophils to migrate and detect infection in-vivo. (3) Retention of cell division and cell signaling enables use of isolated autologous progenitor cells (from either bone marrow or mobilized peripheral stem cells) for wound repair. (4) Retention of the immunogenicity of circulating T cells enables isolation and ex-vivo conditioning for autologous immune therapy.

By providing gentle, precise, high throughput and efficient cell enrichment, the LeukoChip™ platform will enhance the performance and quality of information in Clinical Diagnostics, Cellular Therapy and Life Sciences.

LB33

CHARACTERIZATION OF A NOVEL NEUTROPHIL-DEFICIENT MOUSE STRAIN

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Background and objectives: Genetic deletion of specific leukocyte lineages strongly contributes to understanding the role of various leukocyte subsets in physiological and pathological conditions. There have been a number of attempts to generate genetically neutrophil-deficient mouse strains. However, all those strains suffer from substantial limitations such as the limited efficiency of neutrophil deletion, the effect of the mutations on other lineages or the limited survival of the mutant mice. We have previously shown that the antiapoptotic Mcl-1 protein is essential for the survival of neutrophils but not macrophages. Therefore, we tested whether myeloid-specific deletion of Mcl-1 could provide a novel and more suitable genetic model of neutrophil deficiency in experimental mice.

Materials and methods: $LysM^{Cre}$ mice expressing Cre recombinase in the myeloid compartment were crossed with animals carrying the $Mcl\text{-}1^{flox}$ mutation to obtain $LysM^{Cre/Cre}Mcl\text{-}1^{flox/flox}$ (referred to as $Mcl\text{-}1^{\Delta Myelo}$) mice characterized by myeloid-specific conditional deletion of Mcl-1. Leukocyte populations in the peripheral blood, spleen and bone marrow of $Mcl\text{-}1^{\Delta Myelo}$ mice were tested by flow cytometry. The viability and fertility of the mice was monitored for 6 months under SPF conditions. The susceptibility of the $Mcl\text{-}1^{\Delta Myelo}$ mice to infection was tested following intraperitoneal injection of 2×10^7 *Staphilococcus aureus* bacteria/mouse or intravenous injection of 10^5 *Candida albicans* cells/mouse. K/BxN serum-transfer arthritis and the mouse model of the epidermolysis bullosa acquisita

(EBA) were used as neutrophil-dependent in vivo inflammation models.

Results: Mcl-1$^{\Delta Myelo}$ mice were viable, though their long-term survival was somewhat reduced compared to wild type mice. Importantly, Mcl-1$^{\Delta Myelo}$ mice were fertile even in homozygous form. The mice have a strong (>99%) reduction of circulating neutrophil counts and severe deficiency of splenic and bone marrow neutrophils. However, other leukocytes such as circulating eosinophils, monocytes, T and B cells, or splenic dendritic cells and macrophages are not affected. Following infection with *S. aureus*, all Mcl-1$^{\Delta Myelo}$ mice died on the first day. The neutrophil-deficient mice were also significantly more sensitive compared to the control group in the candidemia model. Mcl-1$^{\Delta Myelo}$ mice are completely protected from all signs of arthritis development in the K/BxN serum-transfer model. Following injection of mCVII antibody, the neutrophil-deficient mice, in contrast to control group, showed no skin lesions or blisters in the EBA model.

Conclusions: The Mcl-1$^{\Delta Myelo}$ mutation leads to dramatic reduction of the number of circulating and tissue neutrophils without affecting other blood and splenic leukocyte lineages. This reduced innate immune response makes these mice more susceptible for bacterial and fungal infections. However, Mcl-1$^{\Delta Myelo}$ mice are viable and fertile in homozygous form and are completely protected in two known neutrophil-dependent *in vivo* disease models. Taken together, the Mcl-1$^{\Delta Myelo}$ mice may provide a useful novel model of genetically determined neutrophil deficiency.

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