

#### 2017 Abstracts

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## Phospholipase D2 (PLD2) is crucial for oxidized cholesterol uptake and foam cell formation in atherosclerotic plaques

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The uptake of cholesterol carried by Low Density Lipoprotein (LDL) is tightly controlled in the body. While hepatocytes effectively regulate their function to counteract high cholesterol, macrophages have limited means of dealing with excess cholesterol, which is why it accumulates inside macrophages transformed into foam cells. That hastens inflammation. We have uncovered and characterized a novel mechanism that involves the signaling protein Phospholipase D (PLD) in foam cell formation. Utilizing bone marrow-derived macrophage (BMDM) from PLD genetically deficient mice, we demonstrate that phagocytosis of oxidized LDL (ox-LDL) is dependent on PLD. However, the role of the two mammalian isoforms, PLD1 and PLD2 is very different, as only PLD2-/- macrophages fail to engulf ox-LDL. For the molecular mechanism, we show that PLD2 interacts with the docking protein (Grb2) and with actin-polymerizing protein Wiskott-Aldrich syndrome protein (WASp) during phagocytosis. Further, the uptake of ox-LDL utilizes the scavenger receptor CD36. These results were validated in vivo showing that PLD2, but not PLD1 expression, was elevated in human arterial specimens derived from endarterectomies. Thus, through phagocytosis, PLD2 expression and activation lead to enhanced foam formation, which will hasten progression of atherosclerosis pathology. This knowledge provides new molecular targets to better understand the disease.

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## Neutrophil Superoxide Production is Inhibited by Staphylococcus aureus SaeR/S-regulated Factors Samuel SaeR/S-regulated Factors

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Neutrophils are the first line of defense after a pathogen has breached the epithelial barriers, and unimpaired neutrophil functions are essential to clear infections. Staphylococcus aureus (S. aureus) is a prevalent human pathogen that is able to withstand neutrophil killing, yet the mechanisms used by S. aureus to inhibit neutrophil clearance remain incompletely defined. The production of reactive oxygen species (ROS) is a vital neutrophil antimicrobial mechanism. We have previously shown that the staphylococcal SaeR/S-two component virulence system regulates factors that decrease neutrophil ROS production. However, the specific SaeR/S-regulated effector virulence factors that inhibit neutrophil ROS production remain undefined. Herein, we provide evidence that neutrophil ROS inhibition by S. aureus occurs early in the ROS production chain. Human neutrophils were exposed to secreted toxins from wild-type S. aureus USA300 and the isogenic USA300\Delta saeR/S mutant. Our results suggest that S. aureus secreted virulence factor(s) completely inhibit neutrophil superoxide production within minutes in a SaeR/S-dependent mechanism and is independent of neutrophil lysis or superoxide dismutase. In addition, conditioned media made by exposure of S. aureus to peripheral blood mononuclear cells (PBMCs) enhanced the neutrophil-derived superoxide inhibitory effect. Thus, we hypothesize that early interaction of S. aureus with components of the immune system initiates the production of staphylococcal virulence factors that inhibit early neutrophil antimicrobial mechanisms including superoxide production, which disrupts the neutrophil ROS production chain.

## Metformin rewires the pro-inflammatory metabolic and functional adaptation of human inflammatory airway PMNs

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Background and rationale. In cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and other inflammatory airway diseases, polymorphonuclear neutrophils (PMNs) are recruited to the airways where they mediate disease by releasing reactive oxygen species (ROS) and toxic granules laden with proteases. PMN activation is tightly regulated and requires high consumption of nutrients/ATP, and oxygen. The upsurge in energy metabolism in activated PMNs is supported by anabolic signaling via the mechanistic target of rapamycin (mTOR) pathway. In this study, we sought to explore the use of metformin as a metabolic and functional modulator of activated airway PMNs. Metformin is an agonist of the AMP-activated protein kinase (AMPK), which can inhibit mTOR signaling and downstream anabolic processes by re-balancing the rate of ATP production and oxygen consumption. Methods. Using flow cytometry, we first assessed the activation of AMPK by measuring intracellular levels of phosphorylated AMPKa (pAMPKa). Second, using a novel in vitro model mimicking PMN recruitment to CF airways and subsequent activation, we assessed intracellular levels of pAMPKa, ROS production using the CellRox probe, and the release of the primary and secondary granules using surface CD63 and CD66b expression, respectively. Third, we measured the metabolic activity (glycolysis, oxygen consumption, and oxidative phosphorylation) of in vitro activated PMNs using the Seahorse bioenergetic analyzer. Finally, we tested the effect of metformin on the above-described metabolic and functional phenotypes of in vitro activated PMNs. Results. We observed that:(i) CF airway PMNs have lower pAMPKα levels compared to CF blood PMNs, in vivo; (ii) in vitro activated PMNs recruited to CF airway fluid have lower pAMPKa levels compared to those migrated to LTB4, concomitant with higher rates of glycolysis, oxygen consumption, degranulation, and ROS production; and (iii) metformin treatment increased AMPK activation, reduced oxygen consumption, glycolysis, degranulation, and ROS production by in vitro activated PMNs recruited to CF airway fluid. Conclusion. Our work establishes a key role for altered metabolism in regulating PMN function under inflammatory conditions in CF airways. This work also provides proof-of-concept data for the use of metabolic modulators to target pathogenic airway PMNs. Acknowledgments. This work was funded by the CF Foundation (TIROUV15A0).

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#### Selective Inhibition of T Cells by Myeloid-Derived Suppressor Cells from Septic Patients

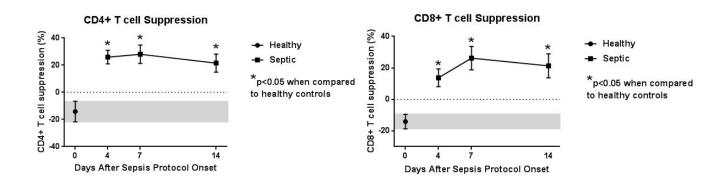
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**Introduction:** Myeloid-derived suppressor cells (MDSCs) have been well described in the cancer literature, but few studies have examined their role in sepsis. Prior human and animal studies from our laboratory demonstrate the increased and persistent expansion of CD11b<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>dim</sup> MDSCs during severe sepsis and septic shock (Mathias B, *Ann Surg*, 2017). Increasing quantities of these cells are associated with the development of chronic critical illness and increased mortality. We sought to further elucidate the mechanism through which these cells influence clinical outcomes after sepsis by quantifying CD4<sup>+</sup> and CD8<sup>+</sup> T cell suppression in both the absence and presence of host MDSCs. We hypothesize that MDSCs are responsible for the adaptive immune suppression after sepsis, rather than direct lymphocyte dysfunction.

**Results**: Within the sepsis cohort, the total number of MDSCs in the whole blood greatly exceeded that of healthy controls  $(1.59 \times 10^5/\text{ml})$  at day 4 versus  $9.15 \times 10^4/\text{ml}$  MDSCs in controls, p=0.02). In the absence of MDSCs, CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation following stimulation with anti-CD3<sup>+</sup>/CD28<sup>+</sup> beads did not significantly differ between septic patients and healthy controls (CD4<sup>+</sup>: proliferation index of  $2.20 \pm 0.13$ ,  $2.28 \pm 0.16$ , and  $2.00 \pm 0.10$  at days 4, 7, and 14 in sepsis patients versus  $1.87 \pm 0.18$  in healthy controls, all p>0.05; CD8<sup>+</sup>: proliferation index of  $2.09 \pm 0.12$ ,  $2.08 \pm 0.11$ , and  $1.94 \pm 0.13$  at days 4, 7, and 14 in sepsis patients versus  $1.95 \pm 0.15$  in healthy controls, all p>0.05). Conversely, in the presence of MDSCs from septic patients, T cell proliferation in CD4<sup>+</sup> and CD8<sup>+</sup> cell subsets was dramatically reduced, whereas proliferation of T cells from healthy subjects was not suppressed by MDSCs from healthy controls (CD4<sup>+</sup>: 25.8%, 28.0%, and 21.5% CD4<sup>+</sup> T cell suppression at 4, 7, and 14 days versus -14.2% in healthy controls, p<0.05; CD8<sup>+</sup>: 13.7%, 26.2%, and 21.3% CD8<sup>+</sup> T cell suppression at 4, 7, and 14 days versus -14.1% in healthy controls, p<0.05). There were no significant differences in CD4<sup>+</sup> or CD8<sup>+</sup> T cell suppression in the sepsis cohort across the three time points, indicating the suppressive function of MDSCs persists for up to 2 weeks after sepsis onset.

**Conclusion:** It is well known that lymphocyte dysfunction occurs after mammalian sepsis. However, in the subacute and chronic periods after sepsis, the presence of MDSCs from septic patients is required to maintain reduced proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. This data implies that adaptive immune dysfunction after sepsis, at least in part, is due to MDSC-T cell interactions rather than intrinsic T cell defects (i.e. "T cell exhaustion"). Therefore, improving post-sepsis outcomes will likely require immunomodulation of MDSCs as opposed to solely focusing on T cells.



Human neutrophils exhibit delayed apoptosis and secrete IL-1B after phagocytosis of Neisseria gonorrhoeae Christine Cho<sup>1,2,3</sup>, Silvie Kremserova<sup>1,2</sup>, Michael A. Apicella<sup>4</sup> and William M. Nauseef<sup>1,2,4</sup>

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An exuberant inflammatory response secondary to recruitment of polymorphonuclear leukocytes (PMN) characterizes infection with N. gonorrhoeae (Ngc), an exclusively human pathogen. Prior studies have shown that Ngc delay apoptosis of adherent PMN, although the underlying mechanism has not been elucidated. Gonococcal opacity-associated proteins (Opa) mediate binding and internalization of Ngc by human PMN through CEA-related cell adhesion molecules (CEACAMs). Human PMN were fed with Ngc at 10:1 MOI for 30 minutes at 37°C, after which unbound bacteria were removed and bacteria-laden PMN were incubated. Phagocytosis of non-opsonized FA1090Δopa by PMN in suspension was profoundly depressed compared to that of the isogenic control  $[FA1090\Delta opa=7.63\% \pm 0.61\% \text{ vs } FA1090 \text{ wild-type}=39.73\% \pm 5.28\%, p<0.01, n=3], consistent with previous$ studies of PMN phagocytosis of Ngc. Opsonization of FA1090Δopa partially restored its ingestion by PMN  $[FA1090 \text{ wild-type}=39.73\% \pm 5.28\% \text{ vs } FA1090\Delta opa+opsonization}=35.03\% \pm 4.28\%, n=3]$  but PMN apoptosis was still delayed, as measured by the change in mitochondrial depolarization [FA1090 wild-type=19.35% ± 3.85% vs FA1090 $\Delta$ opa+opsonization=7.08%  $\pm$  0.81%, p<0.05, n=3]. Ngc also elicited IL-1 $\beta$  secretion from ultra-pure PMN at 10:1 MOI after 2-hour incubation at 37°C [47.82 pg/mL  $\pm$  10.24 pg/mL, n=3]. In contrast to S. aureus, which can both prime and activate PMN to release IL-18, Ngc required a priming step with lipopolysaccharide (LPS), similar to activation of the inflammasome in macrophages. We conclude that: 1) Opa-CEACAM interactions contributed to cell death pathways in human PMN fed Ngc, and 2) human PMN, primed with LPS then fed Ngc, released IL-1\u00ed. Taken together, the IL-1\u00ed release and delayed apoptosis could contribute to the exuberant inflammatory response by PMN in Ngc infection.

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#### Insights into lysis of human neutrophils after S. aureus ingestion

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Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is among the 12 bacteria recently recognized by WHO as posing the greatest risk to human health. CA-MRSA causes a wide range of infections, most of which are characterized by a robust inflammatory response that leads to extensive tissue damage and necrosis, even in apparently healthy individuals. Neutrophils, the first line of host defense against invading microbes, play a key role against *S. aureus* infection. However, despite the potent neutrophil antimicrobial system, 20-50% of ingested *S. aureus* remain viable in neutrophils and cause their lysis. Human neutrophils fed CA-MRSA lyse by an unknown, caspase-independent mechanism that is inhibited by necrostatin-1, an allosteric inhibitor of receptor-interacting serine/threonine kinase 1 (RIPK-1). RIPK-1 figures prominently in necroptosis, a form of programmed necrotic cell death that is dependent on RIPK-1, RIPK-3, and the pro-death effector, mixed lineage kinase domain-like (MLKL).

We initiated studies of alternative forms of programmed cell death pathways and first tested the hypothesis that neutrophils fed CA-MRSA underwent necroptosis. Lysis after CA-MRSA phagocytosis was independent of tumor necrosis factor a, active RIPK-1, and MLKL but dependent on active RIPK-3. *S. aureus* alone, without lipopolysaccharide priming, stimulated ultrapure neutrophils (99.8% pure) to release IL-1b. Furthermore, the CA-MRSA-stimulated IL-1b release was blocked by inhibitors of RIPK-3 or serine proteases. In contrast to the release

of IL-1b, ultrapure neutrophils fed CA-MRSA released very little IL-18, and this low IL-18 activation was not inhibited with inhibitors of RIPK-3 or serine proteases. Interestingly, NLRP3 inflammasome was not detected on protein level, and gene expression of NLRP3 was not upregulated compare to unstimulated neutrophils.

Taken together, our data suggest that human neutrophils fed CA-MRSA undergo a novel form of lytic programmed cell death that is dependent on RIPK-3 and associated with concomitant IL-1b activation. Targeting the molecular pathways that culminate in lysis of neutrophils during CA-MRSA infection may serve as a novel therapeutic intervention to limit the associated tissue damage

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#### Inflammasomes and pathogen sensing

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Inflammasomes are multimeric protein complexes that are formed in a cell to orchestrate host defense mechanisms against infectious agents and physiological aberration. Assembly of the inflammasome complex is initiated by nucleotide-binding domain and leucine-rich repeat receptors (NLRs) or absent in melanoma 2 (AIM2)-like receptors (ALRs). NLRs and ALRs mediate host recognition of pathogen-associated molecular patterns (PAMPs) released during bacterial, viral, fungal, and protozoan infections, or danger-associated molecular patterns (DAMPs) released during cellular damage. Activated NLRs and ALRs, in most cases, recruit a bipartite protein known as ASC to engage caspase-1. NLR- and ALR-mediated caspase-1 activation drives pyroptosis and the release of IL-1 $\beta$  and IL-18. The biological activities of IL-1 $\beta$  and IL-18 and pyroptosis are largely beneficial to the host during an infection. However, these cytokines induced by endogenous danger signals trigger sterile inflammation, a risk factor for the development of autoinflammatory and metabolic diseases. Therefore, activation of the inflammasome must be finely controlled to avoid overt tissue damage. These regulatory activities are governed by scaffolding proteins and post-translational modifications, which together, tightly control and modulate inflammasome activation.

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## The TLR4 agonist Monophosphoryl Lipid A mediates broad protection against infection via augmentation of innate antimicrobial cell functions

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Infection with antibiotic resistant organisms is becoming one of the biggest healthcare threats faced by our world today, leading to the need for alternative immunomodulatory therapies to protect those at high risk from developing life-threatening infections. Priming of mice with the TLR4 agonist monophosphoryl lipid A (MPLA) mediates protection against Gram-negative bacterial infections. Previous studies suggest that priming with TLR4 agonists mediate protection against high dose LPS or Gram-negative bacterial challenge through the induction of endotoxin tolerance, leading to attenuation of the pro-inflammatory cytokine response. However, we recently showed that endotoxin tolerance does not predict the antimicrobial response to infection following TLR agonist treatment. Thus, we hypothesized that MPLA would also be able to protect mice against a non-LPS-containing Gram-positive bacterial infection through augmentation of antimicrobial responses. To test this hypothesis, mice were primed with MPLA, followed by systemic challenge with *Staphylococcus aureus*, a common antibiotic resistant pathogen. We found that MPLA-primed mice had significantly improved resistance to *S. aureus*, leading to reduced mortality, improved bacterial clearance and reduced organ injury. MPLA-mediated protection was maintained in RAG2<sup>-/-</sup> mice as well as CCR2<sup>-/-</sup> mice, indicating that protection was not dependent on lymphocytes or monocytes. However, protection was lost in neutrophil- and macrophage-depleted mice, indicating that neutrophils and mature

phagocytes are required. Furthermore, neutrophils and macrophages, but not monocytes, from MPLA-primed mice had augmented phagocytic capacity. Finally, MPLA was also shown to mediate protection against severe Candida albicans systemic infection. This finding demonstrates the ability of MPLA to protect against a broad spectrum of pathogens, including Gram negative and Gram positive bacteria, as well as fungal pathogens. In conclusion, these data demonstrate that priming mice with the clinically available TLR4 agonist MPLA mediates resistance to a severe Gram-positive infection through augmentation of neutrophil and macrophage antimicrobial functions and support the use of MPLA as a clinical agent to reduce the incidence of broad nosocomial infections caused by a variety of organisms.

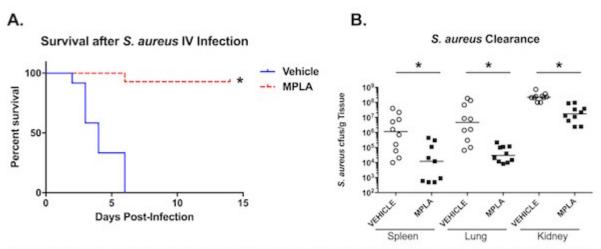


Figure 1. MPLA protects against lethal S. aureus infection. Mice were primed with i.v. MPLA(20 ug) or vehicle (lactated Ringers solution) control for two days. 24 hours after MPLA priming, mice were challenged with 108 cfu S, aureus by i.v. injection, A, Survival was monitored for 15 days, B. Bacterial load in the spleens, lungs, and kidneys was assessed 3 days after S. aureus infection. \*, significantly different from Vehicle control, p ≤ 0.05. Survival n = 15; Clearance n = 10.

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An ocular commensal protects from corneal infection by driving an IL-17 response from mucosal ?d T cells Anthony J. St.Leger<sup>1</sup>, Jigar V. Desai<sup>2</sup>, Rebecca A. Drummond<sup>2</sup>, Abirami Kugadas<sup>3</sup>, Fatimah Almaghrabi<sup>1</sup>, Phyllis B. Silver<sup>1</sup>, Kumarkrishna Raychaudhuri<sup>1</sup>, Mihaela Gadjeva<sup>3</sup>, Yoichiro Iwakura<sup>4</sup>, Michail S. Lionakis<sup>2,2</sup> and Rachel R. Caspi<sup>1</sup>

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The eye is a structurally and immunologically complex organ, in which commensals can play a dual role, depending on the context. The inside of healthy eye is an immunologically privileged site, devoid of microorganisms. Our previous study showed that gut commensals can trigger autoreactive T cells specific to retina through a process of antigenic mimicry, precipitating a blinding autoimmune uveitis. In contrast, the surface of the eye (conjunctiva) is a mucosal site exposed to the environment. Mucosal sites (intestine, oral cavity, nasopharynx, vagina) all have their associated commensal flora, but existence of a resident microbiome on the eve has been highly controversial due to the profoundly antimicrobial nature of the ocular surface environment. We used a mouse model of ocular surface disease to reveal that commensals are present in the ocular mucosa and have functional immunological consequences. We isolate, purify and fulfill Koch's postulates defining a causative agent for one such candidate commensal, Corynebacterium mastitidis. This organism elicits a commensal-specific IL-17 response from (Vγ4) γδ T cells in the ocular mucosa that is central to local immunity, including neutrophil recruitment and release of antimicrobials into the tears, and protects the eye from pathogenic Candida albicans or Pseudomonas aeruginosa infection. Our findings provide direct evidence that a resident commensal microbiome exists on the ocular surface and identify the cellular mechanisms underlying its effects on ocular immune homeostasis and maintenance of local host defense.

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## The mRNA and microRNA transcriptome of lung neutrophils during Streptococcus pneumoniae pneumonia in mice $\mathbb O$

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**Background:** The complex role of neutrophils in modulating the inflammatory response is increasingly appreciated. This study tested the hypothesis that mRNA and microRNA (miR) expression is altered in lung neutrophils during acute pneumonia induced by *Streptococcus pneumoniae* (*S. pneumoniae*), the most common cause of community-acquired pneumonia. Our studies profiled the expression of mRNAs and miRs in lung neutrophils in mice during *S. pneumoniae* pneumonia and performed in depth *in silico* analyses.

**Methods:** Adult female C57BL/6J mice were given either *S. pneumoniae* (suspended in PBS) or sterile PBS by intratracheal instillation into the left lung. After 24 hours, the lungs were harvested and lung neutrophils were isolated using magnetic microbeads. Total RNA was isolated from lung neutrophils, and mRNA and miR expression was profiled using microarrays. Differential expression between PBS and *S. pneumoniae*-treated groups was evaluated using one-way ANOVA after normalization and transformation of microarray signal intensity values. Differentially expressed (DE) miRs or mRNAs were filtered at Benjamini-Hochberg FDR adjusted p value < 0.05, and fold change > 2. Candidate key regulatory miRs were identified *in silico* by constructing networks consisting of DE conserved miRs and their DE predicted mRNA targets, and by using the mirHub algorithm to determine whether the predicted regulatory effect of any given miR on a set of DE genes is significantly greater than expected by chance.

Results: Lung neutrophils from mice with *S. pneumoniae* pneumonia contained over 4000 DE mRNAs, about a third of which (36%) were upregulated at least 2-fold. The ability of neutrophils to respond to inflammatory stimuli is enhanced during pneumonia, as evidenced by the increased expression of pattern recognition receptors and receptors for cytokines and other inflammatory mediators. Their response is seen in their increased expression of transcription factors (including canonical and noncanonical NF-κB signaling molecules, AP-1 and Nrf2), cytokines, chemokines and other inflammatory mediators. Interestingly, neutrophils responded to Type I interferons but did not produce these cytokines, whereas they produced and responded to Type II interferon. Gene and pathway analysis also show that neutrophils have the potential to shape the subsequent adaptive immune response to *S. pneumoniae*, in part by affecting lymphocyte recruitment and activation. Increased mRNA expression of TNF, IFNγ, CD103, CD64 and CD54 was confirmed at the protein level using flow cytometry of lung digests at 24 hours after instillation of *S. pneumoniae* or PBS. CD45 and Siglec-F, two genes whose mRNAs were not increased during the immune response to *S. pneumoniae*, showed no change in protein expression. Of approximately 1100 miRs queried, 31 increased and 67 decreased significantly more than 2-fold in neutrophils from *S. pneumoniae* pneumonia. Network analyses of potential miR-target mRNA interactions revealed candidate key regulatory miRs.

**Conclusions:** Our results demonstrate that *S. pneumoniae* modulates mRNA and miR expression by lung neutrophils. Gene expression of molecules within signaling pathways important in the immune response is enriched, and these signaling pathways are likely to be turned on. Computational analyses suggest that changes in miR expression are predicted to modulate mRNA expression of their targets. Changes in mRNA and miRs may regulate host defense against this pathogen.

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#### Helicobacter pylori modulates the lifespan and cell death mechanisms of human neutrophils

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Helicobacter pylori infects the human stomach and causes a spectrum of disease that includes gastritis, peptic ulcers, and gastric adenocarcinoma. A chronic, neutrophil-rich inflammatory response characterizes this infection. We recently published that H. pylori-infected neutrophils undergo subtype differentiation defined by profound nuclear hypersegmentation, a CD62L<sup>dim</sup>, CD16<sup>bright</sup>, CD11b<sup>bright</sup>, CD66b<sup>bright</sup>, CD63<sup>bright</sup> surface phenotype, and predominantly proinflammatory cytokine secretion. We demonstrate here that the infected neutrophils also have a significantly prolonged lifespan and do not acquire apoptotic nuclear morphology, externalize phosphatidylserine, or exhibit caspase-3, -8, or -9 activation over 72 hours post-infection. This apoptosis inhibition requires direct neutrophil-H. pylori contact as well as neutrophil transcription and translation. We utilized RNA sequencing to probe the neutrophil transcriptome at 6 hr and 24 hr post-infection and identified that mRNA encoding MNDA, a proapoptotic protein, is significantly downregulated in the H. pylori-infected neutrophils. We validated these results by qPCR and are investigating the hypothesis that MNDA downregulation decreases the turnover of MCL-1, inhibiting apoptosis and prolonging the lifespan of H. pylori-infected PMN. Moreover, our preliminary data suggest that H. pylori-infected neutrophils may ultimately undergo a form of 'vital NETosis', incrementally releasing their DNA into the extracellular space where it can be used by H. pylori as a purine source. H. pylori may therefore exploit neutrophil lifespan plasticity and alternative cell death mechanisms as part of a virulence strategy to create a milieu that favors bacterial persistence.

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#### Novel Pro-Resolving receptor axis activates intracellular signaling and phagocytosis

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Sepsis is the leading cause of death in patients with infectious diseases, caused by overwhelming immune response to infection leading to widespread inflammation, organ failure and septic shock. It remains as unmet clinical challenge with high mortality rates and increasing incidence despite the advances in modern medicine such antibiotics and insensitive care. Human phagocytes (neutrophils, monocytes and macrophages) play critical role during acute inflammation by controlling host defense mechanisms, thru temporal regulation of classical proinflammatory eicosanoids and the production specialized proresolving mediators (SPMs). SPM are sub-nanomolar potent, stereoselective agonists that have both anti-inflammatory and pro-resolving properties stimulating efferocytosis and phagocytosis through g-protein coupled receptor pathways. Resolvin D2 (RvD2), a potent agonist that governs local phagocyte resolution responses via novel pro-resolving receptor DRV2 (GPR18) that promotes homeostasis and effective microbial clearance. DRV2 is expressed in human phagocytes neutrophils, monocytes and macrophages (Chiang, JI 2016). Here we present, the RvD2-DRV2 intracellular signaling pathways using state of art single cell mass cytometry (CyTOF) in macrophages from either wild-type (WT) or receptor deficient mice (DRV2-KO). High dimensional data analysis of (CD11b+F4/80+) macrophages reveals that RvD2 timedependently increased phosphorylation of signaling molecules pAKT, p-p38 MAPK, pCREB, pS6, pERK1/2, pSTAT1, pSTAT3, and pSTAT5, each with different kinetics. In macrophages from DRV2-KO mice, upregulation of these phospho-proteins by RvD2 was abolished. Monitored by real-time imaging, RvD2-DRV2 interaction significantly enhanced phagocytosis of live E. coli, an action dependent on PKA and STAT3 signaling cascade in macrophages. In polymicrobial sepsis initiated by cecal ligation and puncture (CLP), RvD2 (~2.7 nmol/mouse)

significantly increased survival (>50%) of wild-type (WT), reduced hypothermia and bacterial titers compared to vehicle-treated CLP mice that succumbed at 48h. Protection by RvD2 was abolished in DRV2-KO mice. Mass spectrometry-based lipid mediator metabololipidomics demonstrated that DRV2-KO infectious exudates gave higher pro-inflammatory leukotriene (LT) B4 and pro-coagulating thromboxane (TX) B2, as well as lower SPM, including RvD1 and RvD3, compared to WT. Results from these studies provide evidence for novel resolution circuits via receptor driven mechanisms (RvD2-DRV2) and intracellular signals for the control of systemic inflammation such as sepsis.

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## The impact of the microbiome on the immuno-modulation and immuno-suppression in severely burned patients

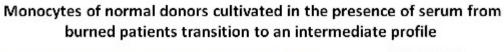
Zeid Kuzbari<sup>1,2,3</sup>, Paméla Thébault<sup>1,3</sup>, Nicolas Boily<sup>1,2,3</sup>, Cecile Grange<sup>1</sup>, Réjean Lapointe<sup>1,2,3</sup> and Jean-François Cailhier<sup>1,2,3</sup>

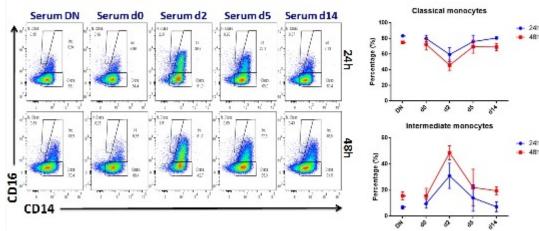
<sup>1</sup>CRCHUM; <sup>2</sup>Université de Montréal; <sup>3</sup>Institut du cancer de Montréal

- A) CONTEXT. Burns induce an intense activation of the immune system of patients resulting initially in a proinflammatory activation which can lead to the exhaustion of the immune system. This second anti-inflammatory phase generates an immunosuppressed state that renders patients susceptible to infections and sepsis. However, the exact sequence of leukocyte activation and potential interactions between the various immune cells are unknown.
- B) HYPOTHESIS AND OBJECTIVES. We hypothesize that burns lead to over activation of antigen presenting cells (APC) that generate an immunosuppressive state affecting multiple leukocyte subsets. Also, these immunosuppressive alterations of APCs are influenced by the microbiome. Furthermore, we propose that the microbiome may have an effect on the immune system of burned patients depending on the species of bacteria present in the blood or on the burned skin. Our goals are to evaluate the immunological activation of peripheral blood leukocytes, especially APCs, and in parallel detect and identify the bacterial strains in patients' blood and skin.
- C) METHODOLOGY. -We used flow cytometry to examine the phenotype of all immune cells of burned patients. We also perform *in vitro* manipulations with monocytes and B cells of normal donors cultivated in presence of burned patients' serum. In addition, the concentrations of inflammatory plasma proteins (cytokines, chemokines, immunoglobulins) in the patients was measured by ELISA assays. -In tandem, the composition of the microbiome in the blood and on the skin (burned and normal) of patients is evaluated via the expression of the gene coding for the RNA 16S of bacteria which is detected by qPCR. The bacterial strains will be identified by next generation sequencing (Illumina Miseq PE).
- D) RESULTS. We studied 25 burned patients. We found an increase in non-classical monocytes at early time points followed by an increase in regulatory T cells, a deregulation of B cell maturation, a decrease in circulating immunoglobulines and in soluble CD40L.

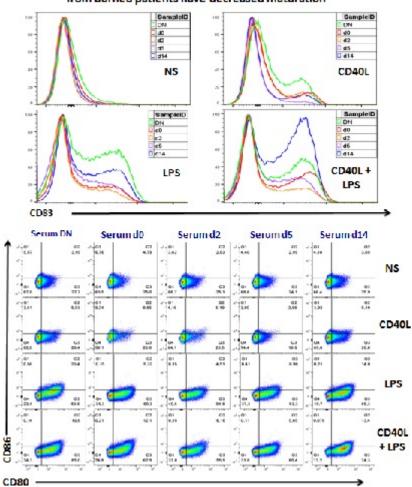
To decipher the complex interactions between leukocytes, leukocytes from normal donors were cultured in sera from burned patients. These experiments revealed that CD40L expression by T cells is unchanged, monocytes adopt an anti-inflammatory profile, dendritic cell maturation and activation are altered, and finally B cells proliferate less and this is partially corrected by the presence of monocytes/macrophages. Bacterial DNA from the blood and the skin of normal volunteers is detectable by qPCR.

E) IMPACT OF THE STUDY. This project will shed light on the fundamental interactions between the microbiota and the modified immunological parameters in burned patients. It will highlight immunological pathways that could be modulated by various immunotherapies to restore the inflammatory homeostasis of burned patients and reduce the infection burden in this population in need.

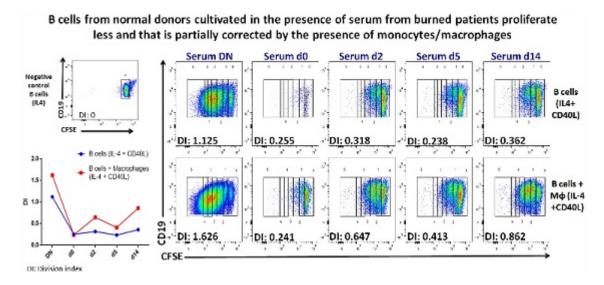




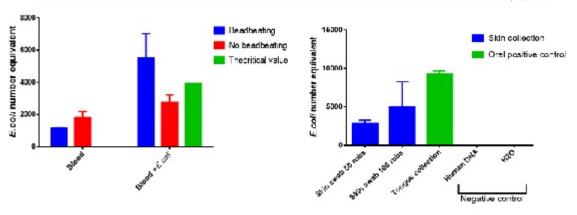
## Dendritic cells of normal donors cultivated in the presence of serum from burned patients have decreased maturation



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Bacterial DNA from the blood and the skin of normal volunteers is extracted and detected by  $\alpha PCR$ .



# Dyslipidemic Diet-Induced Monocyte "Priming" and Dysfunction in Non-Human Primates is Triggered by Rising Plasma Cholesterol and Accompanied by Altered Histone Acetylatiot

John S. Short<sup>1</sup>, Sina Tavakoli<sup>1</sup>, Huynh N. Nguyen<sup>1</sup>, Ana Carrera<sup>1</sup>, Chelbee Farnen<sup>1</sup>, Laura Cox<sup>2</sup> and Reto Asmis<sup>1</sup> University of Texas Health Science Center at San Antonio; <sup>2</sup>Texas Biomedical Research Institute

Monocytes and the recruitment of monocyte-derived macrophages into sites of inflammation play a key role in atherogenesis and other chronic inflammatory diseases linked to cardiometabolic syndrome and obesity. Previous studies from our group have shown that metabolic stress promotes monocyte priming, i.e. enhanced adhesion and accelerated chemotaxis of monocytes in response to chemokines, both *in vitro* and in dyslipidemic LDL-R<sup>-/-</sup> mice. We also showed that metabolic stress-induced monocyte dysfunction is, at least to a large extent caused by the *S*-glutathionylation, inactivation, and subsequent degradation of MKP-1. Here, we analyzed the effects of a Westernstyle, dyslipidemic diet, which was composed of high levels of saturated fat, cholesterol, and simple sugars, on monocyte (dys)function in non-human primates. We found that, similar to mice, a dyslipidemic diet enhances monocyte chemotaxis in non-human primates within four weeks, occurring concordantly with the onset of hypercholesterolemia but prior to changes in triglycerides, blood glucose, monocytosis or changes in monocyte subset composition. In addition, we identified transitory decreases in the acetylation of histone H3 at the lysine residues 18 and 23 in metabolically primed monocytes, and we found that monocyte priming was correlated with the acetylation of histone H3 at lysine 27 after an eight-week dyslipidemic diet regimen. Our data show that

metabolic stress promotes monocyte priming and hyper-chemotactic responses in non-human primates. The histone modifications accompanying monocyte priming in primates suggest a reprogramming of the epigenetic landscape, which may lead to dysregulated responses and functionalities in macrophages derived from primed monocytes that are recruited to sites of inflammation.

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## Systemic approach analysis of long noncoding RNAs' function in infection

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Long noncoding RNAs (lncRNAs) are important regulators of gene expression; however, their functions in inflammatory responses to infection are almost unknown. We used screening approach and identified lncRNA MEG3 as a tissue specific modulator of inflammatory responses during bacterial infection. We also identified a novel role for microRNA-138 in regulating inflammation by critical interaction with lncRNA MEG3. Importantly, we revealed that lncRNA MEG3-4 functions as a competing endogenous RNA to bind miR-138 and release the miRNA's target IL-1b mRNA. This intensified inflammatory responses in cells and in mice. Hence, we extend the decoy modulation mechanism for lncRNAs to anti-bacterial immunity, impacting phenotype and disease progression in a sepsis model following *Pseudomonas aeruginosa* infection. This newly characterized regulatory axis provides a balanced inflammatory response, thereby alleviating the progression of sepsis. Collectively, our work signifies a powerful regulator in pulmonary inflammatory responses through transcriptional regulation of immune response genes, which may help design effective control for infectious diseases.

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# Transition of NAD Salvage to De Novo Biosynthesis Sustains Memory of Endotoxin Tolerance During an Acute Inflammatory Response

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TLR4-dependent acute inflammatory response in monocytes sequentially switches from an initial immune effector phenotype to an immune repressor/adaptive TLR4 deactivated state, which varies in duration if and when resolution occurs. We previously reported that the Nampt-NAD-SIRT1-RelB axis epigenetically coordinates the effector to repressor phenotype, which may persist as profound immune and metabolic suppression in poor outcome and high mortality animal and human sepsis. The mechanism responsible for the sustained repressor phenotype of sepsis is unknown and important for designing molecular treatment targets. Using the THP-1 human monocyte cell model of the acute inflammatory response that simulates sepsis, we show that NAD+ de novo biosynthesis from tryptophan degradation sustains the tolerant repressor phenotype. Mechanistically, TLR4 first triggers the Nampt-dependent NAD+ salvage pathway to activate SIRT1 and drive early epigenetic silencing of pro-inflammatory and proimmune gene expression. However, Nampt-dependent NAD+ generation is soon replaced by increased expression and activation of indole dioxygenase (IDO), which controls tryptophan-dependent increases in quinolinic acid to 10 times its basal level; quinolinic acid is a rate-limiting precursor of de novo NAD+ synthesis. Newly synthesized NAD+ increases in the nucleus and sustains SIRT1 and RelB support of the endotoxin tolerant innate immune repressor phenotype. Inhibiting IDO activity by its specific inhibitor 1-methyl-tryptophan or gene specific CRISPR-Cas9 gRNA prevents accumulation of nuclear NAD+ and breaks innate immune memory of endotoxin tolerance by disassociating SIRT1 and RelB promoter binding. Re-stimulated endotoxin tolerant cells induce TNF-a gene expression in the absence of IDO activity. We conclude that persistent IDO-dependent tryptophan degradation to NAD+ may prolong the immune repressor state associated with severe acute inflammation and sepsis.

## Maternal pregravid obesity remodels the DNA methylation landscape of cord blood monocytes disrupting its inflammatory program

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Pre-pregnancyobesity is associated with adverse health outcomes for the offspring, including increased incidence of neonatal bacterial sepsis and necrotizing enterocolitis. We have recently reported that umbilical cord blood monocytes (UCBM) from babies born to obese mothers (obese group) generate a dampened response to Toll-like receptors 1, 2 and 4 ligands compared to those collected from lean mothers (lean group). These observations suggest altered in utero development of the offspring's immune system. We therefore investigated transcriptional differences within resting and LPS-activated UCBM stratified by pre-pregnancy body mass index. Despite the absence of any gene expression differences between resting cells, the obese group failed to transcriptionally respond to ex vivo LPS stimulation, both at coding and non-coding loci. In addition, UCBM from the obese group produced reduced levels of cytokines, chemokines, and growth factors following LPS stimulation.

We next examined the role of epigenetic mechanisms in mediating this potentially tolerant phenotype. Several studies have identified DNA cytosine methylation as a stable regulatory mechanism that affects immune cell development, fulfills the criterion of heritability, and establishes long term epigenetic memory. Using a targeted bisulfite approach, we show that resting UCBM from obese group present a starkly different methylome compared to the cells from the lean group, with significant differences in genes involved in immune and inflammatory response, adhesion, and defense response. Additionally, we report notable differences in methylation levels overlapping promoter regions of genes involved in myeloid cell differentiation. To quantify the relationship between these epigenetic differences and expression levels, we developed a novel systems biology based approach measuring sample-wise association between transcriptional and epigenetic readouts for every gene of the genome. Using this model, we show that pregravid obesity associated methylation profile of UCBM is highly predictive of LPS inducible gene expression levels. The most striking example of a robust association was detected in *PPARG*, a metabolically sensitive regulator of LPS signaling. To establish the functional consequences of these molecular differences, we are currently characterizing the impact of these changes on monocyte differentiation, and polarization. These data highlight potentially critical role of pregravid obesity on immune competence of the offspring at birth. These findings, along with additional epigenetic readouts such as chromatin accessibility and histone modifications would further our understanding of mechanisms that explain the increased risk of infection in neonates born to obese mothers.

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## BCL6 Inhibition Represses HIV Infection ex vivo by Suppression of Immune Activation: Implication for Viral Clearance in Secondary Lymphoid Tissue of HIV-infected patients undergoing cART treatment

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**Background**: CD4 T follicular helper (Tfh) cells in the germinal center (GC) are highly susceptible to HIV-1 infection *in vivo* and *ex vivo*. Viral persistence within CD4 Tfh cells remains a central barrier to HIV/AIDS cure efforts. B-cell lymphoma 6 (BCL6) is a master transcriptional factor for Tfh cells. BCL6 binds to interferon

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stimulated genes (ISG) and inhibits the expression of antiviral resistance factor (MX2 and IFITM3) in CD4 Tfh cells. BCL6 also promotes inflammatory sequelae in VSV-infected mice. Recently, a small inhibitor molecule specifically targeting N-terminal BTB domain of BCL6, FX1, was developed against lymphomas and effectively decreased the frequency of Tfh cells in the lymphoid tissue of mice*in vivo*. Therefore, we tested these hypothesis in this study: (1) inhibition of BCL6 via FX1 in activated CD4+T cells would enhance the expression of antiviral responses genes and reduce their susceptibility to HIV infection, (2) inhibition of BCL6 via FX1 in innate immune response cells would limit immune activation.

**Methods**: We used small inhibitor molecule (FX1) to block BCL6 activity in Phytohaemagglutinin (PHA)-activated CD4 T cells and human peripheral mononuclear cell (PBMC) *ex vivo*. HIV (NL4-3 and BaL) infection was performed by spinoculation with or without 50umol/ml FX1. HIV infection rate was determined by intracellular staining of HIV capsid protein (p24) and flow cytometry analysis at 4 days after infection or HIV p24 ELISA assay. We use 50ng/ml LPS to induce TLR4-mediated immune activation in human PBMC, and 1umol/ml GS9620 for TLR-7-mediated immune activation. The expression of inflammatory cytokines and genes were examined by intracellular cytokines staining (ICS) and Nanostring assay.

**Results**: We found *ex vivo* BCL6 inhibition via FX1 resulted in: (1) significantly reduced HIV infection and replication in PHA-activated peripheral CD4 T cells, (2) suppressed CD4 T cell activation as evidenced by down-regulating activation marker (HLA-DR) expression, (3) repressed LPS-mediated TNF-a, IL6 and IL1beta expression in monocyte and monocyte derived macrophage (MDM), (4) downregulated GS9620-mediated TNF-a expression in pDC. Furthermore, we observed lower IFN-a, IFN-b, and ISGs (MX1, MX2, IFIT1, IFIT2, IRF7) expression in activated human peripheral CD4 T cells and CD4 Tfh cells from human tonsil.

Conclusions: Our results suggest: (1) BCL6 is associated with CD4 T cell activation and innate immune activation, (2) BCL6 promotes the susceptibility of CD4 T cells to HIV infection, (3) BCL6 potentiates HIV replication and reactivation in CD4 helper T cells. Our data also indicates anti-Bcl6 can restrict the inflammatory response associated with persistent HIV, limit viral replication and lymphoid reseeding, and prevent viral reactivation in HIV-infected CD4 T helper cells during cART treatment. Together, these data highlight the potential application of BCL6 inhibition *in vivo* to reduce HIV persistence in cART-suppressed infected subjects.

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#### Easy isolation of particle-free human ILC2s from peripheral blood mononuclear cells

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<sup>1</sup>STEMCELL Technologies, Vancouver, BC, Canada; <sup>2</sup>Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada

Type 2 innate lymphoid cells (ILC2s) play a key role in the regulation of various immune processes. ILC2s are implicated in the development of allergic airway diseases, airway tissue repair, protection against helminth infection, and maintenance of metabolic homeostasis. A highly pure ILC2 population is needed for in-depth analysis of these cells. The isolation of ILC2s can be challenging due to their low frequency, as the percentage of lineage CRTH2+ CD127+ CD161+ ILC2 in peripheral blood mononuclear cells (PBMCs) is only around 0.05%. The most common method to isolate ILC2s is by fluorescence activated cell sorting, which can be time-consuming when working with rare cell types. To address this concern, we have developed a faster immunomagnetic selection method (EasySep<sup>TM</sup>) to obtain particle-free ILC2s from human PBMCs. Additionally, our column-free method makes it practical to process the large numbers of PBMCs required to obtain sufficient ILC2s for downstream assays because there is no inherent limit imposed by column capacity.

Here we describe a simple two-step protocol to isolate ILC2s. In the first step, CRTH2 (CD294) positive cells are labelled with CRTH2-PE monoclonal antibody. These cells are then positively selected using an anti-PE antibody complex and EasySep Releasable RapidSpheres<sup>TM</sup>. After separation, the magnetic particles are released from the

positively selected cells using our release buffer. In the second step, non-ILC2 cells (lineage-positive) within the CRTH2 positively selected population are immunomagnetically labeled with a cocktail of antibody complexes and magnetic particles. The unwanted cells are retained in the magnet, whereas the CRTH2 positive ILC2s are simply poured off and ready for subsequent analysis. The entire separation process takes 2.5 hours.

Using this method, the purity of lineage CRTH2<sup>+</sup> CD127<sup>+</sup> CD161<sup>+</sup> ILC2s isolated from PBMCs was 90.8 +/- 2.9 % (mean+/- SD, n=10), with recovery of 17.8 +/- 13.3% (mean+/- SD, n=10). EasySep<sup>TM</sup> isolated ILC2s are functional, as shown by their ability to produce IL-13 upon in vitro stimulation with IL-33 and IL-2. Overall, EasySep<sup>TM</sup> Human ILC2 Isolation Kit allows researchers to easily and efficiently isolate highly pure and functional ILC2s, which are ready for downstream applications.

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#### DISSECTING MECHANISMS OF BETA-GLUCAN TRAINED IMMUNITY

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Critically ill and burned patients suffer from immunoparalysis that prevents innate leukocytes from combating invading pathogens. Novel treatments that bolster immune responses to infections in these patients is required to decreases sepsis mortality. In vitro treatment with β-glucan rewires immune cell metabolism to boost effector function for several days. The efficacy and mechanism of β-glucan in vivois unknown. We hypothesized that prophylactic β-glucan will improve survival after P. aeruginosa infection in burned mice through its primary receptor, dectin-1. Wildtype, dectin-1 KO and Toll-like receptor 2 KO mice were treated either with 2 doses of 1mg β-glucan or saline intraperitoneally (IP) as a control, then injected IP with 10<sup>8</sup> CFU/mL of P. aeruginosa. Six hours after infection, the mice were assessed for core temperature and sacrificed. Subsequently, IP bacterial counts and macrophage, neutrophil and monocyte recruitment were quantified by culture and flow cytometry, respectively. Peripheral blood IL-6 and TNF-α were assessed in each group by BioPlex. Beta-glucan treated mice had a higher average temperature and decreased bacterial burden in the peritoneal cavity, regardless of receptor knockout status. In keeping with this, significantly more neutrophils and macrophages were recruited to the peritoneal cavity in βglucan-treated mice. No significant difference in monocyte recruitment was seen. Finally, β-glucan treated mice had lower peripheral blood IL-6 and TNF- $\alpha$  levels. Mice trained with  $\beta$ -glucan mounted a robust response to P. aeruginosa infection while naïve mice showed significant illness. Indeed, untreated mice became hypothermic, failed to recruit leukocytes and clear local infection and engaged a global cytokine response typical of sepsis. In vivo protection by β-glucan does not require either known receptor for the molecule, suggesting either compensation or a novel signaling mechanism.

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## The effect of anti-CTLA-4 on tumor-infiltrating effector T cells **O**

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Despite the relative success of immune checkpoint inhibitors, a majority of patients fail to respond to these therapies. Thus it is necessary to understand mechanisms of resistance in order to extend the benefits of immunotherapy to more patients. Checkpoint inhibitors are designed to prevent the interaction of immune checkpoints with their ligands, thereby allowing the body's anti-tumor immune response to be reactivated. Recently, it has been appreciated that anti-CTLA4 therapies, including Ipilimumab, have a second mode of action. They can mediate antibody-dependent cell-mediated cytotoxicity (ADCC). This leads to death of cells bound by anti-CTLA4. While CTLA4 is largely intracellular, it is expressed on the surface of tumor-infiltrating T regulatory cells (Tregs). Thus anti-CTLA4-mediated ADCC can deplete tumor-infiltrating Tregs. In mice, ADCC of tumor-

infiltrating Tregs is required for anti-CTLA4 efficacy. Likewise, Ipilimumab can mediate Fc-receptor dependent depletion of human Tregs. We have found that a portion of effector T cells infiltrating mouse and human tumors express surface CTLA-4 at levels similar to tumor-infiltrating Tregs. We will discuss the possibility that anti-CTLA4 can lead to Fc-receptor-dependent depletion of effector T cells and the implications for therapy resistance.

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#### Acute Activation of CD4+ Memory Tregs by Traumatic Injury

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CD4+ regulatory T cells (Tregs) are rapidly activated by traumatic injury suggesting that T cells may react to injury much like memory T cells. In this study, we used a mouse burn trauma model to screen for injury-induced memory T cell and Treg responses. T cells from sham or burn CD45.1 mice were transferred into CD45.2 mice and recipient mice were exposed to secondary burn or sham injuries. Among all T cell subsets examined, we found that Tregs were the only T cell subset to expand in response to secondary injury. Furthermore, the expanded Tregs expressed markers indicative of a memory T cell phenotype, CD44<sup>hi</sup>/CD62L<sup>low</sup>. To better characterize this Treg subset, we used CyTOF mass cytometry to profile their marker expression. CyTOF staining indicated that CD44, CTLA-4, ICOS, GITR, and Helios were highly expressed by the injury-reactive memory Tregs (mTregs). Next, we tested whether a similar population of mTregs might react acutely to primary injury. An identical subset of Tregs were activated by 6 hours after injury. We then investigated whether the MyD88 signaling pathway or antigen presentation by MHC class II were required for mTreg activation by injury. Normal activation of mTregs occurred in MyD88-/- mice, but not in MHC class II-/- mice or in mice treated with anti-MHC class II antibody. Collectively, we demonstrate that a subset of Tregs react acutely to injury in an antigen-specific fashion and that this subset of Tregs function like memory T cells by rapidly responding to antigens or factors associated with traumatic injuries.

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#### Germline deletion of CD47, "a don't eat me signal", promotes pro-inflammatory adaptive immunity and atherosclerosis.

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CD47 is a transmembrane protein with multiple physiological functions including regulation of efferocytosis, leukocyte homeostasis and apoptosis, and leukocyte integrin-dependent trafficking, CD47 interacts "in cis" (the same cell plasma membrane) with LFA-1 and VLA-4 integrins in T lymphocytes, and "in trans" with Signal Regulatory Protein (SIRP)alphaand SIRPgamma. A recent study has reported antibody blockade (MIAP410) of CD47 as a therapeutic to limit cardiovascular disease. Another study suggested CD47 deficiency protects mice from diet-induced obesity and improves whole body glucose tolerance and insulin sensitivity. Here we investigated the effect of Cd47-deficiency on atherosclerosis using a model of adeno-associated virus (AAV)-induced PCSK-9 mutant hypercholesterolemia. Surprisingly, we observed increased plaque formation in Cd47<sup>-/-</sup> mice with decreased numbers of lesional T lymphocytes. Investigating the immune phenotype, Cd47<sup>-/-</sup> mice exhibited signs of CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation along with increased dendritic cell activation. However, depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells did not rescue this phenotype. Instead we observed that Cd47 deficiency resulted in NK cell activation associated with increased expression of CD25, and IFN-gamma production, suggesting that this cell population may be driving the increased atherosclerosis observed in the Cd47<sup>-/-</sup> mice. Studies were supported by National Institutes of Health grants (HL125780 and HL121363).

Characterization of a novel subset of tissue-resident NKp46pos Vd1 intestinal intraepithelial lymphocytes playing a key role in gut immune homeostasis and in the physiopathology of colon-cancer.

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gd T cells can display a broad array of anti-tumor functions by combining their rapid innate-like cytotoxic response with secretion of immune-regulatory cytokines, such as IFNg. Intestinal intraepithelial lymphocytes (IELs) are particularly enriched with tissue-resident T gd cells. The present study identify and charaterize for the first time a large subset of human colon-resident T gd IELs expressing the natural cytotoxic receptors (NCRs) NKp46. These NKp46<sup>pos</sup> Vd1 IELs are gut-specific as we could not find any similar population in several other human compartment such as skin, liver, uterus, cervix or lymph nodes. As expected as tissue-resident lymphocytes, NKp46<sup>pos</sup> Vd1 IELs mostly carry the Vd1 TCR and are striking different from their NKp46<sup>neg</sup> circulating that are predominately Vd2 restricted in their TCR. Even though tissue-resident murine T gd cells share several features with human Vd1 cells, we did not find any subset of NKp46<sup>pos</sup> T gd subset in different anatomical gut sites of BALB/c and C57BL/6 mouse strains. The NKp46<sup>pos</sup> phenotype reflects a functionally higher anti-tumor potential in vitro compared to their NKp46<sup>neg</sup> counterpart. Indeed, NKp46<sup>pos</sup> Vd1 IELs are GranzymeB<sup>pos</sup> and strongly degranulate and produce IFN-g when co-coltured with K562 leukemia cell line. These important effector-functions are also relevant in vivo as we found that higher frequencies of tumor-infiltrating NKp46<sup>pos</sup> Vd1 IELs in the coloncancer specimens from patients undergone surgical gut resection significantly correlates with lower tumor progression and better prognosis. Taken together, our characterization of human gut-specific NKp46<sup>pos</sup> Vd1 T gd IEL pave the ground to better understand intestinal immune-homeostasis and immune-surveillance against coloncancer tumor in order possibly develop novel clinical/prognostic markers and to dissect still unknown important pathogenic aspects of this disease.

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## The decisive role of TNF-TNFR2 pathway in the activation of CD4+Foxp3+ regulatory T cells: implications in tumor immunotherapy

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CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) play an indispensable role in the maintenance of immune homeostasis and prevention of autoimmune diseases, and represent a major cellular mechanism of tumor immune evasion. Targeting of Tregs has great potential in the treatment of some major human diseases, including autoimmunity, transplant rejection, GvHD, and cancer. Understanding the biological pathways crucial for the regulation of Treg activity is a prerequisite for harnessing the immense therapeutic potential of Tregs.

Previously, we reported that pertussis toxin, which has immunologic adjuvant activity, reduced the number of Tregs in WT mice. Paradoxically, the number of Tregs in IL-6 KO mice was markedly increased by the treatment of pertussis toxin. In an effort to identify the factor responsible for the expansion of Tregs in mice deficient in IL-6, we unexpectedly found that TNF preferentially activates Tregs, resulting in the expansive proliferation, phenotypic stability, and enhanced suppressive capacity of these immune suppressors. This effect of TNF was mediated by

TNFR2, which is preferentially expressed at higher levels by human and mouse Tregs. Furthermore, expression of TNFR2 is able to identify the most functionally suppressive subset of Tregs. In mice deficient in TNFR2 ligands, or TNFR2, or IKK $\alpha$  which is a major component of the TNFR2 signaling pathway, the number of Tregs was reduced markedly in the thymus and peripheral lymphoid organs. Tregs derived from TNFR2 KO mice or IKK $\alpha$  KO mice failed to inhibit inflammation in vivo, due to their impaired capacity to proliferate and inability to consistently express Foxp3. Although counter-intuitive, and contrary to some previous reports, our discovery of the suppressive role of TNF-TNFR2 interaction has been supported by other investigators. Treg-promoting effect of TNF-TNFR2 interactions presumably act as a feedback suppressive response subsequent to proinflammatory effects of TNF.

TNF can be induced by various immunotherapies, including dendritic cell (DC)-based interventions, tumor vaccines and toll-like receptor (TLR) agonists. Such immunotherapy-induced TNF can additionally up-regulate TNFR2 expression on Tregs, resulting in the expansion and activation of tumor-associated Tregs through TNFR2. For example, CpG oligodeoxynucleotides (ODN), the TLR9 agonists, by activating DCs, have the capacity to induce anti-tumor immune responses in mouse models. Nevertheless, CpG ODN can also induce human or mouse Tregs with potent immunosuppressive function, presumably mediated by the elevated levels of TNF, which in turn dampen immune responses against tumor. Therefore, blockade of TNFR2 may enhance the anti-tumor effect of an immunotherapeutic such as CpG ODN by the elimination of Treg activity.

In this study, we tested this hypothesis and found that the combination of an antagonistic anti-TNFR2 Ab and CpG ODN more potently inhibited mouse CT26 colon cancer development and induced greater tumor regression, resulting in a long-term tumor-free survival of up to 80% of mice. Furthermore, tumor antigen-specific immunity developed in the surviving mice, since they completely and selectively resisted a re-challenge by CT26 tumor cells. This effect was associated with a decrease in the proportion of Tregs in tumor-infiltrating leukocytes, down-regulation of TNFR2 expression by Tregs, and with the up-regulation of IFN $\gamma$  expression by tumor-infiltrating CD8 $^+$  T cells. Our data thus clearly indicate that the combination of TNFR2 antagonism and immunotherapy provides a promising cancer treatment, and that TNFR2 acts as a target of checkpoint inhibitors.

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### Mitochondrial size/function controls level of viral immunity in young children

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Infants and toddlers exhibit higher frequency of respiratory viral infections than older children/adults that also often lead to enhanced morbidity in this age group. Due to limitations placed on immunity by immunological maturation, the T-cell responses to these pathogens are unique from neonates or older children/adults and they frequently have lower memory T-cell numbers after vaccination or infection. Here, we sought to understand the metabolic associated mechanisms that limit the full antiviral T-cell response in this age group using influenza as a prototypical respiratory viral pathogen of young children using a novel young mouse model. We found that despite highly proliferative T-cell responses, young mice had a higher frequency of apoptosis in the lungs as compared to older "adult" mice that directly correlated with differences in mitochondrial mass and function. Specifically, using Mass-Spec tissue imaging for histologic localization of metabolites and paper spray Mass-Spec, differences in key metabolites that facilitate T-cell function and/or proliferation were evident between uninfected and infected mice and also between young and adult infected mice. After viral resolution, these mediators correlated with lower T-cell memory absolute numbers. *In vivo and in vitro* manipulation of mitochondrial function through drug mediators allowed for rescued T-cell function in our infected infants and for higher numbers of immunological T-cell memory to persist in the mucosal and draining lymph nodes after virus was cleared from the lungs. Thus, mitochondrial

function appears strongly associated with how immunological maturation limits the full antiviral T-cell response in young children during a primary, and possibly a secondary encounter, with influenza. Furthermore, adjuvants that act through the mitochondria may be key to bolstering immunological memory in young children after vaccination or infection.

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#### Tissue-Resident Myeloid-Derived Suppressor Cells in Adipose from Lean Mice

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Under steady-state conditions, adipose tissue (AT) is highly metabolic, using fatty acid (FFA) oxidation reactions to maintain an appropriate energy balance within the body. Even at relatively low concentrations, reactive oxygen species (ROS) produced by these reactions can damage tissue. If left unchecked, this can develop into the chronic AT inflammation characteristic of obesity. In healthy AT, a network of resident anti-inflammatory immune cells modulates this ever-present FFA oxidation and ROS-induced inflammation in order to maintain immune homeostasis within the tissue. During obesity however, inflammatory cells infiltrate the adipose, disrupting this homeostatic balance. This abject phenotypic switch of AT-resident immune cells has brought to light the importance of understanding how these cells work to maintain the initial, immunosuppressive microenvironment of healthy tissue and what signals lead to its eventual degradation as obesity develops. Characterization of the stromal and vascular cellular fraction (SVF) of AT from lean mice, using flow cytometry, identified a functionally important population of CD11b+ Gr1+ stromal cells residing in the adipose. This surface marker combination, along with the relatively non-inflamed nature of adipose from lean individuals, has led us to hypothesize that these cells are myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogeneous collection of immunosuppressive cells typically associated with cancer or chronic inflammatory diseases. We used flow cytometry to analyze the surface marker expression profiles of these CD11b+ Gr1+ resident myeloid cells in the SVF. We found that approximately 2-3% of the adipose stromal cells expressed the classic CD11bhiLy6ChiLy6GSSClowMDSC surface marker phenotype. In CFSE- and MTT-based assays of splenocyte proliferation, this putative MDSC population attenuated activated T cell proliferation. This suppressive activity was blocked by the arginase inhibitor nor-NOHA. Additionally, these cells produced the immunosuppressive cytokine IL-10. Altogether our findings suggest that there is a population of MDSCs resident in healthy (i.e. non-inflamed) AT, which could contribute to the antiinflammatory adipose microenvironment, by preventing aberrant ROS-induced inflammation, either directly through their own immunosuppressive functions, or indirectly by promoting the suppressive phenotypes of other AT resident cells such as macrophages and T regulatory cells. These endogenous adipose MDSCs could also represent a new therapeutic target for diabetes and obesity, as studies have already demonstrated that injections of culture-derived MDSCs into obese mice increases insulin sensitivity and delays the development of metabolic syndromes.

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#### Oxidation Modulates NAD+-sensor Sirtuin 2 during Obesity with Sepsis

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<u>Introduction</u>: Obesity increases morbidity and mortality in sepsis patients. Sepsis transitions from early/hyper-inflammatory to a late/hypo-inflammatory and immunosuppressive phase. Sustained expression of NAD+ and metabolic sensor-family of proteins, namely sirtuins, modulate this shift. Obese mice show an exaggerated hyper-and prolonged hypo-inflammatory phases of sepsis compared to lean mice. We have recently shown that sirtuin 2 (SIRT2) plays a critical role in modulating the shift from exaggerated hyper- to a prolonged hypo-inflammatory phase during obesity with sepsis. We also showed that SIRT2 deficiency during early sepsis is responsible for

exaggerated hyper-inflammatory response in obese with sepsis via increased acetylation and activation of NFκB p65 in immune cells. Evidence suggests that obesity with sepsis is associated with increased oxidative stress. In this project, we further elucidated the mechanism of how oxidative stress of obesity with sepsis affects direct SIRT2 oxidation and function and modulate inflammation.

Methods: To study changes in oxidized SIRT2 expression in obese-sepsis mice, we isolated splenocytes from diet induced obesity (DIO) and genetically obese (leptin deficient B6.Cg-Lepob/J: ob/ob) mice with sepsis and studied oxidized SIRT2 vs. total SIRT2 expression as sepsis progresses from hyper- and hypo-inflammatory phase. To further elucidate the mechanism of oxidation, we studied the effect of cysteine modification on redox-sensitive cysteines (cysteines from zinc –finger motif) on SIRT2, cys221 and cys224, to serine (C221S and C224S respectively). We transfected HEK 293 cells with wild type (WT) and mutant SIRT2 (C221S and C224S) to study: 1) SIRT2 enzymatic activity, 2) deacetylation function, specifically NFκB p65 deacetylation by WT SIRT2 vs. SIRT2 mutants (C221S and C224S) and finally, 3) anti-inflammatory activity of WT and SIRT2-mutants, using RAW 264.7 cells stimulated with LPS and studied TNF and IL-1β mRNA expression during hyper-inflammatory phase.

Results: We observed that in both the models of obesity, namely ob/ob and DIO mice, the oxidized SIRT2 expression increased during hyper-inflammation and decreased during the hypo-inflammatory phases. In contrast, the total SIRT2 expression increased *only* during the late/hypo-inflammatory phase of sepsis in obese mice. Mechanistically, we showed that the site-directed mutations of both the redox-sensitive cysteines studied (cys221 and cys224) modulated SIRT2 function. Specifically, we observed that the SIRT2 mutants (C221S and C224S) showed: 1) significantly decreased enzymatic activity vs. WT SIRT2, 2) significantly decreased NFκB p65 deacetylation function (increased acetylated NFκB p65 expression) vs. WT SIRT2 and 3) significantly increased pro-inflammatory cytokine TNF-α and IL-1β mRNA expression in response to LPS vs. WT SIRT2

<u>Conclusion</u>: These data suggest that during hyper-inflammatory phase of obese-sepsis with increased oxidative stress, direct oxidation of SIRT2 protein contributes towards exaggeration of hyper-inflammatory response via decreased SIRT2 enzymatic activity and decreased NFκB deacetylation function (increased acetylation of NFκB). Mechanistically, redox sensitive cysteines cys221 and cys224 are critical to SIRT2 activity and function. Thus, the oxidative stress regulates SIRT2 function during exaggerated hyper-inflammatory phase of obesity with sepsis.

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#### Modification of macrophage metabolism and memory by TLR4 agonists

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Treatment with the toll-like receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) augments resistance to common nosocomial pathogens such as *P. aeruginosa*, *S. aureus* and *C. albicans*. The antimicrobial phenotype persists for at least 15 days after MPLA treatment and is independent of T and B lymphocytes since MPLA-induced antimicrobial immunity is equally induced in Rag2KO and wild type mice. The MPLA-induced antimicrobial phenotype is dependent, in part, on modification of macrophage functions since depletion of macrophages with clodronate liposomes partially ablates the antimicrobial phenotype. However, it is unclear how MPLA changes macrophage physiology to achieve the protective effect. To address this, mouse bone marrow derived macrophages (BMDMs) were primed with MPLA for 24 hours, washed and rested for 3 days. Glycolysis (extracellular acidification rate, ECAR) and oxidative metabolism (oxygen consumption rate, OCR) were assessed using glycolytic stress and mitochondrial stress tests, respectively, on a Seahorse XFe96 metabolic analyzer. Cytokine secretion, phagocytosis and respiratory burst were measured as functional endpoints. During the priming period, MPLA inducedcytokine secretion and increased macrophage glycolytic rate but decreased oxygen consumption. However, 3 days following the removal of MPLA, BMDMs became refractory to LPS-induced pro-inflammatory

cytokine (TNF $\alpha$ , IL-6) secretion yet sustained elevated glycolysis and showed recovery of oxygen consumption. 1,2-13C glucose metabolic flux analysis confirmed that the TCA cycle corrected by 3 days after MPLA treatment since glucose-derived carbon was shuttled into the mitochondria promoting succinate generation, increased oxygen consumption and elevated mitochondria-derived ATP production. Elevations in TCA cycle function were confirmed by increased expression of succinate dehydrogenase and citrate synthase. Functionally, MPLA-primed macrophages displayed elevated phagocytosis and respiratory burst functions. RNAseq analysis showed increased expression of phagocytosis-associated receptors such as FcRy, Marco and complement receptors. Treatment with MPLA induced activation of mTOR signaling as indicated by increased phosphorylation of Akt and p70S6kinase. Blockade of mTORC1 signaling with rapamycin ablated the MPLA-induced increase in glycolysis and phagocytosis. Blockade of mTORc1 in vivo ablated MPLA-induced protection for systemic S. aureus infection as indicated by loss of the survival benefit. Priming of macrophages with MPLA also induced accumulation of the mTOR-regulated transcription factor HIF-1α, which appears to be functionally important since MPLA-primed HIF-1α KO macrophages were unable to fully induce MPLA-augmented glucose consumption and phagocytosis. Thus, MPLA initially induces aerobic glycolysis (Warburg effect) in macrophages but this phenotype then switches to one characterized by increased glycolysis and a functioning TCA cycle that facilitates key antimicrobial functions. The memory phenotype is induced through activation of mTOR signaling and is dependent, in part, on stabilization of HIF-1α. Interestingly, the antimicrobial phenotype parallels a classic LPS tolerant phenotype indicating that decreased LPS-induced cytokine production after MPLA priming does not predict impaired antimicrobial immunity. In fact, it is just the opposite.

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#### Regulation of inflammation by Notch signaling

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The Notch signaling pathway is conserved from *Drosophila* to mammals and is critically involved in developmental processes. In the immune system, it has been established that Notch signaling regulates multiple steps of T and B cell development in both central and peripheral lymphoid organs. Relative to the well documented role of Notch signaling in lymphocyte development, less is known about its role in regulating myeloid lineage development and function, especially in the context of inflammation and autoimmune disorders. Interestingly, the gene encoding the master transcription regulator of the Notch pathway, *Rbpj*, is among the recently identified new rheumatoid arthritis risk loci yet the functional significance of Notch-RBP-J signaling in pathogenesis of rheumatoid arthritis is unclear. During the past several years, we and others have described a key regulatory role of the Notch pathway in innate immune and inflammatory responses. Notch1-RBP-J axis promotes inflammatory macrophage polarization while a Notch target gene Hes1 dampens inflammation by attenuating macrophage chemokine production. Such regulatory patterns impose positive and negative checkpoints on inflammatory responses and may have potential implications for pathogenesis and therapy of autoimmune and inflammatory disorders such as rheumatoid arthritis.

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#### Ocular Immune Privilege Selects for Programmed Innate Memory-Tolerance

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The ocular immune privileged microenvironment programs tolerance in macrophages and microglial cells. Soluble molecules found in the conditioned media of healthy retinal pigment epithelial cell (RPE) eyecups program activated macrophages to be anti-inflammatory, and promote immune tolerance. One of the molecules produced by RPE is the neuropeptide alpha-Melanocyte Stimulating Hormone ( $\alpha$ -MSH). Macrophages stimulated with endotoxin and treated with  $\alpha$ -MSH suppress inflammation. As APC, the  $\alpha$ -MSH treated macrophages promote counter-conversion of antigen-specific effector T cells into regulatory T cells. Along with another RPE neuropeptide, Neuropeptide Y,  $\alpha$ -MSH suppresses the phagocytic pathway possibly altering antigen processing, and

preventing inflammation mediated by phagocytosis. The  $\alpha$ -MSH treated macrophages carry out anti-inflammatory and tolerogenic activity long after treatment, or exposure to the ocular microenvironment, and in tissues outside the eye. This suggests that ocular immune privilege induces innate memory to suppress inflammation within the eye, and to systemically maintain tolerance to ocular antigens.

When the RPE conditioned media is depleted of α-MSH, the conditioned media induces apoptosis in the macrophages. Since soluble FasL and TRAIL suppress induction of apoptosis, we examined for the possibility that membrane FasL and TRAIL are delivered by RPE derived exosomes. From the RPE conditioned media the extracellular vesicles were isolated using dehydration buffer and centrifugation. The isolated vesicles were washed, resuspended in PBS, and added to cultures of macrophages. Caspase 3 activity was assayed at 1, 3 and 18 hours later. In addition, exosomes were collected from the conditioned media of RPE eyecups of mice with eyes suffering from autoimmune uveitis. Also, exosomes were collected from in vitro cultures of ARPE-19 cells, a RPE cell line, grown to a confluent monolayer, or cultures where the monolayer was bisected to stimulate a wound response. Only the exosomes from the RPE eyecups, of healthy and uveitis eyes, activated Caspase-3 activity. Immunoblotting for FasL and TRAIL revealed that the exosomes from the RPE eyecups, and not the ARPE-19 cell line have membrane FasL and TRAIL. This corresponded to the exosome activation of Caspase-3 in macrophages. The exosomes from the RPE eyecups were further assayed for microRNA that can influence apoptotic signaling, and pro-inflammatory activity. The exosomes from RPE eyecups contain the pro-apoptotic miR204, which is also a constitutively expressed miRNA of differentiated RPE cells. In contrast, the anti-apoptotic, pro-inflammatory miR155 was found only in the exosomes from uveitic RPE eyecups, but as a pre-microRNA.

These results with our previous findings suggest that as the RPE program tolerance in macrophages through  $\alpha$ -MSH, the exosomes select for macrophages that respond to  $\alpha$ -MSH. These findings may be part of the reason for the observed uniformity in microglial cell characteristics, and functionality in the healthy retina. Having an apoptotic signal delivered by RPE of uveitic eyes may contribute to the mechanisms that mediate self-resolution of autoimmune uveitis in mice. Also, this is associated with the induction of tolerance to retinal antigens mediated by an  $\alpha$ -MSH induced tolerogenic APC found in the spleen of recovering mice. This means that within the healthy immune privileged eye there is in the retina not only programming of memory-tolerance in the macrophages, there is a mechanism to select the macrophages that have been programed.

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## The Role of MALT1 in Macrophages S

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At BC Children's Hospital, a patient with severe combined immunodeficiency accompanied by dramatic inflammation along the gastrointestinal tract was diagnosed with a homozygous mutation in the intracellular signalling molecule, MALT1 MALT1 plays two distinct roles within cells:

- 1) it acts as a scaffolding protein required for Nuclear Factor kappa B (NFkB) activation; and
- 2) it has protease activity, which can enhance or inhibit NFkB-driven transcription, depending upon its target.

The consequences of MALT1 immunodeficiency have largely been attributed to its role in T and B cells, in which MALT1 acts downstream of the T or B cell receptor to drive lymphocyte proliferation and activation. MALT1 is also activated in macrophages downstream of the C-type lectin receptors, dectin-1 and dectin-2, and toll-like receptor 4 (TLR4). However, the role of MALT1 in macrophage-mediated inflammation has not been explored.

Intestinal inflammation is largely driven by macrophages, suggesting that macrophage Malt1 may contribute to this inflammation. Based on this, we hypothesized that Malt1 deficiency in macrophages causes intestinal inflammation by increasing inflammatory cytokine production. In fact, we found that Malt1 deficient murine macrophages actually had lower inflammatory cytokine production than wild type macrophages. In contrast, pharmacological inhibition of Malt1 protease activity increased pro-inflammatory cytokine production in response to innate immune stimuli. *In vivo*, Malt1 deficiency (Malt1<sup>-/-</sup>) exacerbated DSS-induced colitis in mice. Consistent with that, depletion of macrophages from Malt1<sup>-/-</sup> mice protected them from intestinal inflammation during DSS-induced colitis. However, adoptive transfer of wild type macrophages did not confer protection against DSS-induced colitis.

Taken together, our studies are consistent with a model in which MALT1 activity reduces pro-inflammatory macrophage responses, but its scaffolding function increases macrophage inflammatory responses. In future studies, we will investigate the cell-specific contribution of MALT1 deficient macrophages to inflammatory disease using mice with myeloid-specific MALT1 deficiency. These studies will provide critical information about the cell specific role of MALT1 and possible side effects of MALT1 inhibitors currently used for lymphoma treatment.

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## PEGYLATED IFN-ALPHA-2B DECREASES LATENT HIV MEASURES IN ART-SUPPRESSED SUBJECTS

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Pegylated interferon (Peg-IFN)- $\alpha$ 2a resulted in viral suppression and reduction in integrated proviral HIV DNA in 9 of 20 antiretroviral therapy (ART)-suppressed subjects undergoing analytical ART interruption (ATI; NCT00594880). To confirm the first pilot study, here we evaluated if Peg-IFN- $\alpha$ 2b, in the presence of HIV reactivation (ATI), would be safe, maintain viral suppression during ATI and decrease latent viral reservoir in chronic HIV infection. 20 individuals with well controlled HIV infection (on ART, VL <50 copies/ml) received weekly 1  $\mu$ g/kg Peg-IFN- $\alpha$ 2b sc for 20 weeks, with a 4 week ATI (weeks 5-9 of IFN treatment). In addition to safety monitoring, several HIV measures (integrated HIV DNA, TILDA, rectal tissue measures etc.) were assessed at baseline and week 20. Final statistical analysis: we used Wilcoxon Signed rank test to test differences between time points; exact Fisher tests to compare frequency of viral suppression during ATI; Spearman tests, mixed effect models and hierarchical clustering to test relationships between HIV reservoir measurements.

At completion study participants were 20% females, 70% AA. Median age was 47. 18 subjects completed treatment (2 early terminations) with 7 serious events (neutropenia). Peg-IFN- $\alpha$ 2b suppressed plasma HIV RNA during the 4 week ATI in 52% (95% CI= 32-73%), similar to NCT00594880 and higher than historical controls (13%; 95% CI= 3-36%, p= 0.0127; NCT00051818). At week 20, we observed a significant reduction in HIV RNA-expressing GALT cells (p= 0.012) and a reduction in integrated HIV DNA in circulating CD4s (p= 0.0797). As a group response other markers did not change significantly, yet a higher baseline level of rectal mucosa RNA, integrated DNA, TILDA, p24 and 2LTR were associated with a greater decrease after the intervention (p<0.05) to suggest higher baseline levels may best reflect changes. Reservoir measurements were weakly correlated at baseline and their changes over time did not correlate to one another. Amount of HIV rebound during the 4-week ATI was not associated with a change in reservoir measures.

Treatment with Peg-IFN-α2b (20 weeks, 4-week ATI) 1) is safe and well tolerated, 2) maintains viral suppression during a 4-week ATI in half of the subjects and 3) is associated with significant decrease of rectal mucosa HIV RNA and a decrease trend in integrated HIV DNA (PBMC). As a third study, ongoing randomized study incorporating an ART-only arm, repeated sampling and multiple latent reservoir assessments under NCT02227277 will allow to conclusively interpret the pilot study reduction in HIV reservoir measures observed in subjects with

high baselines . The latter would confirm both pilot study observations indicating that Peg-IFN- $\alpha$ 2 may be an HIV cure-directed strategy to reduce HIV beyond ART.

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#### Regulation of macrophage function by the adhesive microenvironment

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Macrophages are essential regulators of wound healing, and are involved in both advancing inflammation and promoting tissue repair processes. Initially, macrophages adopt a pro-inflammatory phenotype and secrete cytokines and reactive species to combat infection. At later stages, these same cells polarize towards a pro-healing phenotype and mediate processes that facilitate tissue repair. Despite a wealth of information known about how soluble factors including cytokines and chemokines influence immune cell function, little is known about how physical and adhesive cues regulate their behavior. The goal of our work is to understand how adhesive properties of the cellular microenvironment regulate the function of macrophages. Using cell micropatterning and surface topography, we have previously found that geometry of adhesion, or cell shape, plays a critical role in regulating their polarization towards pro-inflammatory versus pro-healing states. Cells that are forced to elongate adopt a geometry similar to cells treated with pro-healing cytokines, and more importantly begin to express markers representative of prohealing cells without the addition of exogenous cytokines. More recently, we have examined how the composition and architecture of the provisional extracellular matrix influences macrophage adhesion and function. We discovered that fibrin matrices are protective and inhibit macrophage inflammatory activation, whereas its soluble precursor fibringen potentiates inflammation. Current work is focused on understanding how adhesion and Tolllike receptors, as well as epigenetics regulate these responses, and leveraging these findings to design new materials to encourage macrophage-mediated wound healing.

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#### Immunological Explorations of Ex Vivo Lung Perfusion and the Pall LeukoGuard® Filter

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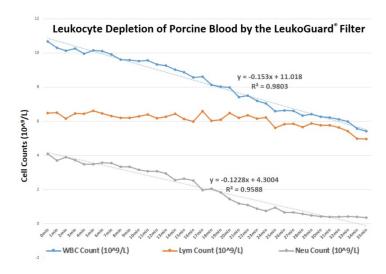
**Introduction:** Marginal donor lungs are clinically evaluated while attached to an ex vivo lung perfusion circuit (EVLP) that incorporates the Pall leukocyte reduction filter (LeukoGuard®). The filter was originally designed for use with cardiopulmonary bypass, and the filter's function during EVLP is very poorly understood. The purpose of this research is to investigate and quantitate leukocytes during EVLP and to determine what the LeukoGuard® filter may or may not be doing during EVLP.

Methods: Lungs were harvested from male Yorkshire pigs (n=8) following 6 h of brain death and were flushed with Perfadex. The lungs were stored for 16 h on ice before being connected to EVLP, and were run on a LeukoGuard® equipped EVLP circuit for up to 12 h, or until the lungs were edematous and nonfunctional. Porcine complete blood counts (CBCs) were obtained hourly from the acellular Steen solution that is used to perfuse the lungs during EVLP. An experimental drug was added to the perfusate that is not used during clinical EVLP. To directly determine how the LeukoGuard® equipped EVLP circuit affects circulating leukocyte counts, we collected blood from a pig immediately before lung harvest. We took baseline CBC readings, and then loaded the blood into the LeukoGuard® equipped EVLP circuit. Blood was pumped through the circuit at 3 L/min, and a CBC was taken every minute for 35 min. We wondered if the leukocyte percentages in the peripheral blood at the time of harvest were the same as that found in the perfusate during EVLP, so we took a peripheral CBC prior to harvesting lungs, and then following the first 10 min of EVLP without a LeukoGuard® filter, we subsequently collected another CBC. At 11 h of EVLP we wanted to determine if leukocytes were still being released from lungs, and also if the LeukoGuard® filter was still removing leukocytes from the perfusate, so we took a CBC of the perfusate at 11 h,

removed the lungs from the circuit, and then ran the lungless LeukoGuard® equipped circuit for an additional 15 min before taking another CBC.

Results: The leukocyte and lymphocyte count in the perfusate of the eight averaged EVLP experiments decreased from 2 h to 12 h, but the neutrophil count initially decreased until hour 3-4, and then increased until hour 7-8, before decreasing for the remainder of the EVLP. There was no further improvement in lung gas exchange after EVLP hour 4, and many lungs began to show signs of deterioration and increasing interstitial edema shortly after 4 h of EVLP. When porcine blood was added to the EVLP circuit, neutrophils were significantly removed by the Pall LeukoGuard<sup>®</sup> filter in a remarkably linear fashion. Lymphocytes were also removed, but to a lesser extent than neutrophils. When we took a CBC measurement of the perfusate 10 min after initiating EVLP without a LeukoGuard<sup>®</sup> filter, we found that the percentages of neutrophils and lymphocytes were much different than that found in the peripheral blood of the donor pig immediately before lung harvest. Following 11 h of EVLP, 15 min of lungless EVLP circulation caused the circulating neutrophil count to drop 4.86 fold and the lymphocyte count to drop 2.75 fold, as compared to the 11 h time point.

Conclusions: We have demonstrated that lungs release leukocytes into the perfusate throughout the duration of EVLP, and we have modeled leukocyte depletion by the Pall LeukoGuard® filter, which provides insight into its function during EVLP and cardiopulmonary bypass. We also found that the leukocytes circulating during EVLP are from the lungs not from residual donor blood. Removing donor immune cells with the LeukoGuard® equipped EVLP circuit prior to transplantation could decrease direct allorecognition (Stone et. al. Am. J. Transplant, 2016) and graft rejection, and a new filter could be designed specifically to remove leukocytes during EVLP. Four hours of EVLP prior to transplantation may be optimal to remove donor leukocytes without damaging donor lungs.



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# TLR signaling in macrophages is regulated by extracellular substrate stiffness and Rho-associated coiled coil kinase (ROCK1/2)

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During inflammation and repair, tissues undergo extensive changes in the composition and structure of extracellular matrix. These matrix changes increase tissue stiffness, and increased stiffness is associated with a number of pathologies including fibrosis and cancer. Furthermore, increased matrix stiffness directly promotes processes like cancer through direct regulation of non-immune cell function. This process is called mechanotransduction. Little is known about how changes in stiffness affect immune cell function, especially macrophages, which reside within

tissues and play fundamental roles in host defense, inflammation and repair. A key function of macrophages is as first responders detecting microbial infection through Toll-like receptor (TLR) recognition of microbial-associated molecular patterns. To investigate whether changes in stiffness directly regulate macrophage functions, we cultured murine primary bone marrow-derived macrophages (BMMs) and RAW264.7 cells on fibronectin-coated polyacrylamide (PA) gels of defined stiffnesses (1kilopascal [kPA], 20kPa, 150kPa) that approximate physiologic tissue stiffnesses and disease states. This system permits precise control of stiffness and delivery of consistent biological ECM composition and cytokine cues. Our studies demonstrate that macrophages adapt to different stiffness microenvironments morphologically and functionally. BMMs on all PA gels are smaller and more circular than those on rigid glass. Macrophages on intermediate stiffness 20kPa PA gels are slightly larger and less circular than those on either 1kPa or 150kPa. Interestingly, there was an inverse correlation between surface stiffness and pro-inflammatory tumor necrosis factor alpha (TNF) cytokine secretion. Inhibition of the rho-associated coiled coil kinase (ROCK1/2), a key mediator in mechanotransduction, enhanced the release of TNF in response to stimulation of TLR4 and TLR9. Inhibition of ROCK1/2 also enhanced the phosphorylation of the TLR downstream signaling molecules, p38 and ERK1/2. Our data demonstrate that the extracellular microenvironment regulates macrophage morphology and TLR-mediated signaling, at least in part through ROCK1/2. These data provide a novel regulatory mechanism by which extracellular physical cues determine the magnitude of TLR signaling and resulting inflammation. Identifying the mechanisms and pathways in this process may offer therapeutic targets for the regulation of auto- and sepsis- mediated inflammatory pathology.

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## Quantifying effects of neutrophil memory on migration patterns using microfluidic platforms and ODE modeling of the mechanistic molecular pathways

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During sepsis, the current leading cause of death in hospitals, neutrophils migrate and accumulate in healthy organs instead of migrating toward the infection. Previous work from us described a dysfunctional phenotype, including oscillatory and spontaneous migration, in neutrophils isolated from septic burn patients [1]. In this study, we present a microfluidic platform to measure neutrophil chemotaxis in an opposing chemoattractant gradient to quantify neutrophil decision-making, with single-cell resolution. We use two chemoattractants: a pro-resolution (fMLP) and pro-inflammatory (LTB<sub>4</sub>) chemoattractant to model how a neutrophil makes a decision to move toward a bacterial infection versus an inflammatory signal. Our hypothesis, is that pro-inflammatory 'programming or training' signals, such as lipopolysaccharide, have a central role in determining the final neutrophil phenotype and in the development of sepsis. Furthermore, the concentration of the LPS exposure is critical in determining the impending neutrophil phenotype. Leukocyte memory in relation to primary low-levels of LPS may lead to a dysfunctional immune response to a secondary bacterial infection. On the other hand, exposure to high-levels of LPS lead to insufficient and dysfunctional migration and neutrophils are unable to contain infection. Despite tremendous advances in the understanding of signaling molecules and pathways acting inside neutrophils, our understanding of the directional decision-making process of neutrophils is limited, and consequently, our abilities to modulate the activity of neutrophils restricted.

In our microfluidic platform, opposing linear chemoattractant gradients are formed along migration channels, and neutrophils (HL-60s) in the central loading chamber must make the decision to migrate toward a preferred chemoattractant. Time-lapse images were taken every 2 min. and cell mazes were incorporated to measure directional migration and capture oscillatory behavior of the cells. To test the importance of leukocyte memory, we stimulate the cells overnight with a pro-inflammatory mediator (LPS) at both high (100 ng/mL) and low (10n g/mL) doses to determine if LPS plays a role in neutrophil decision-making. We show that unstimulated cells migrate toward fMLP over LTB<sub>4</sub> in a 2:1 ratio, with 15% of total cells migrating. Cells stimulated with high-dose LPS show migration in a similar ratio, but with a higher total percentage of migrating cells (22%). Surprisingly, cells

stimulated with a low dose of LPS migrate toward fMLP and LTB<sub>4</sub> in a 1:1 ratio, showing an increase in migration toward LTB<sub>4</sub>, as well as higher migration (18%). We see higher velocity toward LTB<sub>4</sub> (11.0  $\mu$ m/min vs. 7.1  $\mu$ m/min) and lower velocity toward fMLP (12.1  $\mu$ m/min vs. 14.9  $\mu$ m/min) compared to unstimulated cells. This study suggests that low-dose LPS stimulation can alter the decision-making properties of the neutrophil to migrate toward an inflammatory signal over a bacterial infection.

To understand the molecular mechanism of this cell memory, we combined our wet-bench work with an ODE-based dynamical framework to model the interaction of the mutually inhibitory GRK2 and GRK5 proteins and its role in neutrophil decision-making. GRK2 and GRK5 were of interest due to importance as drug targets as well as their interactions with the chemoattractant receptors and LPS. Our computational model results show a bimodal switch between high and low levels of GRK2. We are currently using open microfluidics to extract neutrophils on-chip after migration, to measure receptor levels to determine the underlying molecular mechanism and determine biologically accurate parameter values for our computational model. In the future, this platform can be used for early diagnosis of sepsis or to test the effect of pro-resolving mediators on neutrophil function.

Reference: [1] Jones CN, PLoS One, 2014, 9(12), e114509

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## Novel Pyrrole-Based N-Formyl Peptide Receptor 1 (FPR1) Antagonists

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Formyl peptide receptors (FPRs) are G protein-coupled receptors (GPCRs) expressed on a variety of cell types involved in host defense and sensing cellular dysfunction. Because FPRs play an important role in the regulation of inflammatory reactions implicated in disease pathogenesis, FPR antagonists may represent novel therapeutics for modulating innate immunity and treating inflammatory diseases. Previously, 1H-pyrrol-2(5H)-ones were reported to be potent and competitive FPR1 antagonists. In the present studies, 42 additional 1H-pyrrol-2(5H)-one analogs were evaluated for FPR1 antagonist activity. We identified a number of novel competitive FPR1 antagonists that inhibited N-formylmethionyl-leucyl-phenylalanine (fMLF)-induced intracellular Ca<sup>2+</sup> mobilization in FPR1transfected HL60 cells and effectively competed with WKYMVm-FITC for binding to FPR1 in FPR1-transfected RBL cells. Several pyrroles inhibited human neutrophil Ca<sup>2+</sup> flux, chemotaxis, and adhesion to human epithelial cells with low micromolar IC<sub>50</sub> values. In addition, the most potent FPR1 antagonists inhibited fMLF-induced phosphorylation of extracellular signal-regulated kinases (ERK1/2) in FPR1-RBL cells. Most of the antagonists were specific for FPR1 and did not inhibit WKYMVM/WKYMVm-induced intracellular Ca<sup>2+</sup> mobilization in FPR2-HL60 cells, FPR3-HL60 cells, or interleukin 8-induced Ca<sup>2+</sup> flux in human neutrophils. Moreover, molecular modeling showed that the active pyrroles had a significantly higher degree of similarity with the FPR1 antagonist pharmacophore template as compared to inactive analogs. Thus, the 4-aroyl-3-hydroxy-5-phenyl-1H-pyrrol-2(5H)one scaffold represents an important backbone for the development of novel FPR1 antagonists and could provide important clues for understanding the molecular structural requirements of FPR1 antagonists.

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#### Site-specific anti-cancer immune response to bacterial-derived Immunotherapies

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Immune stimulation due to bacterial infection has been linked to spontaneous cancer remission throughout history. The mechanisms driving this phenomenon are not fully understood, and there has been limited success in translating this approach into safe and efficacious immunotherapies. To overcome these limitations, a novel treatment strategy involving repeated subcutaneous delivery of bacteria-derived immunotherapies was tested. Site-

Specific Immunomodulators (SSIs) are derived from inactivated bacteria and are hypothesized to cause a specific innate immune response in the particular organ or tissue that the bacteria commonly causes infection in. For example, QBKPN, produced from Klebsiella, a common lung pathogen, was designed to activate an anti-cancer lung response. In contrast, QBECO, produced from the gut pathogen E. coli, was designed to target the intestinal track.

Subcutaneous QBKPN treatment, but not QBECO treatment, was efficacious at reducing tumor burden and increasing survival in mouse models of lung cancer. In contrast, in murine models of intestinal cancer, subcutaneous QBECO treatment, but not QBKPN treatment, was efficacious at increasing survival. The therapeutic efficacy in the lungs with OBKPN treatment was largely driven by innate immune mechanisms, including increased systemic phagocytic leukocytes, recruitment of interstitial macrophages and NK cells into the lungs, M1 macrophage polarization, upregulation of NKG2D ligands, and enhanced production of cytotoxic molecules. Although OBKPN and OBECO both similarly stimulated an inflammatory response in the blood that included increased circulating inflammatory monocytes, neutrophils, and pro-inflammatory cytokines, the immune response was tissue specific in the lungs and in the gut. In the lungs, QBKPN treatment caused a large increase in lung interstitial macrophages and NK cells, but this was not seen with QBECO treatment. Additionally, QBKPN treatment, but not QBECO treatment, increased the levels in the lungs of the NKG2D ligand RAE1, plus multiple pro-inflammatory cytokines. This organ specific anti-cancer efficacy was dependent on prior species-matched exposure, as seen by the loss of efficacy with QBKPN treatment in mice sourced from Klebsiella exposure free facilities. Remarkably, the QBKPN efficacy in lung cancer was not dependent on the adaptive immune response, potentially suggesting innate immune memory was responsible for this site-specific response. Collectively, these results suggest that there is a site-specific immune response to immunotherapies derived from specific inactivated bacteria that commonly cause infection at that site. This data demonstrates a novel immunotherapeutic approach, SSIs, that exploits innate anti-tumor immune mechanisms in a site-specific manner.

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Involvement of MrgX2 in the internalization of LL-37 and degranulation of LAD2 human mast cells. Taisuke Murakami, Kaori Suzuki and Isao Nagaoka

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A human bactericidal cathelicidin peptide LL-37 is known to modulate the various immune functions of leucocytes and other cells. In addition, LL-37 induces degranulation of mast cells, which is triggering host defense mechanisms against pathogenic microorganisms; however, the mechanism for LL-37-induced mast cell activation remains unclarified. Recently, a GPCR, Mas-related gene X2 (MrgX2) has been identified as a receptor for LL-37. This finding suggests that LL-37 interacts with cell surface MrgX2 to activate G protein signaling cascade. In contrast, some pruritogenic basic peptides (such as substance P) are reported to induce degranulation by internalization into the cells. Therefore, we investigated the role of MrgX2 in the internalization of LL-37 and mast cell activation (degranulation).

LL-37 dose-dependently induced degranulation of LAD2 human mast cells, and the degranulation was suppressed by pertussis toxin, confirming the involvement of a G-protein in the degranulation. Interestingly, LL-37 dosedependently internalized into the cells, and the extent of internalization and degranulation are correlated. Moreover, the knockdown of MrgX2 in LAD2 reduced the internalization of LL-37 and degranulation by LL-37. Furthermore, transiently MrgX2-expressing HEK293 cells showed the enhanced LL-37 internalization, suggesting that MrgX2 is required for internalization of LL-37. Together these observations suggest that the internalization of LL-37 is correlated with mast cell activation (degranulation), and MrgX2 is required for the internalization of LL-37 and mast cell activation (degranulation).

#### Natural Sesquiterpene Lactones Inhibit T-cell Antigen Receptor (TCR) Activation

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Inhibition of the T cell antigen receptor (TCR) pathway represents an effective strategy for the treatment of T cellmediated inflammatory and autoimmune diseases. To identify natural compounds that could inhibit inflammatory T cell responses, we screened 13 sesquiterpene lactones, including achillin, arglabin, argolide, argracin, 3βhydroxyarhalin, artesin, artemisinin, estafiatin, grosheimin, grossmisin, leucomisine, parthenolide, and taurin for their ability to modulate activation-induced Ca<sup>2+</sup> mobilization in Jurkat T cells. Five of the compounds (arglabin, grosheimin, argracin, parthenolide, and estafiatin) inhibited anti-CD3-induced mobilization of intercellular Ca<sup>2+</sup> i) In Jurkat cells, with the most potent being parthenolide and argacin (IC<sub>50</sub> = 5.6 and 6.1  $\mu$ M, respectively). Likewise, phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in activated Jurkat cells was inhibited by these five compounds, with the most potent being parthenolide and estafiatin (IC<sub>50</sub> = 13.8 and 15.4  $\mu$ M, respectively). These compounds also inhibited ERK1/2 phosphorylation in primary human T cells and depleted intracellular glutathione. In contrast, none of the sesquiterpene lactones inhibited ERK1/2 phosphorylation in HL60 cells transfected with formyl peptide receptor 2 FPR2 cells stimulated with WKYMVM, indicating specificity for T cell activation. Estafiatin, a representative sesquiterpene lactone, was also profiled in a cell-based phosphokinase array for 43 kinase phosphorylation sites, as well as in a cell-free competition binding assay for its ability to compete with an active-site directed ligand for 95 different protein kinases. Besides inhibition of ERK1/2 phosphorylation, estafiatin also inhibited phosphorylation of p53, AMPKα1, CREB, and p27 elicited by TCR activation in Jurkat cells but did not bind to any of 95 kinases evaluated. These results suggest that arglabin, grosheimin, agracin, parthenolide, and estafiatin selectively inhibit initial phases of TCR activation and may be natural compounds with novel immunotherapeutic properties.

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## IVIg and IVIg Activated Macrophages as an IL-10 Dependent Novel Therapeutic Strategy for Inflammatory Bowel Disease

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Inflammatory Bowel Disease (IBD) is a chronic inflammatory disease characterized by inflammation along the intestinal tract. Current treatment for IBD relies on non-specific immune suppression. However, up to 40% of people are predicted to become refractory to all available therapies so development of new therapeutic strategies to treat people with IBD is urgently needed. Macrophages initiate the innate immune response and contribute to the inflammation that characterizes IBD, but they also play an equally important role in turning off the inflammatory response. Intravenous Immunoglobulin (IVIg) is a drug made up of pooled polyclonal IgGs isolated from the blood of more than 1000 donors. It is used to suppress immune responses in autoimmune or inflammatory conditions. We have reported that macrophages stimulated with IVIg produce high levels of the anti-inflammatory cytokine, IL-10, and low levels of pro-inflammatory cytokines, in response to the inflammatory stimulus, lipopolysaccharide (LPS).

To determine whether IVIg-activated, IL-10 producing macrophages can be used to treat intestinal inflammation; we adoptively transferred (IVIg+LPS)-activated macrophages into mice, or treated mice with IVIg, during DSS-induced intestinal inflammation. Adoptive transfer of (IVIg+LPS)-activated macrophages or IVIg treatment reduced clinical disease activity during DSS colitis, including weight loss, rectal bleeding, and stool consistency. Histological evidence of inflammation was also reduced in treated mice, including loss of tissue architecture,

immune cell infiltration, muscle thickening, ulceration, and edema. Colons were longer in the (IVIg+LPS)-activated macrophages or IVIg treated mice, which is also a measure of reduced inflammation.

Consistent with a central role for macrophage-derived IL-10 in IVIg-mediated protection during DSS-induced colitis, IVIg treatment increased IL-10 and decreased pro-inflammatory cytokine production in excised colon cultures. In IL-10-GFP reporter mice, IVIg treatment increased the number of GFP-expressing cells, suggesting that IVIg treatment induces IL-10 expression *in vivo*. Importantly, IVIg did not reduce DSS-induced colitis in IL-10 receptor deficient mice. Finally, adoptive transfer of  $II10^{+/+}$  (IVIg+LPS)-activated macrophages reduced DSS-induced inflammation, whereas  $II10^{-/-}$  (IVIg+LPS)-activated macrophages did not.

In conclusion, IVIg activated macrophages have potent anti-inflammatory activity that can be used to reduce intestinal inflammation *in vivo*. Adoptive transfer of *in vitro*-derived (IVIg+LPS)-activated macrophages, or activating macrophages with IVIg *in situ*, may provide novel effective therapies to treat intestinal inflammation in people with IBD.

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#### Adherent human neutrophils depend on accessory cells for their survival response to LPS

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The survival response of human neutrophils to LPS enhances innate immunity to Gram-negative bacteria, but it may also prolong or exacerbate septic shock, chronic inflammation and other consequences of endotoxemia. TLR4 signaling pathways involved in the survival effect of LPS may be useful targets for therapeutic manipulation if they can be understood in detail. We studied neutrophil survival after TLR4 stimulation in order to test the hypothesis that TLR4 signaling is anti-apoptotic in some but not all cellular environments, including partially or highly purified neutrophil populations (PP and HP, >90% and >99% pure, respectively) cultured under conditions that favor suspension (polypropylene tubes with gentle movement) or adherence (flat-bottom polystyrene plates). We found that the survival response to a pure TLR4 agonist, synthetic E. coli lipid A, was intact when neutrophils were plated as PP populations but not when highly purified. The HP neutrophils remained competent for GM-CSF survival signaling and for TLR4 activation as determined by lipid A induced CD62L shedding. Culture supernatants from PP neutrophils exposed to lipid A improved HP neutrophil viability, suggesting that accessory cells are needed as a source of secreted survival factors. IL-8, reportedly a survival factor for neutrophils, was five-fold more abundant in supernatants from PP versus HP populations. However, verified IL-8 blockade did not inhibit survival activity present in PP culture supernatants indicating that additional factors are involved. HP neutrophils in suspension culture tubes, unlike adherence plates, were competent for TLR4-stimulated survival signaling; this intrinsic TLR4 survival activity correlated with phosphorylation of ERK, a MAPK that promotes neutrophil survival. We speculate that TLR4 signaling may directly promote survival of circulating neutrophils until they reach infected tissue, after which the intrinsic survival response to LPS is inactivated such that neutrophils become dependent on accessory cells for survival.

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## Staphylococcus aureus SaeR/S-regulated Factors Decrease Monocyte-derived TNF-a to Reduce Neutrophil Bactericidal Activity

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The ability of *Staphylococcus aureus* (*S. aureus*)to cause human disease is based in part on its ability to evade killing by human neutrophils. Herein, we use a co-culture model of human peripheral blood mononuclear cells

(PBMCs) and neutrophils to characterize an influential role for the *S. aureus* SaeR/S-two component gene regulatory system in mediating monocyte production of TNF-α. TNF-α production from monocytes, within whole blood and PBMCs, was significantly reduced following challenge with wild type *S. aureus* compared to challenge with an isogenic *saeR/S* deletion mutant. We demonstrate that this decrease in monocyte-derived TNF-α by SaeR/S-regulated factors is independent of cell lysis and significantly contributes to a reduced ability of neutrophils to kill *S. aureus*. We observed that priming of neutrophils using conditioned medium from PBMCs stimulated with *saeR/S* mutant, significantly increased neutrophil bactericidal activity against wild type *S. aureus* relative to untreated neutrophils and neutrophils primed with conditioned medium from wild type *S. aureus* stimulated PBMCs. The increased neutrophil bactericidal activity was associated with enhanced neutrophil reactive oxygen species production and this increase was in part attributed to elevated TNF-α concentrations. Taken together, this study provides insight into an evasion strategy used by *S. aureus* during staphylococcal disease by which SaeR/S-regulated factors impede neutrophil priming by decreasing monocyte-derived TNF-α.

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## Lactic acid upregulates VEGF expression in macrophages and endothelial cells, facilitating choroidal neovascularization $\otimes$

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As one of the immune-modulating agent, lactic acid has been investigated in terms of interconnection between metabolism and immunity in various diseases. However, metabolic changes and its influence on the choroidal neovascularization (CNV) pathogenesis have not been elucidated yet. Therefore, we hypothesized that metabolic by-product 'lactic acid' would be accumulated in eye of age-related macular degeneration (AMD) and affect VEGF expression of different cell components of the eye, facilitating CNV. To investigate the metabolite lactic acid on the CNV pathogenesis, we developed laser-induced murine model of choroidal neovascularization and studied the interaction between metabolites and immune cells. Lactic acid level was significantly elevated in RPE-choroid region of CNV mouse (p<0.05). Through FACS analysis, we revealed that CD11b+Gr1-F4/80+ macrophages were one-hundred times more infiltrated, and the numbers of VEGF-expressing macrophages were also significantly increased in CNV mouse compared with naïve mouse (p<0.05). In vitro experiments with ARPE-19, HUVEC, and THP-1 cell lines showed that lactic acid-treated THP-1 macrophages exhibited increased VEGF mRNA and protein expression approximately 2 times more than untreated controls and facilitates tube formation of endothelial cells. Notably, intravitreal injection of mono carboxylate transporter-1 (MCT1) blocker, \(\alpha\)-CHC, showed significant decrease in VEGF contents in RPE/choroid regions and declined co-localization of macrophages and VEGF signals in flat mount immunofluorescence staining (p<0.05). These findings suggest that metabolite regulation can be a good strategy for treating neovascularization. This study is the first report verifying the effect of metabolite 'lactic acid' on AMD pathogenesis.

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Serpinb6a cooperates with Serpinb1a in protecting myeloid cells from cathepsin G-mediated death in vivo Sabrina Sofia Burgener<sup>1,2</sup>, Philip Ian Bird<sup>3</sup> and Charaf Benarafa<sup>1</sup>

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**Background:** Serpinb6a is an inhibitor of leukocyte cathepsin G and various chymotrypsin-like proteases. Deficiency in Serpinb6a and its human counterpart Serpinb6 are associated with cell death of epithelial cells of the inner ear leading to deafness. Serpinb6a is highly expressed in the cytoplasm of myeloid cells but its function in these cells is not apparent in Serpinb6a. We have previously shown that Serpinb1a is essential in the

regulation of a cathepsin G-dependent neutrophil death and here we investigated the additive role of the two serpins in neutrophil homeostasis.

**Results:** First, Serpinb1a. Serpinb6a double knock-out mice showed a more severe neutropenia in the bone marrow compared to the Serpinb1a<sup>-/-</sup> mice. Strikingly, Serpinb1a. Serpinb6a<sup>-/-</sup> mice also presented reduced blood neutrophils as well as lower blood and bone marrow monocyte percentages, which were not observed in Serpinb1a<sup>-/-</sup> mice. In a zymosan-induced peritonitis model, Serpinb1a. Serpinb6a, mice showed severely reduced neutrophil numbers in the exudate and delayed clearance of the yeast particles. Accordingly, Serpinb1a. Serpinb6a<sup>-/-</sup> neutrophils had a considerably higher kinetic of cell death induced by granule permeabilization in vitro. Caspase and RIPK1 inhibition did not rescue neutrophil death in cells lacking serpins. Increased cell death in Serpinb1a. Serpinb6a. neutrophils was independent of reactive oxygen species production by the NADPH oxidase and was not associated with cleavage of mitochondrial inner complex proteins NDUSF1 and NDUSF3. Pyroptosis mediated by Caspase 1/11 was also ruled out. In contrast, cathensin G deletion was sufficient to rescue cell death in neutrophils suggesting a novel death mechanism independent of classical apoptosis, necroptosis and pyroptosis pathways.

Conclusion: Our results demonstrate that Serpinb6a complements Serpinb1a in providing an additional cytoprotective shield in neutrophils in steady state and during sterile inflammation. Serpinb1a and Serpinb6a are essential in protecting necrosis induced by granule leakage by inhibiting the serine protease cathepsin G, which induces death independently of RIPK1, the NADPH oxidase and apoptotic and inflammatory caspases.

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#### Identifying an Immune Signature Characteristic of Fetal Alcohol Spectrum Disorders

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Fetal alcohol spectrum disorder (FASD) comprises the broad range of structural, neurocognitive, physiological and behavioral abnormalities/deficits that can occur following prenatal alcohol exposure. Of relevance, children with FASD show a wide range of deficits in both innate and adaptive immunity, with a higher incidence of major and minor infections and an increased incidence of cancers. However, clinical and pre-clinical immune-related research remains an understudied area in FASD.

A leading hypothesis suggests that exposure to adverse environmental factors, such as alcohol, during sensitive periods can result in an enhanced proinflammatory phenotype, which is embedded in the functioning of immune cells in both the periphery and brain. Importantly, cytokines act as potent CNS neuromodulators during early life, affecting neuronal migration, synaptogenesis, and synaptic pruning. Alterations in cytokine profiles may alter these processes, resulting in aberrant brain development and immune dysregulation, and, in turn, increased vulnerability to diseases/disorders later in life. Indeed, alterations in immune factors during early-life have been implicated in the pathophysiology of other neurodevelopmental disorders including autism spectrum disorders and schizophrenia.

The hypothesis of the current study is that changes in circulating cytokine levels following prenatal alcohol exposure may impact fetal development, altering both brain and immune system development and contributing to some of the long-lasting changes seen in children with FASD. Here, we performed a comprehensive examination of immune parameters in plasma samples from alcohol-exposed and unexposed children (2 - 3.5 years) obtained from banked samples collected at two ONMI-Net sites in Western Ukraine, as part of the ongoing Collaborative Initiative on FASD (CIFASD) Ukrainian longitudinal study. Cytokine assays were performed using the Meso Scale Discovery (MSD) human biomarker kit, which allows for the measurement of 40 proteins including cytokines and chemokines, as well as vascular and angiogenesis markers.

Our preliminary results show differential patterns of pro- and anti-inflammatory cytokines in alcohol-exposed compared to unexposed children. Moreover, alcohol-exposed children show higher C-reactive protein (CRP) levels compared to unexposed children, which is indicative of chronic, low-grade inflammation. Overall, changes in cytokines during critical developmental periods may underlie some of the long-term effects of prenatal alcohol exposure on cognitive, physiological, and immune function, as well as on sensitivity/vulnerability to alcohol and other drugs in later life. Finally, as cytokines do not operate independently, our ongoing analyses are utilizing data reduction techniques to identify the impact of prenatal alcohol exposure on the complex and interconnected networks of cytokines.

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## Blunted neutrophil priming in children with type 1 diabetes and diabetic ketoacidosis

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Type 1 diabetes mellitus (T1D) is a chronic inflammatory condition sometimes complicated by acute diabetic ketoacidosis (DKA). DKA is a leading cause of morbidity in the pediatric T1D population and is a common indication for intensive care management. DKA is often initiated by non-compliance with insulin management, but infectious etiologies are also common triggers. Whether the etiology is infectious or otherwise, there exists an acute inflammatory state during DKA. Global sterile inflammation triggering an innate immune response may also increase the likelihood of development of DKA in subjects with T1D. We hypothesized that neutrophils from subjects with T1D will exhibit priming, and, furthermore, the primed phenotype will be distinct in subjects with acute DKA as compared with well-controlled T1D. Neutrophils were isolated from 3 groups: 1) subjects admitted with DKA (n=20), 2) subjects encountered in the clinic with well-controlled T1D (n=21), and 3) healthy controls (n=17). Neutrophil priming was assessed in terms of NAPDH oxidase production of reactive oxygen species (ROS) measured by lucigenin-enhanced chemiluminescence, elastase release measured by activity assay, and mobilization of intracellular stores of receptors measured by flow cytometry. In some assays, neutrophils were exposed to TNF- $\alpha$ as a standard priming stimulus. Neutrophils from subjects with DKA or well-controlled T1D display a trend toward enhanced fMLF-elicited ROS production and elastase release compared to healthy controls (p=0.09 and p=0.07, respectively). Interestingly, neutrophils from subjects with both DKA and well-controlled T1D exhibit a marked inability to be primed by TNF-a. This priming resistant phenotype includes minimal enhancement of the fMLFstimulated respiratory burst following priming with TNF-a in both DKA and well-controlled T1D subjects compared with healthy donors (p<0.01). Neutrophils from healthy donors experience a significantly larger fold increase in ROS production following priming with TNF-α (37.05 ± 4.5) compared with subjects with wellcontrolled T1D and DKA (8.33± 2.4 and 4.35± 0.92, respectively; p<0.0001). In addition, there is also a significantly larger mean fold increase of neutrophil elastase release following priming with TNF- $\alpha$  in healthy donors and subjects with well-controlled T1D ( $3.53\pm0.12$  and  $3.81\pm0.61$ , respectively) compared with neutrophils from subjects with DKA (2.44± 0.15; p<0.05). Neutrophils from subjects with DKA display significantly less cell surface L-selectin at baseline compared with well-controlled T1D neutrophils (p<0.01). Likewise, neutrophils from subjects with DKA have a lower fold reduction of surface L-selectin following priming with TNF-α compared to subjects with well-controlled T1D (mean fold reduction = 9.32±1.57 and 19.48±2.84, respectively; p<0.01). Using linear regression analysis, none of the aforementioned differences in neutrophil phenotype from subjects with diabetes are correlated with hemoglobin A1c, a well-established marker of long-term glucose control. In summary, neutrophils from both DKA and well-controlled T1D subjects demonstrate alterations in neutrophil phenotype as compared with healthy controls. Specifically, neutrophils from DKA subjects exhibit blunted priming response to TNF-a and display lower basal surface levels of L-selectin compared with well-controlled T1D subjects. Our findings suggest that neutrophils from children with diabetes exist in an activated state at baseline in both DKA and well-controlled T1D subjects, although this phenotype is distinct between the two conditions. Further studies are

necessary to evaluate the mechanism underlying decreased responsiveness to TNF- $\alpha$  in these cells, but we speculate that the blunted response may be secondary to down-regulation of the TNF- $\alpha$  receptor at the cell surface.

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## Protective Role of ILC2 in Sepsis-Induced Acute Lung Injury

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**Introduction:** Group 2 innate lymphoid cells (ILC2), a recently identified population of innate immune cells, were suggested to be protective in infection. However, the underlying mechanism remains unknown. The current study aimed to determine the protective role of ILC2 in sepsis-induced acute lung inflammation and the underlying mechanism.

Methods: WT and IL-33 knockout mice were subjected to cecal ligation and puncture (CLP) to induce sepsis. The number of ILC2 and the concentration of cytokines including IL-4, IL-9 and IL-13 in the lung tissue were measured by flowcytometry and ELISA, respectively, at different time after CLP. In the *in vitro* studies, lung-derived ILC2 were co-cultured with mouse lung endothelial cells (LEC) and macrophages (Mf), respectively, and challenged with LPS and/or TNF-α for up to 48 h. The LEC necroptosis and the ability of Mf phagocytosis of bacteria were then detected by flowcytometry and confocal microscopy.

**Results:** Sepsis induced IL-33-dependent recruitment of ILC2 in the lung after CLP. The levels of ILC2-derived IL-9 and IL-13 significantly increased in the lung tissue of WT septic mice and in the supernatant of cultured ILC2 after LPS stimulation. IL-9 protects LPS/TNF-a-induced LEC necroptosis, whereas, IL-13 decreased Mf necroptosis in response to LPS. In addition, both IL-9 and IL-13 promote Mf phagocytosis of bacteria.

**Conclusions:** IL-33-mediated ILC2 recruitment in the lung following sepsis protects lung injury through reducing necroptosis of LEC and Mf and promotes bacteria clearance by Mf. Increasing ILC2 and/or ILC2-derived cytokines in the lung may present a new therapeutic strategy for acute lung injury in sepsis.

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#### Monocytic Glutaredoxin 1 Protects Mice Against Obesity, Hyperglycemia and Atherosclerosis

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The goal of this study was to determine the role of monocytic Grx1 in mice and in the development of atherogenesis and obesity. To this end, transplanted bone marrow (BM) from either wild-type (WT) or Grx1<sup>-/-</sup> donor mice into atherosclerosis-prone LDLR<sup>-/-</sup> mice and fed these mice a high-fat diet (HFD) for up to 20 weeks. Grx1<sub>Leuko</sub> -/- mice showed accelerated weight gain after 9 weeks followed by early onset of hyperglycemia. After 6 weeks on HFD, atherosclerotic lesions were slightly larger in Grx1<sub>Leuko</sub> -/- mice than in WT mice, but the differences did not reach statistical significance. However, after 20 weeks, Grx1<sub>Leuko</sub> -/- mice showed 36% larger lesions than WT-BM recipients, and monocyte chemotaxis *in vivo* was increased 1.6-fold. Adipose tissues and livers of Grx1<sub>Leuko</sub> -/- mice also showed increased macrophage content and elevated tissue inflammation as determined by IHC and qRT-PCR-based gene array. Adipose tissue showed significant increases in the expression of proinflammatory genes in addition to an increased abundance of proinflammatory "crown-like" structures. In contrast, genes associated with inflammation resolving macrophages were significantly suppressed. Macrophages isolated from Grx1<sup>-/-</sup> mice and stimulated with INFg+TNFa also showed increased expression of pro-inflammatory M1-associated genes, whereas M2-associated genes were suppressed in Grx-1<sup>-/-</sup> macrophages activated with IL-4. Furthermore, macrophages from Grx1<sup>-/-</sup> mice exposed to metabolic stress also display increased protein *S*-glutathionylation, enhanced hypersensitization to chemokine, and impaired autophagy compared to macrophages from wild-type mice. We

conclude that monocytic Grx1 is critical for maintaining macrophage function and immunometabolic homeostasis in mice and protects mice against obesity and atherogenesis.

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#### **Alcohol Potentiates Bacterial Overgrowth in Setting of Colitis**

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Ulcerative colitis (UC) is an inflammatory disease of the large intestine with unknown etiology that affects more than three million individuals globally. These patients experience periodic episodes of disease reactivation characterized by severe abdominal discomfort and bloody diarrhea, often requiring hospitalization. Triggers of flares appear to be multifactorial but can be precipitated by certain foods. Alcohol is a likely factor affecting both onset and reactivation of UC but a mechanism for this is not established. A recent study carried out in our laboratory in a mouse model of dextran sulfate sodium (DSS)-induced colitis suggests that alcohol can potentiate the inflammatory process and other symptoms associated with colitis. In the current study, we assessed whether alcohol influences bacterial populations and barrier integrity, providing a basis for the enhanced inflammatory effects. Male C57BL/6 mice received either 2% DSS or normal drinking water ad libitum for 5 days. On day 5, 6, and 7, mice were gavaged with either alcohol (~3g/kg) or water and subsequently euthanized on day 7, three hours after the last gavage. The large intestine was harvested and the most distal end preserved in formalin for histological analysis. Fecal pellets were removed from the large intestine and processed for bacterial DNA extraction. Intestinal epithelial cells (IECs) were isolated from the remaining large intestine and processed for total RNA. qPCR of bacterial DNA demonstrated that mice treated with DSS and ethanol gavage had a 10-fold increase in Enterobacteriaceae populations compared to mice treated with DSS and water gavage. Histologic examination of H&E stained large intestine sections from mice receiving DSS plus ethanol demonstrated significantly increased pathological scores (p<0.05) as well as increased infiltration of polymorphonuclear leukocytes when compared to mice receiving DSS plus water gavage. Analysis of tight junction proteins from IECs by qPCR, revealed that occludin, claudin 4, and zonula occludens-1, were all significantly decreased (p<0.05) in mice receiving DSS regardless of their alcohol exposure compared to mice receiving water alone. Similarly, expression of alcohol dehydrogenase-1 was significantly decreased (p<0.001) in IECs from mice receiving DSS treatment with either water or alcohol gavage when compared to mice receiving water or ethanol only. The decrease in tight junction proteins coupled with alcohol persistence due to decreased alcohol dehydrogenase 1 might contribute to intestinal permeability, while increased Enterobacteriaceae load could lead to the progressive inflammation and pathology observed in combined DSS and alcohol treatment. (Support: R21AA022324, T32AA013527 and F31AA025536-01).

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## Neutrophil MPO-HOCl System Regulates Inflammation

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Myeloperoxidase (MPO), an enzyme predominantly expressed in neutrophils, catalyzes two-electron peroxidation of chloride anion to produce hypochlorous acid (HOCl). It is well studied that the MPO-HOCl system plays a critical role in host anti-infection immunity. However, whether this system has any other biological significance is not clearly defined. Here we report that the MPO-HOCl system is indispensable to down-regulation of host inflammatory response. MPO-deficient (MPO-/-) and wild-type (WT) mice were challenged with a lethal dose of zymosan intratracheally to induce non-septic pneumonia. MPO-/- mice had 100% mortality, while WT mice were

fully protected. Similarly, a peritoneal challenge with a lethal dose of zymosan led to ~40% more mortality in MPO-/- mice than in WT counterparts. The peritonitis induced by a sub-lethal dose of zymosan demonstrated that MPO-/- mice were retarded in inflammation resolution, reflected by a protracted inflammation with sustained neutrophil prevalence, and significantly higher levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, MIP2 and KC). Because MPO deficiency causes HOCl production deficiency in neutrophils, we predicted that the excessive inflammatory response must result from the lack of HOCl. Pre-treatment of zymosan with dose-escalation levels of bleach conferred protection of MPO-/- mice in a bleach-dose-dependent manner upon peritoneal challenge. The zymosan, pre-treated with a physiologically relevant level of bleach and administered into the peritonea of MPO-/- mice, gave rise to a substantially attenuated neutrophilic inflammation and a significantly reduced cytokine production, as compared to the non-treated zymosan. Taken together, our data indicate that the MPO-HOCl system provides a novel mechanism for host control of inflammation.

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## Adaptation to complex host niches by fungal pathogen Candida albicans drives resistance to neutrophil attack $\mathbb Q$

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Candida albicans is a successful commensal and pathogen on human mucosal tissues which has evolved to optimally adapt to its host niche. It is widely believed that environments shape niches to which inhabitants adapt. However, there is an increasing body of evidence that microorganisms in turn adapt by changing their environment by anticipating environmental stimuli to improve their own fitness. Here, we propose that C. albicans thrives for hypoxic or anoxic niches which support competitiveness of the yeast and at the same time reduces immune defense effectiveness. Upon infection, circulating neutrophils are rapidly infiltrating to mucosal tissues. High numbers of infiltrating cells coupled with the formation of multicellular structures, such as biofilms, result in hypoxic and anoxic micro niches. We have characterized the range of effects of anoxia on neutrophil responses encountering C. albicans both as planktonic morphotypes and as biofilms. We found that persistent anoxic environments do not affect neutrophil viability per se, however severely hamper neutrophil responses by affecting PAMP sensing and consequent neutrophil responses - phagocytosis, degranulation, NET formation - against C. albicans. On the other hand, anoxia contributes to increased fungal growth, a trait which seems to be conserved in among Candida species. Adaption to low oxygen may therefore be an evolutionary advantage for Candida species in commensal niches and moreover a pre-requisite for a successful infection of the host.

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# Cytokine analysis may be used to diagnose and monitor response to therapy in animal model of S. epidermidis CSF shunt infection

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Bacterial infection is a frequent and serious complication of CSF shunt placement for the treatment of hydrocephalus. Diagnosis of these infections relies on the gold standard of CSF culture; however, culture may not always be reliable in the setting of biofilm, slow growing or fastidious organisms and antibiotic pretreatment. CSF or serum chemokines and cytokines may prove useful as alternative strategies for diagnosis of CSF shunt infection. We hypothesized that *Staphylococcus epidermidis* CNS catheter infection has a distinct chemokine and cytokine profile when compared to baseline CSF and CSF from animals with sterile CNS catheters. To evaluate this hypothesis, we adapted our previously published murine CNS catheter infection model to generate infection with *S. epidermidis* in Lewis rats. The rats tolerate the procedure well and catheter placement in the lateral ventricle was

verified visually. Chemokine and cytokine analysis was performed on CSF at 1 day post infection. As expected there was an increase in the anti-inflammatory cytokine IL -10 which we saw in human samples. This likely represents a compensatory response to the elevation of pro-inflammatory cytokines. There were higher levels of the pro-inflammatory CSF chemokines and cytokines IL-1β, IL-6 and CCL3 in rats implanted with S. epidermidis infected CNS catheters. Interestingly there was no increase in the chemoattractants CCL2, CXCL1 and CX3CL1. At day 5 post-implantation, the levels of pro-inflammatory mediators also decreased, suggesting that these values may be useful for monitoring the course of infection over time. Importantly this demonstrates the role of proinflammatory IL -β, IL-6 and CCL3 in differentiating infection from trauma even at early time points. Coupling inflammatory mediator analysis with bacterial detection strategies may be a useful tool for diagnosing shunt infection in early post-operative time periods.

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#### Production of AIM by peritoneal macrophages and its role in phagocytic activity

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Accumulated evidences demonstrate that macrophages are classified into different populations based on their gene expression profiles, which are determined by the tissue localization as well as by their origins. Apoptosis inhibitor of macrophage (AIM), encoded by Cd5l gene, is one of the signature molecules expressed by specific resident macrophage subsets, including peritoneal macrophages, liver Kupffer cells and splenic red pulp cells, under the control of the LXR/RXR heterodimer. AIM is a circulating protein, harboring three scavenger receptor cysteine-rich (SRCR) domains. In blood, most of AIM molecules associate with IgM, which prevents renal excretion of AIM, maintaining its average serum level at approx. 5 µg/mL in human. Recently, we have observed notable features of AIM related to various diseases. AIM is endocytosed by types of cells, e.g. macrophages, adipocytes, hepatocytes and specific epithelial cells, via scavenger receptors such as CD36. In addition, AIM binds to a variety of internal hazardous substances including dead cell debris, Lipopolysaccharides, or multiple modified proteins. With such characteristics, AIM appears to play a role in rapid clearance of necrotic debris and/or toxic materials, particularly in an injured tissue, e.g. duringacute kidney injury<sup>1</sup>, thereby preventing secondary inflammation and subsequent fibrosis, also facilitating tissue recovery. Here, we analyzed the precise activity of AIM in the peritoneum and found that AIM is principally produced by F4/80<sup>high</sup> large peritoneal macrophages (LPMs). Interestingly, AIM is preferentially ingested by LPMs, resulting in an increased phagocytic activity of AIM-associated dead cell debris by LPMs. In contrast, the phagocytosis observed in other types of cells in the peritoneal cavity, such as MHC-II<sup>high</sup> cells including small peritoneal macrophages (SPMs) or monocyte-derived inflammatory macrophages, was not accelerated by AIM. Thus, it is plausible that AIM might contribute to the prevention of inflammatory responses by enhancing rapid removal of harmful or antigenic materials by non-inflammatory phagocytes, especially in injured tissue, thereby suppressing disease development.

<sup>1</sup>Arai S et al. Nat Med 22:183, 2016

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Alveolar macrophage Nox2 protects against lung injury in a murine model of systemic inflammation Renee Potera, Mou Cao, Lin F. Jordan, Jessica S. Hook and Jessica G. Moreland University of Texas Southwestern Medical Center

Acute lung injury (ALI), occurring as a component of the systemic inflammatory response syndrome (SIRS), leads to significant morbidity and mortality. The production of reactive oxygen species (ROS), in part by the neutrophil NADPH oxidase 2 (Nox2), has been implicated in the pathogenesis of ALI. We have previously demonstrated the development of pulmonary inflammation following induction of SIRS in Nox2-deficient (gp91<sup>phox-/y</sup>) mice that was absent in WT mice. Given this finding, we hypothesized that Nox2 in a resident cell in the lung, specifically the alveolar macrophage, is essential in regulating the inflammatory balance in the lung. Utilizing the zymosan-induced

generalized inflammation model, we examined whole lung digests and bronchoalveolar lavage fluid (BALf) from WT and gp91<sup>phox-/y</sup> mice one and two hours after intraperitoneal injection with zymosan. We found that both genotypes developed systemic inflammation with sequestration of neutrophils in the lung following zymosan injection, but neutrophil infiltration to the alveolar space was present only in the gp91<sup>phox-/y</sup> mice. MIP-1 alphagene expression and protein secretion was higher in the whole lung digest of uninjected gp91<sup>phox-/y</sup> mice compared to the WT mice. Gene expression of MIP-1 alpha, MCP-1 and MIP-2 was up-regulated in alveolar macrophages isolated from the BALf of gp91<sup>phox-/y</sup> mice at baseline compared with WT mice. In parallel to our in vivo studies, alveolar macrophages and bone marrow derived macrophages (BMDM) were cultured and stimulated with PAM3CSK4 in vitro. We found higher gene expression of MIP-1 alpha and MIP-1 beta in cultured unstimulated alveolar macrophages isolated from gp91<sup>phox-/y</sup> mice compared with WT mice. When alveolar macrophages were treated with PAM<sub>3</sub>CSK<sub>4</sub>, there was significantly higher expression of MCP-1, MIP-1 alpha, MIP-1 beta and MIP-2 in the gp91<sup>phox-/y</sup> mice compared with WT mice. Unstimulated BMDM isolated from gp91<sup>phox-/y</sup> mice demonstrated higher gene expression of MCP-1 and MIP-2 compared to WT mice. There was higher gene expression of MIP-2 following stimulation with PAM<sub>3</sub>CSK<sub>4</sub>, although the magnitude of the change was less than that seen in alveolar macrophages. Interestingly, gene expression of MCP-1 was decreased following stimulation withPAM<sub>3</sub>CSK<sub>4</sub> in BMDM isolated from the gp91<sup>phox-/y</sup> mice with no significant differences in the expression of MIP-1 alpha or MIP-1 beta. Our results suggest that Nox2 in the alveolar macrophage is required to protect against the development of ALI in the setting of SIRS. We speculate that Nox2-derived ROS signaling functions in a homeostatic role to repress inflammation in the lung in part by modulating chemokine expression by the alveolar macrophage.

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### Inflammatory modulation induced by crystalloid intravenous infusion in healthy subjects

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Introduction: More than 100 000 patients are admitted and treated in intensive care units every year across Canada and many more to hospital wards. Many of these patients will be administered intravenous fluids during the course of their stay. These fluids differ mainly in their electrolyte composition and concentration, osmolarity and pH. While these solutions are indispensable in resuscitation and in fluid balance management, recent evidence suggests that they may not be as inert as once thought. Animal models have shown that fluids rich in sodium have a tendency to induce more pro-inflammatory effects, whereas those rich in chloride rather favour acidosis and hemodynamic instability. The aim of our research is therefore to evaluate the immunomodulatory effects of the common intravenous fluids administered in clinical medicine initially in healthy subjects.

Methods: A cohort of healthy patients is being recruited for an interventional study. Each patient receives a bolus infusion of one of three commonly used solutions (Normal Saline, Ringer's Lactate, and Plasma-Lyte) over 15 minutes. There is at least one week which separates each infusion. Blood is taken at two time points: before infusion and 6h post. Analysis of the phenotype of neutrophils is done at 6h by flow cytometry. CD14+ monocytes are also differentiated into macrophages and stimulated by different concentrations of the fluids in vitro. Their activation is determined by phosphorylation of p38 map kinase, phenotype by flow cytometry and upregulation of cytokine profiles by ELISA. Analysis of the cytokine milieu is also performed on total serum and laboratory tests determine any clinical effects on the patients.

Results: Our preliminary results show that neutrophils stimulated with normal saline have a more pro-inflammatory phenotype than those stimulated with Ringer's Lactate. The activity of these cells is demonstrated by the differential phosphorylation of p38 according to varying concentrations of the intravenous fluids. The phenotype of tissue monocytes differentiated into macrophages is unaffected by stimulation with the solutions. Pro-inflammatory cytokines are secreted and induced by infusion of the solutions.

Conclusion: Although many clinical studies have evaluated the outcomes of patients receiving different types of intravenous fluids, their inflammatory impacts remain poorly understood. This is the first study which looks

specifically at their immunomodulatory and inflammatory effects. Our preliminary data corroborates our hypothesis that infusion of these solutions have an effect of immune cell activity. With better understanding of these effects, our hope is that a more judicial use of intravenous fluids will lead to better outcomes for patients.

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### Human NETs synthesis contributes to angiopoietins-mediated pro-inflammatory and pro-angiogenic activities

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Neutrophils act as the first line of defense against pathogens by releasing proteolytic granules and bacterial phagocytosis. In addition to the bacterial-induced inflammatory response, neutrophils play a role in the pathogenesis of many acute and chronic inflammatory diseases by their capacity to promote the release of cytokines, chemokines, reactive oxygen species (ROS) and the formation of neutrophil extracellular traps (NETs). The latter is a novel pathogen killing mechanism (NETosis) that has been described as the extracellular release of neutrophil nuclear DNA, which binds to pro-inflammatory proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO). We previously reported the expression of the angiopoietin receptor (Tie2) on human neutrophils and the capacity of both angiopoietins (Ang1 and Ang2) to induce pro-inflammatory activities, such as the synthesis and release of platelet-activating factor (PAF), up-regulation of β<sub>2</sub>-integrin complex (CD11b/CD18) and neutrophil chemotaxis. More recently, we reported a differential capacity between angiopoietins to promote transcriptional activities in human neutrophils. Indeed, only Ang1 at high concentrations, can support transcriptional and translational activities associated to synthesis and/or release of specific cytokines (e.g. IL-1\beta, IL-1RA, IL-8 and MIP-1β) while Ang2 has no such effect. Since we observed that both angiopoietins can activate Tie2 on the neutrophils and mediate pro-inflammatory activities, we wanted to examine whether angiopoietins can induce NETs formation, assess the underlying mechanisms, and interrogate the role of these particular NETs on pro-inflammatory and pro-angiogenic activities under in vitro conditions.

Using freshly isolated neutrophils from human healthy volunteers, we observed that a treatment with Angl and Ang2, alone or combined (0.1 - 10 nM; 3 hours) were equipotent to promote the release of NETs ( $\approx$ 2.5-fold) as compared to PBS-treated neutrophils. We also observed that the release of NETs is Tie2-dependent and requires the intracellular participation of PI3K, p38 and p42/44 MAPK pathways, ROS production, intracellular calcium release, and protein arginine deiminase 4 (PAD4) activation. These angiopoietin(s)-induced NETs can also modulate in a concentration-dependent manner (100 - 800 ng/ml) a rapid up-regulation of  $\beta_2$ -integrin complex (CD11b/CD18) on neutrophils and P-selectin translocation onto human umbilical vein endothelial cells (HUVEC), contributing to increase neutrophil adhesion onto human extracellular matrix (hECM) (up to 3.3-fold) and activated HUVECs (up to 3.4-fold). In addition, we observed that these effects were abrogated by treating NETs for 1 hr with DNAse I (10 U/mL), whereas DNAse I alone does not interfere with the activation of neutrophils and ECs by pro-inflammatory agonists. We also assessed the effect of NETs on *in vitro* HUVEC capillary-like tube formation on a 2-dimensional Matrigel assay to mimic intussusceptive angiogenesis. Treatment with VEGF (40 ng/mL; 4 hrs positive control) increased the length, number and area of capillary-like tube loops by 58, 265 and 157% respectively as compared to basal PBS-treated HUVEC. In comparison, NETs isolated from treatments with angiopoietins alone or combined increased capillary-like tube length by 23% to 33%, the number of closed loops by 100 to 140% and tubules area by 56 to 80% as compared to basal PBS-treated HUVEC.

Our study is the first one to report the capacity of both angiopoietins to promote the release of NETs and to demonstrate that NETs formation contributes to angiopoietin(s)-mediated pro-inflammatory and pro-angiogenic activities.

## Antimicrobial cathelicidin peptide LL-37 protects septic mice by modulating macrophage pyroptosis and neutrophil NETosis

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LL-37 is the only known member of the cathelicidin family of antimicrobial peptides in humans. In addition to its broad spectrum of antimicrobial activities, LL-37 can modulate various inflammatory reactions. We previously revealed that LL-37 improves the survival of a murine cecal ligation and puncture (CLP) sepsis model. In the present study, we elucidated the mechanism for the protective action of LL-37 on the CLP model, focusing on the effect of LL-37 on macrophage pyroptosis and neutrophil NETosis. Interestingly, LL-37 inhibited the CLP-induced caspase-1 activation and pyroptosis of peritoneal macrophages. Moreover, LL-37 suppressed the levels of inflammatory cytokines (IL-1B, IL-6 and TNF-a) in both peritoneal fluids and sera, and inhibited the activation of peritoneal macrophages (as evidenced by the increase in the intracellular levels of IL-1B, IL-6 and TNF-a). Furthermore, LL-37 suppressed the increase of DAMPs (such as histone-DNA complex and HMGB1) and soluble TREM-1 in peritoneal fluids and sera. In contrast, LL-37 increased the level of antimicrobial NETs (myeloperoxidase-DNA complex) in peritoneal fluids and sera. Finally, LL-37 reduced the bacterial burdens in both peritoneal fluids and blood samples. Together, these observations suggest that LL-37 improves the survival of CLP septic mice by suppressing inflammatory cytokine production and bacterial growth through at least two mechanisms, i.e., the inhibition of macrophage pyroptosis and the induction of neutrophil NETosis (release of antimicrobial NETs). Thus, LL-37 can be a promising therapeutic candidate for sepsis because of its various functions, including the modulation of cells death (macrophage pyroptosis and neutrophil NETosis), inflammatory cytokine production and bactericidal activity.

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## 6-Formylindolo (3,2-B) Carbazole (FICZ) Improves Intestine Motility And Prevents Bacteria Growth Following Ethanol And Burn Injury

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6-Formylindolo (3,2-b) Carbazole (FICZ) is a ligand of aryl hydrocarbon receptor (AHR) which regulates Th17 release of IL-17 and IL-22 production. Both IL-22 and IL-17 are known to play an important role in maintaining mucosal barriers including the intestine barrier. Earlier, we have shown that acute alcohol (ethanol) intoxication combined with burn injury suppresses Th17 responses and disrupts the intestinal barrier leading to an increase in gut bacterial growth and bacteria translocation. Others suggest that increases in gut bacterial growth could result from a decrease in intestinal motility/transit. We determined whether ethanol combined with burn injury influences intestine transit and whether the treatment of mice with FICZ has any effect on intestine motility and bacterial growth. To accomplish 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) four hours prior to receiving ~12.5% total body surface area burn. Mice in the FICZ group were administered intraperitoneally with FICZ (200 µg/Kg BW) at time of injury. Intestine motility was measured by FITC-dextran method. In short, one day after injury, mice were gavaged with 0.4ml of 22mg/ml FITCdextran. After three hours of gavage, mice were euthanized and luminal contents from stomach, small intestine (proximal, middle and distal) and large intestine were collected to assess the accumulation of FITC-dextran. In addition, feces from the distal small intestine was collected to determine total bacteria and enterobacteriaceae by RT-PCR using specific primers. We observed that there was a significant delay in the intestine transit in mice receiving a combined insult of alcohol and burn injury compared to sham vehicle mice. The accumulation of FITCdextran in large intestine feces was 2.95±0.66 µg/ml in burn ethanol mice and 1234.11±226.91µg/ml in sham

vehicle mice. This accompanied an increase in total bacteria  $(8.97 \times 10^6 \pm 2.78 \times 10^6 \text{ copy} \# \text{ in burn ethanol mice} \text{ and } 0.47 \times 10^6 \pm 0.28 \times 10^6 \text{ copy} \# \text{ in sham vehicle mice})$  and enterobacteriaceae  $(16.18 \times 10^4 \pm 5.63 \times 10^4 \text{ copy} \# \text{ in burn ethanol mice} \text{ and } 0.018 \times 10^4 \pm 0.011 \times 10^4 \text{ copy} \# \text{ in sham vehicle mice})$ . However, treatment of mice with FICZ improved intestine transit time and prevented bacterial growth. These data suggest that ethanol intoxication combined with burn injury decreases intestine motility, and the treatment of mice with FICZ improves intestine motility and prevents the increase in bacteria growth following ethanol and burn injury. (Support: R01 AA015731, F31 AA024367, T32 AA013527).

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### The R753Q Polymorphism in TLR2 Attenuates Innate Immune Responses to Mycobacterial Infection and Impairs Recruitment of MyD88 to TLR2

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TLR2 plays a critical role in host defense against mycobacterial infections. The Arg753Gln (R753Q) TLR2 polymorphism has been associated with increased incidence of tuberculosis and infections with non-tuberculous mycobacteria in human populations, but the mechanisms by which it affects TLR2 signaling are unclear. In this study, we determined the impact of the R753Q TLR2 polymorphism on macrophage sensing of mycobacteria. Upon infection with *M. smegmatis* or *M. bovis BCG*, macrophages from knock-in mice harboring R753Q TLR2 expressed lower levels of TNF-α, IL-1β, IL-6 and IL-10 compared to cells from wild-type mice but exhibited comparable bacterial burdens. The decreased cytokine responses in R753Q TLR2-expressing macrophages were accompanied by impaired phosphorylation of IRAK1, p38 and ERK1/2 MAPKs and p65 NF-κB, suggesting that the R753Q TLR2 polymorphism alters functions of the MyD88-IRAK-dependent signaling axis. Supporting this notion, HEK 293 cells stably transfected with YFP-tagged R753Q TLR2 showed reduced recruitment of MyD88 to TLR2, decreased NF-κB activation and impaired IL-8 expression upon exposure to *M. smegmatis*. Collectively, our results indicate that the R753Q polymorphism renders TLR2 signaling-incompetent, leading to an impaired MyD88-TLR2 assembly, phosphorylation of IRAK1, activation of MAPKs and NF-κB and deficient induction of cytokines in macrophages infected with *M. smegmatis* 

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#### Identification and characterization of adenosine deaminase 2 variants in pediatric vasculitis

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**Background** Deficiency of adenosine deaminase 2 (DADA2) is a recently recognized, autosomal recessive genetic disease. Patients present with various, early-onset systemic vascular and inflammatory manifestations, and often recurrent strokes. The clinical features and histological findings of DADA2 overlap with those of early childhood onset polyarteritis nodosa (PAN), a primary "idiopathic" systemic vasculitis, characterized by necrotizing inflammatory lesions of small and medium-sized vessels. Despite similar clinical presentation, individuals with PAN and DADA2 may benefit from different therapy. While treatment of primary chronic vasculitis is usually with toxic immunosuppressive drugs, there has been clinical indication that DADA2 patients respond better to less toxic IL-6 receptor antagonists and anti-TNF $\alpha$  therapy. We aimed to screen patients with PAN, cutaneous PAN, unclassifiable phenotype, or chronic vasculitis of any type with onset-age less than 5 years for variants in adenosine deaminase 2 (*ADA2*).

**Methods** Of the 493 pediatric patients included in our international, multi-ethnic cohort, ARChive (A Registry of Childhood Vasculitis), there were 99 patients who provided DNA samples, and 41 of these fulfilled screening criteria. We sequenced the coding exons of *ADA2* in these 41 patients. Identified variants in *ADA2* were characterized by quantifying their effect on ADA2 expression, secretion, and enzymatic activity by qPCR, standard ELISA, and kinetic colorimetric assays, respectively. RNA sequencing of whole blood was done to enable transcriptomic profiling of patients with DADA2 versus PAN and other types of chronic vasculitis.

**Results** We have identified variants in ADA2 with known and novel association with DADA2; four patients were found to be homozygous or compound heterozygous for rare (MAF < 0.01), predicted pathogenic variants. An additional two patients were found to be heterozygous for rare, missense variants; further analysis of expression and enzymatic activity may guide the identification of additional pathogenic variants that could contribute to the observed phenotype.

**Conclusion** At present, the gold standard for direct diagnosis of vasculitis is histopathological examination of biopsy specimens from involved organs. Screening *ADA2* among patients with possible early-onset chronic vasculitis or PAN phenotype may identify and diagnose patients, perhaps without requirement for biopsy. Early diagnosis of DADA2 patients may spare them treatment with toxic systemic immunosuppressive drugs, and allow more effective intervention with targeted biologic and/or gene therapy.

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### Cross-talk between two DNA sensing pathways in human plasmacytoid dendritic cells

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Plasmacytoid dendritic cells (pDCs) function as the most potent producers of type-I and type-III interferons in the human body and they play key roles in antiviral immunity. The innate sensing of viral nucleic acids through tolllike receptor (TLR)7 and -9 remains the major initiating factor for interferon production by pDCs. TLR-mediated sensing of nucleic acid via the endosomal pathway has been studied and documented in detail. However, some pathogens do not enter the pDC through the endosomal pathway and may be delivered directly to the cytoplasm or may infect the pDC, creating cytoplasmic replication intermediates, including dsDNA.And yet, for the most part, the sensing of DNA in the cytosolic compartment in human pDCs remains unexplored. Here, we demonstrate the existence and functionality of the components of cytosolic DNA sensing pathway comprising of cGAMP synthetase (cGAS) and stimulator of interferon gene (STING) in human pDCs. Both cGAS and STING were found to be transcribed and translated in primary human pDCs as determined by qPCR and both traditional and imaging flow cytometry. cGAS was found to be located in the cytosolic compartment of pDCs and to time-dependently colocalize with non-CpG double-stranded DNA (dsDNA), the initiating signal for this pathway. Upon co-localization of dsDNA with cGAS, the downstream pathway was triggered in pDC as STING was seen to be dissociated from its location at the endoplasmic reticulum. Upon direct stimulation of pDCs by the STING agonist 2'3' cyclic AMP-GMP (cGAMP) or dsDNA, pDCs produced type-I and type-III interferon, albeit at much lower levels than typically triggered by viruses through the TLR7/9 pathway. We have also documented via flow-imaging experiments that cGAS/STING-mediated interferon production is mediated by nuclear translocation of IRF3 while TLR9 and -7mediated activation in response to viral nucleic acid stimulated operates via IRF7 without involvement of IRF3. Our data also indicate that pre-stimulation of the cGAS/STING pathway in pDCs dampens a subsequent TLR9mediated response to herpes simplex virus (HSV), resulting in much lower levels of IFN-a production than seen with HSV alone. Thus, our study indicates that the cGAS-STING pathway exists in parallel to the TLR9-mediated DNA recognition in human pDCs with some possible cross-talk between these two pathways.

#### Understanding the regulation of virulence in Staphylococcus aureus during innate immune evasion

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Staphylococcus aureus (S. aureus) is a prevalent human pathogen responsible for a range of diseases spanning from superficial skin and soft-tissue infection to life-threatening septicemia. S. aureus tightly regulates the production of major virulence factors by sensing and adapting to its surroundings. This regulation is, in part, orchestrated by twocomponent gene regulatory systems (TCS). S. aureus has been shown to up-regulate a specific TCS, namely the SaeR/S system, during neutrophil phagocytosis. Previous work has demonstrated an essential role for SaeR and SaeS in evading innate immunity. In contrast, essentially nothing is known about how accessory genes saeP and saeQ influence S. aureus pathogenesis. Herein, we show a potential role for saeP within both in vitro and in vivo host-pathogen systems. Using a clinically-relevant wild-type S. aureus LAC strain (USA300) and its isogenic mutant LAC $\Delta$ saeP, our results show a potential regulatory role for SaeP. To begin characterizing the role of these gene products, we utilized *in vitro* phenotypic neutrophil assays, as well as, an *in vivo* mouse skin infection model. Compared to wild type LACUSA300, our data suggest the LACΔsaeP mutant has increased survival after neutrophil phagocytosis. Additionally, this mutant demonstrates increased cytolytic activity towards neutrophils. We hypothesize that the LAC $\triangle$ saeP mutant has increased toxin production. Initial studies support this idea and demonstrate increased transcript abundance of lukG/H, a predominant neutrophil cytolytic factor, in the LAC $\Delta saeP$ mutant compared to LAC. We also show an increased production of virulence factors important in mouse skin infection, manifesting as increased skin abscess size from LAC $\Delta saeP$  infection compared to LAC. These preliminary findings suggest SaeP may be involved in regulation of S. aureus pathogenesis by controlling overexpression of key virulence factors.

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#### Plasma interleukin 10 concentrations in Standardbred racehorses by means of a novel validated ELISA Jinwen Chen<sup>1</sup>, Zibin Jiang<sup>1</sup>, Larry R. Soma<sup>1</sup> and Mary A. Robinson<sup>1,2</sup>

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This studyaimed to assess plasma interleukin 10 (IL-10) concentrations in Standardbred racehorses by means of a novel ELISA following validation of the assay for use with equine plasma samples. Plasma samples were obtained from 23 Thoroughbreds for use in assay validation and from 318 Standardbred racehorses at rest 2 to 2.5 hours prior to warm-up and racing. The sandwich ELISA method was developed with equine anti-IL-10 polyclonal antibody and biotin-streptavidin chemical interaction to enhance sensitivity. The assay was validated for specificity, sensitivity, precision, and accuracy by use of both recombinant and endogenous proteins. The assay cross-reactivity with other human and equine cytokines was very low or absent (<0.1%) and serial dilution of plasma samples resulted in proportional decreases in reactivity, both indicating high specificity of the method. Partial replacement of detection antibody with capture antibody caused assay signals to significantly decrease by 46%. The inter- and intra-assay precisions were <12.9% and< 10.6%, respectively; inter- and intra-assay accuracies were within ranges of  $\pm$  18.2% and  $\pm$  11.7%, respectively, from 94 to 6,000 pg/mL, and the sensitivity was 21 pg/mL. Plasma IL-10 concentrations varied widely among the 318 Standardbreds at rest (range, 0 to 430 ng/mL; mean, 3,806 pg/mL; median, 382 pg/mL). ELISA method proved suitable for quantification of IL-10 in equine plasma samples. Plasma IL-10 concentrations in the Standardbred racehorses were high (> 10 ng/mL) in 13% of the horses, which warrants further investigation. Key words: horse, interleukin 10, ELISA, plasma concentrations

### Human gastric epithelial cells recruit dendritic cells for luminal H. pylori uptake in a novel gastric organoid co-culture model.

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<u>Background & Aims</u>: Gastric dendritic cells (DCs) control the adaptive response to infection with *Helicobacter pylori*, the causative agent for gastritis, peptic ulcer disease and gastric cancer. We hypothesize that DC interactions with the gastric epithelium position gastric DCs for uptake of luminal *H. pylori* and promote DC responses to epithelial-derived mediators, thereby impacting the outcome of *H. pylori* infection. The aim of this study was to develop and evaluate a co-culture model based on primary human gastric epithelial cells and DCs to study the mechanisms of gastric DC – epithelial cell interactions *in vitro*.

<u>Methods</u>: Human gastric organoid lines were derived from adult human gastric mucosal samples following standard protocols and were maintained as 3-dimensional spheroids in a collagen matrix. *H. pylori* infection was established by luminal microinjection of the gastric organoids. To set up co-cultures, immature human monocyte-derived DCs were added to gastric organoid cultures. We then analyzed DC interactions with the organoids by live imaging, confocal microscopy and electron microscopy. Digital image analysis and particle tracking were used to quantify DC recruitment.

<u>Results</u>: Human monocyte-derived DCs co-cultured with gastric organoids spontaneously migrated through the Matrigel matrix to associate with the basolateral side of the gastric epithelium. *H. pylori* infection of the organoids significantly increased epithelial chemokine expression, leading to enhanced DC recruitment. Recruited DCs established tight interactions with the basolateral side of the gastric epithelium, closely representing the *in vivo* situation. Moreover, migrated MoDCs extended dendrites between the gastric epithelial cells and acquired *H. pylori* bacteria injected into the organoid lumen.

<u>Conclusions</u>: The present study shows that gastric organoid – DC co-cultures closely model the *in vivo* situation in that DCs respond to epithelial cell signals, form tight interactions with the epithelial layer and acquire bacteria from the spheroid lumen. Our primary cell co-culture model is suitable for studies of gastric infection and epithelial-immune cell interactions and will yield important insights into human gastric immunobiology.

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#### Molecular mechanisms underlying the evolution of the slp76 signalosome

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The well-defined mammalian slp76-signalosome is crucial for T-cell immune response, yet whether slp76-signalosome exists in invertebrates and how it evolved remain unknown. Here we investigated slp76-signalosome from an evolutionary perspective in amphioxus Branchiostoma belcheri (bb). We proved slp76-signalosome components bbslp76, bbGADS and bbItk are present in amphioxus and bbslp76 interacts with bbGADS and bbItk, but differences exist between the interaction manners within slp76-signalosome components of amphioxus and human (h). Specifically, bbslp76 has a unique WW-domain blocked its association with hItk and decreased TCR-induced tyrosine-phosphorylation and NFAT-activation. Deletion of WW-domain shifted the constitutive association between bbslp76 and hPLCy1 to a TCR-enhanced association. Among slp76-signalosome, the interaction between slp76 and PLC<sub>γ</sub>1 is the most conserved and the binding between Itk and slp76 evolved from constitutive to stimulation-regulated. Sequence alignment and 3D structural analysis of slp76-signalosome molecules from keystone species indicated slp76 evolved into a more unfolded and flexible adaptor due to lack

of WW-domain and several low-complexity-regions (LCRs) while GADS turned into a larger protein by a LCR gain, thus preparing more space for nucleating the coevolving slp76-signalosome. Altogether, through deletion of WW-domain and manipulation of LCRs, slp76-signalosome evolves from a rigid and stimulation-insensitive to a more flexible and stimulation-responding complex.

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#### Dendritic cell and monocyte immune tolerance following coronary artery bypass graft surgery

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**Introduction** Coronary artery bypass grafting (CABG) is a common cardiac surgical procedure which triggers an overall systemic inflammatory response that is thought to contribute to poor patient outcomes including infection. Dendritic cells (DC) and monocytes play a central role in the initiation and regulation of the immune response and changes in their functional capacity may contribute to poor patient outcomes post-CABG. Immune tolerance is a form of innate memory which renders immunoregulatory cells less responsive to a secondary insult. Immune tolerance has been well described in sepsis patients (i.e. non-sterile inflammation. In contrast, immune tolerance in the setting of surgery (i.e. sterile inflammation), has been poorly described, and it may be a contributor to the increased risk of infection reported post-cardiac surgery. Therefore, this study aimed to investigate immune tolerance in CABG patients by characterising changes in DC and monocyte activation status and production of inflammatory mediators.

**Method** Blood samples (EDTA) were collected from CABG patients (n=33) at five time-points (admission, intraoperative, ICU, day (D) 3, D 5). Blood from each patient at each time-point was mixed with RPMI culture media and incubated (37°C, 5% CO<sub>2</sub>) for 6 hours. Leucocytes were harvested and flow cytometry was used to measure surface markers and intracellular cytokine and chemokine expression. In parallel, lipopolysaccharide (LPS) was added to the culture to model responses to a secondary insult (i.e. a bacterial infection). A repeated measure one-way ANOVA with Dunnett's post-test was used for the analysis of the percentage positive cells detected by flow cytometry with the admission sample used as the comparator baseline. P values <0.05 were considered significant.

**Results and Discussion** Significant suppression of DC and monocyte activation/adhesion markers were observed post-CABG in these patients. Expression of CD9, CD38, CD40, CD80, CD80 on DC and CD9, CD38, CD40, CD80, CD86 monocyte expression was significantly suppressed compared to admission, particularly in the surgery and ICU samples (all P<0.05). In the case of CD9, CD40, CD80, CD83 on DC and CD38, CD80, CD86 on monocytes, the suppression was transient and recovered to baseline levels by D5 post-CABG. In contrast, expression of CD83 on monocytes was increased over the entire post-operative period. CABG also resulted in suppression of DC and monocyte intracellular inflammatory responses: IL-10, MIP-1α, IL-8, IL-12, MCP-1, IL-1α, IP-10, IL-6, MIP-1β in DC and IL-10, MIP-1α, IL-8, MCP-1, IL-12, IL-6, TNF-α, IL-1α, MIP-1β in monocytes (all P<0.05). This suppression persisted to D5 post-CABG.

In the parallel cultures where LPS was added to model CABG patient responses to a secondary insult, there was evidence of immune tolerance post-CABG. Patient DC and monocytes were less responsive to LPS stimulation for all aforementioned activation/adhesion markers as well as for all intracellular inflammatory cytokine and chemokine responses measured (all P<0.05). The evident immune tolerance was transient for CD83, CD40 on DC and for all monocyte surface markers except CD40, recovering by D5. In contrast the immune tolerance persisted to D5 post-CABG for all other DC markers and for DC and monocyte intracellular inflammatory responses.

Conclusions These data demonstrate that CABG supressed DC and monocyte activation/adhesion marker expression and inflammatory mediators. Furthermore, CABG resulted in the development of immune tolerance, as demonstrated by the reduced ability of patient DC and monocytes to respond to LPS as a secondary insult following CABG. Despite some recovery, the majority of these changes were still apparent at D5 post-CABG, indicating impaired functionality and immune tolerance does not resolve quickly. Such immunosuppression and immune tolerance post-CABG may predispose patients to increased risk of infection and contribute to poor patient outcomes following cardiac surgery.

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Comparison of Gene Segment Usage in Complementarity-Determining Regions of Unchallenged Mice Bailey A. Bye<sup>1</sup>, Trisha A. Rettig<sup>1</sup>, Michael J. Pecaut<sup>2</sup> and Stephen K. Chapes<sup>1</sup>

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The immune system responds to antigen in a number of ways, including a genetic rearrangement process that is part of the adaptive response called somatic recombination. This process produces unique immunoglobulin (Ig) molecules in B-cells. In the Ig heavy chain, much of the genetic diversity caused by somatic recombination occurs in complementarity-determining region three (CDR3), which is composed of a portion of the variable (V), all of the diversity (D), and a portion of the joining (J) gene segments. The Ig kappa chain does not contain a D-gene segment. These three gene segments are cut and rearranged with n- and p-nucleotide additions to produce an immunoglobulin molecule capable of binding a specific antigen with the greatest affinity possible. We investigated the diversity of CDR3 segments of unchallenged mouse B cells that express the same V-, D-, and J-gene segment utilizing high-throughput sequencing and nucleotide sequence alignment methods. Splenic RNA was obtained from three independent groups of four 9- to 11-week-old female C57BL/6J mice housed in specific pathogen-free environments and sequenced using Illumina MiSeq. Sequencing results were imported into CLC genomics workbench (http://www.clcbio.com), quality cleaned, and potential antibody sequences were isolated. These sequences were then imported into the ImMunoGeneTics (IMGT) High V-Quest tool for analysis. After IMGT analysis, productive antibody sequences were imported into CLC again to determine constant region identity. Microsoft Excel was used for V-/D-/J-gene combination analyses. One heavy and kappa chain V/D/J combination displaying a CDR3 region of variable nucleotide length was selected from the 15 most common gene combinations among the three mouse pools and aligned to its germ line nucleotide sequence using the MAAFT alignment program. From each full alignment, three nucleotide sequences of different length were isolated and compared. In the Ig heavy chain, although each sequence was encoded by the same V-, D-, and J-gene segment, gene representation across the entire sample set was variable. The kappa chain alignments exhibited less sequence variability than the heavy chain alignments. Overall, heavy chain CDR3 length was dependent on D-gene segment representation. D-gene segment representation decreased in correlation with decreased CDR3 length. In the Ig the heavy chain, the V-gene segment appeared to vary the least of the V-/D-/J-gene contributors, while, in the kappa chain, V- and J-gene segment usage appeared to be equally uniform. These results suggest somatic recombination in the heavy chain can cause V-/D-/J-gene segment representation to differ widely within the same gene combination, while the kappa chain remains more homogenous overall. This work was supported by NASA grants NNX13AN34G and NNX15AB45G, NIH grant GM103418, the Molecular Biology Core supported by the College of Veterinary Medicine at KSU, and the KSU Johnson Cancer Research Center.

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Characterization and consensus of the conventionally housed C57BL/6J antibody repertoire

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Each B cell produces a single immunoglobulin (Ig) idiotype composed of two identical heavy and two light chains. The heavy chain consists of single variable (V), diversity (D), and joining (J) gene segments coupled to one of five

constant (C) regions. The light chain is encoded by one of two separate loci, kappa or lambda, and consists of single V and J gene segments coupled to a constant region. These gene segments are recombined randomly during B cell differentiation and differences in gene segment usage, random nucleotide additions, splicing differences, and subsequent mutations generate a unique population of B cells and antibodies, termed the antibody repertoire. While antibody repertoires have traditionally been characterized using amplification of immunoglobulin segments, it is well understood that there are issues with this methodology that can influence the obtained repertoire data. To avoid amplification biases, we characterized the unamplified antibody repertoire of C57BL/6 mice

Total splenic RNA was collected and pooled from three groups of four female, conventionally housed, specific pathogen free, unimmunized, unchallenged C57BL/6J mice, nine to eleven weeks of age. The RNA was size selected and sequenced on the Illumina Mi-Seq platform without antibody specific amplification. We analyzed the V, D, J, and C gene segment usage of the heavy (IgH) and kappa-light (Igk) chain B cells present in whole splenic tissue.

We recovered between 22,985 and 39,096 IgH sequences and between 25,463 and 51,852 for Igκ. Between 8,714 and 11,200 sequences were labeled productive by IMGT for IgH and 12,199 and 15,111 for Igκ. The remaining sequences did not have enough information to determine functionality and are considered "unknown".

For IgH we found that a global level, V, D, J, and C usage showed high levels of correlation ( $R^2$  of V gene segment usage=0.8481 to 0.5842, p<0.0001) among pools and this trend was mirrored Igk for V and J ( $R^2$ =0.6848 to 0.4701, p<0.0001). When the randomness of V gene usage was assessed, we found a non-random distribution of frequencies along the genome for IgH and Igk (p<0.0001). We analyzed the use of constant regions for IgH and determined that IgM was the most common. We also examined the V, (D), J combinations and found low to moderate levels of correlation of V-J pairings for heavy-chain ( $R^2$ =0.5547 to 0.3933, p<0.0001) and kappa chain ( $R^2$ =0.4543 to 0.2319, p<0.0001).

We have shown that in the conventionally housed, specific pathogen free, C57BL/6J mouse, gene segment usage for heavy and light chain is correlated across pools, however the V/J pairings are more unique across pools; emphasizing the uniqueness of antibody generation even among inbred animals. Furthermore, this characterization of an unamplified dataset will allow for additional RNA-Seq analyses by our lab and provide a basis for other studies including comparisons to immunized animals.

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## Identification of S100A14 as a Potential Mechanism of Innate Immune Resistance in HIV-exposed Seronegative Injection-drug Users (HESN-IDU)

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Despite practicing needle-sharing behavior in areas of high HIV-1 prevalence, some injection-drug users remain sero-negative despite many years of high-risk activity (HESN-IDU). Previously, we observed that HESN-IDU had approximately 3.5 times more activated DCs and NK cells when compared to no-risk control donors or low-risk non-sharing injection-drug user controls (NS-IDU). To determine the potential mechanisms of NK activation in the HESN-IDU that may lead to immune resistance, we examined the proteomic profile of NK cells in HESN-IDU and control donors. Proteomic analysis of NK cells from HESN-IDU subjects revealed significantly higher expression of interferon-related proteins and proteins from the S100 family compared to controls. S100A14 was the proteins with the highest fold change and lowest p-value. S100A14 can be secreted and therefore represents a potentially immuno-modulatory protein. We investigated if S100A14 was secreted into the plasma of subjects from our cohort

and observed that it was indeed present at significantly higher levels in the plasma of HESN-IDU subjects (n=15) when compared to non-sharing IDUs (n=15, p>0.05) or control donors (n=10, p>0.01). To assess if S100A14 activates NK cells in normal PBMCs, we measured by flow cytometry the surface expression of CD69 after overnight stimulation. When added to PBMCs from control donors, recombinant S100A14 activated NK cells (n=12, p>0.01) and it was in a dose dependent fashion. Activation of NK cells was absent when isolated NK cells were incubated directly with S100A14 (n=3), but in co-culture with monocyte, activation was observed. S100A14 stimulated production of TNF-alpha after 5hrs in monocytes in a PBMC mixture (n=7, p>0.05) as well as in isolated monocytes (n=5, p>0.05), suggesting monocytes are important in the S100A14-mediated activation. Together, this data identifies S100A14 as a novel NK stimulatory protein that crosstalk to monocytes and may be part of the resistance phenotype observed in HESN-IDU subjects.

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#### Efficient enrichment of functional ILC subsets from human PBMC by immunomagnetic selection

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Innate lymphoid cells (ILCs) are exceedingly rare but important regulators of homeostatic and disease-associated immune processes. These cells are resident in barrier organs and their frequency and relative proportions change dramatically from tissue to tissue. The frequency of ILCs in peripheral blood of healthy humans is approximately 0.07% of CD45<sup>+</sup> leukocytes. However, this frequency is highly variable between individuals. ILCs promptly produce effector cytokines in response to local stimulatory signals that modulate the surrounding cells. ILCs are divided into distinct subsets (ILC1, 2 and 3) based on their differential expression of effector cytokines and master transcription factors.

Studying ILCs is challenging due to their low frequency and their lack of specific cell surface marker expression for isolation. Currently, fluorescence activated cell sorting is the only effective method to isolate ILCs, but sorting rare cells is time-consuming, expensive and often results in low purities and recoveries. Pre-enrichment of ILCs would allow for reduced sorting times and improved purities for all three subsets. Accordingly, we have developed a fast immunomagnetic negative selection method to pre-enrich all ILCs from human leukapheresis samples. Briefly, unwanted cells are labelled with antibodies and magnetic particles and placed into an EasySep<sup>TM</sup> magnet. Unwanted cells are retained in the magnet and the enriched ILC fraction is simply poured off into a new tube. These cells are now ready for downstream applications. We find that total ILCs (defined as Lineage CD45 CD127) are enriched from an initial frequency of 0.01-0.23% (n=28) to a final frequency of 17-86%, an enrichment of 200-1500 fold with virtually no loss of ILCs. ILC1 (defined as Lineage CD45+ CD127+ CRTH2 CD117- CD161+/-) were enriched from 0.01-0.2% to 4.5-14%. ILC2 (defined as Lineage CD45 CD127 CRTH2 CD117 CD161) were enriched from 0.01-0.1% to 5.8-51% and ILC3 (defined as Lineage CD45+ CD127+ CRTH2- CD117+ CD161+) were enriched from 0.01-0.1% to a frequency of 6-16% (n=28).

This pre-enrichment takes only 21 minutes and drastically decreases sort time, allowing sorting over 3.7 x 10<sup>5</sup> ILCs from 2 x 10<sup>9</sup> PBMCs in only 12 minutes. In addition, we found that all subsets were functional; when stimulated, sorted ILC1s produced IFNy, ILC2s produced IL-13 and ILC3s produced IL-22. Our newly developed methods of pre-enrichment of ILC should aid human ILC research.

## Reduced chronic pain by rubber flooring alters leukocyte populations, phenotypes, and response to substance P of dairy cows

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Cows on concrete flooring (vs. rubber) have classical immunological indicators of chronic pain; increased PBMC numbers, more IL-1β, and lower IL-1Ra. We hypothesized that a profile of cell phenotypes and biomarkers on lymphocytes and monocytes could be associated with chronic pain and that responses to additional substance P (a neurotransmitter associated with pain) would be reduced. 1st-calf heifers were assigned to rubber or concrete flooring. On day -3, 7, 21, 45 and 90 of the 2nd lactation, 5-part differential counts of whole blood and buffy coat leukocyte marker expressions were analyzed. Buffy coat cells were further stimulated with substance P (SP) in vitro. Cells were labeled with CD4, CD8, CD3, CD14, WC-1 (χδ), and DEC-205 (dendritic cells, DC). Lymphocyte and monocyte cell populations were analyzed separately. Only % cells positive of d 90 of lactation 2 are shown here. Number and percentage of lymphocytes were greater for cows on concrete particularly on d 21. Monocyte counts were greater for cows on concrete than rubber on d 7 but the percentage of monocytes was greater for cows on concrete on d 44. However, neutrophil % had a flooring by day effect such that rubber flooring caused a greater percentage of neutrophils on all but d 90. Neutrophil numbers were greatest for cows on rubber flooring on d 7 (P = 0.01). Lymphocyte numbers and percentages were greater for cows on concrete through d 21. Basophil numbers and percentages increased sharply at d 7 for cows on concrete; however, cows on rubber had a substantial increase on d 44. Eosinophils increased on d -3 and d 44 for cows on rubber, but decreased compared to those on concrete on d 7. Monocyte CD3 % was greater for cows on rubber and increased more with cows on rubber with SP stimulation (P < 0.001). CD14 and CD4 % were greatest for cows on rubber and with SP stimulation they each decreased for cows on concrete, but increased for cows on rubber (P < 0.001). In contrast, CD8 % was greater for cows on rubber flooring, but only tended (P = 0.06) to show the same pattern. CD14 % was greatest on the monocyte population and had a floor \* SP effect (P < 0.001). The response to substance P was opposite for concrete and rubber flooring housed cows, decreasing for those on concrete and increasing for those on rubber. The WC1 marker only responded to SP stimulation in both flooring treatments by increasing the percentage of positive cells (P < 0.01). The dendritic cell marker had greater percentages with cows on the rubber flooring (P < 0.002). Lymphocytes did not have altered CD14. The percentage of cells with CD3 expression was greater (P < 0.001) for cows on rubber, but both responded positively to the SP stimulation. CD8 % was greater (P < 0.001) for cows on rubber, but did not respond differently to SP. In contrast CD4 % positive cells was greater for cows on concrete and decreased with SP stimulation, but increased with stimulation for cells from the cows on rubber flooring (P < 0.01). WC1 percentages were not affected by flooring or by SP treatment. Dendritic cell positive cells were greater for cows on the rubber flooring, and the percentage increased with SP, but did not change for the cows that were housed on concrete (P < 0.01). These results showed that leukocyte populations and their phenotypes that enable functions are altered by concrete flooring and that responses to additional SP are altered in cows under those housing conditions. These results will help us to identify a minimally invasive way to determine housing conditions that may be reducing well-being of cattle.

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### Establishment of non-resolving low-grade inflammatory monocytes due to autophagy disruption by super-low dose LPS

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Chronic inflammation is an underlying feature of a variety of highly prevalent diseases such as diabetes, atherosclerosis, inflammatory bowel disease, and cancer. Subclinical endotoxemia has emerged as a common factor correlated with these diseases, potentially contributing to the establishment of chronic inflammation by programming monocytes to a non-resolving inflammatory state. We observed that injection of subclinical dose LPS

exacerbated atherosclerosis as reflected in elevated aortic lipid deposition, enlarged necrotic core, and reduced plaque collagen content. Adoptive transfer of *in vitro* programmed monocytes by subclinical dose LPS rendered the similar effect on atherosclerosis. At the mechanistic level, we found that super-low dose LPS programs the non-resolving low-grade inflammatory monocytes/macrophages through TRAM-dependent disruption of autophagy completion, and accumulation of inflammatory mediators such as p62 and Pellino-1. Treatment with an autophagy enhancer such as LiCl can attenuate the effects of low-dose endotoxin by reducing the levels of Pellino1. Together, our data suggest that super-low dose LPS may preferentially program a non-resolving inflammatory state conducive to the pathogenesis of chronic disease, through disruption of autophagy and cellular homeostasis.

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#### Rab17 mediates differential antigen sorting following efferocytosis and phagocytosis

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**Introduction:** Macrophages engulf pathogens (phagocytosis) and apoptotic cells (efferocytosis). Both phagocytosis and efferocytosis share a common degradative pathway in which the target is engulfed into a membrane-bound vesicle, respectively termed the phagosome and efferosome, where they are degraded by fusion with endosomes and lysosomes. However, despite this shared maturation pathway, macrophages are immunogenic following phagocytosis but not efferocytosis, indicating that differential processing or trafficking of antigens must occur. The maturation of phagosomes is regulated primarily by the Rab family of GTPases. However, it is unknown whether Rab GTPases play a similar role in efferosome maturation and how efferocytosis maintains immunological silence.

**Hypothesis:** We hypothesized that Rab GTPases regulate the maturation of efferosomes, and that differences in the recruitment of specific Rab GTPases to efferosomes are responsible for differences in cargo processing and antigen trafficking in efferocytosis.

Materials and Methods: To determine whether Rab GTPases regulated efferosome maturation, we utilized live cell microscopy of macrophages ectopically expressing Rab5 and Rab7 as they engulfed mimics of pathogens or apoptotic cells to study dynamics of Rab recruitment. To identify Rab proteins that are differentially recruited to efferosomes, we performed mass spectrometry on efferosomes and phagosomes isolated using a magnetic bead-based pull-down assay. Mass spectrometry analysis identified Rab17 as being preferentially recruited to efferosomes. We studied Rab17 recruitment using both bead-based mimics, and then examined the fate of phagocytic and efferocytic cargo by utilizing immunofluorescence microscopy to examine the co-localization of cargo and Rab17 either with TfR—a marker of the recycling endosome compartment—or with MHC II. Finally, we created a dominant negative variant of Rab17 and studied the effects of ectopic expression of this variant on cargo trafficking. All statistics were performed using GraphPad Prism 6, and ANOVA with Tukey post hoc analysis was performed to determine statistical significance.

**Results:** We determined that Rab5 and Rab7, key regulators of phagosome maturation, were recruited to efferosomes in identical fashion to phagosomes. Mass spectrometry analysis revealed several proteins that were differentially recruited to efferosomes in comparison to phagosomes, with Rab17 being amongst these. Rab17 was found to be selectively recruited and retained on efferosomes. It was also found to co-localize selectively with vesicles containing apoptotic cells but not bacterial pathogens, and, significantly, to the recycling endosome compartment rather than the MHC II loading compartment. Expression of a dominant negative variant of Rab17 resulted in disruption of efferosome co-localization with recycling endosomes and resulted in mis-trafficking of efferocytic cargo to the MHC II loading compartment.

**Discussion and Conclusions:** Combined, these results indicate that macrophages prevent presentation of apoptotic cell-derived antigens by preferentially trafficking efferocytosed, but not phagocytosed, cargoes away from the MHC II loading compartment via the recycling endosome. Given that defects in efferocytosis result in the

development of autoimmune diseases such as SLE and rheumatoid arthritis, our results identify Rab17 as a potential therapeutic target in the treatment of these diseases.

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#### Alcohol Increases Susceptibility to Enteropathogens in Mice with DSS-Induced Colitis

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Ulcerative colitis (UC), one of the two main forms of Inflammatory Bowel Disease (IBD), is a cyclical, life-long disease characterized by disease remission and active disease flares. Since no cure exists, UC patients experience symptoms of abdominal pain, increased weight loss, intestinal inflammation, and rectal bleeding. Recently, patients presenting with an IBD diagnosis reported increases in their IBD symptoms after drinking alcohol. Alcohol alone not only induces a systemic pro-inflammatory response, but can also be directly harmful to gut barrier integrity. We observed that alcohol exacerbates DSS-induced inflammation, weight loss, and colon shortening in mouse model of alcohol and DSS-induced colitis. For this study, we assessed whether mice receiving alcohol and DSS are more susceptible to the mouse enteropathogen, C. rodentium. Male C57BL/6 mice received DSS for 5 days to induce UC. On day 5, mice were divided into two groups: mice gavaged with alcohol (~3g/kg) or mice gavaged with water on days 5, 6, and 7. Three hours after the last gavage on day 7, mice were orally administered C. rodentium at  $1 \times 10^5$ CFUs. Body weight and mortality were regularly monitored over the course of the experiment. On day 11, mice were euthanized and colons harvested to measure length and the tight junction proteins claudin-2, 4, and 8 and occludin via qPCR. Additionally, colonic tissue sections were stained by H&E and alcian blue. C. rodentium infection in mice following DSS and alcohol treatment resulted in increased weight loss compared to those receiving DSS alone and C. rodentium. We observed a 50% reduction in survival in DSS alcohol treated mice following C. rodentium infection compared to 100% survival with DSS alone and C. rodentium. This accompanied a significant decrease (p<0.0001) in colon length in DSS alcohol treated mice following C. rodentium infection compared to DSS alone and C. rodentium. Mice infected with C. rodentium following DSS and alcohol treatment had significant decreases in both claudin 8 and occludin compared to all other groups. H&E staining revealed prominent colonic damage and inflammatory infiltrate in DSS alcohol plus C. rodentium infected mice. Hitstopathology scores were based on exudate, epithelial damage, polymorph nuclear leukocytes (PMNs) infiltration, necrosis, submucosal edema and crypt dilation and scored in a blinded fashion by a pathologist. We found statistically significant increases in histopathology scores in DSS alcohol and C. rodentium treated mice (p<0.001) compared to DSS alone and C. rodentium. Finally, upon analysis of the mucus layer via alcian blue staining, 8/12 mice in the DSS ethanol and C. rodentium group showed a demonstrable decrease in the mucus layer lining colonic epithelial cells along with a decrease in the appearance of goblet cells. Together, along with our previous findings, these data suggest alcohol increases susceptibility to enteropathogens in mice with DSS-induced colitis by further impairing intestinal defense mechanisms. (Support: R21AA022324, T32AA013527 and F31AA025536-01).

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## Neutrophil Binding To ICAM-1 Expressed On Both Endothelial Cells & Pericytes Contributes To Pericyte Dissociation & Development of Indirect ARDS

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Central to the development of extrapulmonary/indirect acute respiratory distress syndrome (iARDS) is loss of pulmonary vascular endothelial cell barrier integrity. We have previously shown that the Angiopoietin/Tie pathway plays a significant role in modulating endothelial cell (EC) activation. Angiopoietin(Ang)-1 and Ang-2 share affinity for the tyrosine kinase receptor, Tie2, on ECs; Ang-1 (synthesized by pericytes) and Ang-2 (stored preformed and released by activated ECs) function to maintain vascular homeostasis. Ang-1/Tie2 binding is associated

with Tie2 phosphorylation and a quiescent vasculature, and promotes an anti-inflammatory, pro-survival, and stable phenotype, while Ang-2, found elevated in plasma from ARDS patients, is associated with decreased barrier function, increased inflammation and micro-vascular leakage when bound to Tie2. We have shown that the Angiopoietin/Tie signaling pathway is mediated through direct EC/neutrophil interaction. In our murine hemorrhagic shock/sepsis double hit model for the development of iARDS, depletion of peripheral blood neutrophils significantly reduces indices of ARDS and in *in vitro* experiments direct EC/neutrophil interaction is critical for EC activation and release of Ang-2. To further investigate the mechanisms contributing to EC loss of barrier function, in this study we focused on the role of pulmonary vascular pericytes. Pericytes are cells of vascular smooth muscle cell lineage that form a single cell sheath around microvascular endothelial cells providing stability and anchoring to the vascular basement membrane. In addition to EC/pericyte interactions (through PDGFb/PDGFR-b interaction for example), neutrophils migrating from the blood to tissue interact with both ECs and their associated pericytes. The significance of these interactions and their contribution to EC function has not been well characterized. In this study we show that, in isolated mouse lung ECs, hemorrhage-primed neutrophil interaction with ICAM-1 decreases PDGF-beta release into culture supernatant. In addition, when hemorrhageprimed neutrophils are added to isolated mouse lung pericytes a significant decrease in PDGFR-beta is observed. These findings suggest that through ICAM-1 binding, hemorrhage-primed neutrophils contribute to EC loss of barrier function by decreasing the stabilizing impact of EC/pericyte interactions and decreasing Ang-1/Tie2 binding. (JLN Research supported by NIH-NIGMS GM103652)

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## Epigenetic Programmer SIRT1 Bridges Innate and Adaptive Immunity during Acute Inflammation from Sepsis.

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Profound and persistent defects in both innate and adaptive immunity often develop within hours of the onset of acute systemic inflammation from sepsis, and contribute to its high mortality by promoting persistent and acquired bacterial and fungal infection and reactivating latent viruses. We previously reported that the switch from activated to deactivated monocytes during sepsis in mice and humans depends on nuclear NAD+ sensing by epigenetic programmer Sirtuin 1 (SIRT1), which reversibly creates silent heterochromatin at pro-immune gene promoters and that inhibiting SIRT1 pharmacologically during sepsis immunosuppression reverses heterochromatin structure to competent euchromatin in monocytes and prevents death in septic mice. Here, we show that SIRT1 bridges innate and adaptive immune reprogramming during sepsis by concomitantly reprogramming CD11c dendritic and CD4+ T-cell polarity. To develop this unifying concept, we administered the SIRT1 specific inhibitor EX527 or DMSO control intraperitoneally after sepsis transitioned to immunosuppression 24 h after inducing sepsis in a standardized 60% lethal model by cecal ligation and puncture (CLP), and assessed polarity in isolated splenocytes by flow cytometry at 6 and 24 h. We found: 1) reduced sepsis-induced increases in the proportion of CD4+ Foxp3+ CTLA4+ T regulatory (Treg repressor type) vs. non-repressor CD4+ T cells, without observing a significant effect on the number of Treg repressor cells; 2) decreased expression of IL-10 and TGFb proteins and increased expression of interferon g in CD4+ T-cells; 3) decreased expression of CD11c+ CD80+ antigen-receptor repressor and increased expression of IL12p40 and TNFa protein in CD11c+ dendritic cells; 4) no significant effect on sepsisinduced decreases in total splenic CD4+ T cells and CD11c+ dendritic cells; and 5) decreased plasma IL17 protein levels without finding evidence for IL17 producing CD4+ T cells in the spleen. Taken together with our reports that NAD+-dependent SIRT1 controls innate immune monocyte effector and repressor polarity by regulating the acetylation state of gene-specific nucleosome histones and NFkB p65 transcription activator, we suggest that persistent increases in NAD+ and SIRT1 activation during sepsis epigenetically sustain both innate and adaptive immune repressor polarity, which can be reversed by SIRT1 targeting to improve sepsis prognosis. (Supported by NIH R01 AI065791 and R01 GM102497 (cem) and R01GM099807 (vv).

Staphylococcus aureus-induced neutrophil-derived ectosomes alter neutrophil-Staphylococcus interaction Benjamin S. Avner $^{1,2,3}$  and William M. Nauseef $^{1,2,3}$ 

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Ectosomes - also called plasma membrane-derived microparticles/microvesicles - are membrane-bound vesicles of  $0.1\text{-}1~\mu\text{m}$  in diameter shed from the plasma membrane of intact cells, which transport protein and lipid signals between cells. Ectosomes derived from neutrophils (PMN) have been isolated from the bloodstream of septic patients, leading to speculation of a role in regulating inflammation. PMN ectosomes triggered by phagocytosis of SA (hereafter PMN-SA ectosomes) are produced within 20 minutes of phagocytosis of SA. Depending on their source, different types of PMN ectosomes have been described as having either pro- or anti-inflammatory effects on other cell types. To date, signaling between PMN by means of PMN ectosomes is largely uninvestigated.

We generated PMN-SA ectosomes from human PMN fed opsonized USA300 LAC (a community-associated strain of methicillin-resistant SA) at 10:1 MOI. Phagocytosis was allowed to proceed for 10 min, after which we centrifuged at 500 x g to isolate PMN, and resuspended and incubated at 37°C for 20 min to allow ectosomes to be generated. We isolated the ectosomes by centrifuging first at 4,000 x g to remove intact bacteria and PMN, and then ultracentrifuging the supernatant at 200,000 x g in a swinging-bucket rotor to sediment and concentrate the ectosomes.

Fresh human PMN in suspension (10M cells/mL) were incubated together with varied concentrations of PMN-SA ectosomes (0.2 to 20  $\mu$ g/mL). To determine if ectosomes alter phagocytosis of SA by PMN, we first incubated PMN with ectosomes at 37°C for 5-20 min. We then added opsonized GFP-labelled USA300 LAC SA at 1:1 MOI along with 10  $\mu$ M diphenyleneiodonium (DPI), a flavoenzyme inhibitor used to inhibit the NADPH oxidase and prevent bleaching by HOCl generated in PMN phagosomes. Suspensions were tumbled for 10 min at 37°C to promote phagocytosis. We centrifuged suspensions at 500 x g to isolate PMN and used flow cytometry to quantitate phagocytosis of the GFP-labelled SA by the PMN. Treatment of PMN with PMN-SA ectosomes promoted a concentration-dependent increase in phagocytosis. A 10-min pre-treatment with 20  $\mu$ g/mL of PMN-SA ectosomes increased phagocytosis, with 6.1  $\pm$  2.7% more PMN displaying fluorescent signal (n = 4, p-value 0.02). As little as 5 min of pre-treatment with ectosomes was sufficient to augment phacotytosis of SA by PMN, while pre-treatment times of up to 20 minutes did not increase the magnitude of this effect.

When PMN-SA ectosomes and SA were combined with PMN simultaneously, ectosomes did not increase phagocytosis of SA (change of  $2.1 \pm 2.0\%$ , n = 3, p-value 0.21). Since PMN-SA ectosomes promoted increased phagocytosis with pre-treatment but not co-treatment, we speculate that they had a priming effect on PMN to promote phagocytosis. Ongoing studies will explore the contribution of priming to the observed effects on phagocytosis as well as the possibility that direct interactions between ectosomes and SA when both were presented simultaneously interfere with the ability of ectosomes to alter PMN responses.

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## The role of neutrophils and extracellular adenosine in shaping age-driven susceptibility to Streptococcus pneumoniae lung infection

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Streptococcus pneumoniae (pneumococcus) remain a leading cause of life-threatening infections such as pneumonia, bacteremia and meningitis in the elderly. Neutrophils are innate immune cells that are key determinants of disease following infection because their initial presence is required to control bacterial numbers, but their

persistence in the lungs can lead to tissue destruction and bacterial spread. We previously found that extracellular adenosine (EAD) production was crucial for PMN antibacterial function, resolution of pulmonary inflammation, and host resistance following pneumococcal pneumonia. EAD is produced by the sequential action of two exonucleosidases, CD39 and CD73, and can signal via four known receptors, i.e. A1, A2A, A2 and A3, that can be pro- or anti-inflammatory. The objective of this study was to explore the role of the EAD-pathway in both agedriven changes in PMN responses and host susceptibility to S. pneumoniae. Timed pharmacological inhibition of the A1 receptor during infection revealed that this receptor was required for pulmonary PMN recruitment and control of bacterial numbers following pneumococcal lung infection. However, signaling via this receptor after 18 hours post-infection was detrimental to host resistance. Reflecting the age-driven susceptibility to pneumococcal pneumonia in humans, we and others showed that aged (e.g. 19 month old) mice fail to control pulmonary infection as efficiently as young (e.g. 2-month old) mice, resulting in enhanced systemic spread of the bacteria to the blood and brain. We show here that this age-associated susceptibility correlated with a delay in the initial protective PMN recruitment into the lungs followed by an over-exuberant and extended acute inflammatory response. We also found that PMNs from old mice failed to efficiently kill pneumococci ex vivo. Importantly, adoptive transfer of PMNs from young mice prior to lung challenge decreased bacterial burdens in the lungs and significantly mitigated disease symptoms in old mice. Age-associated changes in the EAD pathway may contribute to the susceptibility of old mice to pneumococcal infection because aged mice, in comparison to young mice, exhibited elevated basal pulmonary levels of the adenosine-degrading enzyme adenosine deaminase and depressed levels of the EAD-producing enzyme CD73 at the peak of inflammation, suggesting that old mice may generally have lower levels of EAD than aged mice. Old mice also expressed significantly higher levels of the pro-inflammatory A1 receptor on their PMNs. These age-driven alterations in the EAD pathway could account for the dysregulated PMN influx and defective function associated with aged hosts and contribute to the heightened susceptibility of the elderly to invasive pneumococcal disease.

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## Oxidative Signals in Bone Marrow Hematopoietic Niche Regulate Innate Immune Responses after Ischemic Tissue Damage

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Ischemic tissue damage activates hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM) and persistent HSPC activity may drive chronic inflammation and impair tissue recover. However, the negative regulatory signals for the tissue damage-activated HSPCs during tissue recovery from ischemic damage are not well understood. Here, we report that deletion of Nox2 NADPH oxidase in mice results in persistent elevations in BM HSPC activity and levels of inflammatory monocytes/macrophages in BM and ischemic tissue in a model of hindlimb ischemia. In culture with TLR2/4 stimulation, Ly6Chi monocyte differentiation was enhanced from multipotential Lineage-cKit+Sca1+ (LSK) cells but not from myeloid committed progenitors in Nox2-deficient cells. The sustained elevation in myelopoiesis of HSPCs appeared to result from loss of oxidative signal originated by Nox2 generating reactive oxygen species (ROS) in the extracellular space. Specifically, we found that tissue damage-induced elevation of hydrogen peroxide and oxidized phospholipids and activation of the ROS sensor Lyn kinase are Nox2-dependent in the BM hematopoietic niche, and that addition of hydrogen peroxide, oxidized phospholipid, Lyn activator and Nox2-intact neutrophils inhibit the activation of LSK cells and their inflammatory monocytes outputs in Nox2-deficient conditions. These data suggest that oxidative signals in the bone marrow hematopoietic niche are mediated by activation of the ROS sensor Lyn kinase, oxidized phospholipids and increased BM homing of neutrophils from the blood. Furthermore, our data show that Nox2-intact neutrophils that home to the BM induce inflammation resolution and promote tissue recovery. In summary, oxidative signals in BM

hematopoietic niche regulate innate immune memory of LSK cells linked with resolution of inflammation and regeneration of damaged tissue.

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#### Anti-Microbial Resistance via Enhancement of Innate Immunity Using Novel Synthetic TLR4 Agonists

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Objective: The TLR4 agonist monophosphoryl lipid A (MPLA) is an FDA-approved vaccine adjuvant with properties that augment innate immunity and bacterial resistance. However, MPLA is unavailable as a stand-alone immunotherapeutic agent for patients at high risk of infection. We identified three synthetic TLR4 agonists termed phosphorylated hexaacyl disaccharides (PHAD) which are structurally similar to MPLA, yet have translatable potential for stand-alone human use. We sought to identify whether PHADs are capable of priming innate immunity and enhancing resistance to bacterial infections.

Methods: To evaluate priming of innate immunity, BALB/C mice received either intraperitoneal (IP) MPLA, PHAD, 3D PHAD, 3D-6 acyl PHAD, or vehicle control 48 and 24 hours prior to IP infection with 10<sup>8</sup>CFU of *P. aeruginosa*. Six hours later plasma and peritoneal lavage fluid were collected and analyzed for bacterial load, cytokines and growth factors. The total number of neutrophils and monocytes recruited to the peritoneal cavity was determined. A second cohort of BALB/c mice were primed with either intravenous (IV) MPLA, PHAD, 3D PHAD, 3D-6 acyl PHAD, or vehicle 48 and 24 hours prior to IV inoculation with 10<sup>8</sup> CFU IV *S. aureus*. Mice were monitored for survival over 14 days.

Results: MPLA and all PHAD treated mice demonstrated lower *Pseudomonas* burden in peritoneal lavage fluid, as well as increased recruitment of neutrophils and monocytes to the peritoneal cavity post IP priming and infection. Plasma levels of pro-inflammatory cytokines were attenuated in all treatment groups in the IP infection model. Importantly, survival was significantly higher in all treatment groups when compared to vehicle at 14 days after systemic *S. aureus* infection.

Conclusions: Priming with PHADs augmented innate immunity and rendered rapid mobilization and recruitment of neutrophils and monocytes to the peritoneal cavity, leading to improved bacterial clearance of IP *P. aeruginosa*. Also, priming with PHADs improved survival from IV *S. aureus* infection. PHADs are ultrapure synthetic lipids with the ability to protect against both Gram-negative and Gram-positive organisms that commonly lead to severe nosocomial infections. As such, further investigation is warranted to evaluate PHADs as prospective immunotherapeutic agents for populations at increased risk of infection.

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# Mitochondrial Pyruvate Dehydrogenase Complex Directs Immunometabolic and Bioenergy Reprogramming and is a Druggable Target for Improving Sepsis Survival in Mice.

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A gap in understanding sepsis survival mechanisms remains unfilled after many years of research and repetitive treatment failures. We are testing a unifying theory of sepsis where severe systemic inflammation rapidly departs an anabolic immune effector state and enters a persistent, low energy, catabolic state, which prevents the immune system and failing organs from regaining anabolic energy needed to restore homeostasis and survive. In early support of this "bioenergy depletion" theory, we previously reported that nuclear SIRT1 and 6 within hours of sepsis onset epigenetically convert the sepsis-initiating anabolic and effector immune state to a persistent low energy catabolic state by limiting glucose flux and oxidation. We further showed that pharmacologically inhibiting

SIRT1 restores glucose oxidation, reverses immune suppression, and improves survival in septic mice. Here, we further elucidated the mechanism by which glucose oxidation is limited during catabolic state of sepsis. We show that mitochondrial pyruvate dehydrogenase complex PDC inhibition by pyruvate dehydrogenase kinase (PDK) during sepsis plays a crucial role in supporting the low energy catabolic state by its rate-limiting control over glucose oxidation; PDC is a critical contributor of acetyl CoA and reducing hydrogens (H+) needed to fuel the electron transport chain, support redox signaling, and generate ATP. Importantly, we find that activating PDC by blocking PDK phosphorylation of PDC E1a with a single dose of 25 mg/kg dichloroacetate (DCA) administered intraperitoneally to septic mice at sepsis onset, 12h and 24h post-sepsis onset decreases PDC phosphorylation, increases glucose oxidation decreases lactic acid levels in splenic monocytes and improves sepsis survival by up to 40%. Inhibition of PDK by DCA treatment during sepsis significantly improved: A. mean arterial blood pressure and microvascular immune support assessed by leukocyte-endothelial adhesion; B. intestinal pathology, as assessed by histology; C. immune cell function, as assessed by circulating mononuclear cells and tumor necrosis factor a (TNFa) mRNA levels in spleen; and D. liver function, as assessed by serum alanine transaminase (ALT) aspartate transaminase (AST) and alkaline phosphatase transaminase (AST); and E) expression anabolic cell growth and differentiation mediators b catenin and IGF growth factor in small intestine by immunohistochemistry. This reprogramming is detectable within 6 h of single dose DCA treatment. We suggest that impediments in glucosedependent mitochondrial fueling compromises anabolic pathways needed to restore organ and immune homeostasis, and that PDC is a potential druggable target for reversing bioenergy depletion during sepsis. (Supported by NIH R01 AI065791 and R01 GM102497 (cem) and R01GM099807 (vv).

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USP18 tunes the host antiviral response to favor HIV replication in macrophages Mark A. Wallet, Jared P. Taylor, Katherine Santostefano, Naohiro Terada and Melanie Cash University of Florida

The interferon (IFN) stimulated gene (ISG) response is critical for host defense against viral infection. Type 1 interferons (IFNa and IFNB) are expressed and secreted from cells in response to immune detection of viral infection. T1 IFNs cause cells to express ISGs and their protein products leading to blockade of viral infection/replication through diverse mechanisms. Most viruses are highly sensitive to the effects of T1 IFN, however we found in previous work that HIV has adopted certain elements of T1 IFN signaling to favor its own replication in macrophages – a process that also leads to unwanted inflammation. For example, HIV triggers signal transducer and activator of transcription 1 (STAT1) phosphorylation in macrophages and this is required for HIV mRNA transcription. Typically, STAT1 is phosphorylated in response to IFNs and contributes to antiviral immunity or inflammation. The paradoxical utilization of STAT1 by HIV led to our hypothesis that HIV usurps elements of the antiviral response to favor its own replication.

In the current study, we sought to understand how HIV replicates efficiently in macrophages – an innate immune cell type that is both capable of producing T1 IFN and responsive to T1 IFN. Gene array analysis of macrophages infected with HIV identified a distinct antiviral gene signature including numerous genes known to encode proteins that restrict HIV replication (ISG15, IFIT1, RSAD2 [viperin], HERC5, MX1, TRIM5, etc.). HIV also caused expression of USP18, a counter-regulator of T1 IFN signaling and negative regulator of the anti-viral protein ISG15. Knock down of USP18 by shRNA in THP-1 myeloid cells resulted in augmented and sustained IFNBinduced STAT1 phosphorylation, enhanced ISG expression, and increased "ISGylation" - ISG15 conjugation to host proteins. USP18 knockdown also markedly reduced HIV replication in THP-1 cells. Next, USP18 expression was knocked down by siRNA in human monocyte derived macrophages (MDMs). Here the effect mirrored THP-1 cells where enhanced STAT-1 phosphorylation and increased ISG expression were observed. A challenge arose when trying to infect MDMs that had been transfected with siRNA. Even delivery of non-targeting control siRNA (using several different platforms) rendered the MDMs un-infectable by HIV. Thus, an alternative method was required to confirm the role of USP18 in HIV replication.

Induced pluripotent stems cells (iPSCs) were derived from peripheral blood CD34 hematopoietic stem cells of a healthy donor. Next, *USP18* was knocked out in iPSCs by CRISPR/Cas9 gene targeting. *USP18*+/+ or *USP18*-/- iPSCs were used to differentiate monocytes and then macrophages. iPSC macrophages (iMacs) were infectable by HIV and normally responsive to IFNs or other inflammatory stimuli. Notably, *USP18*-/- iMacs were resistant to HIV replication and showed augmented ISG expression in response to T1 IFN or HIV infection. These data support that the IFN/ISG response limits HIV replication, in contrast to our previous findings showing a critical role for STAT1 in HIV replication. To investigate this further, MDM were infected with HIV in the presence or absence of a T1 IFN receptor-blocking antibody (α-IFNAR). When α-IFNAR was included, HIV replication was suppressed rather than increased. In addition, when very low does T1 IFN (or T3 IFN) were included in cultures, MDM actually supported higher viral replication. Thus, there is a bimodal response to IFNs where weak signals enhance HIV replication but strong signals potently block replication. USP18 balances the magnitude of IFN signaling allowing HIV adequate STAT1 signaling for replication while preventing the full antiviral response that would halt replication. Thus, USP18 is a rational target for drugs to control viral replication and possibly to modulate vaccine responses.

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## Evidence of potential immune tolerance in sheep: Reduced sheep neutrophil respiratory burst function post-orthopedic trauma surgery

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Background: Immune tolerance has been described as one form of innate immune memory. For instance, macrophages that have been stimulated by lipopolysaccharide (LPS) have been reported to become more or less responsive to subsequent activation signals. Similarly, during the later immune paralysis phase of sepsis an impairment of neutrophil respiratory burst function results in deficient neutrophil phagocytosis and an increased risk of secondary and/or recurring infections. Similar reports of impaired neutrophil respiratory burst function have been reported in in humans in response to a range of settings: with anesthesia, following abdominal hysterectomy, during clinical infection, in active Crohn's Disease and post-cardiac surgery.

Previous novel treatments for sepsis have been developed based upon findings in murine models; however, unsurprisingly given the immunological differences between mice and humans, these novel treatments have been unsuccessful in clinical trials. Sheep, an outbred animal as compared to inbred mice, potentially offer a more immunologically relevant model in which sepsis can be investigated. Indeed, sheep models of sepsis based upon bacterial or LPS administration are common. However, there remains uncertainty around the efficacy of novel treatments that address the later immune paralysis phase of sepsis. This potential remains unexplored in sheep models as this later immune paralysis phase of sepsis and the associated immune tolerance has not been well characterised. In an opportunistic study, we collected neutrophils from an orthopedic trauma surgery study and aimed to investigate, as a proof-of-principle for the development of a sheep sepsis model, whether immune tolerance was evident in sheep post-surgery.

Methods: Neutrophils were isolated via hypotonic lysis and density gradient separation from healthy sheep (n=22) or from sheep after orthopedic trauma surgery (week 1: n=9; week 2: n=7). Respiratory burst was investigated using a cytochrome c reduction assay that measured superoxide anion generation (i.e. respiratory burst function) of isolated neutrophils in response to either buffer, platelet activating factor, phorbol 12-myristate 13-acetate or platelet activating factor and phorbol 12-myristate 13-acetate together.

Results: The maximal neutrophil respiratory burst in all groups of sheep was evident following stimulation by platelet activating factor and phorbol 12-myristate 13-acetate together. Using the healthy sheep as a control, there was no evidence of endogenous neutrophil activation (unchanged response to buffer) or priming (unchanged responses to either platelet activating factor or phorbol 12-myristate 13-acetate) at either week 1 or week 2 post-surgery. The maximal neutrophil respiratory burst was reduced at week 1 post-surgery (P < 0.05), but not at week 2 compared to healthy control sheep.

Discussion and Conclusion: This study reports an impairment of sheep neutrophil respiratory burst function post-orthopedic trauma surgery that resolved by two weeks post-surgery. This immune tolerance could predispose towards increased risk of infection in the immediate interval post-surgery. Evidence of potential immune tolerance in sheep neutrophils post-orthopaedic trauma surgery demonstrates an important similarity between sheep and human neutrophils. Furthermore, it supports the hypothesis that neutrophil immune tolerance may also be present in the later immune paralysis phase in sheep models of sepsis, and supports the development of such models to investigate this question.

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Mammary tumor cells release soluble factors that modulate phagocytic activity of macrophages in vitro Débora M. Silvestro, José Renildo Carvalho, Thiago Albuquerque, Toshie C. Konno, Maria Anete Lallo and Elizabeth C. Perez

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Considering that tumor microenvironmental signals can affect macrophages functions, we investigate the influence of mammary tumor supernatants on phagocytic activity of macrophages. For this, RAW 264.7 macrophages were cultivated alone or with 4T1 mammary tumor cells supernatants for 48 hours on glass coverslips in a 24 well plates. The heat inactivated bread yeast particles were added at a 1:10 effector target ratio and incubated for two hours. Coverslips were collected at 0, 6, 24 and 48 hours and submitted to Giemsa stained. Phagocytic index was calculated considering % of macrophages that internalized at least yeast X average number of fungal cells in these macrophages. Results show that macrophages cultivated with 4T1 mammary tumor cells supernatants have lower average number of yeast particles ingested when compared to macrophages cultivated alone. These results indicate that soluble factors released by mammary tumor cells can affect phagocytic activity of macrophages in the tumor microenvironment.

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### Differential regulation of SOCS1 and SOCS3 gene expression by interferon-beta and IL-10 in endotoxinstimulated macrophages

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Activation of macrophages by bacterial endotoxin, lipopolysaccharide (LPS), induces co-expression of many genes, including *IFNB1* (interferon-beta) and *IL10*. Thecytokines encoded by these genes can in turn induce activation of STAT1 and STAT3, and expression of STAT1- and/or STAT3-responsive genes such as *SOCS1* and *SOCS3*. STAT3 activation is required for IL-10-mediated induction of *SOCS3* expression by macrophages, and it is also essential for IL-10-induced inhibition of pro-inflammatory cytokines such as TNF-alpha, IL-1and IL-6. In this study, we examined the roles of IFN-beta and IL-10 in the induction of *SOCS1* and *SOCS3* expression by LPS-stimulated human monocytes and murine macrophages. We found that LPS treatment induces co-activation of STAT1 and STAT3 and induction of *SOCS1* and *SOCS3* expression by monocytes and macrophages. The induction of *SOCS1* but not *SOCS3* expression by LPS was IFN-beta- and STAT1-dependent. In contrast, the induction of *SOCS3* expression by LPS was IFN-beta- and STAT1-independent, but was associated with activation of STAT3.

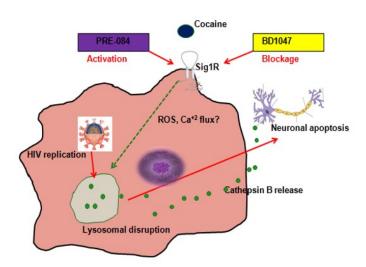
The induction of *SOCS3* expression by LPS was significantly reduced, but not abrogated, in macrophages derived from *Il10* knockout mice. These findings indicate that intermediate production of IFN-beta is essential for *SOCS1* expression by LPS-stimulated macrophages; whereas, induction of *SOCS3* expression is predominantly mediated by endogenous IL-10.

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### SIGMA-1R AND COCAINE INTERPLAY IN CATHEPSIN B SECRETION IN HIV-1 INFECTED MACROPHAGES

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**Background:** Pathogenesis of HIV-1 associated neurocognitive disorders (HAND) is mediated through the infiltration of perivascular macrophages into the brain that secrete viral proteins, neurotoxic and inflammatory factors. One of these factors is cathepsin B (CATB), a lysosomal cysteine protease that induces neuronal apoptosis. CATB increases significantly in plasma and in cerebrospinal fluid from HIV-1 infected patients (Cantres-Rosario *et al.*, 2013). Interestingly, cocaine potentiates CATB secretion and neurotoxicity *in vitro*. *Cathepsin B* also increases in the plasma of HIV-1 cocaine abusers. Cocaine modulates sigma-1 (Sig1R), a non-opioid receptor, and correlates with several effects in macrophages: increased viral replication, oxidative stress and inflammatory cytokines. However, the role of Sig1R in CATB secretion and HIV-1 replication in macrophages is unknown. *Our goal is* to determine if Sig1R modulation with pharmacological agents can reduce CATB secretion from HIV-1 infected macrophages.

**Methods:** Monocyte derived-macrophages (MDM's) from seven (n=7) seronegative human donors were isolated from blood samples by Ficoll. MDM's were differentiated and infected with HIV-1ADA, treated with Sig1R antagonist (BD1047) prior to cocaine treatment for 3, 6, 9 and 11 days post-infection. The same procedure was followed with PRE-084, a specific Sig1R agonist. HIV infection and cathepsin B secretion levels were assessed from supernatants by ELISA.

**Results:** Treatment of infected macrophages with BD1047 10  $\mu$ M prior to cocaine decreased infection levels and CATB secretion when compared to cells treated with cocaine (p24=100 ng/mL vs. 10 ng/mL; 450 ng/mL vs. 200

ng/mL). However, BD1047 10 µM in absence of cocaine had no effect on infection levels or CATB secretion. On the other hand, activation of Sig1R by PRE- 084 1µM significantly decreased HIV-1 infection (p24=90,000 ng/mL vs. 10 ng/mL) and CATB secretion (p24=420 ng/mL vs. 200 ng/mL) at 11 days post-infection in HIV+ vs. HIV+PRE-084 groups (p≤0.05) in absence of cocaine. Though, when PRE-084 was added prior to cocaine, no significant differences in p24 levels and CATB were found. Statistical Analyses were done by Two-way ANOVA and Tukey's *post-hoc* tests using an alpha ( $\alpha > 0.05$ )

Conclusions: These results demonstrate that Sig1R plays a role in CATB secretion and infection levels. Sig1R antagonist (BD1047) might be used as potential therapeutics for reducing cocaine's effect on HIV-1 infection and CATB secretion in macrophages. Future projects should investigate this effect in proteomics studies in HAND and in drug abusing populations.

Keywords: HIV-1, cocaine, sigma-1 receptor, HAND, cathepsin B

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### Differential Expression of the Check-point protein, VISTA, both in Response In vitro Stimuli and In vivo Septic Insult.

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Sepsis refers to an aberrant/dysfunction immune/organ system response to an infection and/or suspected infection. It is a life-threatening condition that affects many critically ill patients, however, there is no currently FDAapproved therapeutic treatment. Thus, a better understanding of the patho-biology of sepsis is needed if we are to identify novel therapies.

V-domain Ig-containing Suppressor of T Cell Activation (VISTA) is an immune checkpoint protein found on mature myeloid cells as well as T-cells that suppresses T cell mediated immune responses such as proliferation and cytokine production. Due to its role in T cell regulation, and the success of other checkpoint proteins, VISTA has been studied as an immunotherapy in cancer research. However, VISTA has not been studied in the context of sepsis. To better understand the role of VISTA in experimental sepsis we initially chose to characterized VISTA on cell types known to play a role in the immune response to septic infection; macrophages and T cells, under the hypothesis that VISTA plays a central role in macrophage and T cell function in response to experimental septic challenge in mice.

We first examined VISTA expression in culture/in vitro on J774 (mouse necrophage cell line) as well as Jurkat T cell lines using cell selective stimulation. VISTA expression was measured by both flow cytometry as well as western blot. After stimulation, Jurkat T cells displayed increased VISTA expression; while after stimulation, J774 cells, showed decreased VISTA expression as compared to unstimulated cells. Additionally, we induced sepsis in adult C57BL/6 male mice via cecal ligation and puncture (CLP). VISTA expression was measured in CD3+ splenocytes as well as peritoneal macrophages 24 hrs post-CLP via flow cytometry and Western blot assay. After 24 hours VISTA expression was increased in the spleen in CLP mice compared to sham, while in peritoneal macrophages VISTA expression was decreased following CLP compared to sham mice.

In conclusion, this study demonstrates that unlike the check point proteins, PD-1 and BTLA, where we documented rises in both lymphoid and macrophage expression in response to sepsis, VISTA expression is altered differentially in these two immune cell sub-populations. And while the significance of these septic changes VISTA's expression remain to be defined in a causal/ pathogenic sense, it is tempting to speculate that such a unique expression pattern might explain the non-redundant functionality of VISTA as compared to these more classic check-point proteins.

### Cytokine analysis may be used to diagnose and monitor response to therapy in an animal model of S. epidermidis CSF shunt infection

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Bacterial infection is a frequent and serious complication of CSF shunt placement for the treatment of hydrocephalus. Diagnosis of these infections relies on the gold standard of CSF culture; however, culture may not always be reliable in the setting of biofilm, slow growing or fastidious organisms and antibiotic pretreatment. CSF or serum chemokines and cytokines may prove useful as alternative strategies for diagnosis of CSF shunt infection. We hypothesized that Staphylococcus epidermidis CNS catheter infection has a distinct chemokine and cytokine profile when compared to baseline CSF and CSF from animals with sterile CNS catheters. To evaluate this hypothesis, we adapted our previously published murine CNS catheter infection model to generate infection with S. epidermidis in Lewis rats. The rats tolerate the procedure well and catheter placement in the lateral ventricle was verified visually. Chemokine and cytokine analysis was performed on CSF at 1 day post infection. As expected there was an increase in the anti-inflammatory cytokine IL -10 which we saw in human samples. This likely represents a compensatory response to the elevation of pro-inflammatory cytokines. There were higher levels of the pro-inflammatory CSF chemokines and cytokines IL-1B, IL-6 and CCL3 in rats implanted with S. epidermidis infected CNS catheters. Interestingly there was no increase in the chemoattractants CCL2, CXCL1 and CX3CL1. At day 5 post-implantation, the levels of pro-inflammatory mediators also decreased, suggesting that these values may be useful for monitoring the course of infection over time. Importantly this demonstrates the role of proinflammatory IL -B, IL-6 and CCL3 in differentiating infection from trauma even at early time points. Coupling inflammatory mediator analysis with bacterial detection strategies may be a useful tool for diagnosing shunt infection in early post-operative time periods.

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#### The role of alternative polyadenylation in the antiviral innate immune response

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Alternative polyadenylation (APA) is an important regulatory mechanism of gene functions in many biological processes. However, the extent of 3' UTR variation and the function of APA during the innate antiviral immune response is unclear. The goal of this study is to explore the global APA profile and to characterize the dynamic APA-mediated regulation of antiviral responses using in vitro transcription-sequencing APA sites (IVT-SAPAS). We show genome-wide poly(A) sites switched and average 3' UTR length shortened gradually in response to vesicular stomatitis virus (VSV) infection in macrophages. We then performed gene ontology (GO) analysis of human APA genes between two consecutive time points on the DAVID website and found that genes with APA and mRNA abundance change are enriched in immune-related categories such as the TLR, RLR, JAK-STAT and apoptosis-related pathways.

To evaluate whether the variation in 3' UTR usage affects protein production, thirteen genes that exhibited poly(A) site switching during the antiviral immune response, TOLLIP, FOS, NFKB1, DDX58, RIPK1, DDX3Y, TRIM25, JAK2, SOS1, N4BP1, SIRPA, SPSB1, and PLSCR1, were selected for further analysis. The results suggested that tandem 3' UTRs could affect protein expression both by influencing mRNA abundance and translation efficiency. Finally, the relationship between 3' processing factors and the antiviral response was analyzed. Results showed the down-regulated expression of 3' processing factors after VSV infection. When the core 3' processing factors are knocked down, viral replication is affected. In all, this study reports the annotation of genes with APA in antiviral immunity and highlights the roles of 3' processing factors on 3' UTR variation upon viral infection.

#### The JNK-mediated NADPH oxidase-independent NETosis

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**Introduction:** Neutrophils are the most abundant leukocytes present in human peripheral blood, and play an important role in controlling bacterial and fungal infection. The two most studied neutrophil effector functions have been phagocytosis and granules release. In 2004, a new neutrophil function was demonstrated - NETosis and NET release that have the capacity of trap and kills extracellular pathogens. To date, two main pathways of NET formation were described: NADPH oxidase-dependent and -independent NETosis. Despite the differences in NADPH oxidative activity, these pathways share similarities. Recently we demonstrated that transcriptional firing is important to both pathways of NETosis, although different transcriptional factors was observed in each pathway. These pathways are also distinctive by ERK activation (NOX-dependent) and calcium influx, citrullination of histone and mitochondrial ROS production (NOX-independent). JNK activation is important to drive the LPSmediated ROS production and subsequent NETs release. Our transcriptomics study suggested that JNK activation could be involved in calcium inonophore A23187-induced NETosis. However, the role of JNK in Nox-independent pathway is not clearly established.

**Objectives:** To identify whether JNK activation is important for NOX-independent NETosis driven by the calcium ionophores A23187 and Ionomycin.

Methods: Human neutrophils were isolated using the PolymorphPrep (blood of healthy adult donors). To induce NETosis, we used the calcium ionophores A23187 and ionomycin in the presence or absence of JNK inhibitor SP600125. The fluorescence intensity of Sytox-Green or MitoSOX in neutrophils was measured over 1-4 hours to quantify NETosis or mitochondrial ROS. Western blots were performed to determine the amounts of citrullinated H3 and GAPDH. Confocal imaging was used for determining the presence of myeloperoxidase, citrulinattied H3, p-JNK and DNA.

Results: We observed an impaired A23187 and Ionomycin-induced NET release after treatment with JNK inhibitor. Neutrophils treated with JNK-inhibitor showed less production of mitochondrial-ROS and citrullination of histone 3. However, the calcium influx were not affected after treatment with JNK-inhibitor.

Conclusions: These data show the importance of JNK-activation in A23187 and ionomycin-mediated NOXindependent NETosis. The JNK-activation seems to be involved in mitochondrial ROS production and citrullination of H3, two main steps involved in NOX-independent NETosis.

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Immunological Changes in Host Lung Environment during Antecedent Influenza A Infection Lead to Expression of S. aureus SaeR/S Dependent Virulence Expression during Secondary Bacterial Pneumonia Timothy R. Borgogna, Bennet Hisey, Teresa Hoyt, Eli Sward, Kyler Pallister, Nicole Meissner and Jovanka Voyich Department of Microbiology and Immunology, Montana State University

A infections typically present mild morbidity and can be resolved by the immune system in healthy individuals. However, influenza A infections are associated with increased susceptibility to secondary bacterial infections, wherein morbidity and mortality increase significantly. In the previous decade, influenza co-infections have displayed mortality rates ranging from 11 to 15-fold higher than those of influenza alone, and have consistently ranked within the top 10 leading causes of death in the US. Though largely recognized for its versatile roles as a primary infectious agent, Staphylococcus aureus (S. aureus) has become the most prominent secondary infectious agent in fatal cases of influenza A bacterial co-infections. The drastic increase in mortality observed in these coinfections has warranted the attention of numerous studies aimed at describing the immunomodulatory effects of an antecedent influenza A infection that contribute to increased host susceptibility to secondary bacterial infection; yet, little attention has been directed towards describing how these changes in cellular recruitment and signaling may directly impact bacterial pathogenesis. The innate ability of S. aureus to sense and respond to environments encountered during interactions within a human host is dependent on tightly controlled gene regulatory systems. SaeR/S is a virulence regulatory system demonstrated to be essential in the transcriptional regulation of S. aureus virulence genes involved in evasion of human neutrophils and toxin production. To that end, we hypothesize that immunological modulations in the host lung environment during an antecedent influenza A infection trigger S. aureus virulence expression via the SaeR/S regulatory system. Using a murine model of bacterial co-infection mice were infected with antecedent influenza A (WSN or PR8) followed by S. aureus challenge 6 days post-influenza infection. Morbidity and mortality assays of mice challenged with USA300 (LAC) and an isogenic gene deletion mutant USA300ΔsaeR/S (ΔsaeR/S) indicate secondary infection by S. aureus is saeR/S dependent. Transcriptional analyses revealed saeR-target genes are upregulated within hours during co-infection compared to mice infected with S. aureus alone, suggesting the trigger(s) responsible for induction of saeR/S are present prior to bacterial challenge or rapidly recruited. Differential staining of bronchial alveolar lavage fluid (BALF) in mice infected with influenza A alone display increased recruitment of cells morphologically similar to lymphocyte populations. Preliminary findings using flow cytometry to identify these populations revealed an increased recruitment of CD11b low/mid, Ly6G(-), Ly6C(-) cell population during primary influenza A infection that was markedly reduced upon S. aureus challenge. Hematoxylin and eosin (H&E) staining of lung sections at time points concurrent with observed upregulation of virulence genes demonstrate increased inflammation and infiltration of neutrophils in mice co-infected with LAC compared to mice co-infected with ΔsaeR/S or infected with S. aureus and influenza A alone. Consistent with previous findings, analysis of cytokine profiles demonstrates influenza A leads to differential production of IFNg in co-infected mice compared to mice infected with S. aureus alone. In addition, our data suggests antecedent influenza A promotes the induction of saeR/S leading to an inhibition of TNFa which may further exacerbate the co-infection. Taken together, our studies suggest influenza A infection causes immunological modulations within the lung environment that trigger S. aureus virulence factor production leading to increased host morbidity and mortality. On-going efforts aim to further characterize and identify host responses to influenza A that lead to the activation of the SaeR/S virulence regulatory system.

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Extracellular Alkaline pH Promotes NADPH Oxidase-Independent NETosis: A Matter of Calcium Influx Cristiane Souza<sup>1,2</sup>, Leandro Breda<sup>1,2</sup>, Meraj Khan<sup>1</sup>, Niels Camara<sup>2</sup>, Sandro Almeida<sup>2</sup>, Hartmut Grasemann<sup>1,3</sup>, Neil Sweezey <sup>1,4</sup> and Nades Palaniyar<sup>1,5</sup>

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Toronto, Toronto, ON, Canada.; <sup>4</sup>Department of Physiology University of Toronto, Toronto, ON, Canada; Department of Pediatrics University of Toronto, Toronto, ON, Canada; <sup>5</sup>Department of Laboratory Medicine & Pathobiology, University of Toronto, Toronto, ON, Canada; Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada.

**Introduction:** Neutrophils are key innate immune cells important for the first line of host defense. Several studies have been demonstrated that lowering intracellular pH leads to impaired neutrophil functions. However, the influence of pH on NETosis is still poorly understood. In this study, we describe a potential molecular mechanism by which pH mediates the NOX-independent NETosis.

**Methods:** Neutrophils from human blood were isolated using Polymorprep. Different pH conditions (pH range 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8) were adjusted using HCl and NaOH in RPMI/HEPES. Neutrophils were then activated with A23187 or ionomycin, and NETosis kinetics assessed by Sytox Green assay and confirmed by confocal microscopy. Mitochondrial ROS (mROS) production was measured by Mitosox. Calcium mobilization/influx was determined by the fluorescent probe Fluo4-AM, using a plate reader assay and confocal microscopy.

**Results:** We confirmed that extracellular pH can change the neutrophil intracellular pH, during basal conditions. Stimulation of neutrophils using A23187 or ionomycin caused increase in intracellular pH. During alkaline conditions, NETs release was increased at baseline and after stimulation with agonists. We also observed an increase in calcium influx and PAD4 amount in higher pH. Consistent with this result, citrullination of histone 3 (hallmark of PAD4 activity) was increased during alkaline conditions, in baseline controls and afterstimulationwith A23187 or Ionomycin. PAD4 is a calcium-dependent enzyme responsible for the citrullination of histone 3, an important step in NETosis. We also found that mROS production was increased at higher pH levels, corroborating with increased calcium influx, since calcium facilitates mROS production.

**Conclusion:** Our results suggest that alkaline pH promotes the increase in intracellular calcium concentration, facilitates citrullination of histone 3, mROS levels and consequently NETosis.

**Financial support:** CIHR and FAPESP.

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# IL-4 signaling is required for optimal production of IFN-gamma by T cells during Leishmania infection Prajwal $Gurung^{1,2}$

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Leishmania is a global health problem that affects more than 2 million people every year with 300 million people at risk worldwide. It is well established that a dominant Th1 response (IFNgamma (IFN-g), a hallmark Th1 cytokine) provides resistance, while a dominant Th2 response (IL-4, a hallmark Th2 cytokine) confers susceptibility during Leishmania infections. Given the important role of IL-4 during Leishmania infections, we used IL-4 neutralizing antibody to study and investigate the cellular and molecular events regulated by IL-4 signaling. As previously published, neutralization of IL-4 in Leishmania infected BALB/c mice (a Leishmania susceptible strain) provided protection when compared to PBS treated-Leishamania infected BALB/c mice. Interestingly, despite the protection, the IFN-g production by T cells was dramatically reduced. This demonstrates two important and previously unknown points- 1) the protection against Leishmania may not depend on the amount of IFN-g, but rather on the amount of IL-4, and 2) IL-4 is required for optimal production of IFN-g by T cells. In conclusion, our studies break the preexisting dogma of resistance/susceptibility to Leishmania infections and open new research grounds to investigate the unsuspecting role of IL-4 in controlling Th1 responses. A complete understanding of these cellular and molecular crosstalks will hopefully uncover a much-needed novel therapeutic target to treat Leishmania

### The role of ADAM17-mediated shedding of leukocyte cell surface proteins in inflammation and gut homeostasis

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Activation of integrin-mediated adhesion and induction of leukocyte migration into sites of inflammation involves various leukocyte cell signaling proteins that modulate trafficking, including L-selectin, fractalkine, and tumor necrosis factor  $(TNF)\alpha$ .

These cell adhesion molecules and cytokines are synthesized as membrane anchored molecules that are released and activated by the membrane-anchored metalloproteinase TNF $\alpha$  convertase enzyme (TACE, also known as ADAM17).

We recently identified a crucial regulator of the maturation and function of TACE and thus the release of these leukocyte cell signaling proteins. This molecule, which is called iRhom2 has seven trans-membrane spanning domains and is a catalytically inactive member of the Rhomboid family of intra-membrane serine proteinases. Mice lacking iRhom2 are unable to release TNF $\alpha$  from their immune cells and are therefore protected from inflammatory arthritis. In inflammatory bowel disease, increased amounts of soluble TNF $\alpha$  are released by various immune cell populations, such as macrophages, dendritic cells, neutrophils, as well as effector T cells and play a key role in the activation and perpetuation of the inflammatory response, but the adhesion molecules fractalkine and L-selectin have received very little attention. We are currently testing how the expression of iRhom2 and/or TACE by these immune cell populations affect their function in mouse models of disease. This work will provide important new information on the role of iRhom2 and of the release of cell-adhesion molecules such as fractalkine, L-selectin and proinflammatory cytokines in the context of inflammation and gut homeostasis. We hope that these studies will help uncover new targets for treatment of this debilitating disease in humans.

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## NADPH oxidase deficiency increases pro-inflammatory signaling and cytokine production in neutrophils challenged with sterile fungal PAMPs $\odot$

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Invasive Pulmonary Aspergillosis (IPA) is a severe life-threatening infectious pulmonary disorder by the opportunistic fungal pathogen, Aspergillus fumigatus (AF) in immunocompromised patients, including the primary immunodeficiency chronic granulomatous disease (CGD). CGD results from inactivating autosomal or X-linked recessive mutations in the genes encoding subunits of the phagocyte NADPH oxidase that generates superoxide (O<sub>2</sub>). Lack of reactive oxygen species (ROS) leads to recurrent bacterial and fungal infections and also dysregulated inflammation. This includes the response to fungal pathogen-associated molecular patterns (PAMPs), which is typically neutrophilic and associated with abscess formation. Whether ROS generated from polymorphonuclear leukocyte (PMN) play a crucial role in regulating inflammation, in addition to their well-known microbicidal effects, is still elusive. We hypothesized that the CGD PMN lacking of ROS are directly linked with the neutrophilic hyperinflammation by overexpression of neutrophil chemoattractants and pro-inflammatory cytokines as a feed forward loop upon exposure to fungal PAMP stimulation. To investigate this hypothesis, we measured cytokine levels and confirmed that CGD mouse PMN produce substantially more of the major chemotactic cytokine CXCL2 and TNF-α and IL-1β compared to WT control PMN upon challenge of sterile yeast particle, zymosan, Recognition of zymosan β-glucan by Dectin-1 and its downstream signaling via spleen tyrosine kinase (Syk) is a well-known mechanism for zymosan-induced leukocyte innate immune activation. In neutrophils, zymosan also signals through CD11b/CD18. However, whether these are critically involved in CGD

hyperinflammation is not well-understood. Cell surface expression of CD11b was significantly upregulated in basal and zymosan-stimulated CGD PMN. We assessed activation of Syk and Extracellular signal-regulated kinase (Erk1/2). Interestingly, we found significantly increased Syk and Erk1/2 kinase phosphorylation in zymosan-stimulated CGD PMN compared to WT PMN. Taken together, we concluded that pro-inflammatory cytokines were significantly upregulated in CGD, and hypothesize this is linked to CD11b upregulation and Syk tyrosine kinase and Erk1/2 overactivation. ROS may modulate PMN cytokine production by negatively regulating these intracellular signaling pathways. This research is anticipated to advance understanding of key regulatory roles of NADPH oxidase in host PMN responding to fungal pathogens as well as suggest novel role of PMN in PAMPs-induced inflammation.

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## The emerging oral pathogen, Filifactor alocis, inhibits PMA and bacteria-induced formation of neutrophil extracellular traps.

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Filifactor alocis is a gram-positive anaerobic rod which has emerged as an important periodontal pathogen. Neutrophils are a major component of the periodontal innate host response, and they employ different antimicrobial strategies to control the oral microbial community. The formation of neutrophil extracellular traps (NETs) is one of the cell's antimicrobial strategies to both kill and entrap pathogens. NETs are detected in periodontal pockets as well as in epithelial tissue from periodontitis patients. The hypothesis that F. alocis would manipulate human neutrophils ability to form NETs was tested. Human neutrophils were unstimulated or stimulated with PMA (50 nM, 180 min), or challenged with F. alocis (multiplicity of infection (MOI) 10-50-100, for 15-180 min), or Streptococcus gordonii (MOI 10-50-100, for 180 min), and their ability to form NETs was assessed by confocal microscopy. NETs were determined by overlay of DAPI and myeloperoxidase and the quantification of the confocal images analyzed by ImageJ software. The formation of NETs was minimal, less than 5 %, in F. alocischallenged neutrophils independent of time and MOI. In contrast, when neutrophils were challenged with S. gordonii-a commensal oral bacterium-a dose dependent induction on NET formation was observed. S. gordonii challenge induced a robust respiratory burst response. Moreover, S. gordonii-induced NETs were dependent on reactive oxygen species production. Pre-treatment of neutrophils with F. alocis (MOI 10, 60 min) resulted in a 37 % inhibition of PMA-induced NETs. In addition, F. alocis pre-treatment significantly inhibited S. gordonii induced NETs albeit the inhibitory effect was less pronounced compared to the PMA condition. Neutrophils pre-treated with F. alocis cultured supernatants did not inhibit either PMA or S. gordonii-induced NETs, which indicates that the live organism, not secreted factors, is required to inhibit the signaling pathway that promotes the formation of NETs. These results indicate that F. alocis does not induce NET formation; but is able to significantly inhibit both the PMA and S. gordonii response. The ability of F. alocis to dampen neutrophil responsiveness to other stimuli could be an evasion strategy to avoid killing.

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### Regenerating Islet-Derived Protein 3 Enhances Innate Respone to Influenza A Virus Infection

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Influenza A virus (IAV) is an annual pathogen affecting an average of 200,000 Americans every year. Due to the high mutation rate and genetic re-assortment of IAV, a new vaccine is needed every year as a preventative measure. However, new strategies for combating infection are needed, especially those that enhance your own body's methods of fighting pathogens. Regenerating Islet-Derived protein 3 alpha (REG3A), an antimicrobial peptide

typically expressed in the gut epithelia of mammals, helps maintain host-bacteria homeostasis in our gastrointestinal tract. REG3A is classified as a C-type lectin and our lab focuses on anti-microbial peptides as well as C-type lectins (mannose-binding lectin and surfactant protein D) and their function against Influenza A virus. For that reason we decided to look at any possible interactions of REG3A and IAV in vitro to determine any capability as an anti-IAV peptide. Although it is classified as a C-type lectin, REG3A does not show the ability to aggregate the virus unlike surfactant protein D. Thus we tested REG3A against a the A/Philippines/2/82 strain of Influenza A virus (IAV) and observed that REG3A consistently decreases the fluorescent focus count (FFC) of influenza in vitro indicating a reduction of infectivity of IAV in the presence of REG3A. Furthermore, after looking at electron microscopy images of IAV after treatment with REG3A, we observed that the envelope of virus particles had been damaged in a similar fashion to LL-37, a known anti-IAV peptide. Neutrophil peroxide activity was slightly increased with increasing doses of REG3A alone, and showed greater activity when pre-incubating IAV with REG3A compared to IAV alone. Currently, it is unknown whether REG3A is expressed by epithelial cells in other tissues such as the lungs and innate cells, so in the near future we will explore the expression pattern of REG3A in these cell types. In conclusion, REG3A is demonstrating capacity as an anti-IAV peptide, thus further studies are required to determine other effects of REG3A on innate immunity, developing methods of inducing expression in various cell types, and testing the activity of REG3A against other strains of IAV in order to reveal the full potential of REG3A as a future IAV treatment.

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## RIP2 is Important in the Response to House Dust Mite and Attenuates Allergic Airway Inflammation in Response to Dermatophagoides farinae

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Allergens are comprised of a heterogeneous group of substances, which, despite being structurally and functionally diverse, elicit very similar downstream type 2 adaptive immune responses. How this is accomplished is not completely understood. Elucidating the innate immune mechanisms involved in responding to these stimuli may guide the development of therapies for type 2 disorders. One of the innate immune receptors that have been reported to elicit type 2 adaptive immune responses is the peptidoglycan sensor NOD2. In this study, we examine the role played by RIP2, the downstream effector kinase for NOD2, in the response to house dust mite. We utilized an *in vitro* culture model of primary murine tracheal epithelial cells and *in vivo* model of *Dermatophagoides farinae*-mediated allergic airway inflammation. We show that in airway epithelial cells, RIP2 is tyrosine phosphorylated in response to exposure with extracts of *D. farinae* and is important for dust mite-induced inflammatory cytokine and chemokine production. Using the *D. farinae* allergic airway model, we demonstrate that, *in vivo*, local airway inflammation, systemic cytokines, and dust mite-specific antibody production were all reduced in RIP2 KO mice compared to WT animals. These data illustrate that RIP2 can be activated by a relevant allergic stimulus and that such activation can contribute to allergic airway disease. These findings also suggest that RIP2 inhibitors might have some efficacy in downregulating the inflammatory response in type 2-mediated diseases.

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#### **Involvement of RIP2 in signaling mediated through the FcgR**

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Receptor interacting protein 2 (RIP2) is a kinase which mediates signaling downstream of the bacterial peptidoglycan sensors NOD1 and NOD2. Engagement of these receptors leads to activation of NF-kB, MAP kinase, and type I interferon pathways which ultimately result in the secretion of cytokines and antimicrobial peptides to eliminate the recognized pathogens. Dysregulation of NOD2/RIP2 signaling has been associated with the development of various inflammatory disorders, and genetic loss or pharmacologic inhibition of RIP2 has been

demonstrated to alleviate disease in murine models of inflammatory bowel disease (IBD), multiple sclerosis (MS), and allergic airway inflammation. These findings point to RIP2 as an emerging therapeutic target in such disease settings.

Despite the fact that RIP2 interacts with numerous proteins and adaptors, this kinase has only been shown to be functionally important downstream of NOD1/2 signaling. Previous work identifying the RIP2 tyrosine autophosphorylation site suggests that this site could be a potential Src family kinase (SFK) SH2 binding motif. This prompted us to explore whether signaling through other receptors which utilize SKFs, might also utilize RIP2. In this work, we investigated whether RIP2 is involved downstream of the Fc gamma receptor (FcgR). Using raw 264.7, we found that RIP2 is tyrosine phosphorylated (and, therefore, likely activated) upon cross linking of the FcgR. To determine the role of RIP2 downstream of FcgR, we generated bone marrow derived macrophages (BMDMs) from wild type and RIP2 knock out mice and assessed different signaling pathways after FcgR cross linking. We show that loss of RIP2 leads to deficient FcgR signaling, and dysregulated iNOS and pro-inflammatory cytokine and chemokine production upon FcgR cross linking. In order to determine the mechanism by which RIP2 is involved in this pathway, we investigated whether different members of the SFKs can phosphorylate RIP2. We found that Hck and Fgr can mediate RIP2 tyrosine phosphorylation in a manner which correlates with their activation status. Altogether, our data demonstrate that RIP2 is activated in the course of FcgR signaling through members of SFKs. This finding will have important implications for future therapies designed to target this kinase.

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### MafB /GATA-1 axis bares the potential mechanism for immune suppression and anemia of critical illness - a neuro-immune link

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Patients who survive initial burn injury are susceptible to nosocomial infections. Anemia of critical illness is a compounding factor in burn patients that necessitates repeated transfusions, which further increases their susceptibility to infections and sepsis. Robust host-response is dependent on adequate number and function of monocytes/macrophages (MØ) and dendritic cells (DCs). In addition to impaired red blood cell (RBC) production, burn patients are also prone to depletion of DCs and an increase in deactivated monocytes. In steady state hematopoiesis, RBCs, MØs and DCs are all generated from a common myeloid progenitor (CMP) within the bone marrow (BM). We hypothesized in a mouse model of burn injury, increase in myeloid specific transcription factor MafB at the CMP stage steers their lineage potential away from MEP (megakaryocyte erythrocyte progenitor) production and drives the terminal fate of CMPs to form MØ versus DCs, consequences being anemia, monocytosis and DC deficits. Results indicate, even though burn injury stimulated BM hematopoiesis by increasing multipotential stem cell production (LSKs; linneg Sca1+cKit+), the BM commitment is shifted away from MEP and towards GMP (granulocyte monocyte progenitors) with corresponding alterations in peripheral blood components such as Hgb, Hct, RBCs, monocytes and granulocytes. Furthermore, burn induced MafB in CMPs act as a transcriptional activator of MCSFR and a repressor of TfR, promoting MØ and inhibiting erythroid differentiations while dictating a pDC phenotype. Results from siRNA and gain of function (gfp-GATA1 retrovirus) studies indicate, targeted interventions to restore MafB/GATA-1 balance can mitigate both immune imbalance and anemia of critical illness. Our study further illustrates how conditions resulting in high catecholamine microenvironment such as burns can instigate myelo-erythroid reprioritization influenced by beta-adrenergic stimulation leading to anemia. In mouse model of scald burn injury we observed, along with a 3-fold increase in bone marrow LSKs, the myeloid shift is accompanied with a significant reduction in MEPs. Beta-blocker administration (propranolol) for six days post burn not only reduced the number of LSKs and MafB+ cells in multi potent progenitors but also influenced myelo-erythroid bifurcation by increasing the MEPs and reducing the GMPs in the bone marrow of burn mice. Furthermore, similar results were observed in burn patients' PBMC derived ex-vivo culture system demonstrating that commitment stage of erythropoiesis is impaired in burn patients and intervention with propranolol (non-selective beta 1,2-adrenergic blocker) increases MEPs. Also, MafB+ cells that were significantly

increased following standard burn care could be mitigated when propranolol was administered to burn patients establishing the mechanistic regulation of erythroid commitment by myeloid regulatory transcription factor MafB. Overall, results demonstrate that beta- adrenergic blockers following burn injury can redirect the hematopoietic commitment toward erythroid lineage by lowering MafB expression in multi potent progenitors and be of potential therapeutic value in restoring overall immune cell trafficking.



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