ABSTRACTS

1

Comparative Genomics for the Identification of Novel Innate Immunity Regulators

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The innate immune response is critical to combat infection. However, an acutely or chronically overactive innate immune response can contribute to the pathogenesis of many diseases with an inflammatory component including sepsis, atherosclerosis, asthma, cancer, and many others. Thus, it is critical that innate immunity be tightly regulated, activated when needed and otherwise held in check. To identify novel regulators of innate immunity that could affect infectious or inflammatory disease, we have used a comparative genomics screening approach. In this approach, we developed a pipeline in which we (1) use rapid RNA-interference screens in the nematode C. elegans and mouse macrophages to identify candidate innate immunity regulators, (2) use C. elegans infection models to obtain in vivo validation of these RNAi data, and (3) use knockout mice and human patient cohorts to determine the effect of these genes in disease on mammals. This approach has been very fruitful, and we have uncovered several novel genes that regulate innate immunity in C. elegans and mammals. We speculate that these genes are therefore likely to regulate innate immunity in humans. In my talk, I will provide an overview of the innate immune response in C. elegans, outline our comparative genomics screening approach, and discuss one key pathway uncovered by this comparative genomics screening, a Tbc1d23 (RABGAP)-RAB-IRAK2-XBP1 pathway that regulates the maintenance of Toll-like receptor signaling.

2

Damage Associated Molecular Patterns (DAMPs)-Activated Neutrophil Extracellular Trap Formation Exacerbates Sterile Inflammatory Liver Injury

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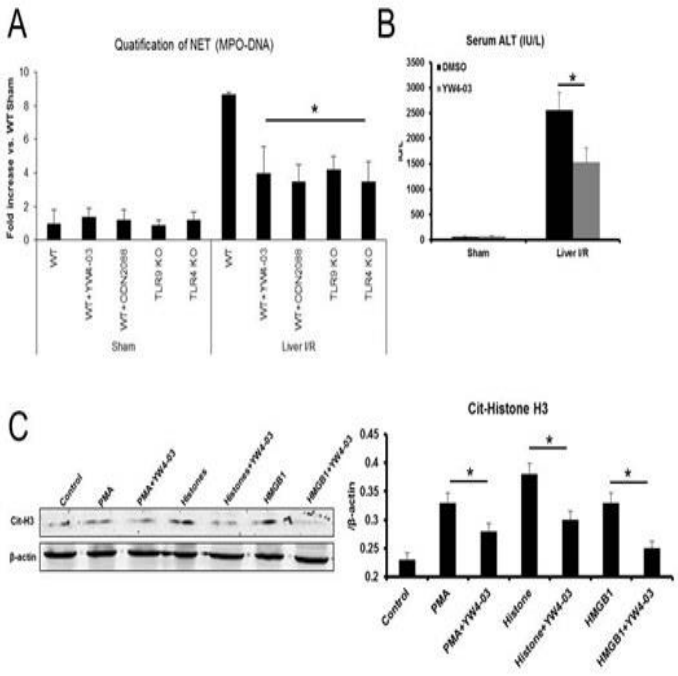
Background Liver ischemia/reperfusion (I/R) injury unavoidably occurs after transplantation, elective hepatic surgery, massive trauma, and hypovolemic shock. The initiation of liver I/R injury results in the release of damage associated molecular patterns (DAMPs) such as HMGB1 and histones. DAMPs subsequently trigger innate immune and inflammatory cascade via pattern recognition receptors such as Toll-like receptor (TLR)-4 or TLR9. Neutrophil infiltration and accumulation in the ischemic liver lobe after reperfusion further contributes to the organ damage, innate immune and inflammatory responses after liver I/R injury. Formation of neutrophil extracellular trap (NET) which is composed of decondensed chromatin fibers lined with neutrophil antimicrobial proteins has been recently found to be a novel response to various stimuli. Whether infiltrated neutrophils form NETs and what their potential roles during liver I/R injury remain unknown. The purpose of our study is to determine the mechanisms by which NETs are stimulated and how they contribute to the innate immune and inflammatory response during liver I/R injury.

Methods TLR4 and TLR9 knockout (KO) mice, and WT counterparts, and TLR9 antagonist (ODN2088)-treated were subjected to a non-lethal partial (70%) warm liver I/R model. Mice received the histones, or HMGB1, with/without treatment of peptidylarginine deiminases (PAD) 4 (a nucleoprotein that mediates NET formation by citrullinating histones) inhibitor (YW4-03). Mouse bone marrow neutrophils were harvested and stimulated with PBS (negative control), or phorbol-myristate-acetate (PMA; positive control), histones, or HMGB1, with/without PAD4 inhibitor.

Results NET formation was identified in the sinusoids of ischemic liver lobes by confocal immunofluorescence. This was associated with increased NET markers, serum level of myeloperoxidase (MPO)-DNA complexes (Fig A), free DNA and nucleosomes, and tissue level of citrullinated histone H3 compared to mice undergoing sham operation. Treatment with PAD4 inhibitor conferred significant protection (Fig B) compared to controls after liver I/R evidenced by inhibition of NET formation (Fig A), indicating the pathophysiological role of NETs in liver I/R injury. In vitro, stimulation of neutrophils directly with DAMPs, HMGB1 or histones resulted in significant NET formation and was inhibited by adding PAD4 inhibitor respectively (Fig C). Gene deletion or inhibition of TLR4 or TLR9 significantly prevents the NET formation both in vivo (Fig A) and in vitro.

Summary Our study demonstrates that DAMPs released during liver I/R promotes NET formation through TLRs signaling pathway. Development of NETs subsequently exacerbates organ damage and initiates inflammatory responses during liver I/R injury. Targeting NET

formation may be a new therapeutic strategy to reduce ischemia-related liver damage.



3

The Melanocortin-Adenosinergic Pathway Mediates Immune Tolerance through a PD-1/PD-1L Dependent Mechanism

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Mice that have recovered from experimental autoimmune uveoretinitis (EAU) are resistant to relapse. One mechanism that contributes to this resistance to relapse is the induction of retinal antigen-specific regulatory immunity in the spleen of EAU-recovered mice. This post-EAU regulatory immunity requires T cell expression of the adenosine 2A receptor (A2Ar), and melanocortin 5 receptor (MC5r) expression on APC. The ligand for MC5r,α-melanocyte stimulating hormone (α-MSH), induces a regulatory APC that promotes Treg cell activation. It is not known what type of Treg cells are induced, and whether other contact-dependent activation signals are involved in activating the Treg cells. Neuropilin-1 (NRP-1) has been reported to distinguish between inducible (iTreg) and natural (nTreg) nTreg cells, and the contact-dependent PD-1/PD-L1 pathway has been implicated in the contra-conversion process to generate Treg cells. C57BL/6 (WT) and $A2Ar^{(-/-)}$mice were immunized with IRBPp 1-20 in CFA to induce EAU. Since $A2Ar^{(-/-)}$mice do not develop post-EAU Treg cells, they are used as a negative control. T cells from the spleen of WT and $A2Ar^{(-/-)}$post-EAU mice were re-stimulated with IRBP for 48 hours, and stained for CD4, CD25, NRP-1, and PD-1. $A2Ar^{(-/-)}$ post-EAU mice had significantly less $NRP\text{-}1^-$ T cells in their spleens compared to T cells from WT post-EAU mice, and sorted $NRP\text{-}1^-$T cells suppressed EAU when transferred to recipient mice. In contrast, mice that received sorted WT $NRP\text{-}1^+$T cells, $A2Ar^{(-/-)}$ $NRP\text{-}1^+$, or $A2Ar^{(-/-)}NRP\text{-}1^-$ T cells were unable to suppress disease in recipient mice. $NRP\text{-}1^-$ Treg cells also expressed PD-1, and sorted $PD\text{-}1^+$ T cells suppressed EAU. WT APC from unimmunized mice were collected from the spleen, treated withα-MSH, washed, and used to activate IRBP-specific primed T cells. Before the activation step, PD-L1 was blocked onα-MSH treated APC, and its ability to activate post-EAU Treg cells was assessed by measuring IFN-γ, TGF-β, and IL-17 production. Blocking PD-L1 on the APC resulted in an inflammatory cytokine profile when used to activate post-EAU Treg cells. In contrast, a regulatory cytokine profile was observed when regulatory APC is used to activate the same Treg cells. These results show that the post-EAU Treg cell is $CD4^+$ $CD25^+$ $NRP\text{-}1^-$ $PD\text{-}1^+$, and that their activation is possible through a PD-1L mediated contra-conversion of effector T cells. Therefore, as EAU resolves the melanocortin-adenosinergic induces autoantigen-specific iTreg cells that may include PD-1/PD-1L mediated contra-conversion of effector T cells into iTreg cells.

4

Sensing Death: How the SaeR/S System Hijacks Cell Fate to Promote Disease

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Successful resolution of staphylococcal infections is heavily dependent on the abundance and proper function of neutrophils, or polymorphonuclear leukocytes (PMNs), at the infection site. In addition to a proper antimicrobial response generated by the PMNs, the mechanisms by which neutrophils undergo cell death after pathogen encounter dictates the outcome of infection. To this end, we examined both host and pathogen contribution to regulation of the neutrophil life span by determining how the *Staphylococcus aureus* (*S. aureus*) SaeR/S two component system modulates PMN cell fate. With the use of cell death and cytokine assays, we show that accelerated PMN death is SaeR/S-

dependent and results from NF-kB inhibition. Furthermore, we show that neutrophil-derived cytokines inhibited by the SaeR/S system are necessary for both PMN survival as well as *S. aureus* clearance.

5

MHCII+ PMN Arise during Acute Cutaneous Leishmania Infection and Can Influence T Cells

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The protozoan parasite *Leishmania braziliensis* is the causative agent of the disease cutaneous leishmaniasis (CL) which can cause ulcerated skin lesions in individuals living in endemic regions. Skin pathology is thought to result from overabundant inflammatory myelocytic and lymphocytic cell responses. The effect of acute CL infection on neutrophil (PMN) activity and how PMN, which are thought to be short-lived effector cells, might influence other cells of the immune response during disease remains unclear. In collaboration with investigators from the Federal University of Bahia (UFBA) we studied PMN from peripheral blood of patients with acute CL encountered in a rural leishmania-treatment center in Corte de Pedra, Bahia. We identified, in both a subset of human CL patients and in murine models of leishmaniasis, the presence of neutrophils that express MHCII (called HLA-DR in humans), a molecule thought to be restricted to professional antigen presenting cells. In murine models of cutaneous leishmaniasis, MHCII+ PMN were identified in the site of parasite infection, draining lymph nodes and circulation. Analysis of CL patient blood smears for immature granulocyte forms (metamyelocytes and bands) showed that CL patients have greater numbers of immature PMN than healthy controls, possibly suggesting a connection between undifferentiated PMN forms and the presence of the MHCII+ PMN population. Further characterization of MHCII+PMN in CL patients by flow cytometry showed that, when compared to patient MHCII- PMN, there were increased markers of PMN activation (decreased surface CD62L), increased degranulation (increased surface CD63, CD66b and CD11b) and increased ROS production (as measured by DHR) following stimulation. In addition to antigen-presentation machinery, MHCII+ PMN in CL patients also showed increased amounts of co-stimulatory molecules (CD80 and CD86). Co-incubation of PMN and T cells isolated either from CL patients or from *Leishmania*-infected mice in the presence of soluble *Leishmania* antigen resulted in T cell proliferation *in vitro*, suggesting that MHCII+ PMN could potentially present antigen and stimulate T cells. These data suggest that *Leishmania* infection results in a subset of PMN with a unique activation and immune profile.

6

Isolation of Oligopotent Granulocyte-Monocyte Progenitors (GMPs) and Lineage-Committed Granulocyte Progenitors (GPs) and Monocyte Progenitors (MPs) from Mouse Bone Marrow

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Accurate identification of specific progenitors is essential for mapping cell fate choices at the single cell level and for precise definition of mechanisms that underlie hematopoietic cell production in the steady-state, during an emergency response (infection/inflammation), and in leukemogenesis. Models of hematopoiesis are currently undergoing extensive revision to incorporate newly discovered progenitor populations and hematopoietic lineage maps remain controversial. In the current study we focused on the production of granulocytes (neutrophils) and monocytes/macrophages. Granulocyte-monocyte progenitors (GMPs), which have the potential to produce both granulocytic and monocytic cells, are contained in the Lin^- c-Kit^+ $Sca\text{-}1^-$ (LKS^-) $CD34^+$ $Fc\gamma R^{hi}$ fraction of mouse bone marrow. However, not all LKS^- $CD34^+$ $Fc\gamma R^{hi}$ cells have the potential to produce both granulocytes and monocytes. Only about 40% of colonies formed in methylcellulose under permissive conditions contain both cell types. The rest are either pure granulocyte or pure monocyte colonies, indicating the presence of large proportions of lineage-committed granulocyte progenitors (GPs) and monocyte progenitors (MPs) in the LKS^- $CD34^+$ $Fc\gamma R^{hi}$ fraction. Using *in vitro* and *in vivo* assays, we now show that initiation of Ly6C surface expression marks lineage commitment by these progenitors and can be used to separate "true" oligopotent GMPs ($Ly6C^-$) from lineage-committed GPs

and MPs ($Ly6C^{+}$). Furthermore, CD115 levels permit the separation of GPs ($CD115^{lo}$) from MPs ($CD115^{hi}$).

Our characterization of these progenitors now permits precise investigation of molecular mechanisms that regulate steady-state myelopoiesis, the emergency response to infection or inflammation, and the development of myeloid cell diseases. Indeed, we show by analysis of single cell gene expression data and by flow cytometry that the transcription factor IRF8 (ICSBP), a known regulator of myelopoiesis, is expressed by GPs and MPs, but not by GMPs. Our data indicate that it exerts its control of myeloid fate specification in the lineage-committed progenitors rather than the oligopotent GMPs. Mutations in IRF8 are associated with acute and chronic myeloid leukemia (AML and CML) in humans. $IRF8^{-/-}$ mice are defective in monocyte production and exhibit CML-like disease characterized by accumulation of immature neutrophils. In ongoing studies we are using $IRF8^{-/-}$ mice to determine how IRF8 regulates myelopoiesis by defining its influence on the survival, proliferation and differentiation of GPs and MPs.

7

Massive Neutrophil Extravasation during Sepsis and TLR2-Induced Cytokine Production are Regulated by Integrin a3ß1.

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Neutrophil migration to sites of infection is vital for pathogen clearance, but massive neutrophil infiltration during systemic inflammation leads to tissue injury and host mortality. While β_2 integrins are well known to mediate neutrophil firm adhesion and crawling, the role of β_1 integrins in vascular extravasation has been less explored. In this study, we evaluated the surface expression kinetics of β_1 and β_3 integrin heterodimers on neutrophils during sepsis using mouse models (CLP and endotoxemia) and human samples. We show that only integrin $\alpha_3\beta_1$ becomes significantly upregulated. Moreover, human subjects with sepsis, but not SIRS (systemic inflammation w/o infection) upregulated $\alpha_3\beta_1$. The relative expression level of $\alpha_3\beta_1$ on neutrophils correlated with their activation state, as based on myeloperoxide secretion and cytokine mRNA levels. Administration of synthetic peptide (LXY2), which binds at the ligand (laminin) binding site on $\alpha_3\beta_1$, and conditional gene deletion of the integrin in granulocytes significantly reduced the number of extravasated neutrophils and improved mouse survival. It is interesting to note that, thru a compensatory mechanism, naive α_3 conditional knock-out (cKO) mice upregulated their integrin $\alpha_6\beta_1$ (another laminin-binding integrin) surface expression by approximately 5-fold. This $\alpha_6\beta_1$ upregulation, however, could not functionally compensate for the deficiency in $\alpha_3\beta_1$-mediated extravasation of neutrophils from α_3 cKO mice during CLP- and LPS-induced sepsis. Therefore, our study provides a mechanism for massive neutrophil infiltration during bacterial sepsis, which is different from the $\alpha_6\beta_1$-dependent mechanism neutrophils use during IL-1β–triggered sterile inflammation previously reported by the Nourshargh group. Peritoneal bacterial clearance was unaffected between CLP-treated α_3 cKO and control mice in our study.
Interestingly, LXY2 binding to $\alpha_3\beta_1$ on neutrophils was associated with a reduction in TLR2-, but not TLR4-induced IL-10 secretion *in vitro*. Co-treatment of wild-type mouse neutrophils with LXY2 and TLR2 ligand Pam_3CSK_4 resulted in markedly reduced FAK phosphorylation from whole cell lysates after 4h *in vitro*, compared to FAK phosphorylation induced by Pam_3CSK_4 alone. Previous studies suggested a role for IL-10 as a regulator of the transition from mild sepsis to irreversible septic shock. Thus, integrin $\alpha_3\beta_1$ upregulation promotes enhanced neutrophil migration and, in turn, could regulate sepsis progression by modulating neutrophil IL-10 release.

8

Intrabronchial Infection of Rhesus Macaques with Simian Varicella Virus Results in a Robust Mucosal Immune Response.

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Varicella zoster virus (VZV) is the etiological agent of varicella (chickenpox) and herpes zoster (shingles). Primary VZV infection is believed to typically occur via the inhalation of virus either in respiratory droplets, from shedding varicella lesions or by direct contact with

infectious vesicular fluid. However, the ensuing immune response in the lungs remains incompletely understood. We have shown that intrabronchial inoculation of rhesus macaques with simian varicella virus (SVV), a homolog of VZV, recapitulates the hallmarks of acute and latent VZV infection in humans. In this study, we performed an in-depth analysis of the host immune response to acute SVV infection in the lungs and peripheral blood. We report that acute SVV infection results in a robust innate immune response in the lungs, characterized by the production of inflammatory cytokines, chemokines, and growth factors. We also detected an increased frequency of plasmacytoid DCs that corresponded with an increase in IFNa levels and correlated with an early decrease in viral loads in the lungs. This innate immune response is followed by T and B cell proliferation, antibody production, T cell differentiation and T cell cytokine production, which correlate with the complete cessation of viral replication. Although terminally differentiated CD8 T cells became the predominant T cell population in bronchoalveolar lavage cells, a higher percentage of CD4 T cells were SVV-specific. Together with our recent observations that only CD4-depleted rhesus macaques experience severe varicella, and that CD4 T cell immunity seems to wane faster than CD8 T cell immunity, our data collectively suggest a critical role for CD4 T cell responses in the resolution of primary SVV infection in the lungs. Given the homology between SVV and VZV, our data provide novel insight into the host mucosal immune response to VZV, which in turn will facilitate development of novel vaccines aimed at enhancing protective immunity in the elderly.

9

Regulating the Regulators: Regulatory Programs of Dendritic Cell Migration

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The primary antigen presenting cell that primes a naive T cell during infection or vaccination is the dendritic cell (DC). DCs function as sentinels to detect invasion and respond by upregulating requisite T cell priming signals and also by migrating to the site of naïve T cell priming, the lymph node (LN). Although this latter step is crucial for a DC to interact with T cells, the molecular regulation of DC exit from sites of immunization is not known. We recently discovered, using a murine system, that one member of the NOD-like receptor family of innate immune molecules, NLRP10, controls the ability of DCs to reach draining LNs. Accordingly, loss of NLRP10 results in a global defect in adaptive immunity upon immunization with an antigen in multiple adjuvants. Using in vivo tracking techniques we determined that this loss of adaptive immunity was due to a DC-intrinsic failure to emigrate out of inflamed tissues following antigen capture. Actin polymerization and migration of NLRP10-deficient DCs in multiple 2D assays revealed no defect in DC movement or chemotaxis. Therefore, we engineered a 3D artificial extracellular matrix that recapitulates inflamed tissue; we can image active DC movement through these matrices towards LN-homing chemokine gradients such as CCL19. Use of these systems uncovered a failure in leading edge coordination by activated DCs deficient in NLRP10 during directed movement, suggesting defective signaling in pathways that control the Rho family of small GTPases. We are delineating the DC-intrinsic regulatory networks that control when DCs emigrate from an infected or damaged tissue in order to relay this information to cells in the draining LN. Further we are testing whether the same regulatory pathways exist in human DCs and ultimately whether alterations in DC migration potentially affect the response to the seasonal influenza vaccine.

10

The Role of CXCR2 in the Host Response to Salmonella Typhimurium Colitis

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Neutrophils are the immune system's first responders to infection and are known to be a critical part of human immune responses. While some of their functions and mechanisms are well-established, the specific signals regulating neutrophil antimicrobial function and the mechanism(s) whereby they are recruited to the mucosa are not well elucidated. Most neutrophils express the chemokine receptor CXCR2, which helps the neutrophil identify and migrate toward inflamed or infected tissues.
To better understand the role of CXCR2 neutrophils during infection, mice were depleted of neutrophils that express CXCR2, and changes in the immune response to *Salmonella* Typhimurium were quantitated. *Salmonella* Typhimurium is a food-borne pathogen that causes severe diarrhea when it infects the intestines and can cause fatal bacteremia if it gets into the bloodstream.

When a healthy individual is infected with *Salmonella*, the immune system responds with a massive influx of neutrophils to the intestines; this response is important in preventing dissemination of *Salmonella* into the bloodstream. The hypothesis driving this research is that signaling through the CXCR2 receptor regulates both the recruitment of CXCR2+ neutrophils and the expression of antimicrobial proteins in the mucosa during infection with *S.* Typhimurium.

BL/6 Mice were depleted of CXCR2+ neutrophils in two ways: injection with an antibody against CXCR2 or genetic deletion of the Cxcr2 gene. Consistent with a protective role for neutrophils, disseminated *Salmonella* numbers were higher in CXCR2+ neutrophil-depleted mice than non-depleted mice. Surprisingly, $Cxcr2^{-/-}$ mice showed lower numbers of disseminated *Salmonella*. Furthur analysis of the knockout mice revealed retention of 129/SvJ genes near the Cxcr2 locus, including Nramp1, an ion transporter that is important for host defense against intracellular pathogens and is non-functional in BL/6 mice. Studies using BL/6 mice with restored Nramp1 function as controls showedsignificantly higher disseminated*Salmonella* numbers in $Cxcr2^{-/-}$mice. Flow cytometric analysis of gut-infiltrating neutrophils revealed the presence of different populations of neutrophils: some that contain the antimicrobial protein calprotectin, and others that do not. Western Blot analysis of intestinal tissue from infected mice shows dramatically reduced levels of calprotectin when CXCR2+ neutrophils are depleted.

This data suggests there is a population of neutrophils that do not express CXCR2 yet are able to migrate to infected gut tissue, and the process of migrating to the gut causes a change in the antimicrobial protein production in some neutrophils, but not others. Understanding the way neutrophils get to sites of infection and how they function as part of the immune barrier of the intestinal tract is important for developing treatments to boost neutrophil presence and antimicrobial activity when people with weak immune systems get sick with bacteria like *Salmonella*.

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Dysregulation of Neutrophil-Maintained Bone Marrow Hematopoietic Niche Induces Inflammatory Myelopoiesis

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Obesity is a form of a chronic inflammatory state that has been implicated in disease inception and progression. Inflammatory myelopoiesis helps to sustain this state and is largely dependent on hematopoietic stem progenitor cells (HSPCs) in the bone marrow (BM), of which function is partly regulated by poorly defined microenvironment called the "niche". However, little is known about the role of neutrophils in the BM hematopoietic niche. Here we show that diet-induced obesity (DIO) increases inflammatory myelopoiesis associated with increased BM granulocyte-monocyte progenitor cells (GMPs) and circulating inflammatory $Ly6C^{hi}$ monocytes. We found that senescent $CD62L^{dim}$ neutrophils, which have high CXCR4 expression and have been shown to favorably home to the BM, are increased in circulation and decreased in the BM in DIO mice. Moreover, adoptive transfer of senescent neutrophils from healthy donors into DIO mice is sufficient to reduce circulating inflammatory monocytes and BM GMPs with an increase in $CD62L^{dim}$ neutrophils in the BM. These suggest that senescent neutrophil trafficking to the BM from circulation is impaired in DIO. In vitro, co-culture of neutrophils suppress toll-like receptor-mediated generation of inflammatory myeloid cells from HSPCs and this effect is partially blocked by a hydrogen peroxidase scavenger, catalase, and NADPH oxidase-lacking ($gp91^{phox-/-}$) neutrophils, indicating that the suppression of inflammatory myelopoiesis is mediated through neutrophil-derived hydrogen peroxide. Consistently, $gp91^{phox-/-}$ mice exhibit increases in inflammatory myeloid cells and BM GMPs, and adoptive transfer of circulating neutrophils from wild-type mice, but not from $gp91^{phox-/-}$ mice, decreases inflammatory myeloid levels in $^{gp91phox-/-}$ mice. In summary, our data uncovers a novel role of neutrophils in maintaining BM hematopoietic niche through suppressing inflammatory myelopoiesis of HSPCs. Dysregulation of this mechanism may be involved in DIO-induced inflammatory myelopoiesis.

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Disruption of the Polyisoprenyl Diphosphate Phosphatase 1 Gene Enhances Neutrophil Dependent Bacterial Clearance in a Murine Model of Pneumonia

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Presqualene diphosphate (PSDP) is an intracellular phosphorylated lipid in neutrophils (PMNs) that controls the actions of key enzymes involved in cell activation. Recent studies have identified polyisoprenyl diphosphate phosphatase 1 (PDP1) as a lipid phosphate phosphatase that serves as a signaling nexus in human PMNs. Exposing cells to inflammatory stimuli initiates a signaling cascade consisting of protein kinase C dependent PDP1 activation, the subsequent conversion of cellular pools of PSDP to its less active monophosphate form, and cell activation. The anti-inflammatory and pro-resolving mediator, lipoxin A4, blocks protein kinase C/PDP1 associations and subsequent cell activation. Here, in work in progress, we present evidence that, similar to human PDP1, its murine homologue displays a distinct substrate preference for PSDP. PDP1 deficient (PDP1-/-) mice were generated and assessed for their ability to clear a bacterial challenge in an experimental model of pneumonia. At 2, 6, and 24 hours after intratracheal E. coli instillation, PDP1-/- mice, relative to littermate controls, displayed significantly lower CFUs in lung homogenates. Of interest, a significant increase in lung interstitial PMNs was detected at baseline and at the 2 hour time point suggesting a regulatory role for PDP1 in other cell types. PMN depletion using an Ly6G antibody injected 24 hours prior to bacterial instillation led to lung bacteria clearance in PDP1-/- that was similar to wild type mice, consistent with an important role for PMN PDP1. Bone marrow derived PMNs from PDP1-/- mice that were exposed to phorbol esters had decreased capacity for superoxide anion generation compared to control cells. PDP1-/- and control PMNs displayed similar levels of bactericidal activity in vitro for E. coli. In addition, PDP1-/- PMNs displayed a dose dependent decrease in neutrophil extracellular traps than wild-type cells in response to E. coli. Decreased NETosis was a characteristic of both murine PDP1-/- PMNs and human PMNs transfected with anti-PDP1 antibodies. Taken together, these data suggest that PDP1 is a pivotal signaling mechanism for cellular responses in bacterial host defense and represents a potential new therapeutic target to regulate acute inflammation.

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Nadph Oxidase Limits Sterile Inflammation by Modulating IL-1/G-CSF-Driven Neutrophilic Inflammation

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The phagocyte NADPH oxidase is a multi-subunit enzyme that generates antimicrobial superoxide in response to multiple agonists. Derivative reactive oxygen species (ROS) are important in many immunological processes including antigen presentation, autophagy and modulation of inflammatory responses. Chronic granulomatous disease patients with ablative NADPH oxidase subunit mutations are susceptible to recurrent microbial infections and chronic inflammatory conditions. Moreover, variant NADPH oxidase alleles causing only partial loss of enzyme activity are also associated with increased susceptibility to autoimmune diseases. The underlying molecular pathways that are altered by NADPH oxidase-derived ROS that cause over inflammation are poorly defined.

We hypothesized that the NADPH oxidase regulates the dynamics and magnitude of inflammatory cell recruitment in response to Danger Associated Molecular Patterns (DAMPs) generated during sterile necrotic tissue injury, early in inflammation.

Mice lacking NADPH oxidase activity due to inactivation of Cybb, in a model of peritoneal inflammation, exhibited exaggerated and prolonged inflammatory response to necrotic cells and to uric acid crystals, a canonical DAMP. IL-1 and IL-1receptor (IL-1R) - driven secondary pro-inflammatory cytokines IL-6, G-CSF were also elevated in the Cybb Knockout (CybbKO) mice and associated with early recruitment of neutrophils into the peritoneal cavity. Transplant studies indicated that NADPH oxidase-deficient hematopoietic sentinel cells are required for the production of exaggerated amounts of peritoneal IL-1. IL-1 further induced local production of G-CSF and enhanced peripheral neutrophil mobilization. G-CSF is a major regulator of neutrophil production, survival, and under inflammatory conditions, augments effector functions of immune cells. Additional data suggest that steady state granulopoiesis was similar in WT and CybbKO. Moreover, in vivo neutralization of IL-1α abrogated peritoneal neutrophilic influx and the increase in G-CSF, while IL-1β neutralization had no effect on ameliorating acute inflammation. Loss of NADPH oxidase activity dysregulated IL-1 production, elevated local G-CSF levels and enhanced neutrophil recruitment in response to tissue injury. Thus, the NADPH oxidase regulates the

dynamics of inflammatory cell recruitment and local cytokine production early in inflammation.

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New Roles for PagP in Bacterial Evasion of Host Immune Defenses

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PagP is an outer membrane enzyme of Gram-negative bacteria that functions to attenuate the host immune response to infections. The *E. coli* homologue is a small beta-barrel with an interior palmitoyl-group binding pocket known as the hydrocarbon ruler. The phospholipid donor must migrate into the external leaflet before diffusing laterally through the beta-barrel wall at a site known as the crenel. It is here that regiospecificity for the *sn*-1 acyl chain is enforced, with the result that C18 acyl chains at the *sn*-2 position are excluded. The main function of the hydrocarbon ruler is to exclude C14 acyl chains esterified at the *sn*-1 position. The acceptor of the palmitate chain is lipid A, or endotoxin, an acylated and phosphorylated disaccharide of glucosamine. Lipid A binds opposite the crenel at another lateral opening in the beta-barrel wall known as the embrasure. The reaction depends on the formation of a ternary complex with the acyltransfer dependent on His33 and Ser77, and with Arg114 functioning to position the head group of the phospholipid donor. Recently, we have observed that phosphatidylglycerol (PG) also functions as a palmitate acceptor for PagP, which leads to the accumulation of palmitoyl PG in the outer membrane. As such, the role of PagP in fortifying the permeability barrier against cationic antimicrobial agents must now be considered to be a consequence of the combined palmitoylation of both lipid A and PG. Additionally, despite the apparent absence in BLAST searches of PagP from the *P. aeruginosa* genome, a divergent homologue does in fact exist, and serves to palmitoylate lipid A on the opposite glucosamine sugar compared to that of *E. coli*. Unlike *E. coli* lipid A palmitoylation, which attenuates innate immune signaling, *P. aeruginosa* lipid A palmitoylation is proinflammatory. *P. aeruginosa* isolated from the airways of chronically infected cystic fibrosis patients are found to possess constitutively palmitoylated lipid A, raising the possibility that inhibition of PagP in the cystic fibrosis lung environment might reduce inflammation leading to chronic infections.

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Targeting TLR4 as a Efficacious Anti-Influenza Strategy

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We previously reported that TLR4$^{-/-}$ mice are refractory to influenza-induced lethality. Therapeutic administration of the TLR4 antagonist, Eritoran, was found to block influenza-induced lethality in mice, as well as lung pathology and clinical symptoms, viral titers and cytokine responses. Both CD14 and TLR2 were also found to be required for Eritoran-mediated protection. We have since extended these findings by showing that administration of a TLR4-specific IgG on day 2 only or on days 2 and 4 after influenza infection (strain A/PR/8/34; "PR8") significantly protected wild-type mice from lethal influenza infection. Thus, TLR4, a known target of Eritoran, is directly involved in influenza-induced lethality. Additionally, PR8-infected mice that received Eriotran therapeutically and survived were refractory to subsequent re-infection by the same virus, indicating that Eritoran treatment does not hinder development of influenza immunity. Interestingly, we found a very different outcome when Eritoran treatment was initiated 3 h prior to PR8 infection and continued daily for 4 successive days. WT mice were not protected from lethality, implying that Eritoran given prophylactically must block early virus induced-signaling required for protection. More surprisingly, this same treatment regimen rendered TLR4$^{-/-}$ mice susceptible to PR8 infection. This implies that a non-TLR4 target of Eritoran is necessary for TLR4$^{-/-}$ mice to survive PR8 infection when administered early relative to infection. Pre-treatment/early administration of Eritoran had no effect on the susceptibility of CD14$^{-/-}$ or TLR2$^{-/-}$ mice. Finally, we observed improved protection against influenza by Eritoran when administered with Oseltamivir starting at days 4 or 6 post-infection. Overall, our data provides further support that the blockade of TLRs and potentially other pattern

recognition receptors in combination with antivirals could provide stronger therapeutic intervention for influenza and potentially other infections. Supported by AI18797 (SV), AI104541 (SV/JB)

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Effect of Core Polysaccharide in Activation of TLR4 by Escherichia Coli Lipopolysaccharide

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Host responses to invading Gram-negative bacteria (GNB) typically include rapid and robust innate immune responses to GNB lipopolysaccharides (LPS) by host cells expressing Toll-like Receptor 4 (TLR4) and MD-2. Maximal host responses to LPS depend on ordered LPS•host protein interactions involving Lipopolysaccharide Binding Protein (LBP), albumin and CD14 which promote extraction and transfer of LPS monomers to CD14 followed by transfer of LPS monomers to MD-2•TLR4, triggering TLR4 activation. LPS consists of up to 3 structurally distinct, covalently linked domains: a Lipid A region embedded within the GNB outer membrane and a core oligosaccharide with or without an O-antigen polysaccharide chain. LPS species that lack an O-antigen polysaccharide chain are known as "Rough" (R) LPS. Escherichia coli LPS is a prototypical LPS, containing hexa-acylated Lipid A and a core oligosaccharide containing up to six different sugar residues. The length and composition of the core oligosaccharide chain varies among species and strains of GNB; if and how this structural variation affects MD-2/TLR4 recognition and response to LPS has not been rigorously studied. We now show that the potency and amplitude of TLR4 activation by E. coli LPS increases with core oligosaccharide chain length (Ra-LPS > Rc-LPS > Re-LPS > Lipid A) , reflecting effects of the core polysaccharide on the TLR4-activating potency of LPS.MD-2 complexes. These findings provide the first description of how the core polysaccharide of TLR4-activating LPS can influence TLR4 activation.

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The Bordetella Saga: Lipopolysaccharides Structure to Activity Relationships

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The Bordetella genus comprises several human and animal respiratory tract pathogens and nine species are currently described. The most extensively studied ones are *B. pertussis, B. parapertussis,* and *B. bronchiseptica*. Despite the availability of vaccines, there still are 400,000 deaths/year caused by whooping cough (*B. pertussis*) and a high morbidity in many countries (*B. pertussis* and *B. parapertussis*). Newly emerged human pathogens (*B. bronchiseptica* and *B. holmesi*i) also represent a serious danger. It is important to note that significant economic losses are associated with infections in poultry and cattle due to Bordetella (*B.avium B.hinzii* and *B. bronchiseptica*) species.
Although LPS molecules share a common architecture, they vary with each species of a genus and each structural detail exerts a strong influence on the biological activities. This was particularly well demonstrated with *B. pertussis* lipids A and in other structures of the genus when discrete modifications strongly impacted signalization.
While studying the endotoxin structural diversity and variability, as factors of bacterial adaptation and virulence, we recently established detailed structures of several laboratory strains and clinical isolates of *B. pertussis, B. parapertussis, B. bronchiseptica B. petrii* and *B. holmesii*. We showed that *B. avium, B. bronchiseptica* and *B. pertussis* were modifying their lipids A by substitution of both phosphate groups with glucosamine residues. Their presence was shown to strongly increase the biological activities of purified endotoxin and whole bacteria. The occurrence of palmitate in the lipid A structures of *B. bronchiseptica*, at one or two sites, was correlated with virulence.The presence of short-chain fatty acids decreased the biological activity of the corresponding LPS.
These structural details strongly impact the recognition of LPS molecules complexed with MD2 at the level of the TLR-4 cellular receptors with consecutive signalling cascades, leading to inflammation.

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Natural Killer Cell Mediated Adaptive Immunity in Trans-Nuclear Mice and Humanized Mice

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NK cells are traditionally defined as cells of the innate immune system, as they lack recombination-activating-gene (Rag) recombinase-dependent clonal antigen receptors. However, we and others have shown that subsets of murine NK cells mediate adaptive immunity to a variety of haptens and experimental anti-viral vaccines. To assess whether the molecular origin of NK cell mediated adaptive immunity is genomically fixed, we used somatic cell nuclear transfer (SCNT) of a nucleus derived from a 2,4-dinitro-1-fluorobenzene (DNFB) sensitized Rag2–/– NK cell, and tetraploid-blastocyst complementation, to generate memory NK cell-derived transnuclear (TN)-ES cells and TN-mice. Cloning by SCNT supports the generation of pluripotent stem cells from somatic cells in which the genetic material of the donor cell remains stable and as established during cell fate determination in development. Lymphocyte-deficient Rag/IL-2Rγc–/– recipients of TN-NK cells, and 4/5 (TN x Rag–/–) F1, and 5/10 (TN F1 x Rag–/–) F2 offspring responded vigorously to DNFB challenge, but failed to respond to a novel Ag, oxazolone (OXA) despite prior vaccination. Hence, the molecular origin of Ag-specific NK cell-mediated immunological memory transferrable via the genome.

Whether human NK cells can mediate adaptive immune responses is currently unknown. We therefore compared NK cell frequency and phenotypes in vaccinated v.s. naïve humanized mice, and asked whether Ag-primed human NK cells exhibit increased killing of Ag-loaded target cells in vitro, and whether HIV gag/env primed human NK cells alter the course of HIV infection in donor-matched humanized mouse recipients. Vaccination resulted in a significant increase in CX3CR1+, CXCR6+, CD90+, and CD16+ human NK cells in spleens and livers of humanized mice, and vaccine primed human NK cells killed Ag-loaded targets significantly better than naïve donor-matched NK cells. Moreover, adoptive transfer recipients of donor-matched HIV-gag/env-primed human NK cells presented with a significant reduction in HIV-1 viral load compared to mock recipients. This reduction in HIV-viral titer may be due to hIL-15-treatment of NK cell donor mice, and/or an increase of NK cell numbers in adoptive transfer recipients, and/or due to HIV-Ag-specific NK cell priming before transfer, and is under further investigation. Whether human NK cells are capable of long-term Ag-specific immunity is currently unknown, however, our data clearly demonstrates that human NK cells remember prior stimuli with anti-viral vaccines, and as such, it provides a strong rationale for further studies that address whether human NK cells can be exploited to mediate clinically meaningful immune responses.

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Mechanisms Regulating Nucleic Acid Sensing Toll-Like Receptors

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The Toll family of receptors has critical roles in microbial recognition and activation of defense responses. Cell surface Toll-like receptors (TLRs) including TLR4/MD-2, TLR1/TLR2, TLR2/TLR6, or TLR5 recognize microbial membrane lipids or flagellin, whereas TLR3, 7, 8, and 9 reside in intracellular organelle and sense nucleic acids. Recent progresses have revealed that self-pathogen discrimination by TLRs is error prone and TLRs have been implicated in a variety of autoimmune diseases. Although the ligand specificity and downstream signaling pathways of each TLR have been extensively studied, much less is known as to how innate immune responses to self-products are controlled. Nucleic acid-sensing TLRs, TLR7, 8 and 9, have a risk of responding to self-derived nucleic acids. To prevent autoimmune responses, these TLRs are thought to be controlled by restricting nucleic acid sensing in endolysosomes, not the cell surface. Extracellular self-nucleic acids are instantly degraded and do not get to endolysosomes, whereas microbial nucleic acids are protected by microbial membranes and get to the endolysosomes. To limit nucleic acid sensing in endolysosomes, trafficking of nucleic acid-sensing TLRs from ER to endolysosomes is tightly controlled. The subcellular distribution of TLR7 and 9 are dependent on a multiple transmembrane protein Unc93B1. Unc93B1 is not just TLR transporter but also has a role in controlling the balance between TLR7 and TLR9. Unc93B1-dependent control of TLR7 and 9 balance prevents TLR7-dependent autoimmunity. TLR7 has a unique risk of autoimmunity, which is counteracted by TLR9. We here focus on a mechanism underlying TLR7-unique risk of autoimmunity and show that TLR7 is a promising target for therapeutic intervention in autoimmune diseases.

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Pellino-1 and Pellino-3b in TLR Signaling and Tolerance

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Endotoxin tolerance reprograms Toll-like receptor (TLR) 4-mediated responses in monocytes and macrophages by attenuating expression of pro-inflammatory cytokines while retaining or enhancing LPS-driven inducibility of anti-inflammatory cytokines and antimicrobial effectors. We previously demonstrated deficient TLR4-induced activation of IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1 as critical hallmarks of endotoxin tolerance. E3 ubiquitin ligases Pellinos regulate TLR signaling pathways via modifications of IRAK kinases. In this study, we examined the impact of endotoxin toleranceon Pellino-1/3b expression and utilized overexpression and gene ablation approaches to establish their functions in TLR2/4 signaling. LPS stimulation increased Pellino-1 mRNA and protein expression in control, medium-pretreated human monocytes, THP-1 and MonoMac-6 macrophage-like cells. Endotoxin-tolerant cells showed attenuated expression of Pellino-1 but maintained increased levels of Pellino-3b. Overexpression of Pellino-1 in 293/TLR2 and 293/TLR4/MD2 cells enhanced Pam3Cys- and LPS-mediated NF-κB activation and cytokine gene expression, while Pellino-1 knockdown reduced these responses, demonstrating Pellino-1 as a positive regulator of TLR4 signaling. In contrast, Pellino-3b overexpression reduced, whereas Pellino-3b gene knockdown increased TLR2/4-driven NF-kB activation and expression of inflammatory cytokines, establishing it as a negative regulator. These data suggest non-redundant functions for Pellino-1 and Pellino-3b within the TLR4 signaling pathways and implicate their role in endotoxin tolerance.

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Allergy-Protective Activation of Dendritic Cells by the Cowshed Bacterium Lactococcus Lactis G121 Requires Endosomal Acidification and Activation through TLR13

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Introduction: Epidemiological studies show that traditional farming environment during fetal development and in early childhood reduces the incidence of allergies later in life. A number of Gram-negative and Gram-positive bacterial strains have been isolated from cowsheds and proven to be allergy-protective in various mouse models. In the human system, we could show that protective immune-modulatory effects of the Gram-negative *Acinetobacter lwoffii* F78 depend on TLR4, whereas the Gram-positive *Staphylococcus sciuri* W620 utilizes mainly TLR2. In addition, both strains engage also NOD2. Knowledge of the identity of mediators of the allergy-protective effect of *Lactococcus lactis* G121, and the pattern recognition receptors involved, however, is largely lacking.

Objectives: Identification of the central receptors and mechanisms by which L. lactis G121 induces an allergy-protective immune response.

Material and Methods: Challenge-induced cytokine release from in vitro cultures of human monocyte-derived dendritic cells (moDCs), from co-cultures of moDCs with autologous naïve $CD4^+$ T-cells as well as from mouse bone marrow-derived dendritic cells (BMDCs) was analyzed. For the *in vivo* experiments, an OVA mouse model using intranasal transfer of BMDCs was used.

Results: Challenge of DCs with *L. lactis* G121 induced release of IL-12p70, IL-23, IL-10 and IFN-β. The inhibitors Cytochalasin D and Bafilomycin A1 (Baf) drastically inhibited cytokine production, indicating the importance of bacteria uptake and endosomal acidification. Baf-treatment of DCs also inhibited L. lactis-induced up-regulation of co-stimulatory molecules and thus L. lactis-induced IFN-γ and IL-10 release in co-culture with T-cells. Using an OVA mouse model with intranasal transfer of differently treated and sensitized BMDCs, we demonstrated the importance of endosomal acidification in L. lactis-mediated allergy protection also *in vivo*.Activation of BMDCs from TLR13-deficient

mice strongly implicates TLR13 as a major host sensor of *L. lactis* G121.

Conclusion: *L. lactis* G121 uptake, endosomal acidification and activation of TLR13 underlie the allergy-protective effect of the cowshed bacterium *L. lactis* G121. Whether the TLR13 activating or other *L. lactis* G121 molecular patterns are similarly operative in humans is a question arising.

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Molecular Requirements for Activation and Antagonism of TLR4

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LPS/MD-2/TLR4 ternary complexes containing TLR4-activating hexaacylated LPS form a symmetric "m-shaped" dimer of the ternary complex, with each TLR4 containing an activating endotoxin (E)/MD-2. Each E/MD-2 bridges the two TLR4 molecules: binding TLR4 within the same ternary complex by agonist-independent interactions and the neighboring TLR4 by agonist-dependent interactions. Whether or not endotoxin-induced dimerization and activation of TLR4 depends on each TLR4 containing an activating E/MD-2 complex is unknown. To address this question, we produced and purified wild-type (wt) and variant E/MD-2 complexes that have equivalent agonist-independent TLR4 binding properties but markedly different TLR4 agonist properties. LOS/MD-2[125-I] of very high specific radioactivity (500,000 cpm/pmol), was used to measure specific, saturable TLR4-dependent binding to HEK/TLR4 cells and establish experimental conditions in which added wt LOS/MD-2 occupied < 10% of surface TLR4 and induced sub-maximal TLR4-dependent cell activation. Under these conditions, added sMD-2 alone or a ligand (eritoran)/MD-2 complex with virtually pure TLR4 antagonist properties produced dose-dependent inhibition of cell activation by wt LOS/MD-2 that paralleled inhibition of wt LOS/MD-2 binding. A mutant LOS/MD-2 complex (LOS/MD-2(F126A)) with ca. 1% the TLR4 agonist activity of the wt complex produced similar dose-dependent inhibitory effects but uniquely, at sub-saturating doses, acted synergistically with wt LOS/MD-2. These findings are consistent with a model of endotoxin-induced TLR4 activation in which agonist-dependent bridging contacts between both ligand/MD-2/TLR4 ternary complexes are necessary and function in a combinatorial manner to permit enhancement of TLR4 activation by intrinsically very weak TLR4 agonists when occupation of TLR4 by strong agonists is limiting.

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Micro- and Nanoscale Spatial Organization of Candida ß-Glucan and C-Type Lectins during Innate Immune Fungal Recognition

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Dendritic cells express DC-SIGN and CD206, C-type lectins (CTLs) that bind a variety of pathogens and facilitate their uptake for subsequent antigen presentation. These receptors are expressed on innate immune cells such as immature dendritic cells (DCs) that are responsible for coordinating immune responses to significant fungal pathogens of the Candida genus. Both CTLs form membrane micro/nanodomains on naïve DCs, and they dramatically rearrange during the formation of fungal recognition contact sites. We have quantitatively analyzed the spatiotemporal distribution of CTLs in host-fungal particle contact sites using confocal microscopy and super resolution fluorescence imaging. We examined the spatiotemporal distribution of CTLs in contacts with various environmental and pathogenic Ascomycetous yeasts (*S. cerevisiae*, *C. albicans*, *C. parapsilosis*) by confocal microscopy. Despite remarkable similarity in the composition and gross structure of the cell walls of these yeasts, we found significant heterogeneity in the amount and rate of CTL recruitment amongst these species. For instance, the

most prevalent pathogen, C. albicans, exhibited the smallest contact site areas and weakest receptor recruitment. This yeast also provoked the poorest phagocytic responses by DCs, suggesting that this species may evade innate immune surveillance by manipulating the process fungal contact site construction to its advantage. Furthermore, we have examined nanostructure of both CTLs and β-glucan, an important immunogenic fungal cell wall ligand, by super resolution imaging and spatial statistical analysis. We found that CTL nanodomains are larger and more closely packed in the contact site relative to non-contact membrane. DC-SIGN nanodomains in fungal contact sites exhibit a 70% area increase and a 38% decrease in interdomain separation. Likewise, contact site CD206 nanodomains possess 90% greater area and 42% lower interdomain separation relative to non-contact regions. Finally, we have also performed the first ever super resolution imaging on C. albicans cell wall β-glucan nanostructure and find that its surface exposure is restricted to small ~100 nm patches. This is consistent with current theories that this immunogenic ligand is largely "masked" from CTL recognition, and the exposure sizes observed are predominantly below the size thought to support DC activation. Interestingly, β-glucan nanodomains do not significant change their size or inter-domain distances after caspofungin treatment, a condition that induces β-glucan "unmasking". These spatial arrangements of ligand and receptor are likely to be important for the avidity of interaction of receptors with fungal pathogens and the availability of ligand that triggers CTL-mediated DC activation.

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Mirroring the Complexity of Human Inflammatory Uveitis in Animal Models

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Human inflammatory noninfectious uveitis, believed to be driven by autoimmune responses to eye-derived antigens, is responsible for up to 20% of legal blindness in western countries and up to 25% in the developing world. Uveitis varies in terms of clinical manifestations and course. As an explanation, associations with Th1 and Th17 responses have been reported but causal relationships and etiological factors are obscure. We have developed and studied three transgenic mouse models of uveitis, which are on the same genetic background (B10.RIII) and are driven by immunity to the same autoantigen (retinal IRBP), but which differ in etiology, clinical characteristics and effector mechanisms: *(i)*experimental autoimmune uveitis (EAU) induced by immunization with IRBP161-180 emulsified in complete Freund's adjuvant (CFA); *(ii)*EAU induced by infusion of IRBP161-180-pulsed dendritic cells (DC); *(iii)*spontaneous uveitis in mice transgenic for the IRBP161-180-specific T cell receptor, in which disease development is in part dependent on commensal microflora. A series of immunological and immunogenetic experiments that included adoptive transfers, genetic crosses and effector cytokine neutralization studies, demonstrated that CFA-EAU was strongly dependent on a Th17 response, whereas the DC-induced as well as the spontaneous disease were dependent on a Th1 effector response. We conclude that either Th1 or Th17 effector responses can drive tissue pathology in uveitis, and propose that the innate immune context in which the autoantigen had initially been encountered by the immune system determines the nature of this response. These findings may help to explain the clinical and immunological heterogeneity of human disease and could have implications for therapy.

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C1q Modulation of Cytokine Production in Human and Mouse Macrophages during Clearance of Atherogenic Lipoproteins Correlates with Suppression of NFkB Activity

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Innate immune protein C1q plays a dual role in the chronic inflammatory disease atherosclerosis. It is known to be involved in activation of the inflammatory complement cascade, which exacerbates pathology and atherosclerotic lesion development. However, in early stages of atherosclerosis C1q is protective, although its precise mechanism has not been elucidated. We hypothesize that complement-independent activities of C1q are reprogramming macrophage responses during clearance of damaged lipoproteins. As a pattern recognition receptor, C1q recognizes and binds atherogenic forms of modified lipoproteins such as oxidized low density lipoprotein (oxLDL). Using human monocyte-derived macrophages, murine bone marrow-derived macrophages from C1q-deficient animals and

the mouse macrophage cell line Raw264.7, we determined that C1q enhances human and mouse macrophage clearance of oxLDL but not native LDL, measured by flow cytometry. In addition, levels of pro-inflammatory cytokines IL-1β and IL-6 were downregulated by C1q during macrophage clearance of oxLDL, whereas anti-inflammatory cytokine IL-10 levels were increased, measured by qPCR and ELISA. An NFkB luciferase gene reporter assay showed C1q suppression of transcription factor NFkB activation during lipoprotein clearance, thereby providing a possible mechanism for the observed modulation of cytokines. C1q-polarization of macrophages towards an M2-like phenotype may help to limit inflammation in early atherosclerosis and slow disease progression.

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New Challenges of Endotoxin Detection in Modern Pharmaceuticals

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Bacterial Endotoxin Testing (BET) is done for several decades and the test principles haven't changed since then. By now, these classical test have been directly transferred to modern fields of application. The Limulus Amebocyte Lysate (LAL) test was originally geared to modest sample conditions. Meanwhile, the substantial progress in Biotechnology and the growing market for bio-pharmaceuticals are challenging requirements for conventional used test methods.

In recent presentations and publications, inadequate endotoxin recoveries in common formulations of biologics were described. Due to the fact, that within certain formulation conditions, no Endotoxin is detectable, it is assumed that the endotoxin is masked and not accessible for factor C of limulus lysate-based detection systems.

Here, we investigated the phenomenon of endotoxin masking in certain ordinary sample matrix compositions. The results show, that buffered samples containing protein and/or surfactant can significantly diminish the active state of endotoxin in common detection systems. Furthermore, we can demonstrate that the endotoxin masking capacity of such compositions is huge and that the disappearance of endotoxin activity in the detection system is dependent on various parameters. Moreover, the velocity and extent of endotoxin masking can be modulated by variation of certain parameters.

In conclusion, endotoxin masking prevents exact determination of endotoxin contaminations using classical detection methods. The detailed understanding of the biophysical principles of endotoxin masking allows the development of dedicated protocols for overcoming inadequate endotoxin measurements in complex formulations.

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Acute Phase Deaths from Polymicrobial Sepsis Are Characterized by Suppression, but Not Exhaustion, of Innate Immunity

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INTRODUCTION

Within 6 and 24hours post-Cecal Ligation and Puncture (CLP) induced sepsis, mice may be stratified as predicted to Live (Live-P) or die (Die-P) based on plasma IL-6. At 6hr, both groups have equivalent peritoneal bacterial CFUs and recruitment of phagocytes. By 24hr, however, Die-P mice have increased bacterial burden, *and*, increased neutrophil (PMN) recruitment. This suggests that Die-P phagocytes have impaired bacterial killing and this leads to mortality.

METHODS

Sepsis was induced by CLP in female outbred mice. Peritoneal Cavity cells (PCs) were harvested by lavage at 6hr or 24hr post-CLP. Mice were stratified into Live-P and Die-P groups by plasma IL-6 levels. Bacterial killing was determined with *E.coli* and the harvested peritoneal cells. Reactive Oxygen Species (ROS) were measured with Dihydrorhodamine-1,2,3, and Luminol chemiluminescence. Total phagocytosis and intra-phagosomal processes were determined with triple-labeled *E.coli*, covalently labeled with ROS and pH sensitive probes, and an ROS/pH insensitive probe for normalization. Data were acquired via flow cytometry or luminometry.

RESULTS

Within 6hr of CLP, Die-P PCs kill significantly less bacteria than Live-P, and this continues to 24hr. Die-P neutrophils and macrophages generate significantly less ROS in response to *E.coli* or PMA stimulation, thus suggesting these cells are exhausted or suppressed. Challenging PCs with a saturating dose of triple-labeled

E.coli revealed that although a similar percentage of total PCs phagocytized *E.coli*, Die-P PCs phagocytized significantly fewer *E.coli*, thus suggesting that Die-P PCs are suppressed but not exhausted. Furthermore, Die-P phagocytizing cells generated significantly less intraphagosomal ROS, and acidified their phagosomes significantly less than Live-P phagocytizing cells. Data is summarized in Table 1.

CONCLUSION

Despite fluid resuscitation and antibiotics, ~50% of mice die in our model of CLP induced sepsis. These data show that although Die-P mice have augmented neutrophil recruitment, they also have deficits in bactericidal functions. Furthermore, Die-P cells are not exhausted, but are instead immunosuppressed. This is important as the prevailing sepsis paradigm posits that deaths in the acute phase of sepsis (<5 days) are due to too much inflammation, whereas chronic phase deaths (>5 days) are characterized by not enough inflammation and immunosuppression. These data suggest that immunosuppression is already prevalent in the acute phase.

	Neutrophils		Macrophages		Total Peritoneal Cells	
	6hr	24hr	6hr	24hr	6hr	24hr
O-E.coli Mediated ROS	<	<	<	<		<
PMA Mediated ROS	<	<	<	<		<
% of cells phagocytizing					≈	≈
Internalized Bacteria Fluorescence	<	<	<	<		<
Phagosomal ROS	≈	<	≈	<		<
Phagosomal Acidification	≈	<	<	<		<
Cell #	≈	>	≈	>	≈	>
Bacterial CFU					≈	>
Bacterial Killing					<	<

Table 1: Results Summary for Die-P mice, as compared to Live-P. O-*E.coli* = Opsonized E.coli. PMNs/macrophages were identified by surface markers or phagosomal processes. Peritoneal cell ROS results (right most column) obtained by Luminol chemiluminescence. Total Peritoneal Cells were assayed without surface marker discrimination, but are predominantly PMNs/macrophages (>90%). All differences are p=<0.05 or less. Data from at least 3 independent experiments.

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Ethanol Intoxication Amplifies Pro-Inflammatory Phenotype on Pre-Alveolar Macrophages after Post-Burn Pulmonary Infection

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Alcohol intoxication is involved in 50% of all burn injuries that require hospitalization, resulting in an increased risk of multi-organ failure and mortality, relative to those who had not been drinking. Experimental models of intoxication and burn have demonstrated alterations in innate and adaptive immunity resulting in marked immune dysfunction and greater susceptibility to pulmonary infection. When an intratracheal Pseudomonas aeruginosa (PA) infection follows intoxication and burn injury, our studies have revealed amplified pulmonary inflammation, relative to either insult alone. The magnitude and duration of pulmonary inflammation are mediated in part by alveolar macrophage (AM) function. Here we sought to identify leukocyte populations within the alveolar space that may be contributing to exacerbated pulmonary inflammation seen after intoxication, burn injury, and infection. Mice were subjected to 1.2 g/kg ethanol by oral gavage 30 minutes prior to a 15% total body surface area dorsal scald injury. Following injury, mice were given an intratracheal infection of 2,000 CFUs of PA. At 24 hrs post-injury, bronchoalveolar lavage cells were collected and leukocyte populations were identified by flow cytometry. Consistent with previously published data, there was no change in the frequencies of AMs or neutrophils, regardless of treatment group at 24 hrs. Interestingly, we identified a F4/80+ Ly6G+ population and found that with burn alone, this population decreased by 40%, relative to sham groups, and with combined injury was further reduced by 50% ($p<0.05$). These cells were subdivided into CD11c+SiglecF- and CD11c+SiglecF+ populations. CD11c+SiglecF- cells were Ly6C+ inflammatory monocytes, while CD11c+SiglecF+ cells were Ly6C- pre-alveolar macrophages (pre-AMs). We observed a trend toward more Ly6C+ monocytes and less Ly6C- pre-AMs in combined injured mice, relative to burn injury alone. Pre-AM phenotype was assessed using the mean fluorescent intensity (MFI) of pro-inflammatory (M1) markers MHC II and CD11b, as well as anti-inflammatory (M2) markers CD206 and CD71. There was no change in the MFI of MHC II after burn alone, relative to shams, however, intoxication and burn triggered a 1.4-fold increase in MHC II, relative to all groups ($p<0.05$). Additionally, cells from burn injured mice had a 1.3-fold increase in CD11b, relative to shams, while combined injured mice had a 1.8-fold increase, relative to shams ($p<0.05$), and a 1.3-fold increase, relative to burn alone. There were no changes in M2 markers at this time point. These data reveal that

while there are less F4/80+ Ly6G+ cells, as well as less pre-AMs in the alveolar space of combined injured mice at 24 hr post injury, these cells express a higher degree of pro-inflammatory markers which may contribute to the elevated levels of pulmonary inflammation in combined injury. [Supported by R01AA012034 (EJK), T32 AA013527 (EJK), F31 AA022566 (JAI), and the Falk Foundation (EJK)].

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Identification of a Second Patient with p40phox Chronic Granulomatous Disease

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Chronic granulomatous disease (CGD) is a rare genetic immunodeficiency that is caused by mutations in *CYBA, CYBB, NCF1, NCF2,* and *NCF4,* encoding for $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ of the NADPH oxidase enzyme complex (NOX2). CGD is characterized by a failure of phagocytes (neutrophils, monocytes, macrophages and eosinophils) to generate superoxide and other related reactive oxygen species (ROS), leading to recurrent infections, granulomatous complications, and premature death. Thus far, only one patient has been reported with biallelic mutations in *NCF4* that resulted in $p40^{phox}$ CGD. $p40^{phox}$ has been shown to play a critical role in phagocytosis-induced NOX2 activity. Recently, a 19-year old male with severe colitis and a suspected genetic diagnosis of $p40^{phox}$ CGD [compound heterozygous for distinct splice mutations in *NCF4* - het c.118-1 G>A and het c.759-1 G>C] presented at the NIH for routine screening prior to hematopoietic stem cell transplantation. Immunoblot analysis of a patient neutrophil lysate confirmed the absence of the $p40^{phox}$ protein. ROS production by patient neutrophils was evaluated in response to soluble ligands, such as phorbol 12-myristate 13-acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (fMLF) and particulate ligands such as opsonized zymosan (OpZ) and heat-killed *Staphylococcus aureus* (HKSA). Extracellular ROS production was evaluated using superoxide dismutase-inhibitable ferricytochrome c reduction while intracellular ROS production was evaluated using either H_2O_2-mediated conversion of dihydrorhodamine 123 (DHR) to rhodamine using flow cytometry or ROS-mediated luminol chemiluminescence. Our findings indicate that, contrary to typical CGD, in $p40^{phox}$ CGD, stimulation of neutrophils with PMA yielded both extracellular and intracellular ROS production that that fell within normal ranges. In addition, analysis of extracellular ROS production using both soluble and particulate ligands also fell within normal ranges. However, in $p40^{phox}$ CGD, intracellular ROS production stimulated by particulate ligands was markedly impaired by both DHR staining and luminol chemiluminescence, demonstrating a functional defect in this patient and providing diagnostic tools to identify other patients with this rare immunodeficiency. Funded by NCI Contract No. HHSN261200800001E.

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Big MAP Kinase 1/ERK5 Mediates Toll-Like Receptor 2- and 4-Dependent Inflammatory Responses in Endothelial Cells and Monocytes

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Background/Rationale: Sepsis is initiated by interactions between microbes and innate immune receptors, including Toll-like receptors (TLRs). Binding of microbial components to their cognate TLR causes the activation of inflammation and ultimately contributes to the development of coagulopathy, increased vascular permeability, and organ failure. We recently made the novel discovery that big mitogen-activated protein kinase 1 (BMK1), also known as extracellular signal-regulated kinase 5 (ERK5), is a key mediator of TLR2 signaling in human endothelial cells and peripheral blood mononuclear cells (PBMCs); ERK5 promotes the TLR2-dependent expression of cytokines, chemokines, and plasminogen activator inhibitor 1 (PAI-1), a coagulation pathway intermediary. ERK5 is a unique MAP kinase, differing from ERK1/2, as it has a large C-terminus, a nuclear localization signal, and relatively few known substrates. We performed the following studies to test the hypothesis that ERK5 broadly mediates microbial-induced endothelial and PBMC activation, and is a central mediator of inflammatory signaling in vivo in sepsis models. **Methods:** *In vitro*: Human lung microvascular endothelial cells (HMVEC-Lung) were either pre-incubated with highly specific ERK5 inhibitors; an inhibitor of MEK5, the only known upstream activator of ERK5; or siRNA specific for ERK5 or MEK5. PBMCs were pre-incubated with ERK5 inhibitor. Cells were then stimulated with Pam3Cys or FSL-1 (TLR2), LPS (TLR4), or IL-1β (IL-1R). Cytokines were quantified in culture supernatants.

Adhesion of calcein-labeled neutrophils to activated endothelial cell monolayers was quantified using a fluorescent plate reader and visualized by microscopy. *In vivo*: Mice were given ERK5 inhibitor and then challenged (i.v.) with LPS or Pam3Cys. Cytokines were quantified in mouse plasmas. For survival studies, mice were given ERK5 inhibitor and then challenged (i.p.) with an LD_{50} of LPS. **Results:** *In vitro*: Bacterial TLR2 and TLR4 agonists, as well as IL-1β, caused ERK5-dependent endothelial cell and PBMC expression of cytokines, including IL-6 and IL-8 ($p<0.05$). ERK5 inhibition in endothelial cells reduced neutrophil adhesion to activated endothelial monolayers ($p<0.05$). *In vivo*: Treatment with ERK5 inhibitor led to improved survival in endotoxemic mice ($p<0.05$; $n = 8$). In mice challenged systemically with LPS or Pam3Cys, ERK5 inhibitor treatment attenuated the induction of PAI-1, and cytokines and chemokines, including IL-6, CCL2, and CCL3 ($p<0.05$; $n = 4$-7). **Discussion:** Our results highlight critical, heretofore overlooked functions for ERK5 in regulating infectious inflammatory signaling in endothelial cells and monocytes. Its role in IL-1β-induced activation suggests ERK5 may be involved in MyD88-dependent responses to exogenous and endogenous agonists. If so, ERK5 and its pathway intermediaries may represent novel targets for sepsis, for which there are currently no effective pharmacotherapies.

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Polyarginine and Hyaluronic Acid-Based Coating for Titanium Implants Suppresses Inflammatory Responses in Macrophages

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Titanium implants are widely used in orthopedics, dentistry, cardiology and otorhinolaryngology. Titanium is a material of choice for implants due to its biocompatibility. However titanium implants can also cause local chronic inflammation that in turn leads to implant intolerance. To overcome these problems a special coating was designed. The coating composed of Polyarginine/Hyaluronic acid (PAR/HA) has antimicrobial effects and can also release an additional antimicrobial peptide, Catestatin (CAT). As macrophages play a central role in regulation of inflammation, their response to these coating materials was analysed. We tested the effect of PAR-HA and PAR/HA+CAT on the activation of M1 and M2 human monocyte-derived macrophages. Human peripheral blood monocytes were isolated from buffy coats by magnetic cell sorting using CD14 beads. M1 and M2 macrophages have been generated by cultivating human monocytes in the presence of IFNγ and IL-4, correspondingly, for 6 days in serum-free medium. Cytokine production on days 1, 3, 5 and 6 of incubation was measured by ELISA. We found that both PAR/HA and PAR/HA+CAT coatings had a strong inhibitory effect on the production of inflammatory cytokines TNFα and CCL18 released by both M1 and M2 human primary monocyte-derived macrophages. Additionally we analysed whether PAR/HA or PAR/HA+CAT can drive M2 differentiation of monocytes by studying the expression of macrophage mannose receptor CD206. On day 6 of incubation, cells were fixed, stained by immunofluorescence and analysed by confocal microscopy. Both types of coating strongly suppressed the expression of CD206. These findings indicate that macrophages respond to PAR/HA and PAR/HA+CAT by suppression of two major types of macrophage inflammatory responses. Therefore PAR/HA and PAR/HA+CAT are promising coating material for titanium implants and can be used to avoid local chronic inflammation and implant intolerance.

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CD14-Independent Receptor Internalization and Signaling Mediated by TLR4/MD2 Agonistic Antibody

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Toll-like receptor 4 (TLR4) and MD2 heterodimerization is critical in regulating both MyD88/TIRAP- and TRIF/TRAM-mediated signaling pathways. MyD88/TIRAP signaling complex occurs at

plasma membrane upon stimulation with microbes or microbial products and activates mainly NF-kappaB and MAPKs. On the other hand, TRIF/TRAM-mediated pathway occurs after receptor endocytosis and leads to downstream activation of tank-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3). Recent studies reported the absolute requirement of membrane-associated CD14 in controlling TLR4/MD2 internalization into endosomes and TRIF/TRAM-mediated signaling pathways. Although CD14 is necessary for lipopolysaccharide (LPS)-induced receptor endocytosis and TRIF/TRAM signaling, we report in this study that CD14 is not required for receptor endocytosis and downstream signaling mediated by the TLR4/MD2 agonistic antibody (UT12) in macrophages. Deficiency of CD14 completely ablated the TBK1/IRF3 signaling axis that mediates the production of IFN-beta/IP-10 in LPS-treated macrophages without affecting MyD88-mediated signaling including NF-kappaB, MAPK activation and TNF-alpha/IL-6 production. However, neither the MyD88/TIRAP nor the TRIF/TRAM pathway and their associated cytokine gene/protein expression/secretion was altered in the absence of CD14 in UT12-treated macrophages. Interestingly, CD14 was not endocytosed in either thioglycollate-elicited or bone marrow-derived mouse macrophages treated with LPS and UT12. LPS and UT12 driven TLR4 endocytosis could be partially blocked by the spleen tyrosine kinase inhibitor (piceatannol). However, piceatannol inhibited both MyD88 and TRIF dependent cytokine production. Although another chemical inhibitor of Syk downstream effector PLC-gamma2 completely blocked LPS- and UT12-mediated endocytosis, it failed to inhibit IFN-beta production. Further, TLR4 endocytosis is seen in cells tolerized with either LPS or UT12, and this is independent of CD14. Cross-tolerance between LPS and UT12 also induced CD14-independent TLR4 endocytosis. These data indicate that receptor endocytosis and downstream signaling pathway can be induced by UT12 and during endotoxin tolerance in the absence of CD14. Supported by AI81797 (SV).

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Francisella Tularensis Schu S4 O-Antigen and Capsule Mutants Induce Programmed Necrosis in Primary Human and Murine Macrophages.

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Francisella tularensis is a facultative intracellular bacterium and the causative agent of tularemia. Characteristic features of this pathogen include the low bioactivity of its LPS and an ability to escape the phagosome and replicate to extremely high density in macrophage cytosol. We previously described the identification of a three-gene locus required for *F. tularensis* Schu S4 growth in human monocyte-derived macrophages (MDMs). *FTT1236-FTT1238* are required for O-antigen and capsule synthesis, and disruption of these genes renders Schu S4 rapidly cytotoxic to MDMs, but the underlying mechanism remained obscure. Published data demonstrate that macrophages infected with wild-type *F. tularensis* eventually undergo apoptosis, which favors bacterial dissemination and disease, whereas the attenuated *F. tularensis* live vaccine strain (LVS) and *F. novicida* activate the AIM2 inflammasome and trigger pyroptosis, which curtails infection. Herein we used pharmacologic agents and mutant macrophages to elucidate the mechanism of cell death elicited by the *FTT1236* mutant. Our data confirm selective activation of caspase-3 by wild-type Schu S4, and demonstrate that the inflammasome components caspase-1 and ASC were critical for IL-1β secretion by human and murine macrophages infected with *FTT1236*. However, despite early activation of caspase-1 in *FTT1236*-infected cells, neither caspase-1, caspase-11, ASC nor NLRP3 was essential for macrophage death; and deletion of RIP3/caspase-8 was also without effect. In marked contrast, macrophage viability was restored by treatment with glycine or the RIP1 inhibitor Necrostatin-1, indicating that *FTT1236* triggers programmed necrosis. We therefore conclude that O-antigen and capsule are required to prevent inflammatory macrophage death during tularemia. At the same time our ability to uncouple inflammasome-dependent cytokine secretion and cell death reveals additional differences between virulent and attenuated strains of *Francisella*, and underscores the ability to pathogens to inform studies of innate immune evasion. Supported by NIAID 2U54AI057160 and P01AI044642.

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TL1A Signaling Enhances the Differentiation of TH9 Cells and Th9-Driven Pathologies via STAT6 Signaling Pathways

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The TNF family member TL1A (TNFSF15) plays an important role in the development of inflammatory bowel diseases (IBD), experimental autoimmune encephalomyelitis, and allergic lung inflammation by modulating TH1, TH17, and TH2 responses. TL1A polymorphisms have been identified through genome-wide association studies to confer susceptibility to IBD and have been associated with disease severity. IBD patients with TL1A risk haplotypes have elevated expression of TL1A in peripheral blood monocytes and transgenic mice overexpressing TL1A develop spontaneous small intestinal inflammation. However, the effects of TL1A on other TH subsets remain unknown. Recently, TH9 cells have been identified as an independent TH cell subset that produces mainly IL-9 has been implicated in allergic lung inflammation, parasitic worm infections, and IBD. In this study, we identified TL1A as a strong inducer of TH9 cell differentiation and IL-9 production in vitro. Mechanistically, TL1A enhanced STAT6 activation via NF-κB signaling pathway that lead to enhanced binding of the transcription factor IRF4 to the Il9 promoter. Utilizing an adoptive T cell transfer model of colitis we demonstrated that TH9 cells differentiated ex vivo in the presence of TL1A are highly pro-inflammatory in vivo and lead to more severe intestinal inflammation compared to TH9 cells as characterized by increased cell numbers in mesenteric lymph nodes and spleens, enhanced proliferation of transferred cells, and increased IL-9, IL-13, and IL-17 production. Using blocking anti-IL-9 antibodies attenuated TL1A-driven intestinal inflammation in this model. Our results demonstrate that TL1A promotes TH9 cell differentiation and function and define a role for IL-9 in TL1A-induced mucosal inflammation and potential therapeutic target in inflammatory diseases.

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Chronic Ethanol Consumption Alters Intestinal Microbiota Impairing Immune Function

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It is well known that chronic alcohol consumption leads to significant organ damage and increased susceptibility to bacterial and viral infections resulting in higher morbidity and mortality. Furthermore, ethanol exposure in the intestinal lumen alters bacterial metabolism leading to disruption of the mucosal barrier. An increased intestinal permeability further leads to translocation of gut-derived endotoxins causing inflammatory responses in the liver that contribute to alcoholic liver disease. Moreover, we have recently shown that chronic ethanol consumption in a rhesus macaque model of voluntary ethanol self-administration results in decreased production of IL-2, IL-17, TNFα, and IFNγ by gut-resident CD4 and CD8 T cells. However, the exact mechanisms underlying impaired cytokine productions have yet to be elucidated. Recent studies have shown that the microbiome modulates immunity in the gut. In order to gain a better understanding of the dose-dependent effects of ethanol on the intestinal microbiome, we performed ribosomal RNA sequencing to determine the differentially expressed bacteria in the small and large intestine. In the colon, we observed a ~2-fold increase in heavy drinkers compared to nondrinkers in the phylum Bacteroidetes that subsequently results in a decrease in Firmicutes, the phylum containing most beneficial lactic acid bacteria. Additionally, in the duodenum, jejunum, ileum, and colon we found a 3-fold decrease in the commensal species *Lactobacillus intestinalis*, a member of the Firmicutes phylum, in heavy drinkers compared to nondrinkers. In the duodenum, jejunum, ileum, and colon we also found a ~2-fold increase in the species *Prevotella copri* in heavy drinkers versus nondrinkers. This species has been previously associated with inflammation, particularly rheumatoid arthritis. Interestingly, in the colon we found a 39-fold decrease in heavy drinkers compared to nondrinkers in the recently identified species *Helicobacter macacae*, which has been associated with chronic diarrhea and colon cancer in macaques. These studies are likely to reveal mechanisms of ethanol-related gut dysregulation and are critical to our understanding of the mammalian immune system.

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Phosphoinositol Lipoarabinomannan Involvement in Mycobacterium Smegmatis-Modulation of Neutrophil Functions

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Mycobacterium smegmatis is a soil and water-inhabiting bacterium with which humans are frequently exposed. Despite being considered non-pathogenic, *M. smegmatis* retains the capacity to manipulate macrophages. The cell surface of *M. smegmatis* is studded with phosphoinositol lipoarabinomannan (PILAM), a characteristic glycolipid of the mycobacterium genus that is associated with virulence. This study tested the hypothesis that PILAM from *M. smegmatis* is involved in the bacteria's ability to modulate neutrophil functional responses. Flow cytometry was used to measure secretory vesicles, specific granules, and azurophil granules exocytosis; and gelatinase granule exocytosis was determined by measuring gelatinase release by ELISA. Neither PILAM nor viable *M. smegmatis* alone induced secretory vesicle or specific granule exocytosis, and had an opposite trend in fMLF-stimulated azurophil granule exocytosis where *M. smegmatis* increased fMLF-stimulated azurophil granule release and PILAM inhibited degranulation. In contrast, both *M. smegmatis* and PILAM alone induced an increase in gelatinase granule release. Moreover, pre-treatment of neutrophils with either *M. smegmatis* or PILAM enhanced subsequent gelatinase degranulation after stimulation with fMLF. PILAM and *M. smegmatis* also shared the ability to increase intracellular respiratory burst activity. Confocal microscopy was used to determine *M. smegmatis* viability after neutrophil infection using GFP-expressing *M. smegmatis* and propidium iodide, a non-permeable dye that stained all non-viable bacteria with a compromised cell surface. After a 120-minute infection of human neutrophils, approximately 80% of the phagocytized bacteria remained viable. Our data suggests that PILAM from *M. smegmatis* is involved in gelatinase granule exocytosis and respiratory burst response. In addition, *M. smegmatis* is able to survive inside neutrophils after 2 hours post challenge, indicating that the interactions between human neutrophils and a non-pathogenic bacterium from the mycobacterium genus are more intricate than anticipated.

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Salmonella Typhimurium Co-Opts the Mammalian Type I Interferon System to Restrict Innate Inflammatory Responses Selectively and Promote Pathogenesis

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Innate immune sensing systems and their induced inflammatory responses are subject to complex layers of negative regulation. Such regulation is critical to balance pathogen recognition and clearance with host tissue integrity and an eventual return to homeostasis. Nowhere is the restriction of innate responses more significant than at mucosal surfaces that are in constant contact with a flux of synthetic and microbial irritants. In particular, mechanisms restricting innate responses are crucial in the intestinal mucosa, as the trillions of microbes composing the commensal microflora must be tolerated to prevent colitis-associated pathologies. However, the strict governance of innate responses at intestinal sites may provide an opportunity for pathogens seeking to gain an advantage over the host. We demonstrate here that mice lacking a functional gene for interferon beta (IFN-β-/-) display significantly enhanced resistance to oral infection with pathogenic Salmonella enterica subsp. enterica serovar Typhimurium (ST). In vivo the resistance to ST in IFN-β-/- mice is associated with markedly increased neutrophil activity in the small intestine. In vitro infection of murine macrophages with Salmonella reveals that autocrine or paracrine action of IFN-β selectively restricts the transcriptional inflammatory responses mediated by both the Toll Like Receptors (TLRs) as well as the NOD-Like Receptors (NLRs). This "sculpting" of the innate response involves the suppression of IL-1 family cytokines as well as key neutrophil chemoattractants. This work provides mechanistic insight into homeostatic roles for type I interferons in the gut, and demonstrates how these effects may actually promote the pathogenesis of enteric pathogens. Supported by AI18797 (SV).

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Jmjd3-Mediated Epigenetic Regulation of Inflammatory Cytokine Gene Expression in Serum Amyloid A-Stimulated Macrophages

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Serum amyloid A (SAA), a major acute-phase protein, has potent cytokine-like activities in isolated phagocytes and synovial fibroblasts. SAA-induced proinflammatory cytokine gene expression requires transcription factors such as NF-κB; however, the associated epigenetic regulatory mechanism remains unclear. Here we report that Jmjd3, a histone H3 lysine 27 (H3K27) demethylase, is highly inducible in SAA-stimulated macrophages and plays an important role in the induction of inflammatory cytokine genes. SAA-induced Jmjd3 expression leads to reduced H3K27 trimethylation. Silencing of Jmjd3 expression significantly inhibited SAA-induced expression of proinflammatory cytokines including IL-23p19, G-CSF and TREM-1, along with up-regulation of H3K27 trimethylation levels on their promoters. Depletion of Jmjd3 expression also attenuated the release of proinflammatory cytokine genes in a peritonitis model and ameliorated neutrophilia in SAA-stimulated mice. Finally, we observed that Jmjd3 is essential for SAA-enhanced macrophage foam cell formation by oxidized LDL. Taken together, these results illustrate a Jmjd3-dependent epigenetic regulatory mechanism for proinflammatory cytokine gene expression in SAA-stimulate macrophages. This mechanism may be subject to therapeutic intervention for sterile inflammation and atherosclerosis.Supported by grants from National Natural Science Foundation of China (Grants 81202316 and 31270941), from National Basic Research Program of China (973 Program, Grant 2012CB518001) and National Institutes of Health grant R01 AI033503 and AI040176.

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NAD-Dependent SIRT4 Participates in the Resolution of Acute Inflammatory Response by Restoring Immunometabolic Homeostasis

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Emerging data support that concurrent shifts in innate and adaptive immunity and metabolism direct the course of acute systemic inflammation, which becomes lethal during dysregulated immunometabolic homeostasis. We previously reported that nuclear sirtuin 1 (SIRT1) in monocytes couples with NFkB RelB, SIRT6 control immunometabolic homeostasis by switching the early proinflammatory anabolic phenotype with high Warburg-type glycolysis to a persistent hypo-inflammatory catabolic phenotype dependent on high mitochondrial fatty acid oxidation. Remarkably, pharmacologically rebalancing the SIRT1-dependent homeostat during early hyperinflammation or late hypoinflammation markedly improves sepsis survival, but how homeostasis is physiologically restored is unknown. Here, we find in a promonocyte cell model that increased mitochondrial SIRT4 expression during adaptive hypoinflammation promotes acute inflammation resolution. Mechanistically, Sirt4 inactivates mitochondrial glutamate dehydrogenase and malonyl CoA decarboxylase and concomitantly activates pyruvate dehydrogenase to balance of glucose and fatty acids as oxidative mitochondrial fuel. In further support of SIRT4 mitochondrial homeostasis function, SIRT4 siRNA knockdown results in sustained activities of glutamate dehydrogenase, lactate dehydrogenase, pyruvate dehydrogenase kinase, and diminished malonyl CoA levels that increase mitochondrial catabolism. These novel findings support that intramitochondrial SIRT4 promotes acute inflammation resolution by rebalancing glucose, aminoacids, and fatty acids as mitochondrial fuel sources.

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Inhibition of Heat Shock Protein 90 Alters Epigenetic Genes Important in Innate Immune Responses and Metabolism in a Mouse Model of Alcoholic Liver Injury

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Acetylation and methylation of chromatin-associated histones that influence transcriptional activity of genes is reported in alcoholic livers. Molecular chaperones like hsp90 are being recognized as mediators of chromatin remodeling under stressful conditions such as alcohol exposure. We hypothesized that alcohol exposure modulates expression of chromatin modifying enzymes chaperoned by hsp90 during liver injury. To test our hypothesis, C57Bl/6 mice were fed Lieber-deCarli diet

with 5% v/v ethanol for 10 days followed by a binge containing 20% alcohol. A single injection of hsp90 inhibitor,17-DMAG [17-Dimethylamino-ethylamino-17-demethoxygeldanamycin] was administered (30-50 mg/kg BW) i.p. before the binge. PCR arrays analyzing chromatin modifying enzyme expression were employed to determine effect of alcohol. Results show significant up-regulation of 5 genes including enzymes responsible in acetylation, ATF2, histone methytransferases PRMT6 and SETD7, phosphorylation enzyme, RPS6KA3, histone deacetylase, HDAC3 and down-regulation of HDAC9 during alcohol exposure in the liver. Analysis of expression in isolated hepatocytes and Kupffer cells exhibits cell type specific regulation of expression. ATF2 was exclusively upregulated (2 fold) in alcohol exposed Kupffer cells while PRMT6 (3.2 fold) increased in hepatocytes. HDAC9, on the other hand, was down regulated (7 fold) in alcohol exposed hepatocytes. HDAC3, SETD7 and RPS6KA3 were increased in KCs and hepatocytes. Inhibition of hsp90 after chronic alcohol exposure significantly alleviated liver injury as noted by reduced serum ALT and decreased liver triglycerides. DMAG altered expression of ATF2, PRMT6, HDAC3 and HDAC9 in the liver. Phosphorylation of ATF2 by LPS in macrophages activates transcription of pro-inflammatory cytokines. 17-DMAG treatment after alcohol feeding prevented an increase in ATF2 expression (P=3.13 e-5) along with decreased pro-inflammatory cytokines such as TNFα. PRMT6, an arginine methyltransferase (p=0.001) and HDAC3 (p=0.001), a regulator of lipid metabolism was reduced during hsp90 inhibition in the liver. Our data indicate that alcohol exposure regulates epigenetic genes in a cell-specific manner likely via molecular chaperone, hsp90 influencing innate immune responses and metabolism. (Supported by the NIH/NIAAA # AA179086)

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Dynamic Programming of Innate Immune Responses by Varying Dosages of Endotoxin

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Innate monocytes and macrophages can be dynamically programmed into distinct states depending upon the strength of external stimuli. Innate programming may bear significant relevance to the pathogenesis and resolution of human inflammatory diseases. However, systems analyses with regard to the dynamic programming of innate leukocytes are lacking. We observed that varying dosages of LPS differentially modulate the expression of selected pro- and anti-inflammatory mediators in human and murine monocytes. Mechanistically, we demonstrated that super-low and higher doses of LPS cause differential activation of GSK3 and Akt, as well as the transcription factors FoxO1 and CREB. Taken together, our study reveals a dynamic modulation of monocytic cells in response to varying dosages of endotoxin, and may shed light on our understanding of the dynamic balance that controls pathogenesis and resolution of inflammatory diseases.

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The Alarmin High-Mobility Group Nucleosome-Binding 1 Protein Contributes to the Induction of Antitumor Immunity

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Alarmins are endogenous mediators that rapidly become available in peripheral tissues in response to danger signals and are capable of enhancing the induction of innate and adaptive immune responses by promoting the recruitment and maturation of antigen-presenting cells (APCs). We have previously shown that high-mobility group nucleosome-binding 1 (HMGN1) protein is a potent alarmin that contributes to the induction of antigen-specific immune response. In this study, we investigated whether HMGN1 contributes to the generation of antitumor immunity. Inoculation of EG7, a mouse thymoma line transfected to express ovalbumin (OVA), into *Hmgn1*$^{-/-}$ and littermate-matched *Hmgn1*$^{+/+}$ mice revealed that the tumor grew much faster in *Hmgn1*$^{-/-}$ mice than in *Hmgn1*$^{+/+}$ mice. Concomitantly, EG7-bearing *Hmgn1*$^{-/-}$ mice generated fewer splenic OVA-specific CD8 cells than EG7-bearing *Hmgn1*$^{+/+}$ mice, suggesting that endogenous HMGN1 contributed to the development of antitumor immune responses. In addition, HMGN1-expressing EG7 tumors grew slower than control EG7 tumors in mice, albeit both cell lines proliferated equally *in vitro*, suggesting the generation of resistance to HMGN1-expressing tumors. Importantly, the antitumor resistance of mice bearing HMGN1-expressing EG7 tumors was dependent on cell-mediated

immunity since depletion of CD4 and CD8 completely abolished the antitumor immunity. Furthermore, mice vaccinated with plasmids encoding HMGN1-gp100 fusion protein manifested gp100-specific, Th1-polarized immune responses, and acquired resistance to challenge with mouse B16F1 melanoma. Overall, the data show that HMGN1 contributes to the generation of antitumor immunity and suggest that the alarmin HMGN1 may prove to be an effective tumor vaccine adjuvant. [Funded in part by NCI Contract HHSN261200800001E].

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Developmental Regulation of Human Immune Responses: Comparative Genome-Wide Profiling of Neonatal Monocytes.

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Background: Neonates, particularly those born prematurely (<32 weeks gestation) are at high risk for infections in their first weeks of life. The vulnerability of these infants to infection is often attributed to a gestational age dependent immaturity of the neonatal immune system. Monocytes play an important role in regulating first-line immune responses to infection. Activation of monocytes can occur through the stimulation of Pattern-Recognition Receptors (such as TLR-4), leading to NF-κB-mediated activation of immune genes. The gestational regulation of genes that control immune function and injury responses likely play a key role in determining neonatal outcomes.

Hypothesis: The vulnerability of preterm infants to infection is due to changes in transcription of key immune genes; the expression of these genes is developmentally regulated.

Objective: To identify age-dependent changes in gene expression upon activation of monocytes using endotoxin (LPS).

Design/Methods: Monocytes were extracted from infants born extremely preterm (Gestational age ≤ 28 weeks, n=8), full term cord blood (gestational age = 37-40 weeks, n=12) and from adult peripheral blood (n=12). Whole genome expression profiling following LPS stimulation versus unstimulated control cells was performed using the Illumina HumanHT-12 v4 Expression BeadChip Array. Statistical analysis was performed using R (v. 3.0.2, LUMI and LIMMA). Gene set enrichment analysis was performed using the curated gene sets and gene ontogeny gene sets from GSEA and visualized using Cytoscape.

Results: Over 400 genes were found to be differentially expressed using a q-value of 0.01, and a fold change cut off value of 2; subsequent gene set enrichment revealed 12 pathways to be highly altered in preterm neonates, comparing to adults. Immune pathways related to cytokine and chemokine activity were differentially expressed (CXCL10, CCL7, IFNGR1, IL1R2), as well as MHC class II activity and antigen processing (HLA-DRB1, HLA-DQA1, HLA-DPA1, HLA-DMA). Surprisingly, oxygen binding proteins were highly up-regulated in both preterm and term infant monocytes, while in contrast genes controlling cytochrome-c oxidase activity were down-regulated. In addition to oxygen transport and processing, these genes play a key role in regulating the response to oxidative stress and damage.

Conclusions: Our study reveals ontogenetic differences at the level of gene expression, which may contribute to the vulnerability of preterm infants to infection. This research also provides unique insights into the role of monocytes in the increased vulnerability of neonates to infection. Our findings also shed light onto the role of both immune and non-immune related genes regulating the human immune response developmentally.

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Do Peripheral Myeloid Cells Contribute to Brain Injury following Neonatal Hypoxia-Ischemia?

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Introduction: Inflammation is believed to be an important factor in the pathophysiology of hypoxic-ischemic injury in the developing brain. The traditional view of an "immune privileged" central nervous system (CNS) is progressively giving way to the concept of an "immune specialized" CNS, which actively interacts with the periphery. However, the contribution of

peripheral immune cells to central inflammation remains unclear.

Method: *Lys*-EGFP-*ki* mice, a strain expressing EGFP in peripheral myeloid cells, but not in microglia (kindly provided by Thomas Graf), were used to investigate the influx of peripheral immune cells following injury in a murine model of neonatal hypoxia-ischemia (HI). To induce HI the left common carotid artery was permanently ligated in postnatal day 9 mice, followed by exposure to 50 minutes of hypoxia (10%). The presence of peripheral myeloid cells in the CNS was investigated using FACS analysis and by immunohistochemistry staining from 24 hours to 86 days post-HI. Invading myeloid cells were identified in tissue sections through their expression of EGFP and classified by FACS analysis as infiltrating monocyte/macrophages (CD11b+EGFP+Ly6G-) and infiltrating granulocytes (CD11b+EGFP+Ly6G+).

Results: We observed a progressive accumulation of EGFP positive peripheral myeloid cells in the ipsilateral injured hemisphere from 2 hours after HI, with the earliest expression found in CD31+ blood vessels, but later cells were identified in the brain parenchyma. FACS analysis demonstrated infiltration of both hematogenous monocyte/macrophages (CD11b+EGFP+Ly6G-) and granulocytes (CD11b+EGFP+Ly6G+) in response to HI, particularly at 3-14 days after HI.

Conclusion: In summary, we have found that a heterogeneous population of peripheral myeloid cells (hematogenous monocyte/macrophages and to a lesser extent granulocytes) infiltrates the brain following HI. These cells progressively accumulate in the injured thalamus, hippocampus, and cortex where they persist for several weeks post-insult, suggesting that they contribute to the inflammatory response after HI and may also modulate post-insult injury mechanisms.

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Are Routine Automated Complete Blood Counts with Manual Differentials for Determining Neonatal Neutrophil Composition Antiquated?

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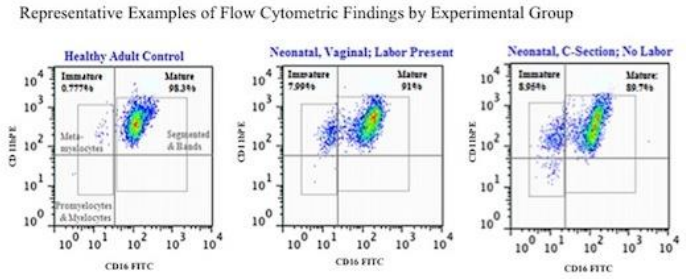
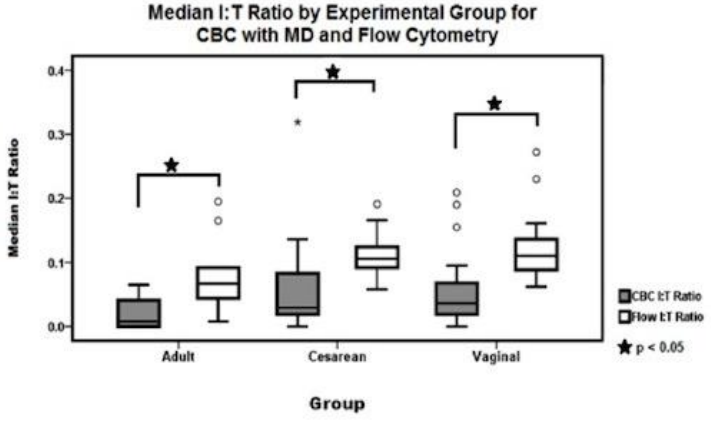
Background: Manual differentials, introduced into clinical practice over 150 years ago, remain the primary method for determining neutrophil composition in neonates. This is because automated counters mistakenly characterize nucleated red blood cells as white blood cells, artificially inflating their numbers. This study was conducted to determine if flow cytometry could be used to accurately determined neutrophil composition in newborns.

Method: 50 µL of whole blood was collected from term, healthy newborns via cord blood and adults by venipuncture. Neutrophil markers including CD45, CD16, and CD11b were used to sort neutrophils into immature ($CD16^-$, $CD11b^-$) and mature ($CD16^+$, $CD11b^+$) groups via flow cytometry. Results were compared to those obtained by routine lab methods using manual differentials.

Results: Flow cytometry more accurately determined neutrophil composition in newborns and adults by identifying more immature neutrophil forms. This is represented as immature to total neutrophil ratios (0.11 vs. 0.07, p=0.04). Gene expression of key granule proteins was used to verify maturational stage.

Conclusion: Results obtained in this study raise concerns regarding the accuracy of routine laboratory methods in determining neutrophil composition in clinical use. Further investigation of flow cytometry should occur.





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The Role of Kupffer Cells in the Response to Intoxication and Burn Injury

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Of the 450,000 burn patients each year in the US, nearly 50% are intoxicated at the time of their injury and have worsened clinical outcomes compared to those without prior alcohol exposure. We have previously demonstrated that the elevated IL-6 observed when intoxication precedes burn in mice plays a causative role in post burn pulmonary inflammation and intestinal barrier breakdown. Similarly clinical observations of trauma patients correlate circulating IL-6 levels to mortality risk. Therapeutic strategies targeting single cytokines have had limited clinical success however, and a deeper understanding of the systemic source and cell type responsible for the over exuberant cytokine production is needed. We recently reported that intoxication before burn enhances bacterial translocation from the gut into the lymphatic and portal systems leading to hepatic damage and cytokine production. This altered "gut-liver axis" then influences pulmonary inflammation through release of IL-6 into the circulation. We now seek to determine the hepatic cell type responsible for orchestrating this aberrant hepatic response. In addition to increasing post burn intestinal permeability, alcohol is known to sensitize Kupffer cells in the liver to lipopolysaccharide (LPS), creating a situation where both the stimulus and inflammatory reaction to gut-derived LPS is increased. We therefore hypothesize that Kupffer cells play a central role in the altered gut-liver axis when intoxication precedes burn. To this end, mice were given 1.2 g/kg ethanol by oral gavage 30 minutes prior to a 15% total body surface area burn. Antecedent depletion of Kupffer cells was achieved via tail vein injection of clodronate liposomes (0.5 mg/kg) two days before injury or empty liposomes were administered as a control. The absence of Kupffer cells (verified by immunohistochemistry) attenuated hepatic damage as measured by a 53% reduction in serum alanine aminotransferase ($p<0.05$), a 44% reduction in serum aspartate aminotransferase ($p<0.05$), a 37% decrease in hepatic triglycerides ($p<0.05$), as well as a 77% reduction in hepatic IL-6 mRNA expression ($p<0.05$) compared to intoxicated burned mice receiving control liposomes. This mitigation of hepatic damage was associated with a 34% decrease in pulmonary neutrophil infiltration ($p<0.05$) and attenuated alveolar wall thickening compared to matched controls. Nearly identical hepatic and pulmonary protection after intoxication and burn was observed in TLR4 knockout mice perhaps suggesting Kupffer cell TLR4 signaling may be altered in this setting. Overall these data implicate Kupffer cells as key players in the detrimental response to intoxication and burn and may serve as a therapeutic target in this common clinical scenario. This work was supported by NIH R01AA012034 (EJK), F30AA022856 (MMC), T32 AA013527 (EJK), F31 AA022566 (JAI), and the Falk Foundation.

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Human Host Defense Peptide Ll-37 Enhances the LPS Uptake by Endothelial Cells without Cell Activation: a Possible Role in the LPS Clearance

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Background: Sepsis is a systemic disease resulting from harmful host response to bacterial infections. In sepsis, LPS is released from the proliferating or dying Gram-negative bacteria, leaks into the blood stream, and binds with the receptors (CD14/TLR4) on blood and vascular cells. Binding of LPS to the receptors induces inflammatory responses through MyD88-dependent and independent pathways. In contrast, LPS is cleared from the blood by endothelial cells and macrophages in the liver (LPS clearance). Such LPS uptake by these cells may protect the host from LPS-induced inflammatory responses.

A human neutrophil-derived host defense peptide LL-37 not only possesses an antibacterial activity, but also has a potent LPS-neutralizing activity by directly binding with LPS. We previously demonstrated that LL-37 protects mice from lethal endotoxin shock through the inhibition of LPS binding to CD14/TLR4 on the host cells, thereby suppressing the LPS-induced inflammatory responses. However, it remains to be clarified whether LL-37 regulates the LPS uptake. Thus in this study, we evaluated the modulatory action of LL-

37 on the LPS uptake by liver sinusoidal endothelial cells (LSECs).
Results: LL-37 enhanced the LPS uptake by LSECs. Importantly, LL-37 was also incorporated into LSECs along with the uptake of LPS; however, LL-37 was incorporated into the cells even in the absence of LPS. Since LL-37 can bind with LPS, LL-37 likely enhances the LPS uptake by LSECs via the complex-formation with LPS. Next, we investigated the effect of LL-37 on the endothelial cell activation during LPS uptake. In the absence of LL-37, LPS upregulated ICAM-1 and IFN-β expression in LSECs via the MyD88-dependent and independent responses, respectively; however, LL-37 suppressed the activation during incorporation of LPS. These observations suggest that the incorporated LL-37-LPS complex does not activate the cells. We further clarified uptake mechanism for the LL-37-LPS complex. LPS uptake was inhibited by endocytosis inhibitors and heparan sulfate in the presence of LL-37, suggesting that the LL-37-LPS complex is incorporated into LSECs via endocytosis, possibly by interacting with membrane-bound heparan sulfate proteoglycans.
Conclusion: Here we reveled a novel function of human host defense peptide LL-37 to enhance the LPS uptake by liver sinusoidal endothelial cells. Importantly, the incorporated LL-37-LPS complex did not activate TLR4 signaling within the cells. Thus, LL-37-mediated LPS uptake likely plays a role in the clearance of LPS without endothelial cell activation.

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The Palmitoylated A-Peptide/ß-Peptoid Hybrid Pam-(Lys-ßNSpe)6-NH2 Is a Novel Host Defense Peptide Mimetic with Potent Anti-Inflammatory Activity

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Host defense peptides (HDPs), and synthetic mimics hereof, are considered to be promising leads for novel anti-sepsis therapies due to their potent immune-modulating properties. However, development of drugs based on HDPs has been hampered by problems with toxicity as well as by poor bioavailability due to enzymatic degradation.

Here we report the identification of a proteolytically stable palmitoylated α-peptide/β-peptoid hybrid oligomer, Pam-(Lys-βNSpe)$_6$-NH$_2$, with potent anti-inflammatory properties. This novel host defense peptide mimetic prevents release of IL-6 from human leukocytes stimulated with LPS or LTA. Moreover, it reduces secretion of IL-6, IL-8, and TNF-α induced by exposure of human leukocytes to UV-inactivated *E. coli* and *S. aureus* whole bacteria. The LPS neutralization potency was comparable to that of polymyxin B, but, in contrast to polymyxin B, the mode of action did not involve direct binding to LPS. Furthermore, Pam-(Lys-βNSpe)$_6$-NH$_2$ was found to be a potent and selective inhibitor of release of oxygen radicals and degranulation in neutrophils mediated by activation of the formyl peptide receptor 2 (FPR2), whereas signaling through other chemotactic receptors including the homologous FPR1 was unaffected. By using imaging flow cytometry we showed that a fluorescently labeled analog of Pam-(Lys-βNSpe)$_6$-NH$_2$ interacts with FPR2. Furthermore the interaction between Pam-(Lys-βNSpe)$_6$-NH$_2$ and FPR2 was found to inhibit binding of FPR2-activating ligands.

We describe an HDP that inhibits both the pro-inflammatory response induced by pathogen associated molecular patterns such as LPS/LTA as well as by agonist of the neutrophil pattern recognition receptor FPR2. Thus, we consider Pam-(Lys-βNSpe)$_6$-NH$_2$ to be a promising anti-inflammatory drug lead that may prove useful for treatment of inflammation driven diseases including sepsis.

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Refined Pathogenesis of S.Aureus in Response to Neutrophil-Derived Signals Is Regulated by the Pathogen at the Single Amino Acid Level.

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The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is a leading cause of skin and soft-tissue infections worldwide. *S. aureus* uses two-component gene regulatory systems (TCSs) to sense and respond to varied environmental conditions. The pathogen's success is due in part to the *S. aureus* exoprotein expression (SaeR/S) system, which is used to sense neutrophil-derived factors and activate transcription of virulence genes essential to evasion of killing by the host innate

immune system. The mechanism by which the sensor kinase (SaeS) recognizes the stimuli and activates the cognate response regulator (SaeR) to modulate gene expression is unknown. In this study, we utilized single amino acid mutants generated in the predicted extracellular loop of the membrane sensor kinase. We discovered that one methionine residue (M31A) is essential for *S. aureus* to sense antimicrobial peptide alpha-defensin-1 and human neutrophils. In addition, M31A mutant was attenuated in pathogenesis as demonstrated by reduced transcription in virulence genes and decreased neutrophil cytotoxicity. Interestingly, we found that two strains with aromatic residue mutations (W32A and F33A) had lower mRNA levels of virulence genes in the absence of inducing signals, but had similar expression patterns of that of the wild-type strain post neutrophil exposure. Taken together, these results indicate that the SaeR/S system is able to refine its pathogenesis at the single amino acid level depending on the external host-derived stimuli. Ongoing research focuses on determining phosphorylation patterns in SaeR in response to neutrophil-derived stimuli. By utilizing the single amino acid mutant strains in the SaeS sensing domain, we are defining molecular mechanisms used by *S.aureus* to promote pathogenesis at the host-pathogen interface, which are of interest for developing novel therapeutics.

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Cytoplasmic LPS Activates Caspase-11: Implications in TLR4-Independent Endotoxic Shock

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Inflammatory caspases, such as caspase-1 and -11, mediate innate immune detection of pathogens. Caspase-11 induces pyroptosis, a form of programmed cell death, and specifically defends against bacterial pathogens that invade the cytosol. During endotoxemia, however, excessive caspase-11 activation causes shock. We report that contamination of the cytoplasm by lipopolysaccharide (LPS) is the signal that triggers caspase-11 activation in mice. Specifically, caspase-11 responds to penta- and hexa-acylated lipid A, whereas tetra-acylated lipid A is not detected, providing a mechanism of evasion for cytosol-invasive Francisella. Priming the caspase-11 pathway in vivo resulted in extreme sensitivity to subsequent LPS challenge in both wild type and Tlr4-deficient mice, whereas caspase 11-deficient mice were relatively resistant. Together, our data reveal a new pathway for detecting cytoplasmic LPS.

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NLRP12 Mediates Neutrophil Recruitment during Pulmonary Bacterial Infection

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Effective neutrophil recruitment to the site of an infection is critical for controlling invading pathogens; however, the specific signals required for neutrophil recruitment are incompletely understood. We show that mice deficient in the nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family member NLRP12 were highly susceptible to infection with *Francisella tularensis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Increased susceptibility of NLRP12-deficient mice to infection correlated with defective migration of neutrophils into the lungs of infected animals. Protective neutrophil recruitment required hematopoietic, but not neutrophilic, *Nlrp12* expression. NLRP12-deficient macrophages also had impaired production of the neutrophil chemotactic chemokine CXCL1 in response to inflammatory stimuli. Finally, reconstitution of NLRP12-deficient mice with wild-type, but not CXCL1-deficient, macrophages corrected the defect in neutrophil recruitment to inflammatory stimuli. These results demonstrate a previously unrecognized role for NLRP12 in host defense against bacterial pathogens through CXCL1-dependent recruitment of neutrophils to the site of an infectious insult.

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Guanylate Binding Proteins Promote Caspase-11 Activation but Not the Release of LPS from Chlamydia-Containing Vacuoles.

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Intracellular, non-endosomal LPS activates the pro-inflammatory enzyme caspase-11. Activation of the caspase-11-containing noncanonical inflammasome requires macrophage priming through Toll-like receptor (TLR) or Interferon (IFN) receptor signaling. We previously demonstrated that TLR-/IFN-inducible

Guanylate Binding Proteins encoded on mouse chromosome 3 (GBPchr3) promote caspase-11-dependent IL-1 secretion and pyroptosis in response to infections with the vacuolar Gram-negative bacterial pathogen Legionella pneumophila. The mechanism by which GBPchr3 proteins promote caspase-11 activation is not fully understood, however, it has been proposed that GBPchr3 proteins target and break down bacteria-containing vacuoles and thereby release LPS into the cytoplasm where it triggers caspase-11 activation. Here, we demonstrate that the Gram-negative, obligate intracellular bacterial pathogens Chlamydia trachomatis and Chlamydia muridarum also promote IL-1 secretion and pyroptosis in a caspase-11- and GBPchr3-dependent manner. We further show that LPS is released from Chlamydia-containing vacuoles and that this release is exacerbated by IFN priming. However, whereas caspase-11 activation in response to Chlamydia infections is impaired in GBPchr3-deficient macrophages, the release of LPS from Chlamydia-containing vacuoles appears unchanged in the absence of GBPchr3 proteins. These data indicate that GBPchr3 proteins are not required for the lysis of Chlamydia-containing vacuoles but instead play a role in the subsequent activation of caspase-11. Current studies investigate the mechanism by which GBPchr3 proteins assist caspase-11 activation.

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The Roles of NLRP10 in Inflammation and Parasite Control in Cutaneous Leishmaniasis

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During cutaneous leishmaniasis (CL), a vigorous adaptive immune response and ulceration precedes healing. In the mouse model of CL using *Leishmania major*, resistant B6 mice control infection with a strong Th1 response and IFNγ release. The innate immune response in the skin, with infiltration of phagocytic cells, is important in determining the outcome of the adaptive response. These responses are not fully delineated. Among the >20 Nod-like receptor proteins, some respond to cytosolic danger signals and activate inflammasomes with consequent IL-β/IL-18 release, while functions of others remain unknown. NLRP10 is a unique NLR lacking a ligand sensing domain that is expressed in myeloid cells, epithelial cells, and keratinocytes. NLRP10 is not involved in inflammasome activation in myeloid cells, but has been shown to be involved in initiating the adaptive immune response by dendritic cells. We hypothesized that NLRP10 is necessary for an adaptive immune response to *Leishmania* and resolution of infection. To examine this hypothesis we intradermally infected NLRP10 knockout (KO) or control mice with luciferase-expressing *L.major* (*Lm*-luc). Surprisingly, infected KO mice developed dramatically larger lesions with more inflammatory cells than infected controls, but harbored lower parasite burdens throughout the first 8 weeks of infection. Despite a vigorous inflammatory response, KO mice also failed to clear the parasite, showing higher parasite loads than WT after 9 weeks, and unlike WT they failed to resolve lesions. Inflammation was accompanied by elevated IL-17 in the local tissue, lymph node, and spleen. Contrary to our hypothesis, KO mice developed vigorous adaptive immune responses with higher antigen specific IFNγ, IL-10, and IL-6 in lymph nodes and spleen than WT controls. Therefore, in the absence of NLRP10, although infected mice mount a vigorous adaptive response and parasite expansion is initially limited, the infection is not cleared. We formulated alternative hypotheses for the role of NLRP10 in CL: Data suggested either that NLRP10 is essential for appropriate myeloid cell migration into and out of infected tissues, or NLRP10 acts as a negative regulator of inflammation. To discern whether myeloid or tissue resident cells are responsible for aberrant cell localization in KO mice, we infected bone marrow chimeric mice with *Lm*-luc. Infusion of WT bone marrow into either irradiated KO or WT mice resulted in a higher parasite burden, suggesting NLRP10 expression in the myeloid compartment is needed to support growth of parasites. In sharp contrast, models in which either WT or KO bone marrow was infused into irradiated KO mice resulted in a dramatic inflammatory response and tissue damage. These data suggest that NLRP10 in resident cells is essential for controlling inflammation at the site of infection, whereas NLRP10 expression in myeloid cells actually allows parasite expansion but also leads to eventual resolution of infection.

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Nod-Like Receptor Protein-3 Inflammasome Important in Early Stages of Wound Healing

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We have previously shown that sustained Nod-like receptor protein (NLRP)-3 inflammasome activity contributes to the persistent inflammatory response and impaired wound healing of diabetic mice. We thus hypothesized that non-diabetic mice lacking components of the NLRP-3 inflammasome would have a downregulated inflammatory response and accelerated wound healing. NLRP-3 null mice, caspase-1 null mice and C57Bl/6 wild type control mice (WT) received four 8 mm excisional cutaneous wounds. Healing and cytokine and growth factor levels were assessed in wounds at 5 days post-injury. Compared to WT mice, wounds from NLRP-3 null mice and caspase-1 null mice exhibited a reduction in neutrophil (Ly6G staining) and macrophage (F4/80 staining) accumulation as well as in levels of the pro-inflammatory cytokines TNF-alpha and IL-1beta. Contrary to our hypothesis, re-epithelialization, granulation tissue formation, and angiogenesis (CD31 staining) assessed in wound cryosections were delayed in NLRP-3 null mice and caspase-1 null mice compared to WT mice. Topically treating excisional wounds from NLRP-3 null mice with IL-1beta (3000 ng/wound) increased re-epithelialization, granulation tissue formation, and expression of TNF-alpha and IL-1beta, while macrophage accumulation was reduced compared to vehicle (PBS)-treated wounds. Despite improvements in healing, angiogenesis and levels of the pro-angiogenic growth factor VEGF were reduced in IL-1beta treated wounds. These findings support that the NLRP-3 inflammasome likely contributes to the early inflammatory phase of wound healing and is important for effective tissue repair.

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Is Neonatal Neutrophil "Immaturity" Explained by Their Composition of Immature Neutrophils?

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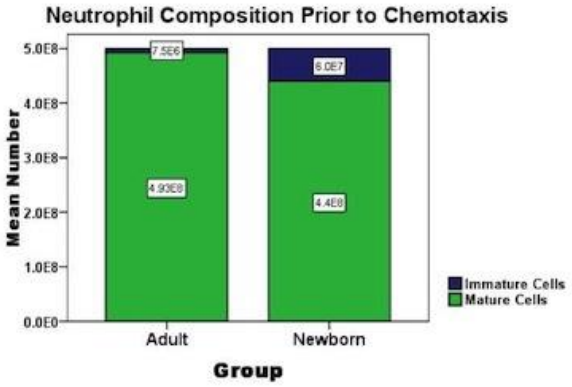
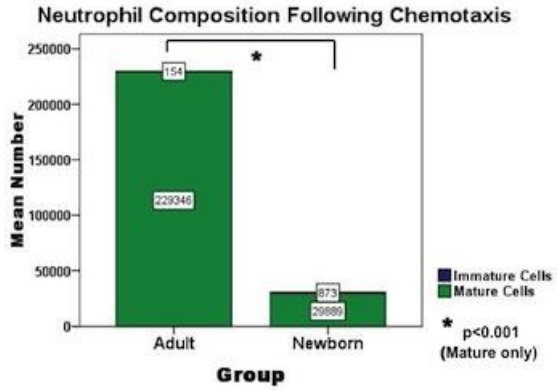
Background: Newborns exhibit deficiencies in neutrophil function but neutrophil composition varies significantly between neonates and adults due to a higher percent of immature forms in neonates (11% vs. 5%; p=0.01). We hypothesize that neutrophil immaturity in newborns directly contributes to differences in chemotaxis and phagocytosis.

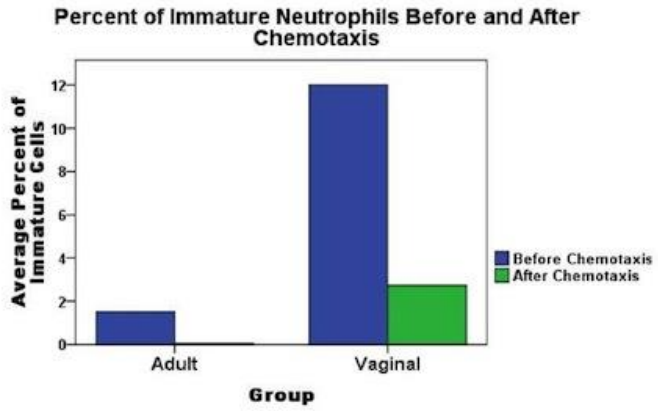
Study Design: Neutrophils were isolated by density gradient from term, healthy newborns via cord blood and adults by venipuncture. Neutrophils were labeled, sorted, and collected using flow cytometry. Chemotaxis was completed using Transwells and collected neutrophils were reanalyzed by flow cytometry to determine final composition. Phagocytosis of GFP-labeled *E.coli* in whole blood was measured using flow cytometry to simultaneously determine cell maturity and fluorescence of engulfed bacteria.

Results: Mature but not immature neonatal neutrophils phagocytose *E.*coli with similar findings between adults and newborns. However, mature neonatal neutrophils have significantly decreased ability to complete chemotaxis as compared to adults ($p<0.001$), while immature forms have similar capabilities.

Conclusion: Neonatal and adult neutrophils at similar maturational stages of development display heterogeneity. That is, differences in function exist despite similar maturational development, which needs to be investigated further.







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IL-10 Is Critical for Myeloid-Derived Suppressor Cell (MDSC) Recruitment and Bacterial Persistence during Staphylococcus Aureus Orthopedic Biofilm Infection

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature monocytes and granulocytes that are potent inhibitors of T cell activation. Although MDSCs are well-recognized for their role in tumor immunosuppression, their immunologic functions during bacterial infection have only recently emerged, and little is known about MDSC function in the context of Staphylococcus aureus (S. aureus) infection. S. aureus biofilms are capable of subverting immune-mediated clearance, and a recent report from our laboratory has identified a population of $Ly6G^{high}Ly6C^{+}$ MDSCs as the main cellular infiltrate during S. aureus orthopedic biofilm infection. Biofilm-associated MDSCs inhibited T cell proliferation and cytokine production, which correlated with a paucity of T cell infiltrates at the infection site. Analysis of FACS-purified MDSCs recovered from S. aureus biofilms revealed increased IL-10 expression, which was confirmed using IL-10-GFP mice where $Ly6G^{high}Ly6C^{+}$ MDSCs represented the main source of IL-10 during biofilm infection. IL-10 secretion by MDSCs has been implicated in inhibiting T cell activation and polarizing macrophages (MΦs) toward an alternatively activated M2 phenotype. To determine the functional importance of IL-10 in shaping the inflammatory milieu during S. aureus biofilm infection, we performed studies in IL-10 knockout (KO) mice. MDSC influx into implant-associated tissues was significantly reduced in IL-10 KO mice at day 14 post-infection concomitant with enhanced monocyte and macrophage infiltrates. The reduction in MDSC recruitment facilitated bacterial clearance as revealed by significant decreases in S. aureus burdens in the tissue, knee joint and femur of IL-10 KO mice. These results demonstrate that IL-10 produced by MDSCs contributes to the persistence of S. aureus biofilm infections by limiting monocyte/macrophage activity.

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Superoxide Induces Neutrophil Extracellular Traps by a TLR4- and Phox-Dependent Mechanism

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BACKGROUND: Neutrophils accumulate in the liver after ischemia-reperfusion injury and contribute to inflammation-associated damage. These cells have recently been shown to extrude select intracellular contents to form a Neutrophil Extracellular Trap (NET). Stimulation of Toll-like receptors (TLRs) initiates a signalling cascade which includes activation of NADPH Oxidase (PHOX), a required step in NET formation. Hydrogen peroxide, a membrane-permeable reactive oxygen species, is an exception that has been reported to induce NETs independently of TLRs and PHOX. A membrane-impermeable species, superoxide, has previously been shown to signal for neutrophil activation and increased proinflammatory cytokine production

through TLR4, but it is unknown whether it also induces NETs.

HYPOTHESIS: In settings of non-infectious inflammation, such as oxidative stress, reactive oxygen species, specifically membrane-impermeable superoxide, can induce NET formation through TLR4.

METHODS: WT and TLR4KO neutrophils were treated with xanthine oxidase and its substrate hypoxanthine to generate extracellular superoxide. We inhibited xanthine oxidase by allopurinol and inhibited NADPH Oxidase (PHOX) by diphenylene iodonium (DPI).

RESULTS: WT neutrophils exposed to superoxide demonstrated elevated levels of citrullinated Histone H3, a specific NET marker, by western analysis; however, TLR4KO neutrophils failed to express cit-H3 despite superoxide treatment. Superoxide-inhibited neutrophils (allopurinol treatment) or PHOX-inhibited (DPI treatment) expressed only basal cit-H3 compared to treatment with phorbol-myristate-acetate (PMA; positive control) in WT and TLR4KO neutrophils. Additionally, superoxide exposure generated characteristic NET fibers in WT neutrophils, but not TLR4KO, as visualized qualitatively by immunofluorescence microscopy and measured quantitatively by mean cytoplasmic spot intensity of extracellular DNA.

CONCLUSIONS: Our finding that extracellular superoxide induces NETs expands upon previous findings that superoxide activates neutrophils and increases proinflammatory cytokine production. Furthermore, in contrast to previous findings that membrane-permeable hydrogen peroxide induces NETs independently of any cell-surface receptor or PHOX, our work demonstrates that superoxide, a membrane-impermeable species, requires both. In summary, our study demonstrates that membrane-impermeable superoxide induces NETs, and that WT TLR4 and functional PHOX are required for this process. This suggests that products of oxidative stress and damage-associated signals released in liver ischemia-reperfusion act via ligand-receptor interactions to promote NET formation.

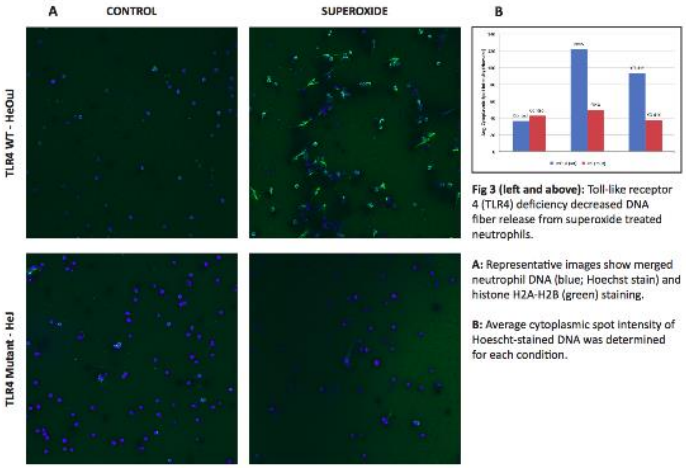

Fig 3 (left and above): Toll-like receptor 4 (TLR4) deficiency decreased DNA fiber release from superoxide treated neutrophils.

A: Representative images show merged neutrophil DNA (blue; Hoechst stain) and histone H2A-H2B (green) staining.

B: Average cytoplasmic spot intensity of Hoescht-stained DNA was determined for each condition.

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Bioregulation of Kallikrein-Related Peptidases by the Kinin B1 Receptor in the Human Neutrophil

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The human kallikrein family comprises a group of 15 trypsin- and chymotrypsin-like proteases (KLK1 to KLK15) that has a differential distribution in cells and biological fluids. The most studied kallikrein (KLK1), is a kininogenase so termed because it has the capacity to release the kinin peptides from endogenous protein substrates called kininogens. Several authors have reported immunolabeling of other KLKs in neutrophils detected on tissue sections of a variety of human tissues, but no previous study has confirmed these isolated observations for any member of the KLK family experimentally. Because kinins are, proinflammatory peptides that modulate neutrophil function by activating the kinin B1 receptor (B1R) and also because the importance of KLK family in inflammation and cancer, we examined whether stimulation of human neutrophils by an agonist of the B1R induces the release of specific KLKs, selected on their functional biology.
Neutrophils, isolated from peripheral blood of clinically healthy donors were used to determine the expression of the 15 KLKs by immunocytochemistry, RT-PCR and Western blotting. When neutrophil homogenates were analyzed by Western blot, most KLKs displayed the expected molecular mass that ranged between 25 to 35 kDa. In contrast, conventional RT-PCR using well characterized primers showed that mRNA expression

was restricted to the presence of *KLK1*, *KLK4*, *KLK10*, *KLK14* and *KLK15* genes; this result indicates that immunoreactive protein levels do not necessarily correspond with the spectrum of mRNAs. Nevertheless, those KLKs that did not have detectable mRNA levels in the neutrophil were easily detected in the non-differentiated HL-60 cells.

Our experiments demonstrate that all investigated KLKs are released into the medium in response to 100 nM of the chemotactic peptide fMet-Leu-Phe. When a B1R agonist was used, KLK1, KLK6, KLK13, KLK14 and KLK10 were released in similar amounts to those obtained with fMLP, whereas KLK2, KLK4 and KLK5 levels were minimal. The fact that these proteases are released by neutrophils following stimulation with fMLP or a B1R agonist suggest that they may be stored in particular granule populations. In fact, images of neutrophils immunostained for each KLK and analyzed by confocal microscopy showed a granular pattern of variable size that seems to be diverse between different kallikreins.

Our results demonstrate, for the first time, the expression of all KLK members of the tissue kallikrein family in the human neutrophil and suggest that secretion of KLK6 and KLK13 after stimulation with a B1R agonist may enhance neutrophil activity since both KLKs can degrade components of the extracellular matrix such as collagen, fibronectin and laminin.

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Allergic Pulmonary Inflammation Accelerates Metastasis via Increased Myeloid Derived Suppressor like Cells

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Disseminated metastasis accounts for majority of breast cancer deaths. Breast tumors often metastasize to the lungs. It is known that myeloid derived suppressor cells in the primary tumor contribute towards tumor growth. Our previous work has demonstrated that myeloid cells are present in the pre-metastatic lungs of mammary tumor bearers and that these cells may contribute towards establishment of infiltrating tumor cells into metastatic foci. Mice with allergic pulmonary inflammation implanted with 4T1 mammary tumors had a 5-fold increase in formation of metastatic foci in their lungs compared to control mammary tumor bearers. Further, allergic mice showed accelerated tumor growth and shorter survival. To understand the host response that contributes to the accelerated rate of metastasis to the lungs, we determined the alterations that occur during allergic pulmonary inflammatory response. Increased levels of chitinase-3-like-1 protein, CCL2, CXCL2, and MMP-9 were found in ragweed sensitized mice. These chemokines may recruit myeloid suppressor-like cells that are known to produce nitric oxide and arginase-1. We observed an increase in myeloid suppressor-like cells in mice sensitized with ragweed allergen and these numbers were further increased upon inoculation with mammary tumor cells. Mammary tumor-bearing mice with allergic pulmonary inflammation had higher levels of pro-inflammatory mediators, CHI3L1, CCL2, CXCL2, and MMP-9 but decreases levels of IFN-? compared to mice with allergic pulmonary inflammation alone. Based on these observations, we hypothesized that allergic inflammation recruits myeloid derived suppressor like cells from the bone marrow and that these cells aggravate the inflammatory milieu to support incoming mammary tumor cells for establishment of metastasis. To test this hypothesis, mice were depleted of myeloid derived suppressor like cells after inducing allergic pulmonary inflammation but prior to inoculation with mammary tumor cells. Since myeloid cells aggravate the allergic inflammatory response, depleting myeloid cells may not only suppress metastasis to the lungs but also dampen the adverse inflammation during allergic asthma response.

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Role of TGFß Signaling in Generation of CD39+CD73+ Myeloid Cells in Tumors

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There is growing evidence that generation of adenosine from ATP, which is mediated by the CD39/CD73 enzyme pair, predetermines immunosuppressive and pro-angiogenic properties of myeloid cells. We have previously shown that the deletion of the TGFβ type II receptor gene (*Tgfbr2*) expression in myeloid cells is

associated with decreased tumor growth suggesting pro-tumorigenic effect of TGFβ signaling. In this study, we tested the hypothesis that TGFβ drives differentiation of myeloid-derived suppressor cells (MDSCs) into pro-tumorigenic terminally differentiated myeloid mononuclear cells (TDMMCs) characterized by high levels of cell surface CD39/CD73 expression. We found that TDMMCs represent a major cell subpopulation expressing high levels of both CD39 and CD73 in the tumor microenvironment. In tumors isolated from MMTV-PyMT/TGFRIIKO mice, an increased level of TGFβ protein was associated with further increase in number of $CD39^{+}CD73^{+}$ TDMMCs compared to MMTV-PyMT/TGFRIIWT control tumors with intact TGFβ signaling. Using genetic and pharmacological approaches, we demonstrated that the TGFβ signaling mediates maturation of MDSCs into TDMMCs with high levels of cell surface CD39/CD73 expression. Disruption of TGFβ signaling in myeloid cells resulted in decreased accumulation of TDMMCs, expressing CD39 and CD73, and was accompanied by increased infiltration of T lymphocytes, reduced density of blood vessels and diminished progression of both Lewis Lung carcinoma and spontaneous breast cancer tumors. We propose that TGFβ signaling can directly induce the generation of CD39+CD73+ TDMMCs, thus contributing to the immunosuppressive, pro-angiogenic, and tumor-promoting effects of this pleiotropic effector in the tumor microenvironment.

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Resveratrol Attenuates Microvascular Inflammation in Sepsis via SIRT1-Induced Modulation of E-Selectin Expression in ob/ob Mice

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Introduction: Sepsis incidence is rising and no effective therapies currently exist to treat its pathophysiology. Obesity increases morbidity and cost of care of septic patients further. Leukocyte-endothelial interaction at the microvascular interface is an important early inflammatory marker in sepsis. We have shown previously that there is exaggerated leukocyte adhesion with adhesion molecule expression in ob/ob mice with early sepsis. NAD+ sensor sirtuin 1 (SIRT1) modifies chromatin structure to epigenetically repress acute inflammatory pathways. Reports indicate that obesity decreases SIRT1 expression. Resveratrol (3, 5, 4'-trans-trihydroxystilbene) found in grapes, berries, peanuts and red wine is a known activator of SIRT1. In the current project we hypothesized that resveratrol induced increase in SIRT1 expression attenuates the leukocyte adhesion and adhesion molecule expression in ob/ob mice during acute phase of sepsis.

Methods: C57Bl/6 (WT) and ob/ob mice were pre-treated with resveratrol vs. vehicle (DMSO: Veh) 18 hours prior to sepsis induction using cecal ligation and puncture (CLP). We studied leukocyte adhesion in small intestinal microcirculation 6 hours post-CLP. We also studied adhesion molecule E-selectin and SIRT1 expression in small intestinal tissue using immunohistochemistry (IHC) 6 hours post-CLP. To further elucidate the mechanism of action of resveratrol via SIRT1 expression, we administered SIRT1 specific inhibitor EX-527 (EX) in a separate group of CLP mice pre-treated with resveratrol and studied leukocyte adhesion and E-selectin expression in the small intestine as described above.

Results: Resveratrol attenuates microvascular inflammation in WT and ob/ob mice: There was a significant increase in leukocyte adhesion in the small intestinal microcirculation in ob/ob vs. WT mice with CLP. Resveratrol pre-treated CLP mice had decreased leukocyte adhesionvs.vehiclein both WT and ob/ob groups (**Figure 1**). Resveratrol decreased E-selectin expression: Resveratrol pre-treatment increased SIRT1 expression and decreased E-selectin expression in intestinal tissue in both WT (data not shown) and ob/ob (**Figure 2**) mice. SIRT1 inhibition abolished resveratrol induced attenuation of microvascular inflammation: SIRT1 inhibition using EX-527 showed increased leukocyte adhesion and E-selectin expression (**Figure 3**) in resveratrol treated ob/ob septic mice compared to resveratrol treatment alone. We also show that resveratrol treated ob/ob septic mice had significantly increased survival compared to vehicle treated mice (data not shown).

Conclusion: Resveratrol induced increase in SIRT1 expression during the acute phase of sepsis reduces microvascular inflammation via modulation of E-selectin expression in and improves survival in ob/ob mice.

Figure 1

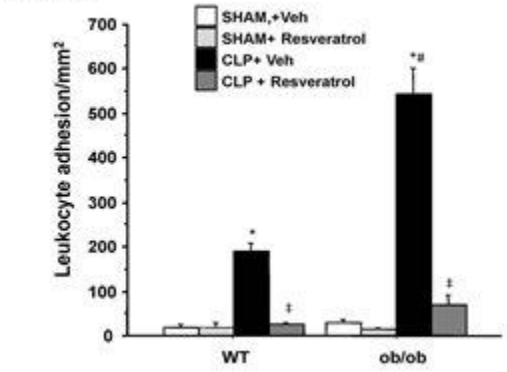


Figure 2

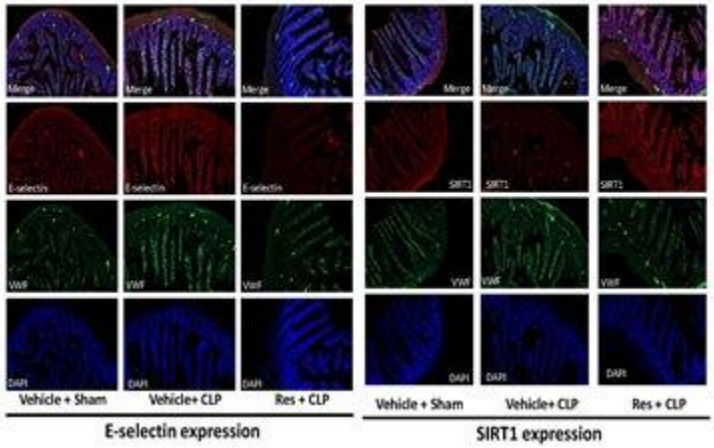
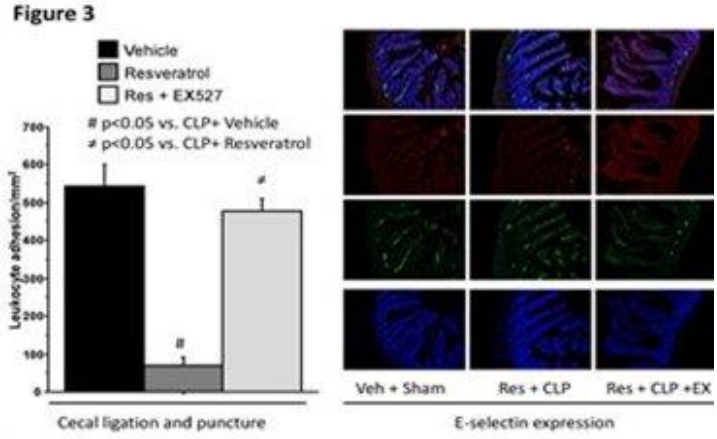


Figure 3



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De-Repression of Macrophage Pathways to Target Persistent HIV Infection

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Permanent integration of the HIV genome into the genomes of host cells presents the greatest barrier to a cure for HIV/AIDS. Despite suppression of viremia to extremely low levels with anti-retroviral therapy, cellular reservoirs for HIV remain for many decades. To eliminate reservoir cells, we must develop new strategies to specifically target these cells for eradication. Through gene array transcriptome profiling of HIV-infected macrophages, we identified ubiquitin specific proteinase 18 [USP18] as one of the most highly up-regulated genes. Because USP18 is known to negatively regulate IFN and NFκB signaling in murine macrophages and T cells, we predicted that USP18 would similarly regulate the host response to HIV infection.

Upon USP18 knockdown by shRNAs, THP1 macrophages showed profoundly restricted HIV replication. Inhibition was observed with reduced frequency of infected cells, but similar production of viral proteins in cells that were infected. USP18 deficient cells also demonstrate an exaggerated interferon-stimulated gene [ISG] response. When THP1 cells were treated with a double-stranded DNA ligand similar to HIV reverse transcription products, knockdown of USP18 resulted in an exaggerated inflammasome/IL-1β response.

To better understand the molecular mechanisms involved, HEK 293 cells were modified with shRNA targeting USP18 or overexpression of USP18. Cells responded to USP18 deficiency with a stronger ISG and NFκB response including increased expression of the key immunoproteasome component LMP2 which facilitates processing and presentation of foreign antigens via MHCI. Overexpression of USP18 inhibited IFN and NFκB pathways and signaling outcomes.

Our findings indicate that USP18 regulates both IFN and NFκB pathways in macrophages. Overall, USP18 serves as a negative regulator of HIV-induced macrophage activation by counteracting virus-induced pathways. We propose that targeting USP18 provides a mechanism to "de-repress" macrophage activation which will result in elimination of persistently HIV-infected macrophages through the inflammasome/pyroptosis pathway or through improved recognition and killing by CD8 cytotoxic T lymphocytes.

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The Role of Ubiquitin Specific Proteinase 18 in HIV Persistence in Macrophages

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Antiretroviral therapy [ART] prevents onset of acquired immune deficiency syndrome [AIDS] in human immunodeficiency virus [HIV] infected patients, but no sterilizing cure for HIV exists. During ART, HIV persists in long-lived cells like memory CD4+ T cells and macrophages. If treatment is halted, plasma viremia becomes detectable within 2-3 weeks and progression to AIDS resumes. Macrophages, which are resistant to the cytopathic effects of HIV, can act as long-term reservoirs for HIV production. These reservoirs must be eliminated for a cure to be possible. Our objective is to understand how HIV-infected macrophages resist the cytopathic effects of HIV and how this process can be counteracted to kill HIV-infected macrophages.

Transcriptional profiling of human monocyte derived macrophages [MDM] showed that ubiquitin specific proteinase 18 **[USP18]** is highly upregulated during HIV infection. USP18 is a negative regulator of type I interferon [T1-IFN] and nuclear factor kappa B [NFκB] signaling pathways. To determine the role of USP18 in HIV infection, we used shRNA to knockdown USP18 in the human acute monocytic leukemia cell line, THP-1.

Partial knockdown (~50%) of USP18 in THP-1 cells resulted in a significant reduction in HIV replication. This suggests that HIV-induced USP18 expression dampens signaling pathways downstream of cytosolic sensors for HIV making the cells less susceptible to cytopathic effects of the virus and/or less visible to cytotoxic T lymphocytes [CTLs]. T1-IFN stimulation induced significantly higher levels of interferon inducible genes with known antiviral functions in the absence of USP18 compared to control. Transcript levels were also higher in USP18 deficient THP-1 cells stimulated with TLR ligands. USP18 deficient cells also had enhanced inflammasome activation after stimulation with LPS or double stranded DNA.

Our data together show that in the absence of USP18, pathways important for inducing cell death by pyroptosis or transcription of antiviral genes are enhanced. We posit USP18 as a potential therapeutic target for HIV-infected macrophages.

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Divergent Effects of BTLA Ligation on the Innate Immune Response to the Combination of Experimental Shock/Sepsis

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Sepsis is a complex inflammatory condition in the presence of infection, in which the balance of proinflammatory and anti-inflammatory responses is crucial to the prognosis. B and T lymphocyte attenuator (BTLA) is an immune-regulatory receptor expressed not only on adaptive immune cells but also on innate cells. Our previous data showed that BTLA knock out mice were protected from septic mortality when compared with WT control mice. Here we used an agonistic anti-BTLA antibody (6A6) to determine whether exogenous antibody treatment could affect the inflammatory response seen following the combined insults of shock (hemorrhage) followed by sepsis. In a C57BL/6 mouse model of hemorrhage followed sepsis induced by cecal ligation and puncture (CLP), we administrated 6A6 intra-venously during hemorrhage resuscitation, and intra-peritoneally right after CLP. 24h later, the serum and peritoneal lavage were harvested. Peritoneal leukocytes' phenotype and number were determined with Gr1 and F4/80 PE antibodies by flow cytometry. Cytokines levels in serum and peritoneal lavage were measured by ELISA. And local bacterial burden was measured using tryptic soy agar blood plates. We found that peritoneal neutrophil and macrophage numbers increased in 6A6 treated group, accompanied with significantly higher TNF-□, MCP-1, KC and MIP-2 levels in peritoneal lavage, higher TNF-□, MIP-2 levels in serum, as well as an elevated local bacterial burden. These results indicate BTLA's role in affecting innate immune response is complex; as it appears capable of aggravating the inflammatory cytokine response while concomitantly suppressing process contributing to the clearance of the infectious agent.

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Cellular and Molecular Mechanisms by Which the TLR4 Agonist Monophosphoryl Lipid a Facilitates Neutrophil Mobilization and Recruitment

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Background. The TLR4 agonist monophosphoryl lipid A (MPLA) is currently used as an adjuvant in FDA-approved vaccine formulations. Our recent studies show

that MPLA will prime the innate immune system and facilitate non-specific antimicrobial immunity against bacterial infection, an effect that is dependent on augmented neutrophil recruitment. Previous studies report that the immunoadjuvant effects of MPLA are mediated through activation of the TRIF-dependent signaling pathway. However, the signaling mechanisms by which MPLA augments neutrophil recruitment are unknown. Our aim was to identify the importance of TLR4-, TRIF-, and MyD88-dependent signaling in facilitating neutrophil mobilization and recruitment upon MPLA administration.

Methods. TLR4, TRIF, and MyD88 knockout mice and wild type control mice received intraperitoneal administration of MPLA (20 µg). At 0, 3, and 6 hours after MPLA challenge, neutrophil numbers in blood and peritoneal cavity were measured. Concentrations of factors that are important for neutrophil mobilization (G-CSF, CXCR4) and recruitment (KC, MIP-2) were measured in plasma and peritoneal lavage fluid at the same time points.

Results. Wild type mice showed a near tripling of blood neutrophil numbers at 3 hours after MPLA challenge compared to baseline. Intraperitoneal neutrophils also increased in wild type mice from less than 0.3 x 106 to 2.3 x 106 at 3 hours after MPLA injection and remained elevated at 6 hours post-challenge. TLR4-deficient C57BL/ScN mice did not exhibit significant changes in blood or intraperitoneal neutrophil numbers at any of the time points studied. Blood neutrophil numbers were increased at 3 hours post-MPLA injection to levels that were similar to that observed in wild type mice, whereas MPLA-induced recruitment of neutrophils into the peritoneal cavity was nearly ablated in MyD88-deficient mice (2.3 vs 0.2 x 106). TRIF knockout mice showed decreased mobilization of neutrophils into blood at 3 and 6 hours after MPLA challenge to levels that were approximately 50% of the peak neutrophil numbers observed in wild type mice. Recruitment of neutrophils into the peritoneal cavity was also attenuated in TRIF knockout mice compared to wild type controls (2.3 vs 1.3 x 106). MPLA induced increased concentrations of G-CSF (6620 ± 230 pg/ml) in plasma at 6 hours after MPLA but was a weak inducer of IL-6 (112 ± 26 pg/ml). Expression of CXCR4 on bone marrow neutrophils was down regulated by MPLA treatment. MPLA induced increased concentrations of MIP-2 and KC in plasma and peritoneal cavity.

Conclusions. MPLA-induced neutrophil mobilization from bone marrow appears to be mediated primarily by activation of the TRIF-dependent signaling pathway whereas neutrophil recruitment to the site of MPLA administration is primarily mediated by MyD88-dependent signaling. MPLA is a potent stimulus for production of the neutrophil mobilization and recruitment factors G-CSF, MIP-2 and KC.

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Regulation of Interferon Pathways by Ubiquitin Specific Proteinase 18

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A major barrier to curing HIV lies with persistently-infected cells such as macrophages, which act as viral reservoirs despite antiretroviral therapy. One strategy to eliminate persistently-infected cells is to activate the cells to enhance HIV replication resulting in cytopathic effects of the virus or elicitation of cellular immunity targeting viral antigens. The answer to eliminating HIV-infected macrophages may lie with the protein ubiquitin-specific proteinase 18 [USP18]. Our preliminary data has shown that in macrophages, HIV induces a minimal IFN-like transcriptional response with relatively high expression of the interferon-stimulated gene USP18. USP18 is a negative regulator of type I [T1-IFN] interferon signaling pathways. Thus, we hypothesize that USP18 is key in masking the presence of HIV in macrophages leading to a decrease in macrophage responsiveness to infection, and allowing the infection to persist.

To better understand the role of USP18 in T1-IFN signaling pathways, we knocked down its expression in human embryonic kidney 293T cells [293T] using shRNA and treated the cells with interferon beta [IFN-β]. To determine the effects of USP18 deficiency in T1-IFN responses, we used quantitative real time PCR (qPCR) to measure the expression levels of key interferon stimulated genes [ISGs]. Our results showed significant increase in ISG15 expression in the absence of USP18, as well as increases in expression of other ISGs such as oligoadenylate synthetase 1 [OAS1], interferon-induced protein with tetratricopeptide repeats 2 [IFIT2 or ISG54], and proteasome subunit, beta type, 9 [PSMB9 or LMP2]. Additionally, we have treated USP18-deficient cells with a double-stranded DNA [dsDNA] mimetic of early HIV reverse transcription

products which activate cells via the cytosolic proteins IFI16 and or cGAS. We found that the innate response to dsDNA, similar to type 1 IFN, was enhanced in the absence of USP18.
Together, these results indicate that USP18 regulates key IFN and innate sensing pathways. As such, USP18 may present a novel target to enhance activation of HIV-infected macrophages, and potentially CD4+ T cells.

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Deciphering Pathways for C1q-Dependent Efferocytosis and Inflammatory Signaling

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Efficient clearance of apoptotic cells and dampening of proinflammatory cytokine production is required for prevention of autoimmunity. Deficiency in complement component C1q is associated with a failure to clear apoptotic cells, inflammation and autoimmunity. We are investigating the mechanisms by which C1q regulates these functions in macrophages. Microarray analysis of C1q-stimulated mouse macrophages revealed that C1q upregulates expression of Mer tyrosine kinase (TK), a receptor on the membrane of macrophages that mediates apoptotic cell clearance and anti-inflammatory signaling. Pathway analysis of the microarray data led to the discovery that C1q and a related protein, adiponectin, trigger MerTK-dependent efferocytosis via activation of AMP-activated protein kinase (AMPK) independently of known C1q and adiponectin receptors. Prolonged stimulation with C1q (18 hrs) resulted in MerTK-dependent efferocytosis and an inhibition of LPS-dependent TNF-α production. A more pronounced C1q-dependent inhibition of proinflammatory cytokine production was observed when macrophages were polarized to an M1 phenotype using LPS + IFN-γ. M1 polarization was confirmed by examining expression of iNOS, which was upregulated with LPS + IFN-γ treatment in the presence or absence of C1q. C1q-dependent inhibition of proinflammatory cytokine production was observed in both primary mouse and human macrophages, however the human macrophages failed to upregulate MerTK suggesting that the mechanism of inhibition did not depend on MerTK signaling. Furthermore, there was no detectable reduction in expression CD14 or TLR4 with C1q suggesting that the mechanism of inhibition is downstream of the LPS receptors. Future studies are aimed at evaluating the role of AMPK and downstream components of the TLR4 signaling pathway in C1q-dependent regulation of proinflammatory cytokine production.

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Opposing Effects of Gram-Positive and Gram-Negative Bacteria on Wound Macrophage Phenotype and Function

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Macrophages are present in nearly every phase of the wound healing response and are essential to repair. Work from this laboratory examines the role of wound macrophages using a polyvinyl alcohol (PVA) sponge implantation model in mice. This model recapitulates the phases of healing and allows for the recovery of a large number of viable cells. Prior studies have shown that monocytes are recruited to sterile wounds from the circulation. Upon entry monocytes become proinflammatory and undergo eventual maturation into macrophages that express Mer tyrosine kinase (MerTK) and release repair cytokines. While initial studies have focused on macrophages in sterile wounds, these do not completely recapitulate the majority of human wounds, which are colonized with bacteria. The role of bacteria in shaping wound macrophage responses is not well understood, and is addressed in work presented here. These studies aim to determine the effect of exposure to the gram-negative bacteria *E. coli*, the gram-positive bacteria *S. aureus*, or their cell wall components, on the function of macrophages and the monocyte to macrophage transition in PVA sponge wounds. Cells isolated from sterile wounds and exposed *ex vivo* to heat-killed *E. coli* or LPS were made elevated levels of pro-inflammatory cytokines and depressed levels of repair-associated cytokines including VEGF and TGF-β compared to untreated cells. These observations, together with earlier work demonstrating accelerated healing in TLR4-deficient animals, suggest that TLR4-mediated signals may restrain healing responses. In contrast, cells recovered from sterile wounds and exposed *ex vivo* to heat-killed *S. aureus*, Pam3CSK4 or LTA were induced to make higher levels of VEGF and TGF-β than untreated cells and cells exposed to *E. coli*.

Based on these data, it was hypothesized that *E. coli*, which is recognized primarily through TLR4, and *S. aureus*, which can activate multiple innate recognition receptors including TLR2, may have distinct effects on development of repair macrophage responses *in vivo*. A model was developed in which PVA sponges are inoculated with live bacteria prior to implantation to induce a low-grade wound infection. Inoculation with *E. coli* induced an inflammatory response in wound monocytes/macrophages but did not alter the balance of proinflammatory monocytes and repair macrophages compared to cells isolated from sterile wounds. The presence of *S. aureus* in wounds resulted in a higher proportion of $MerTK^+$ repair macrophages and a lower percentage of proinflammatory monocytes after wounding compared to cells isolated from sterile wounds. This suggests that *S. aureus*-mediated signals may paradoxically accelerate the monocyte to macrophage transition in wounds. Taken together, these results indicate that *E. coli* and *S. aureus* infection may have opposing effects on the development of wound repair macrophages.

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Alcohol-Induced miR-27a Modulates Innate Immune Responses by Promoting M2 Macrophage Polarization of Human Monocytes

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Background: Alcohol abuse is one of the leading causes of liver disease and it is an independent risk factor for progression to cirrhosis in chronic Hepatitis C Virus (HCV) infection. Activation of monocytes and macrophages (Mo/MØ) contributes to liver fibrosis in chronic HCV infection. Alcohol itself promotes pro-inflammatory monocyte activation, cytokine secretion and expression of microRNAs (miRs), the 18-22 nucleotide sequences that play role in fine tuning immune responses including Mo/MØ differentiation. Thus, we hypothesized that alcohol and HCV have synergistic effects on monocyte activation, phenotype and function. Inflammatory Mo/MØ represent the M1 phenotype while M2, alternately activated MØ have anti-inflammatory effects. Thus, we also aimed to define the effects of alcohol and/or HCV on Mo/MØ phenotype.
Methods: The phenotype (FACS) and function (cytokine mRNA and protein production) of circulating monocytes was evaluated from normal, nonalcoholic individuals before and after acute alcohol binge (2ml vodka/kg body weight). To study the combined effect of alcohol and HCV infection, monocytes from healthy individuals before and 24 hr post-alcohol consumption were co-cultured with HCV infected hepatoma cells (Huh7.5/JFH1). Phenotype and cytokine secretion pattern of the co-cultured monocytes were evaluated by FACS and ELISA. To study the functional role of miRs in these processes we performed knockdown and overexpression experiments.
Results: We found that acute alcohol binge drinking in normal volunteers results in increased frequency of circulating monocytes expressing CD16 and the macrophage (MØ) marker, CD68. Furthermore, expression (MFI) of M2 MØmarkers, CD206, DC-SIGN, and IL-10 secretion were increased in monocytes after alcohol binge drinking. HCV also increased monocyte expression of the MØmarker CD68 and the M2 MØmarkers; CD206 and DC-SIGN and this was further increased by alcohol. MiR-27a was significantly upregulated in monocytes cultured with alcohol alone in combination with HCV compared to HCV alone. To test the functional role of miR-27a, monocytes were transfected with a miR-27a inhibitor that prevented alcohol- and HCV- mediated monocyte differentiation (CD14 and CD68 expression), polarization (CD206 and DC-SIGN expression) and IL-10 secretion. Neutralization of IL-10 decreased CD68, CD206 and DC-SIGN expression demonstrating the functional role of IL-10 in monocyte polarization to M2 phenotype. Overexpression of miR-27a demonstrated its role in IL-10 induction via activating the ERK signaling pathway. Mir-27a induced ERK activation by downregulating the ERK inhibitor, sprouty-2 in macrophages. Functional experiments by overexpressing Sprouty-2 in healthy monocytes demonstrated its role in inhibiting ERK phosphorylation and IL-10 secretion.
Conclusion: In summary, this study demonstrates the important role of miR-27a in alcohol and HCV-mediated effects on monocyte differentiation and macrophage polarization.

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Environment-Host Miscommunication at the Intestinal Epithelial Interface in Type 1 Diabetes – a Tipping Point for Disease?

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The intestinal epithelium serves as a first line of defense and mediates the host-environment interface; moreover, host-environment dialogues have the capacity to influence development of inflammatory and autoimmune disease, including type 1 diabetes (T1D). Various intestinal alterations have been observed in individuals with or at-risk for T1D, but specific mechanisms at play remain elusive. IEC are potent mediators of the host-environment dialogue; they express a wide variety of immune molecules including Toll-like receptors (TLR) and have the capacity to participate in immune tuning. Here, we describe a method for high purity adult human organ donor IEC culture, and use in assays evaluating IEC innate immune function, as well as methods to isolate and characterize intestinal immune cell populations.

Our preliminary data indicate an IFNγ-mediated IEC inflammatory phenotype to microbial ligand stimulation in T1D as measured by IFNγ and TNFα production. To understand the mechanisms behind these responses, we have investigated basal TLR expression and TLR expression following microbial ligand stimulation. In two sex and age-matched cases, IFNγ priming without additional ligand stimulation upregulates TLR4 and TLR5 expression in both T1D and T1D-free cultures. IFNγ priming with additional microbial ligand stimulation further upregulates TLR4 and TLR5 expression in T1D-free cultures to a degree not observed in the T1D culture. Since TLR expression on IEC is considered to largely promote immunoregulatory and homeostatic functions of IEC, these data indicate that T1D-derived IEC cultures may lack proper responses to microbial ligand stimulation following IFNγ priming.

Flow analysis of intestinal immune cells reveals no differences between T1D and T1D-free individuals in frequency of total CD3+, CD19+, CD11b+, or CD11c+ populations. A more detailed analysis of T cell populations reveals similar frequencies of α/β+CD8+ T cells, however the T1D-derived intestinal CD8+ T cells exhibit elevated frequencies of IL-17A+ and IL-17A+IFNγ+, with a decrease in the frequency of α/β+CD8+IFNγ+ when compared to a T1D-free donor. Moreover, the T1D donor exhibited marked increase in α/β+CD4-CD8- double negative cells, which have been implicated in other autoimmune diseases. These "double negative" T cells contain elevated IL-17A+ and IL-17A+IFNγ+ subpopulations, whereas the phenotype of these cells were α/β+CD4-CD8-IFNγ+ in the T1D free donor.

Using these culture techniques to investigate IEC innate immune function in tandem with intestinal immune populations, we can begin to understand the rules and conditions under which the intestinal epithelium tunes both local and distal immune responses, and how these may go awry in inflammatory and autoimmune disease.

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Structural Determination and Synthesis of Isoprenoids as Immune Activators via TLR4/MD-2

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Innate immune receptors are activated by particular components common to microbes, and also by some compounds with different types of backbone structures, including endogenous compounds. The complex of toll-like receptor 4 (TLR4) and myeloid differentiation factor 2 (MD-2) is known as the receptor of lipopolysaccharide (LPS) from Gram-negative bacteria, and the receptor protein recognizes the ligand at the lipophilic terminal moiety, lipid A.
As a TLR4/MD-2 ligand that has different backbone from lipid A, we have recently found a compound, FNC-R-01, from a high-throughput screening. FNC-R-01 is an analog of funiculosin isolated from a soil bacterium, *Penicillium funiculosum*. We then elucidated the chemical structure of FNC-R-01 and established the preparation method of FNC-R-01 from funiculosin. Molecular modeling studies of FNC-R-01 and its derivatives estimated the binding conformation FNC-R-01 and funiculosin with TLR4/MD-2.[1)] Other derivatives of funiculosin and FNC-R-01 were also designed by the molecular modeling. Some of the synthesized derivatives showed immunomodulatory activities and might be useful as immunostimulatnts/adjuvants.

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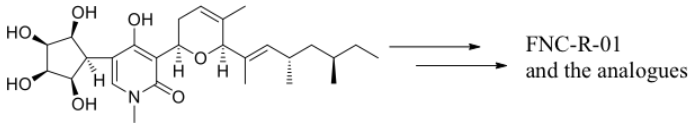

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Serum Amyloid A Induces Interleukin-33 Expression through an IRF7-Dependent Pathway

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Interleukin-33 (IL-33), an IL-1 family cytokine and nuclear alarmin, is constitutively expressed in epithelial barrier tissues and human blood vessels. However, little is known about the induced expression of IL-33 in monocytes and macrophages, which are major cytokine-producing cells of the innate immune system. Here we report the induction of IL-33 expression in both human monocytes and mouse macrophages from C57BL/6 mice by the acute-phase protein serum amyloid A (SAA). SAA induced transcriptional activation of the IL-33 gene, resulting in nuclear accumulation of the IL-33 protein. TLR2, one of the SAA receptors, was primarily responsible for the induction of IL-33. Progressive deletion of the human IL-33 promoter led to the identification of two potential binding sites for interferon regulatory factor 7 (IRF7), one of which (–277/-257) was found to be important for SAA-stimulated IL-33 promoter activity. IRF7 was recruited to the IL-33 promoter upon SAA stimulation, and silencing IRF7 expression in THP-1 cells abrogated SAA-induced IL-33 expression.SAA also promoted an interaction between TRAF6 and IRF7. Taken together, these results identify IRF7 as a critical transcription factor for SAA-induced IL-33 expression in monocytes and macrophages. Supported by grants from National Natural Science Foundation of China (Grants 81202316 and 31270941), from National Basic Research Program of China (973 Program, Grant 2012CB518001) and National Institutes of Health grant R01 AI033503 and AI040176.

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SAA1 Isoforms Display Different Selectivity for TLR2 and FPR2

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Serum amyloid A (SAA) is a major acute-phase protein and a precursor of amyloid A, the deposit of which leads to amyloidosis. Different alleles exit in SAA1, which is a predominant form of the human SAA gene family. Emerging evidence has shown correlation between these alleles and predisposition to diseases including familiar Mediterranean fever and amyloidosis. However, the structural basis for the different properties of these SAA1 variants remains unknown. Here we report the characterization of proteins encoded by SAA1.1, SAA1.3 and SAA1.5, in comparison to that encoded by SAA2.2, for their selectivity of the SAA receptors including Toll-like receptor 2 (TLR2) and formylpeptide receptor 2 (FPR2). SAA1.1 was more efficacious than SAA1.3 and SAA1.5 but equally efficacious to SAA2.2 in calcium mobilization and chemotaxis assays, which measure the activation of the G protein-coupled FPR2. In agreement with this, SAA1.1 and SAA2.2 induced more robust phosphorylation of ERK than SAA1.3 and SAA1.5. Only small differences were observed between the SAA1 variants and SAA2.2 in TLR2-dependent NF-kB luciferase reporter assay. In comparison, SAA1.3 was most effective in stimulating ERK and p38 MAPK phosphorylation. Using bone marrow-derived macrophages from C57BL/10ScN (Tlr4lps-del) mice, we examined the SAA isoforms for their induction of selected pro- and anti-inflammatory cytokines. SAA1.3 was most potent in the induction of TNFalpha and IL-1rn, whereas SAA1.5 induced robust IL-10 expression. These results show differences of the SAA1 isoforms in their selectivity for SAA receptors, which may affect their roles in modulating inflammation and immunity. Supported byNational Natural Science Foundation of China (Grant 31270941), National Basic Research Program of China (973 Program, Grant 2012CB518000), and National Institutes of Health (Grants AI040176 and AI033503).

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Synthetic and Natural Small Molecules That Modulate TLR4 Signal: a New Generation of Therapeutics

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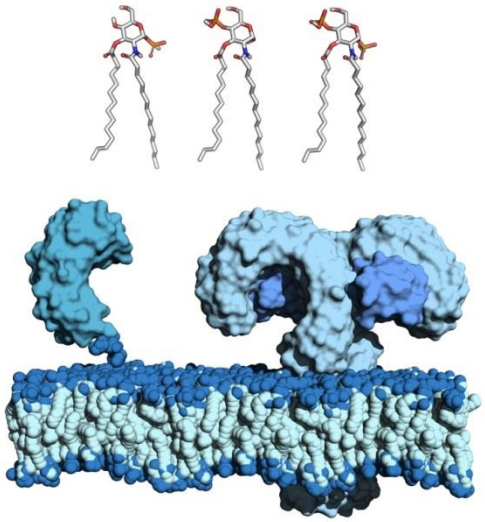

Toll-like receptor 4 (TLR4) detects minute amount of Pathogen Associated Molecular Patterns, PAMPs, namely bacterial endotoxin (lipopolysaccharide and oligosaccharide, LPS and LOS and their bioactive part, the lipid A) and activate the immune and inflammatory responses to pathogen infections. However, deregulated or excessively potent TLR4 activation and signaling generates serious syndromes such as septic shock and sepsis. TLR4 stimulation by endogenous factors also called Danger Associated Molecular Patterns, (DAMPs), has recently been associated to a wide array of diseases ranging from autoimmune, inflammatory and circulatory diseases, to some types of diabetes and tumors.
Small molecules active in modulating TLR4 activity are promising lead compounds for developing specific therapeutics against infectious and inflammatory pathologies. We synthesized glycolipids and other cationic and anionic amphiphiles that are active in modulating the TLR4-mediated inflammatory and innate immunity responses to bacterial endotoxins (lipopolysaccharide, LPS).[1] Cationic glycolipids derived from D-glucose[2] and anionic lipid A mimetics (Figure)[3] inhibit endotoxin-induced cytokine production in innate immunity cells. These molecules are active in vivo in contrasting septic shock and DAMP-dependent syndromes, such as neuropathic pain, caused by microglial TLR4 activation.[4] Some promising preliminary results in neuroinflammation and Amyotrophic Lateral Sclerosis (ALS) animal models will be presented.
The mechanism of action of synthetic compounds and nanoparticles active on TLR4 will be discussed in detail. The activity of some these molecules in inhibiting LPS-dependent TLR4 activation is due to specific targeting of CD14 that chaperones endotoxin association to TLR4.[5]
New perspectives in the modulation of TLR4 signal with synthetic lipid A-like compounds and natural compounds (mainly plant metabolites) will be presented.[6]

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TLR4 Signaling on Dendritic Cells Suppresses Neutrophil CXCR2 Expression and Trafficking during Sepsis.

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Dendritic cells (DC) link innate and adaptive immunity, but the specific role of TLR4 on DC during sepsis is unclear. Here we investigated the role of TLR4 on DC in immune responses during sepsis. C57BL/6 (WT), global TLR4 knockout (TLR4KO), cell specific KO control

(flox), and CD11c-specific TLR4KO (DCTLR4KO) mice underwent cecal ligation and puncture (CLP) for 4h or 18h. Surprisingly bacterial load in peritoneum was three logs lower in DCTLR4KO than control (2.9E+6±3.2E+6 vs 1E+9±1.5E+9CFU/mL) at 18h ($p<0.05$). Neutrophil numbers in peritoneum of DCTLR4KO and TLR4KO were significantly higher than controls at 4h. IL-10 was significantly lower in peritoneal lavage fluid of DCTLR4KO and TLR4KO than controls. Notably, CXCR2 expression on peritoneal neutrophils was significantly higher in DCTLR4KO and TLR4KO mice than in controls at 4h (mean fluorescence intensity: WT vs TLR4KO: 647±47 vs 1243±116; flox vs DCTLR4KO:577±41 vs 1371±101, $p<0.05$), which correlated with increased neutrophil recruitment to peritoneum. To investigate this mechanism further we isolated splenic neutrophils and DC from mice at 4h after CLP and cocultured with LPS (100ng/mL) for 3h. Neutrophil CXCR2 decreased significantly after LPS stimulation when coccultured with WT-DC, but not with TLR4KO-DC. IL10 level in media was inversely correlated with CXCR2 expression. Furthermore, neutrophil CXCR2 expression was persevered in the coculture with WT-DC after LPS stimulation, when anti-IL-10 antibody (1mg/mL) was used to neutralize the IL-10 in media. Neutrophil CXCR2 expression decreased significantly in the coculture with TLR4KO-DC after LPS stimulation, when recombinant IL-10 (20ng/mL) was added into the media. Finally, the adoptive transfer with WT-DC impaired bacterial clearance, neutrophil recruitment and CXCR2 expression in DCTLR4KO mice after CLP. Together our data suggest TLR4-signaling on DC regulates early innate immune responses during sepsis, and the mechanism may involve IL10-mediated suppression of CXCR2 on neutrophils. This novel pathway may suggest new therapeutic strategies for sepsis.

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Treatment with TLR4 Agonist Monophosphoryl Lipid a Enhances Neutrophil Responses to Infection after Burn

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Infection is the leading cause of death in severely burned patients that survive initial injury. Prior sensitization with immune modulator monophosphoryl lipid A (MPLA) is known to induce resistance to subsequent bacterial challenge, associated with an enhanced ability to clear bacteria following systemic challenge with Pseudomonas aeruginosa. MPLA is relatively innocuous and is currently used in human vaccine preparations, making it well-suited for clinical usage to enhance immune responses to infection. Previous studies have shown that treatment of mice with MPLA enhances bacterial clearance, leading to improved survival in various models of sepsis including P. aeruginosa systemic and burn wound infections. The current study was aimed to better define the mechanisms responsible for improved bacterial clearance and survival in MPLA-treated burn-infected mice. Mice underwent severe burn injury to ~35% total body surface area, followed by systemic treatment with MPLA or lactated Ringers (LR; control) for two days. Mice were then inoculated with P. aeruginosa, topically or intraperitoneally, and responding phagocytic immune cells were measured in bone marrow, blood and sites of infection. MPLA increased the number of neutrophils in the blood, at the site of administration and at sites of infection. Conversely, neutrophil numbers in the bone marrow decreased following MPLA. Moreover, MPLA induces G-CSF, a cytokine necessary for the mobilization and expansion of neutrophils in mice. Neutralization of G-CSF prior to MPLA administration ablated MPLA-mediated survival and bacterial clearance in burn wound-infected mice (Fig 1). G-CSF was required for MPLA-induced mobilization of neutrophils out of the bone marrow and into the blood. G-CSF neutralization reversed MPLA-induced attenuation of plasma IL-6 levels in response to infection. This evidence suggests that G-CSF facilitates MPLA-induced mobilization of neutrophils from the bone marrow into systemic circulation, allowing for a more rapid response to infection.

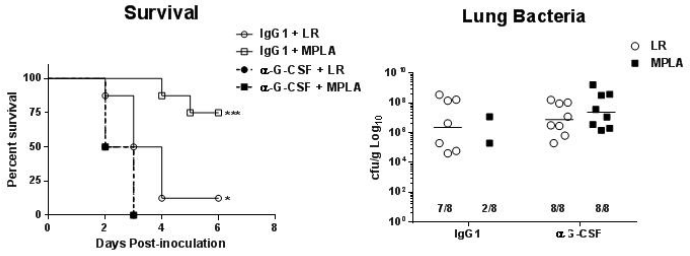

Fig 1. MPLA-induced survival and control of systemic bacterial dissemination in a burn wound infection model is ablated by G-CSF neutralization. Burned mice were treated with anti-G-CSF or IgG1 control 1 hour prior to MPLA administration (day 2 post-burn). Wounds were inoculated with P. aeruginosa on day 4 post-burn. Survival and bacterial dissemination to the lungs after wound inoculation was monitored.

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A-Cubebenoate Isolated from Schisandra Chinesis Shows Therapeutic Effects against Polymicrobial Sepsis

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Approximately 800,000 cases of sepsis are estimated to occur annually in the USA with a mortality rate of about 27% and estimated cost of over $17 billion per year to treat. In this study, we investigated the therapeutic effect of α-cubebenoate, a novel compound isolated from *Schisandra chinesis* against polymicrobial sepsis in a cecal ligation and puncture (CLP) experimental model. Administration of α-cubebenoate strongly enhanced survival in the CLP model. α-cubebenoate administration also markedly blocked CLP-induced lung inflammation and increased bactericidal activity by enhancing phagocytic activity and hydrogen peroxide generation in neutrophils. Expression of two important inflammatory cytokines, IL-1β and IL-6, was strongly increased in the CLP model, and this was dramatically blocked by α-cubebenoate. Lymphocyte apoptosis and caspase-3 activation, which are associated with immune paralysis during sepsis, were markedly attenuated by α-cubebenoate. Taken together, our findings indicate that α-cubebenoate, a natural compound isolated from *Schisandra chinesis*, is a powerful potential anti-septic agent.

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Interaction of the Lipopolysaccharide-Binding Protein (LBP) with Host Cell Membranes: Specificity and Biological Implications

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Lipopolysaccharide-binding protein (LBP) plays a key role in the innate immune response to Gram-negative bacterial infections, based on its dual modulatory effect on Lipopolysaccharide (LPS)-mediated cell stimulation: Low serum levels of LBP sensitize immune cell responses to minute amounts of LPS by enhancing the transport of LPS from aggregates to the cellular receptors CD14 and TLR4/MD-2. High serum levels of LBP promote LPS clearance by transferring LPS from aggregates to serum lipoproteins. These biological functions depend on the transport activity of LBP in serum.
Besides binding to its main ligand LPS, LBP has been demonstrated to interact also with phospholipids. We could show that LBP can mediate a transport of phospholipids into liposomes and in addition, is able to intercalate itself into liposomal membranes. These observations suggest a potential interaction of LBP with biological membranes. However, membrane interaction of LBP on host cells has not been addressed so far.
Employing model membranes resembling the eukaryotic cytoplasmic membrane we have analysed LBP membrane interaction by FRET-spectroscopy, IR-spectroscopy and flow cytometry (FACS). To elucidate the biological significance of LBP cell interaction, we have investigated the interaction of LBP with human macrophages, the primary innate immune cells regulated by LBP. We have analyzed the requirements for LBP binding to the host cell membrane with respect to ligand requirement and the involvement of cellular receptor proteins. Data from flow cytometry experiments and confocal microscopy (CLSM) demonstrate that LBP binding to host cell membranes occurs independent of the LPS-receptor complex and is localized to distinct domains in the cytoplasmic membrane. We have further investigated the effects of LPS on the localization of LBP in the cytoplasmic membrane.
Our data provide the basis for an understanding of the mechanisms of interaction of LBP with host cell membranes and contribute to a deeper understanding of the versatile functions of this central immune regulator.

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Identification of Novel Formyl Peptide Receptor 1 Antagonists

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Formyl peptide receptors (FPRs) are G protein-coupled receptors (GPCRs) expressed on a variety of cell types. Because FPRs play an important role in the regulation of inflammatory reactions implicated in the pathogenesis of various diseases, their antagonists may represent novel therapeutics for modulating host defense and innate immunity. Previously, 4*H*-chromen-4-ones were reported to be potent and competitive FPR1 antagonists. In the present studies, 96 additional 4*H*-chromen-4-one analogs, including related synthetic and natural isoflavones were evaluated for FPR1 antagonist activity. Nine compounds were found to be novel competitive FPR1 antagonists with ability to inhibit fMLF-induced intracellular calciummobilization in FPR1-HL60 cells and effectively displace the fluorescent ligand WKYMVm-FITC from FPR1 in FPR1-HL60 and FPR1-RBL cells. These chromenes inhibited calciumflux and chemotaxis in human neutrophils with nanomolar-micromolar IC50 values. Compound 10 (6-hexyl-2-methyl-3-(1-methyl-1*H*-benzimidazol-2-yl)-4-oxo-4*H*-chromen-7-yl acetate) was found to be the most potent FPR1-specific antagonist, with binding affinity Ki~100 nM. The competitive FPR1 antagonists were specific for FPR1 and did not inhibit WKYMVM/WKYMVm-induced intracellular calciummobilization in HL-60 cells transfected with human FPR2 or RBL cells transfected with murine Fpr1. Moreover, pharmacophore modeling showed that the active 4*H*-chromen-4-ones had a significantly higher degree of similarity with the pharmacophore template as compared to the inactive analogs. Thus, the chromene/isoflavone scaffold represents a relevant backbone for development of novel FPR1 antagonists. Supported byan Institutional Development Award (IDeA) Center of Biomedical Research Excellence grant GM103500.

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Trem-Like Transcript 2 Is Stored in Human Neutrophil Primary Granules and is Upregulated in Response to Inflammatory Mediators

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The Triggering Receptor Expressed on Myeloid (TREM) locus encodes a family of receptors that are emerging as an important class of molecules involved in modulating the innate immune response and inflammation. Of the four conserved members, including TREM-1, TREM-2, TREM-Like Transcript 1 (TLT1) and TLT2, relatively little is known about TLT2 expression and function, particularly in humans. In this study, experiments were performed to determine if TLT2 expression is conserved between mouse and human, demonstrating that human TLT2 is expressed on cells of the lymphoid as well as myeloid/granuloid lineages, similar to murine TLT2. Consistent with studies in the mouse, TLT2 expression is upregulated in response to inflammatory mediators on human neutrophils. Importantly, it was shown that TLT2 in resting human neutrophils is predominantly localized to intracellular vesicles, including secretory vesicles and primary granules; with the majority of TLT2 being stored in primary granules. In contrast to other primary granule proteins TLT2 is not expelled on neutrophil extracellular traps but is retained in the plasma membrane following primary granule exocytosis. In summary, these findings establish that TLT2 expression is conserved between species, and is likely to be important in regulating neutrophil anti-microbial function following primary granule exocytosis.

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Small RNAs Regulate Immunity and Inflammation during Bacterial Infection

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MicroRNAs (miRNAs) have been implicated in a spectrum of physiological and pathological conditions, including immune responses. miR-302b has been implicated in stem cell differentiation and cell proliferation; however its role in infection and immunity remains to be defined. In this study, we showed that miR-302b was induced by TLR2 and TLR4 through ERK-p38- NF-?B signaling upon Gram-negative bacterium Pseudomonas aeruginosa infection. Suppression of inflammatory responses to bacterial infection is mediated by targeting IRAK4, a protein required for the activation and nuclear translocation of NF-?B. Through negative feedback, enforced expression of miR-302b in mice with its “mimics” (or IRAK4 siRNA silencing) inhibited downstream NF-?B signaling and airway leukocyte infiltration, thereby alleviating lung injury and increasing survival in P. aeruginosa-

infected mice. Similarly, IRAK4 siRNA silencing blocked airway leukocyte infiltration and inflammatory cytokine production, thereby increasing survival after infection in mice. On the other hand, miR-302b inhibitors exacerbated inflammatory responses and decreased survival in both P. aeruginosa-infected mice and lung epithelial and macrophage cells. Collectively, our findings revealed that miR-302b is a novel inflammatory regulator of NF-?B activation in respiratory bacterial infections through negative feedback to TLRs-mediated immunity.

Keywords: MicroRNA-302b, P. aeruginosa, K. pneumonia, PAK, TLR4, TLR2, IRAK4, NF-?B
This project was supported by the Flight Attendant Medical Research Institute (FAMRI, Grant #103007), NIH AI101973-01 and AI097532-01A1 to MW.

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Nanoscopic Cell Wall Architecture of an Immunogenic Ligand in Candida Albicans during Antifungal Drug Treatment

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Candida albicans is a human commensal and opportunistic pathogen, and switching between yeast and hyphal forms is thought to be crucial to pathogenesis. β-glucans are a component of cell wall and presented as patches. Dectin-1, a C-type lectin pattern recognition receptor, binds soluble β–glucan as well as purified particulate β–glucan, but can only be activated by particulate ligand. However, the mechanism of this discrimination is unknown. We used direct Stochastic Optical Reconstruction Microscopy (dSTORM) to study the nanostructure of β-glucan exposed on the cell wall before and after a clinically used antifungal drug, caspofungin. β-glucans were labeled with recombinant human Dectin-1 conjugated with Alexa Fluor 647. Getis-Ord analysis was used to quantify β-glucan nanostructure in super resolution localization datasets. Mann-Whitney statistical analysis indicated no significant differences of nanodomain diameter and intradomain β–glucan distance between caspofungin treated and untreated yeast and hyphae. The median nanodomain diameters were ~120 nm and the inter-cluster distances were ~60 nm. Caspofungin treatment increased the β-glucan labeling in confocal microscopy but super resolution datasets analysis indicated no significant change in individual exposure size or separation distance in all conditions. We hypothesize that an increase in ligand density within exposed β-glucan patches may explain the changes seen in confocal microscopy. It is possible that C. albicans avoids the detection of dendritic cells by changing the accessibility of β-glucan to Dectin-1 instead of changing the physical presentation of glucan patches.

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Toll-Like Receptor 10 Is a Negative Regulator of MyD88-Dependent and Independent TLR Signaling

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Toll-like receptors (TLRs) are central components of the innate immune system which provide host defense against infectious pathogens. Following direct recognition of bacterial, fungal or viral components, TLRs activate intracellular signals that lead to inflammatory responses. Among the ten-member human TLR family, TLR10 is the only remaining orphan receptor without a known ligand or signaling function. Murine TLR10 is a disrupted pseudogene, a situation that precludes investigation using a classic gene knock-out approach. We report here that TLR10 suppresses the production of an array of cytokines in stably transfected myelomonocytic U937 cells in response to microbial agonists for TLRs 2, 3 and 4. This broad TLR suppressive activity affects both MyD88 and TRIF-mediated signaling pathways upstream of IκB and MAPK activation. To further explore TLR10 function, we generated transgenic mice expressing TLR10. Compared to non-transgenic littermate controls, whole blood from TLR10 transgenic mouse exhibits blunted IL-6 production when challenged with TLR2, 3 and 4 agonists. Intracellular staining revealed peripheral blood monocytes to be a major target for TLR10 mediated IL-6 suppression. Finally, intraperitoneal injection of a high dose of LPS resulted in significantly lower serum IL-6

levels from TLR10 transgenic mice compared to non-transgenic mice, but did not affect mouse survival in an LPS-induced septic shock model. These results demonstrate for the first time that TLR10 functions as a negative regulator of TLR signaling.

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High Dose LPS Inhibits PMN Recruitment and Leads to Strong Symptoms of Endotoxemia While Low Dose LPS Induces PMN Recruitment and Bacterial Clearance.

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Previous work showed that injection of normal mice with large quantities of LPS (10 μg/mouse, a model of endotoxemia) results in an inhibition of PMN recruitment to the site of injection; inhibition of recruitment was accompanied by a strong proinflammatory cytokine and chemokine response in the blood, decreased CXCR2 receptor on blood neutrophils and increased shedding of PMN L-selectin, all factors that favor inhibition of PMN migration through the endothelium. To further explore the effects of LPS on PMN recruitment, mice were injected with lower amounts of LPS. A reduction to 200 ng/mouse showed little or no differences when compared to the 10 μg dose; a dose of 20 ng LPS/mouse resulted in a decreased systemic response, and a limited recruitment of PMN. However, i.p. injection of a dose of LPS 5000-fold lower than the original dose, 2 ng/mouse, had the opposite effect; it induced chemokine production at the site of injection without inducing a strong systemic response and resulted in strong PMN recruitment and little effect on CXCR2 expression or L-selectin shedding. We have further found that other factors that blunt the systemic response also result in PMN recruitment; these include (1) the absence of the LPS receptor, CD14 and (2) truncation of LPS to its smaller derivatives like Lipid A and MPLA. Furthermore, injection of low dose LPS (2 ng/mouse) or MPLA was protective in a model of severe *E. coli* mediated sepsis as the rapid PMN recruitment resulted in enhanced bacterial clearance and improved survival. These results suggest a therapeutic effect of these agents in severe infection.

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HIV-1 Transgenic Rats Display Immunophenotypic and Cellular Response Alterations Associated with Aging

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Advances in antiretroviral therapy over the last two decades have allowed life expectancy in humans infected with HIV to approximate those of the general population. The process of aging in mammalian species including rats, results in changes in immune response, alterations in immunological phenotypes and ultimately increased susceptibility to infectious diseases. In order to study the effect of age to the immune cells responses in HIV-1 infected individuals, a transgenic rat model was utilized which possessed similar human viral genes, including the HIV-1 provirus along with the deletion of gag and pol. This HIV type 1 (HIV-1Tg) transgenic rat exhibits similar clinical manifestations to human HIV including wasting, skin lesions, cataracts, neurological and respiratory impairment. The HIV-1 rat was therefore chosen as an ideal small animal model for studying the immunological pathologies associated with chronic HIV-1 disease particularly in aged individuals. In this study, peripheral whole blood and splenocytes from young adult, adult and aged wild-type F344 and HIV-1Tg/F344 rats were analyzed using flow cytometry for changes in lymphocyte, granulocyte and monocyte cell populations. Using an endotoxic tolerance model, immune cell responses were assessed for changes in immunophenotypes and cytokine/chemokine release related to age and genotype in each animal group. Our data indicates that the immunophenotype and immune responses can change during aging in HIV-positive individuals. This information may be effective in determining novel therapeutic treatments for HIV patients dependent on individual's age.

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Immune Response of Laying Hens with Thermally Cooled Perches during a Mild Heat Stress

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Heat stress (HS) is a common immune modulator across many species. The objective of this study was to determine if the immune response of hens with cooled perches during the summer months (June through

September of 2013) improved. White Leghorn pullets (n = 162), 16 wk of age, were randomly assigned to 1 of 3 banks of 6 conventional cages each. Each bank was assigned to 1 of 3 treatments from 16 to 32 wk of age: 1) cages with 2 metal circular perches that were cooled with circulating water, approximately 12^{o} C, when temperature within the cage exceeded 25^{o} C (CP), 2) cages with ambient air perches (AP), and 3) cages without perches (NP). Hens were subjected to ambient temperatures throughout the 16 wk experimental period with an additional 4 h of acute HS at about 34.6^{o} C at 27 wk of age. The heterophil to lymphocyte (H/L) ratio and plasma total immunoglobulin G (IgG) concentrations were determined after the acute HS and upon the completion of the study. Interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS), and toll-like receptor 4 (TLR-4) mRNA were examined in the spleen at 32 wk of age. Fixed effects of perch in a randomized design were tested using a PROC MIXED model with SAS 9.2 software. Compared to AP and NP hens, CP hens had a lower H/L ratio ($P < 0.01$) at 27 wk of age. At 32 wk of age, the H/L ratio of CP hens was still significantly lower compared to NP hens ($P < 0.05$). There was no difference in H/L ratio between the AP and NP at both 27 and 32 wk of age ($P > 0.05$, respectively). Plasma total IgG concentrations at 27 wk were not affected by treatments ($P > 0.05$). At 32 wk of age, plasma total IgG concentrations were lower in CP and AP hens compared to NP hens ($P < 0.05$). There were no treatment effects on spleen weight, and the expression of IL-1β, IL-6, TNF-α, iNOS, and TLR-4 mRNA at 32 wk of age ($P > 0.05$, respectively). In conclusion, hen immunity may be improved by using thermally cooled perches during a mild summer after 4 h of acute HS. With the current climate changing, further evaluation is needed to determine the effectiveness of thermally cooled perches on the immune response under more stressful temperatures than what was experienced in the current study.

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Immune Maturity Plays a Significant Role in the Immune Response to CNS Catheter Infection with S. Epidermidis

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Infants are known to be higher risk of ventriculoperitoneal shunt infection, particularly with *S.epidermidis*. As *S. epidermidis* has relatively limited virulence, we hypothesized that this increased infection risk is due to alterations in the immature response that allow for infection.

To evaluate this hypothesis, we adapted our previously published CNS catheter infection model to generate infection with *S. epidermidis* in 14 day old C57BL/6 mouse pups (equating human infants). The pups tolerate the procedure well, with no observed mortality. However, infected pups do experience greater weight loss and worsened clinical illness scores than observed in pups implanted with sterile catheter. In comparison with previously infected adults, the pups have a significantly longer time period of weight loss before they return to normal weight gain patterns. Interestingly, in the immature pups, the infection was more likely to spread from the catheter to the brain parenchyma than we have observed in older mice. Additionally, the younger pups had significantly lower levels of pro-inflammatory mediators CXCL1, CXCL2 and IL6, all of which play a role in recruiting immune cells to the site of infection. As expected, we found a decrease in the number of macrophages and neutrophils recruited to the brain tissue surrounding the catheter in pups as compared to adult mice by flow cytometric analysis. This novel finding could have clinical significance, as the presence of neutrophils is frequently used in children as a marker to determine the presence or absence of infection. There was also a small increase in anti-inflammatory cytokine IL10 noted in the pups implanted with infected catheters on multiplex cytokine analysis and qRT-PCR. Collectively, these findings demonstrate an attenuated inflammatory response in the pups, distinct from that observed in older mice, which may explain the increased parenchymal spread of infection. On-going studies include behavioral studies in the mice to determine long term outcomes of infection on cognitive and motor skills, as these infections in human beings have been associated with IQ loss and other neurologic sequelae. Mice evaluated 2 weeks post-infection, when evidence of illness has resolved and bacterial infection has cleared, have been noted to have abnormal nest building behavior but normal Rotarod performance, suggesting a cognitive but not motor defect that persists following resolution of infection. Better understanding of the immune mechanisms that place infants at higher risk of these infections can be used to guide future screening and adjunctive therapies.

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Dynamic Modulation of Innate Immune Response by Varying Dosages of LPS in Human Monocytic Cells

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Innate monocytes and macrophages can be dynamically programmed into distinct states depending upon the strength of external stimuli. Innate programming may bear significant relevance to the pathogenesis and resolution of human inflammatory diseases. However, systems analyses with regard to the dynamic programming of innate leukocytes are lacking.In this study, we focused on the dynamic responses of human promonocytic THP-1 cells to lipopolysaccharide (LPS). We observed that varying dosages of LPS differentially modulate the expression of selected pro- and anti-inflammatory mediators such as IL-6 and IL-33. Super-low dosages of LPS preferentially induced the pro-inflammatory mediator IL-6, while higher dosages of LPS induced both IL-6 and IL-33. Mechanistically, we demonstrated that super-low and high doses of LPS cause differential activation of GSK3 and Akt, as well as the transcription factors FoxO1 and CREB. Inhibition of GSK3 enabled THP-1 cells to express IL-33 when challenged with super-low dose LPS. On the other hand, activation of CREB with adenosine suppressed IL-6 expression. Taken together, our study reveals a dynamic modulation of monocytic cells in response to varying dosages of endotoxin, and may shed light on our understanding of the dynamic balance that controls pathogenesis and resolution of inflammatory diseases.

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Lymphocyte Response Assay (LRA) and Flow Cytometry Correlation: Clinical Implications

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Objective: Autoimmunity can be defined as loss of host tolerance when more foreign ‘antigens’ present than the innate immune system can recycle and neutralize. A lymphocyte response assay (LRA) is reported that detects specific immune responses and correlates clinically with immune tolerance and intolerance in outcome studies previously reported. LRA is compared to flow cytometry as part of the LRA method validation. Confirming toxic from immunotoxic reactions requires technology such as cell culture and high sensitivity analytics such as flow cytometry reported here that distinguish linear toxicological from amplified immune responses.

Methods: A blood drawing system is used that keeps lymphocytes in whole blood under conditions where cell metabolism is reduced during transport so that ex vivo cell culture can be performed up to three days after phlebotomy. A one-step centrifuge protocol is reported that yields lymphocyte and autologous cell rich plasma used as the LRA incubation media. Incubation is performed in a novel microtiter plate. A negative and positive control is included with each sample. Test antigens are pre-coated onto the surface of specific wells in the microtiter plate. Cells of matched split samples are studied independently by flow cytometry. This LRA can test up to 500 foods, preservatives, medications, molds, toxic minerals, and persisting organic pollutants can be tested on a single 28 ml (one ounce) whole blood sample.

Results: LRA by ELISA/ACT provides an advanced laboratory tool to address the growing clinical need to identify the full repertoire of antigens or substances that may disrupt immune tolerance, provoke delayed hypersensitivity, inflammation and autoimmunity. When split samples are compared (N=100), the LRA assay reproduces a variation less than three per cent (<3%). Lymphocyte activation based on optical microscopy correlates well with flow cytometry using such lymphocyte activation biomarkers as CD107 and CD69 of T and/or B cells.

Conclusion: LRA by ELISA/ACT is an ex vivo cell culture that provides patient specific information about which of up to 500 substances an individual is tolerant to and which show specific T and/or B cell lymphocyte reactivity. This LRA has been performed on over 50,000 people representing over 15,000,000 LRA assays during development. Follow up tests every six months suggest that tolerance can renew when homeostasis is restored in neuroimmunohormonal, digestive, and detoxification control systems. An optional interpretation includes sources of possible exposure and how to substitute for reactive substances. Nutritional, attitudinal and physical activities that evoke human healing responses are included. This assay provides first line diagnostic and therapeutic monitoring of all inflammatory and autoimmune conditions. This LRA is applicable to any

condition where tolerance is lost and delayed hypersensitivity is induced. The assay is cost effective and useful as a primary prevention predictive biomarker as well as a diagnostic and therapeutic outcome monitoring system.

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Abnormal Activation of NK Cells in TTP Patients with Relapse Development.

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Thrombotic thrombocytopenic purpura (TTP) is a rare life-threaten vascular autoimmune disease. There is no effective method to treat it in clinical trials since pathogenesis of TTP has not been fully elucidated. Here we investigate the role of NK cells in relapse development of TTP. Our results showed that the frequencies of CD3-CD56dimCD16- and CD3-CD56briCD16- NK cells are increased in TTP patients with a history of relapse. Expression of CD107a, granzyme A and IFN-γ by CD3-CD56dim NK cells following in vitro stimulation with PMA/ionomycin / monensin is improved in the relapse group, compared with those on NK cells derived from TTP patients without relapse development. Measurement of NK cell surface markers without in vitro stimulation showed more expression levels of LIR-1 and CD52 on the surface of CD3-CD56dim NK cells, but expression of CD16 is down-regulated on NK cells isolated from TTP patient with relapse development. NK cells isolated from TTP patients with a history of relapse indicated stronger cytotoxicity to target K562 cells than those of NK cells derived from TTP patients without relapse development, suggesting prior activation of NK cells in vivo. Treatment with anti-human CD16 antibody up-regulates cytotoxicity of NK cells derived from TTP patients without relapse development through facilitation of CD107a–mediated degranulation. However, Anti-human CD16 antibody treatment does not affect cytotoxicity and CD107a-mediated degranulation of NK cells isolated from TTP patients with a history of relapse, suggesting inability of CD16-mediated signaling in NK cells derived from TTP patients with relapse development. These data provide evidence of altered NK cell activation and/or licensing in TTP patients with a history of relapse modulated by CD16-mediated signaling and a new avenue of investigation into mechanisms of TTP immunopathogenesis.

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Novel Targeting Methods for Pancreatic Cancer

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Pancreatic cancer is the fourth leading cause of cancer deaths in the United States. Despite advances in adjuvant chemotherapy, 5-year survival following surgical resection is only 23% in specialized pancreatic surgery centers. Current systemic chemotherapies for pancreatic cancer are inadequate due to the inability to differentiate cancer cells from healthy tissue. An additional challenge facing pancreatic cancer treatment is the variable patient response from targeted therapies, which emphasizes the need for personalized cancer therapy. Preclinical studies, which are the foundations for clinical trials, rely on studies with established or primary pancreatic cancer cells in vitro and in vivo. Cells in culture do not mimic the physiologic cell in vivo, and pancreatic cancer cells alone in vivo do not reproduce pancreatic cancer and the supporting tumor microenvironment. Therefore, it is not surprising that no targeted therapy to date has been successful in the treatment of pancreatic cancer due to the fact that targets are derived from immortalized pancreatic cancer cell lines.

We have characterized novel, high affinity molecular targeting molecules unique to an individual pancreatic cancer using a high throughput system. Specifically, we utilized fresh surgical specimens incorporating the entire tumor microenvironment along with phage display to develop novel small chain variable fragments (scFv) that are reactive to an individual pancreatic neoplasm, but unreactive to healthy pancreatic tissues. Here we present the characterization of scFv identified using four individual pancreatic neoplasms. We show by ELISA and BioLayer Interferometry that our scFv bind with high specificity to the pancreatic neoplasms from which they were derived, while remaining unreactive to healthy pancreatic tissue. Thus, we present a proof of principle for the development of high affinity molecular targeting molecules unique to individual pancreatic cancer microenvironments using a high throughput system which would easily be translated into clinical cancer care. Future studies will assess the ability of these scFv to home to the original neoplasm using our established patient-derived tumor xenograft models whereby the human tumor and its associated microenvironment is grown in NOD/SCID mice. Together these data will determine whether screening single sets of cancerous

and uninvolved pancreatic tissues using phage display can be used to develop scFv as personalized targeting molecules.

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Pooled Immunoglobulin Induced Regulatory Macrophages Reduce Dextran Sodium Sulfate Induced Intestinal Inflammation

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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 but up to 40% of people are, or will become, refractory to current therapies. Macrophages are key mediators of inflammation initiating the innate immune response. However, macrophages can be skewed to a regulatory phenotype (Mreg) that plays an equally important role in turning off the inflammatory response. High doses of pooled immunoglobulin are used to treat autoimmune diseases and may work, in part, by skewing macrophages to a regulatory phenotype.

My **overarching hypothesis** is that Mregs can be used to reduce intestinal inflammation, like that which characterizes IBD. To address this hypothesis, I propose two specific aims:
Aim 1. To assess the anti-inflammatory properties of Mregs primed with pooled immunoglobulin *in vitro*
Aim 2. To determine whether Mregs can reduce intestinal inflammation *in vivo*.

Methods: Macrophages were derived from mouse bone marrow aspirates. Macrophages were primed to create an Mreg phenotype with high dose pooled immunoglobulin. Inflammatory responses of Mregs were measured *in vitro* and the potential of Mregs to block inflammatory responses was assessed *in vivo* using the dextran sodium sulfate (DSS)-induced mouse model of intestinal inflammation.

Results: Mregs stimulated with high doses of pooled immunoglobulin produce low levels of pro-inflammatory IL-12/23p40 and high levels of anti-inflammatory IL-10 in response to inflammatory stimuli. Interestingly, pooled immunoglobulin reduced IL-12/23p40 in response to TLR agonists 1-9, but only increased IL-10 when stimulated with LPS, a TLR 4 agonist. Importantly, adoptive transfer of *in vitro*-derived Mregs reduces inflammation and clinical disease activity in mice during DSS-induced colitis.

Conclusions: These studies demonstrate that Mregs have potent anti-inflammatory activity that can be used to reduce intestinal inflammation *in vivo*. Skewing macrophages to an Mreg phenotype *in vivo* or adoptive transfer of *in vitro*-derived Mregs may provide a novel immunotherapeutic strategy to treat intestinal inflammation. In future studies, we will investigate the mechanism(s) by which macrophages change their phenotype to become Mregs to determine whether macrophages can be skewed *in situ* to dampen down inflammation in people with IBD.

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Comparison of Structure to Activity Relationships in LPS Isolated from Two Strains of Desulfovibrio Desulfuricans Showing Microdiversity in a Healthy Human Gut

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The role of gut Microbiota in obesity was shown threw the effect of Gram negative increased population in obese patients [1]. More than 10% of the world population is obese and this is resulting in at least 100 millions of type 2 Diabetes cases [2] . Structural disruption of gut microbiota and associated inflammation are considered important etiological factors in high fat diet (HFD)-induced metabolic syndrome (MS) [3, 4].
Recent studies have highlighted that the LPS of Gut Microbiota could contribute to the inflammation process leading to obesity and type 2 diabetes [5]. Same studies showed that the Sulphate-reducing bacteria (SRB) were enriched in high fat diet-fed obese mice and some diabetic humans [6, 7], but their LPS structure had not been fully characterized. This motivated our search for

the detailed lipid A chemical structures related to inflammatory properties.
We isolated 66 isolates of a healthy human gut. Nine strains were characterized by ERIC-PCR, to belong to Desulfovibrio desulfuricans a strict Gram-negative anaerobe. The LPS molecular species of two different SRB isolates were analyzed after complex isolating procedures, and their lipid A moietes characterized. They were found to be similar to relatively common structures but differed in the number of fatty acids and subsequently in their endotoxic activities. The present work confirms and complete previously published data concerning the lipid A chemical composition and gives new insights on the published genes involved in Desulfovibrio lipid A biosynthesis [8].

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Modulation of Microrna Expression by H1N1 Infection in Macaques

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MicroRNAs are small noncoding RNAs that control gene expression by binding to target mRNAs, thereby inhibiting their translation. MicroRNAs play an important role in the regulation of the immune system, including the development and differentiation of B and T cells, proliferation of innate immune cells, and the release of inflammatory mediators. However, the contributions of microRNAs in coordinating an immune response to infection remains poorly understood. Therefore, we studied the changes in microRNA expression in broncho-alveolar lavage (BAL) cells and PBMCs following infection with the 2009 H1N1 virus A/Mexico/4108/2009 in rhesus macaques. Our analysis revealed that MEX4108 replicated efficiently in both the upper and lower respiratory tract. The host immune response was characterized by: 1) an increase in plasmacytoid dendritic cells and IFNα in BAL fluid that correlated with peak viral loads; 2) robust production of several inflammatory cytokines in the BAL; 3) strong T and B cell proliferation especially in the BAL. Overall, the immune response was more robust in the BAL compared to peripheral blood. Our characterization of microRNA gene expression in the BAL showed a downregulation of let-7f, miR-129, and miR-34c as early as 4 dpi and an upregulation of miR-18b and miR-146b at 14 dpi. A database search of validated targets suggest these changes in expression may be associated with 1) an increase in cell proliferation (let-7f targets G-protein RAS and miR-34c targets c-myc); 2) increased production of inflammatory cytokines (let-7f targets IL-6 and IL-10); 3) negative feedback of inflammation (miR-146b downregulates NFκB by targeting IRAK1 and TRAF6); 4) B cell development (miR-18b targets proapoptotic Bim and PTEN and miR-129 targets SOX4). In peripheral blood, we detected a downregulation of miR-132 at 7 dpi and an upregulation of miR-138, miR-192, miR-193b, and miR-451 at 28

dpi. A database search of validated targets suggest these changes may be correlated with an increase in inflammation by activation of NFκB (miR-132 targets IRAK4). In contrast, upregulation of microRNAs late in infection may be associated with the attenuation of inflammation (miR-192 targets MIP-2α and miR-451 targets MIF) and cessation of lymphocyte proliferation (miR-138 targets cyclin D3, miR-192 targets DHFR, and miR-193b targets Mcl-1 and Cyclin D1). Overall, our data provides intriguing new insights in the mechanisms underlying the development of innate and adaptive immune responses following viral infection.

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Single Chain Antibody Fragments with Serine Protease Inhibitory Property Capable of Neutralizing Toxicity of Trimeresurus Mucrosquamatus Venom

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Trimeresurus mucrosquamatus (TM) is one of majorities of snake envenomation with necrotic and hemorrhagic toxin in Taiwan. In this study, chickens were used as an alternative animal model for immunization with TM venom. Using phage display technology to process four rounds of panning, selected single chain variable fragments (scFv) could specifically recognize TM venom proteins, which were later identified as a group of homogeneous venom serine protease. The specific scFv antibodies showed various inhibitory effects on sheep RBC lysis induced by TM venom using an indirect hemolytic assay in vitro. In addition, the survival times of mice were extended to certain degrees when treated with these scFv antibodies individually or in a combination. To elucidate the inhibitory mechanism, we used molecular modeling to build up the serine protease structure to simulate the possible interactions with scFv antibodies. The results suggested that the CDR-loop of the scFv antibodies (3S10 or 4S1) might bind at the 99-loop of venom serine protease so as to affect substrate access due to the partial collapse of the subsite S2 and the partial movement of the subsite S4. It is hoped these chicken-derived antibodies could be applied to develop diagnostic and therapeutic agents against snakebites.

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Toll-Like Receptor Agonists as Potential Adjuvants - in Vitro Studies on Neonatal Dendritic Cells

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Introduction: The susceptibility to infectious diseases of term and especially preterm neonates results partially from their immunological immaturity – functional immunodeficiency of the neonate - of innate and adaptive immunity mechanism. Moreover, immunological immaturity of newborns impair neonatal vaccination responses. Neonatal dendritic cells being important players of an effective vaccine response are less effective to induce an adaptive immune response and to promote the development of immunological memory. Current infant vaccines require several doses to elicit an efficient antimicrobial protection when administered before the sixth month of life. New vaccine adjuvants targeting TLR have been developed to augment vaccine responses. Efficient adjuvantal TLR-triggering may overcome the neonatal immunological impairment and thus render new approaches of early life protection against infection. Accordingly, the aim of this study was to investigate the immunostimulatory action of Alum (TLR- independent adjuvants), R- 848 (TLR-7/8 agonist) and CpG-B (TLR9- agonist) on antigen

presenting cells, here dendritic cells, of preterm (n=10) and term neonates (n=9) in comparison to healthy adults (n=10).

Methods: Activation and maturation markers of dendritic cells were evaluated after incubation of CBMCs/PBMCs with the indicated adjuvants for 8 hours and were analyzed using flow cytometry. Furthermore, the secretion of IL-2, IL-4, IL-6, IL10, TNFα und IL-17 upon adjuvants stimulation was evaluated using cytometric bead arrays.

Results: Stimulation with R-848 and CpG-B resulted in a significant induction of CD40, CD80, CD86, CCR7 and HLA-DR on neonatal plasmacytoid dendritic cells. TLR-specific stimulation of neonatal myeloid dendritic cells resulted in significant induction of CD40 and CCR7. In comparison to TLR- specific adjuvants, the TLR- independent adjuvants Alum had no direct influence on dendritic cells upon incubation. Stimulation with R-848 resulted in significant higher secretion of TNFα, IL-6, IL-10 and IFN-γ. Interestingly, CpG-B resulted in significant higher secretion of TNF and IL-6 but not IL-10.

Conclusion: In summary, the incubation of both R-848 and CpG-B induced activation and maturation of neonatal dendritic cells. These data show that modern TLR-specific adjuvants achieve a direct effect and a more potent upregulation of activation and maturation markers compared to the conventional adjuvants Alum- especially in preterm delivered neonates. Both, R-848 and CpG-B stimulated the secretion of proinflammatory cytokines among all age groups. Interestingly, R-848 exhibited also features of possible immunoregulatory function by the secretion of IL-10. We thus conclude that agents triggering TLR might possibly overcome neonatal lack of vaccine responses.

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Immune Complexes Inhibit Th17 Responses via Suppression of IL-1a and Enhancement of IL-10 Produced by Dendritic Cells

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The precise context in which the innate immune system is activated plays a pivotal role in the subsequent instruction of $CD4^+$ T helper (Th) cell responses. Th1 responses are downregulated when antigen is encountered in the presence of antigen-IgG immune complexes. To assess if Th17 responses to antigen are subject to similar influences in the presence of immune complexes we utilized an inflammatory airway disease model in which immunization of mice with Complete Freund's Adjuvant (CFA) and ovalbumin (Ova) induces a powerful Ova-specific Th1 and Th17 response. Here we show that modification of that immunization with CFA to include IgG-Ova immune complexes results in the suppression of CFA-induced Th17 responses and a concurrent enhancement of Ova-specific Th2 responses. Furthermore, we show the mechanism by which these immune complexes suppress Th17 responses is through the suppression of dendritic cell IL-1 alpha production and the enhancement of IL-10 production. Together these data represent a novel mechanism by which the generation of Th17 responses is regulated.

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Propolis Reduces Expression of Cell Surface CD14 in Phagocytic Cells in Chickens during Heat Stress

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Propolis is a resin produced by honeybees (Extra Green Propolis, Apis Flora Co., São Paulo, Brazil) that has been implicated in induction of H_2O_2 generation by peritoneal MO and inhibition of NO, simulation of antibody production, and enhanced natural killer activity. TLR2 and TLR4 and interleukin (IL) 1 and 6 were enhanced in mice treated with propolis. Chickens had enhanced antibody production when fed propolis. The objective of this study was to determine if basic innate immune cell functions and phenotypes were altered by feeding of propolis to broilers for 42 days during a chronic heat stress. Broilers were assigned to a basal diet only or basal diet supplemented with1000 mg propolis/kg of diet. At 42 d-of-age, total spleen leukocytes were separated and labeled with CD18 (indicative of cell activation), CD14 (component of the LPS recognition molecule, expressed on antigen presenting cells), CD3 (t-cell marker), Bu (b-cell marker), and capability to phagocytize and mount a kill response to bioparticles was determined. The cell populations were separated into 3 distinct populations determined by forward scatter (FS) and side scatter (SS) and analyzed for percentage of cells fluorescing and

fluorescence intensity for the total population and each of the 3 cell populations. Propolis decreased CD14 intensity on the high forward and side scatter population (presumed phagocytic cells), without changing the percentage of cells expressing CD14. Propolis increased the percentage of CD3 expressing cells in that population. Additionally, propolis reduced the percentage of Bu expressing cells in all regions. The percentage of cells expressing CD18 were decreased by propolis in the low SS/FS and lows high FS, but were equal in the high SS/FS region cells. These results showed that propolis may act by altering the ability of the cells to process and present antigen and therefore alter important innate immune functions during heat stress.

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Galectin-3 Modulates Microglia Phenotype under in Vitro Inflammatory Conditions

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Background: Inflammation caused by hypoxia-ischemia (HI) or by infection is important in the development of perinatal brain injury. Activated microglia cells may contribute to injury, but may also have neuroprotective properties. Galectin-3 is produced by activated microglial cells and has immuno-regulatory properties that may affect microglia phenotype and subsequent development of injury.
Aim: To study the effect of galectin-3 on *in vitro* microglia phenotype under normal and inflammatory conditions representing both infectious and sterile inflammation.

Methods: Primary microglia cell cultures were obtained from 1-2 d old C57Bl/6 mice. Conditions mimicking infectious or sterile inflammation were established and evaluated using LPS (10ng/mL) and TNF-α (100ng/mL). The response to galectin-3 was tested in doses from 5-100 µg/mL alone or together with LPS or TNF-α. Supernatants were collected 24 h after treatment and analysed for inflammatory mediators using multiplex protein analyses (BioRad©) including pro-inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-12p40, IL-12p70, IL-17, IFN-γ, TNF-α, GM-CSF) mainly anti-inflammatory cytokines (IL-4, IL-5, IL-9 IL-10, IL-13, G-CSF) and chemokines (eotaxin, KC, RANTES, MCP-1, MIP-1α, MIP-1β) as well as ELISA for MCP-1, IGF-1, and MMP-9.

Results: LPS and TNF-α induced pro-inflammatory (9/11 vs 9/10) and anti-inflammatory (6/6 vs 4/6) cytokines as well as chemokines (6/6 vs 5/6) and MMP-9 in an almost identical manner except generally lower amplitude of the TNF-α induced response. Galectin-3 alone had no effect on any of the proteins analysed. Galectin-3 significantly reduced the LPS-induced microglia response for pro-inflammatory and anti-inflammatory cytokines as well as chemokines and the effect seemed partly dose-dependent. Galectin-3 reduced the TNF-α response in a very similar manner to that seen with LPS, but a larger number of proteins including MMP-9 was affected. LPS decreased baseline IGF-1 levels and the levels were restored by Galectin-3. TNF-α did not affect IGF-1 levels alone or together with Galectin-3.

Conclusion: Galectin-3 has no independent effect on microglia, but modulates inflammatory activation by reducing both pro- and anti-inflammatory cytokine as well as chemokine response under inflammatory conditions *in vitro*. The effect is similar under infectious and sterile inflammatory conditions suggesting that galectin-3 regulates inflammation by mechanisms not related to direct binding of LPS or to toll-like receptor activation. Galectin-3 also restores concentrations of the potentially neuroprotective growth factor IGF-1 reduced by LPS-induced inflammation. Galectin-3 may be important in modulating the inflammatory response and the balance between injurious and protective mechanisms during brain inflammation.

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GAPDH Participates in Posttranscriptional Repression of TNF-a in Endotoxin Tolerant Monocytes

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Background: The immune response to endotoxin is marked by distinct immune/metabolic phases. The initial

proinflammatory phase depends on an increase in glycolysis. The later phase, often called endotoxin tolerance, shows both a repressed response to further inflammatory stimuli and a switch to fatty acid oxidation. Recent work in T-cells showed a surprising role for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a metabolism-sensitive repressor of inflammatory cytokine translation. We have found a similar mechanism present in human monocytes following the metabolic shift away from glycolysis during endotoxin tolerance.

Methods: THP-1 human promonocytes were grown in galactose based media, making their metabolism more oxidative and less glycolytic, as measured by Seahorse XF. TNF-α RNA and protein was measured by RT-qPCR and ELISA in glucose vs galactose-fed cells, with or without LPS stimulation. GAPDH association with TNF-α mRNA was measured through RNA Immunoprecipitation. RNA-IP was then repeated in responsive THP-1 cells and cells given a tolerizing dose of LPS for 24 hours.

Results: Despite there being no difference in TNF-α mRNA expression between glucose and galactose-fed cells, galactose fed cells showed reduced production of TNF-α protein. This decrease in production corresponded with an increased association between GAPDH protein and TNF-α mRNA in galactose-fed cells. A similar increase was seen in tolerant versus responsive cells. Increasing glycolysis through use of oligomycin reversed this association, while blocking glycolysis with 2-deoxyglucose (2-DG) increased the association.

Conclusions: Our findings indicate that GAPDH increases its association with TNF-α mRNA to repress its translation in THP-1 monocytes. This repression primarily occurs when the cells are in a non-glycolytic metabolic state, but that this repression is reversible through increasing the rate of glycolysis. This mechanism represents a previously uncharacterized layer of functional tolerance, one which may have significant implications for the treatment of sepsis.

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Enhanced Human Neutrophil Activation upon Challenge with Filifactor Alocis, a Recently Described Oral Pathogen.

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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 innate host response, and the outcome of the interaction between periodontal pathogens and neutrophils is a key determinant of health status. The hypothesis that F. alocis stimulation manipulates human neutrophil chemotaxis, degranulation and cytokine release was tested. Human neutrophils were challenged with F. alocis and their chemotactic activity towards a potent chemoattractant, fMLF, was assessed using a transwell system. Transmigrated neutrophils were stained with HEMA 3 stain set and visualized on a light microscope. Our data shows that neutrophils challenged with F. alocis had enhanced chemotaxis and chemokinesis activity compared to non-infected cells. Supernatants from F. alocis-challenged cells were used in place of a chemoattractant, in order to determine if there was a factor released by F. alocis infected cells that induced the enhanced chemotactic activity. No chemotactic effect was observed when the supernatant from F. alocis infected cells was used as a chemoattractant source. F. alocis challenge of neutrophils induced a significant degranulation of secretory vesicles and specific granules. In order to determine if neutrophil degranulation plays a role in F. alocis- induced chemotaxis, neutrophils were challenged with F. alocis, or pre-treated with TAT-SNAP-23, a peptide known to block neutrophil degranulation, followed by bacterial challenge. Blocking neutrophil degranulation significantly reduced F. alocis-induced chemotaxis and chemokinesis. As p38 MAPK signaling pathways have been implicated in both chemotaxis and degranulation, next we assessed p38 MAPK activation after challenge with F. alocis using immunoblotting techniques. F. alocis stimulation of neutrophils induced p38 MAPK phosphorylation with maximal induction within 15-30 min stimulation. F. alocis-induced secretory vesicles and specific granule exocytosis was significantly reduced when neutrophils were pre-treated with a p38 MAPK pharmacologic inhibitor, SB-203580. Additionally, supernatants were collected after 20 h from unstimulated, LPS-stimulated, or F. alocis stimulated cells, and IL-1b, IL-1ra, TNF-α and IL-8 levels were measured by a multiplex Luminex-based assay. Our data shows that F. alocis challenge induced a significant increase in IL-8 and IL-1ra levels, similar to LPS-stimulation. However, lower levels of IL-1b and TNF-α were observed in F. alocis-challenged cells compared to LPS stimulation. In conclusion, our results show that F. alocis interaction with human neutrophils resulted in enhanced chemotaxis and

chemokinesis activity, a p38 MAPK-dependent degranulation of secretory vesicles and specific granules, and induction of cytokine/chemokine release. These results indicate that F. alocis can manipulate neutrophil functional responses which result in enhancing their activation.

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Toll-Like Receptor 10 Is a B-Cell Intrinsic Suppressor of Adaptive Immune Responses

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Toll-like receptors (TLRs) play a central role in the initiation of adaptive immune responses. While this role is best appreciated in the context of antigen presenting cells, lymphocyte intrinsic TLR engagement also contributes to adaptive immune responses. Several TLR agonists are known B-cell mitogens and B-cell intrinsic TLR activation is known to promote proliferation, antibody production and class switch recombination (CSR). Importantly, despite thousands of publications on TLRs, the function of TLR10 remains unknown. We have found that antibody mediated engagement of TLR10 on the surface of human peripheral blood B lymphocytes suppresses both B cell proliferation and cytokine production. Interestingly, TLR10 was observed to inhibit responses to a variety of B cell stimulatory signals including those from the BCR, CD40 and other TLRs. To overcome the fact that TLR10 is a naturally disrupted pseudogene in mice, we created a transgenic mouse model using a BAC clone encoding human TLR10 in the context of its native promoter. TLR10 transgenic mice express the receptor in lymphoid tissue with clear expression in B lymphocytes but without measurable effects on B cell development. Compared to non-transgenic littermate controls, TLR10 transgenic mice exhibit diminished antibody responses following vaccination with either T-independent or T-dependent antigens with marked effects on CSR. Adoptive transfer of splenic B cells, from either non-transgenic or TLR10 transgenic mice, into B cell deficient uMT mice revealed that the suppressive effects on antigen-specific humoral immune responses are entirely B cell intrinsic. Our results demonstrate that TLR10 has a functional role within the B-cell lineage that is distinct from that of other TLR family members. Our findings suggest that TLR10 may provide a therapeutic target for certain B cell lymphomas and also autoimmune diseases characterized by dysregulated B cell activity.

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Interaction between NLRP3 Inflammasome and PPAR-Gamma in Regulating Macrophage Phenotype during Wound Healing

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The Nod-like receptor protein (NLRP)-3 inflammasome and the peroxisome proliferator-activated receptor (PPAR)-γ each have been proposed to influence macrophage (Mp) phenotype; however, little is known about the interactions between these pathways, especially during tissue repair. In our studies, wound Mp exhibited downregulation of NLRP3 inflammasome activity and upregulation of PPAR-γ activity as healing progressed, associated the switch from pro-inflammatory to pro-healing phenotypes. However, in the setting of diabetes, these counter-regulatory changes were impaired in both mouse and human wounds leading to a sustained pro-inflammatory Mp phenotype. In vitro experiments demonstrated that the diabetic wound environment is sufficient to sustain inflammasome activity, impair PPAR-γ activity and promote a pro-inflammatory Mp phenotype. In addition, inflammasome inhibitors and PPAR-γ agonists promoted a pro-healing Mp phenotype in both cultured mouse and human Mp, and promoted wound healing in diabetic mice, suggesting that targeting these counter-regulatory pathways represents a novel approach for manipulating Mp phenotype and improving healing of chronic wounds.

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Calcium Oxalate Crystal Induced Activation of NLRP3 Inflamamsome in a Rat Model of Kidney Stone Formation, a Transcriptional Study

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Most common kidney stones are composed of calcium oxalate (CaOx) crystals produced in the presence of increased urinary excretion of oxalate, hyperoxaluria. Recent studies have shown activation of innate immunity through nucleotide binding oligomerization domain-like receptor family, pyrin domain containing -3 (NLRP3) inflammasomes in mice and rats with experimentally induced hyperoxaluria and renal CaOx crystal deposits. What provokes the NLRP3 activation, oxalate (Ox) or CaOx crystals is unknown.

We induced hyperoxaluria in male Sprague-Dawly rats by feeding hydroxy-L-proline (HP) for 28 days. During this time all rats became hyperoxaluric but only half of the 6 rats, developed CaOx crystals in their kidneys, providing an opportunity to compare renal changes during hyperoxaluria as well as hyperoxaluria with CaOx crystal deposition i.e. nephrolithiasis. Rats were euthanized, kidneys explanted, separated into cortex and medulla.Total RNA was extracted for microarray analysis using Illumina bead array readerTM. Gene ontology (GO) and KEGG pathway analyses was performed. Microarray data were verified by quantitative RT PCR and immunohistochemical (IHC) staining for inflammasome associated gene products.

Analysis showed alteration in gene expression in both cortex and medulla in response to hyperoxaluria and CaOx crystal deposition.In hyperoxaluric rats without renal CaOx crystal deposits, genes involved in 17 and 26 pathways, were up regulated in cortex and medulla respectively, and in the presence of renal CaOx crystal deposits, 20 and 33 pathways were up regulated in cortex and medulla. The expression of genes involved in activation of NLRP-3 inflammasome, genes encoding for NLRP-3, TXNIP (thioredoxin interacting protein), caspase-1, PYCARD (ASC: apoptosis –associated spec-like protein containing a CARD), IL-1β, and IL-18 were up regulated in the HP treated hyperoxaluric rats that developed renal CaOx crystal deposits. Same genes were however, down regulated in HP treated rats that developed hyperoxaluria only. Results were confirmed by RT-PCR and IHC analyses.

Results of our animal model study suggest thatOxand CaOx crystals both alter gene expression in the rat kidneys. CaOx crystals appear to play significant role in the activation of NLRP-3 inflammasome in hyperoxaluric kidneys.

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CGD Pathogens Reveal That Inflammasomes Protect against Environmental Microbes

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Chronic granulomatous disease (CGD) disease is caused by a defect in the production of reactive oxygen species (ROS), making patients uniquely susceptible to infection by typically non-pathogenic environmental microbes. Since neutrophils efficiently use ROS to control pathogenic assaults, CGD is often classified as a primary immunodeficiency in neutrophil microbicidal functions. Due to the intracellular nature of several CGD-associated pathogens, we examined the upstream role of macrophages during infection, as they are often the first to encounter microbes. Intracellular pathogens are detected by multi-protein signaling complexes called inflammasomes that result in secretion of proinflammatory cytokines and a programmed, lytic form of cell death termed pyroptosis. Previous work has suggested that pyroptosis is beneficial to the host because it eliminates the intracellular replicative niche and provides the more professional killers, neutrophils, access to the microbes. We hypothesized that CGD patients are susceptible to environmental pathogens that are normally detected and killed via this pyroptosis to neutrophil clearance mechanism.

*Chromobacterium violaceum*infections are extremely rare, but do occur in severely immunocompromised individuals, including CGD patients. However,*C. violaceum*does encode virulence factors, including a type III secretion system. We found WT mice resist even high dose infection with 10^6 CFU of*C. violaceum*, consistent with the notion that*C. violaceum*is minimally pathogenic. In contrast, CGD mice (*p47phox–/–*) and*Casp1–/–Casp11–/–*mice succumb to as few as 100 CFU. NLRC4 is the primary inflammasome protecting against*C. violaceum*, as the*Nlrc4–/–*mice have similar bacterial burdens and survival kinetics as*Casp1–/–Casp11–/–*mice. Importantly, this susceptibility was not phenocopied by*Il1b–/–Il18–/–*mice, implicating pyroptosis as the mechanism of resistance. These results prompted us to examine a variety of CGD pathogens to determine the penetrance of our proposed clearance mechanism. Thus far we have identified two additional CGD pathogens,*Francisella philomiragia*and*Burkholderia thailandensis*, that also require intact inflammasomes and ROS-competent neutrophils for clearance in vivo. Our research on these CGD pathogens reveals a key innate immune pathway that, when intact, protects us from infection with typically innocuous environmental microbes. Additionally, our work demonstrates the value of CGD

pathogens as tools to probe inflammasome pathways and further understand the innate immune responses to intracellular pathogens.

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Antimicrobial Peptide LL-37 Inhibits Pyroptosis of Macrophages and Protects Sepsis in a Murine Model

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Background: The combination of bacterial LPS (lipopolysaccharide) and ATP (extracellularly released from dying cells) induces pyroptosis, a caspase-1 dependent cell death of macrophages. During pyroptosis, inflammatory cytokines (such as IL-1β) are processed and released from the cells. We previously revealed that LL-37 neutralizes the action of LPS. In this study, we investigated the effects of LL-37 on the LPS/ATP-induced pyroptosis of macrophages and sepsis using a cecal ligation and puncture (CLP) model.
Methods: Mouse J774 cells were primed with LPS in the absence or presence of LL-37, and treated with ATP. The effect of LL-37 on pyroptosis was evaluated by detecting IL-1β release, caspase-1 activation and cell death. Furthermore, LL-37 was intravenously administered into mice after CLP, and the survival rate, pyroptosis of macrophages and cytokine levels were evaluated.
Results and Conclusion:
LL-37 suppressed the LPS/ATP-induced IL-1β release, caspase-1 activation, and cell death. Moreover, LL-37 inhibited the binding of LPS to the cells and ATP-induced caspase-1 activation, suggesting that LL-37 inhibits the LPS/ATP-induced pyroptosis by both blocking LPS and ATP actions. Furthermore, LL-37 improved the survival of septic mice. Importantly, LL-37 inhibited pyroptosis of peritoneal macrophages, and suppressed the IL-1β levels in the peritoneal fluid and serum, suggesting the potential inhibition of pyroptosis by LL-37 during sepsis. The present finding provides a novel insight into the modulation of sepsis utilizing LL-37 with an inhibitory action on pyroptosis.

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Mitochondrial Ros Potentiates Indirect Activation of the Aim2 Inflammasome

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Inflammasomes are important complexes present in the cytosol that detect a variety of endogenous and foreign molecules. As such, triggering inflammasomes has been shown to be an essential factor in the control and clearance of cytosolic pathogens. Thus, evasion of detection by host inflammasomes is an important factor for highly virulent pathogens. Here we demonstrate that in contrast to avirulent *F. novicida* (Fn), infection with virulent *F. tularensis* ssp *tularensis* does not trigger activation of the host AIM2 following Francisella infection is due to sensitivity of each isolate to reactive oxygen species (ROS). ROS present at the outset of Fn infection contributes to activation fo AIM2 inflammasome, independent of NLRP3 and NADPH oxidase. Rather, mitochondrial ROS (mROS) is critical for Fn stimulation of the inflammasome. This study represents the first demonstration of the importance of mROS in the activation of the AIM2 inflammasome by bacteria. Together our data suggest that the increased sensitivity of Fn to ROS results in accelerated membrane perturbations of Fn, allowing release of AIM2 activating Fn DNA. Our results also demonstrate that bacterial resistance to mROS is a mechanism of virulence for early evasion of detection by the host.

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The Hematopoietic Stem Cell Transcriptome Illustrates a Failure of Innate Immunity in the Elderly after Trauma and Sepsis

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Introduction: We previously demonstrated that aged mice have increased mortality to primary infections (abdominal sepsis, cecal ligation and puncture (CLP)) as well as secondary infections combined with severe injury (polytrauma (PT) followed by *Pseudomonas* pneumonia). Elderly hematopoietic stem cells (HSCs) that engender myeloid cells after being exposed to either pathogen-associated molecular patterns (PAMPs; infection) or damage-associated molecular patterns (DAMPs; injury) are dysfunctional, including their colony forming ability. We analyzed gene expression profiles in the bone marrow (BM) HSCs after CLP or PT in both young and old mice as it may provide global

insights into the mechanisms behind the increased mortality observed in the elderly populations.

Methods: Young adult (6-12 weeks) or old (20-24 months) C57BL/6 mice underwent CLP or PT. One day later, the mice were sacrificed and their BM HSCs were isolated by negative column isolation followed by flow sorting. RNA was extracted and genome-wide expression analysis was performed. Genomic expression patterns were compared to HSCs from healthy naïve mice at $p<0.001$ (f-test). Ingenuity Pathway Analysis (IPA), and distance from reference (DFR) was calculated.

Results: Unsupervised analysis revealed 3335 genes ($p<0.001$) that could differentiate young and old HSCs from naïve, CLP and PT mice. The first major node of separation was the injury (PT OR CLP vs naive); the second was the age of the animals; and the final major node of separation was between PT vs CLP. Supervised analysis revealed 2344 genes ($p<0.001$) that were able to differentiate all the groups. Young and old HSCs (PT & CLP) had more unique transcriptomic changes (>300 genes) than common genes changes (9). Only HSCs from young PT and CLP mice were both predicted by IPA to have activation (z-score>2.0) of the 'chemotaxis of myeloid cells/phagoytes' and 'recruitment of phagocytes' pathways. In addition, only HSCs from young CLP and PT mice downregulated expression of immunoglobulin genes. Biocarta, and GO pathway analysis (via DFR) revealed that only old HSCs (PT and CLP) were different from control for upregulation of the 'IL22 soluble receptor signaling pathway,' the 'inhibition of matrix metalloproteinases pathway' and the 'negative regulation of myeloid leukocyte differentiation' pathways ($p<0.05$; one-way ANOVA).

Conclusions: Although there are differences in the mammalian response to injury (DAMPs) or infections (PAMPs), age plays a greater role in determining the transcriptomic response of BM HSCs to inflammation. Elderly murine HSCs exhibit a dysfunctional myeloid response to both sepsis and trauma. The genomic expression patterns from their HSCs indicate that more focus should be placed on mechanisms of altering the aged response to inflammation.

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Dynamic and Compartmentalized Response of NK Cells during Endotoxinemia

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Although inflammation is defined as a systemic cascade of events and several experimental models exist, most studies to date focus on one compartment at a time (spleen, peritoneum or lungs). Natural killer (NK) cells have been shown to be key players in systemic inflammation, especially via interferon-γ (IFNγ) production. To gain further insight into the dynamics of NK cell activity during systemic inflammation we performed a serial analysis of NK cells after intraperitoneal administration of lipopolysaccharide (LPS) to C57BL/6 mice. Cells from spleen, lungs, peritoneum, blood and bone marrow were harvested at 1.5, 3, 6, 12 and 24 hours after LPS injection, and Flow cytometry analysis was performed for phenotype, activation markers, cytokine receptors and intracellular cytokines.
NK cells are found in varied proportions and different phenotypes (in terms of CD27/CD11b expression) depending on the compartments they inhabit. While NK cells tend to maintain their organ-specific phenotypes during the course of LPS challenge, their proportions vary considerably with a transient increased being observed in the lung at early time-points in contrast to a steady decrease in the spleen. The dynamics of NK cell activation (CD69, B220, CD11c up-regulation) also showed different patterns between organs with splenic cells expressing the highest degree of activation, the blood cells the lowest. IFNg expression was detected as early as 3 hours and peaked at 6h and steadily decreased by 24h in all compartments maintaining the trend of the activation markers. We also found a transient expression of TNFa positive NK cells at 3h which was specific to the splenic compartment. While NK cell surface IL-12Rβ1 and IL-18Rα. showed little modulation during endotoxin challenge, we observed a progressive down-regulation of the expression levels of IL-2Rβ and to a lesser extent, of Our study shows that during systemic inflammation, NK cells get differentially activated in a dynamic and compartmentalized specific manner. Further efforts are required to reveal multiple roles of NK cells during systemic inflammation and lead to the identification of key locations, moments and markers of NK cells responsiveness during systemic inflammation progression.

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What Neutrophils, Neurons, and Alzheimer's Disease Have in Common: CAP37

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Chronic inflammation associated with increased innate immune system activity is known to be a major underlying factor in Alzheimer's disease (AD). The identification of the specific factors promoting neuroinflammation is critical if therapeutic interventions to attenuate immune mediated neurotoxicity are to be developed. The cationic antimicrobial protein of 37kDa (CAP37) is constitutively expressed in neutrophils (PMN) where its antimicrobial activity is well documented. In addition to its antibiotic activity, CAP37 has strong immunomodulatory effects on monocytes, macrophages and microglia. Since activation of microglia is a key feature in neuroinflammation associated with AD, we queried whether CAP37 might play a role. However, since PMN are not customarily associated with AD brains, we investigated the presence of a non-neutrophilic source of CAP37 in AD brains. We hypothesized that CAP37 is expressed in the brain parenchyma and mediates the neuroinflammatory response in AD through its activation of microglia. To test our hypothesis, we used immunohistochemistry (IHC), Western blotting, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) to determine expression and localization of CAP37 in brains of AD patients and age-matched controls. qRT-PCR and IHC analysis for CAP37 expression was also performed on human primary neurons. To determine factors that might induce CAP37 in AD, primary neurons were treated with two known toxic mediators of AD, tumor necrosis factor alpha (TNFα) and Amyloid β1-40 (Aβ). IHC showed that CAP37 is expressed at a higher frequency in cortical neurons in temporal and parietal lobes in AD patients than in age-matched controls. CAP37 was also found in CA3 and CA4 hippocampal neurons of AD patients. Confirming our IHC results, qRT-PCR showed an increase of CAP37 mRNA in the AD temporal lobe, a region of the brain that is highly impacted in AD. PMN did not contribute to this increase in CAP37 mRNA, since mRNA of other markers of PMN granules remained unchanged. We saw no change of CAP37 in the occipital lobe, a region less affected in AD. In the frontal lobe, we found an increase in CAP37 mRNA but no change in protein. qRT-PCR observations confirmed CAP37 expression in primary neurons, and both TNFα and Aβ were able to increase this expression. We suggest that CAP37 could precede or follow the pattern of atrophy that occurs in AD which begins in the hippocampus and spreads to the temporal and parietal lobes, followed by the frontal lobe, and lastly the occipital lobe. Overall, our findings show that in addition to its expression in PMN, CAP37 is expressed in neurons and its neuronal expression is increased in AD patients. This is supportive of our hypothesis that CAP37 may modulate the innate immune response in AD from within the brain parenchyma.

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The TRPV1 Cation Channel Regulates the Endothelial Cell Toll-Like Receptor 4-Dependent Inflammatory Response

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Background: The transient receptor potential cation channel V1 (TRPV1) is activated by noxious stimuli (e.g. high temperature and low pH), plant compounds (e.g capsaicin), and endogenous lipid mediators (e.g. endocannabinoids, such as N-arachidonoyl dopamine (NADA)). While TRPV1 has been consistently implicated as an inflammatory regulator, reports have attributed both anti-inflammatory and pro-inflammatory roles to TRPV1. Previous work in our lab has shown that the endocannabinoid NADA reduces Toll-like receptor (TLR)-2 and TLR-4-induced inflammation in the endothelium. Therefore, we aimed to test the hypothesis that TRPV1 has a role in endothelial cell inflammatory responses to TLR activation. Endothelial cells are centrally involved in the pathogenesis of organ injury in acute inflammatory disorders, such as sepsis. The unresolved presence of host cytokines and microbial components can constitutively activate endothelial innate immune pathways, including TLR signaling cascades, resulting in excessive inflammation, coagulopathy, and the loss of vascular barrier function.

Methods: Quantitative PCR and immunoblots were used to assess TRPV1 expression in endothelial cells. Calcium flux assays were used to explore the effects of NADA on intracellular calcium levels in human lung microvascular endothelial cells (HMVEC-Lung). To

determine the effects of TRPV1 on cytokine production, HMVEC-Lung were pre-treated with a TRPV1 inhibitor or transfected with TRPV1 siRNA, and then stimulated with lipopolysaccharide (LPS). Cytokines were quantified in culture supernatants. Cytokines were also quantified in supernatants of LPS-treated lung endothelial cells isolated from wild-type and TRPV1 knockout mice. To determine the contribution of TRPV1 to the activity of NADA, HMVEC-Lung were pre-treated with NADA and a TRPV1 inhibitor, and then stimulated LPS.

Results: Primary human endothelial cells from multiple organs express TRPV1, the only known cation channel activated by NADA. NADA also induces calciumflux into HMVEC-Lung, consistent with a physiological function for TRPV1. Pharmacologic inhibition or deletion of TRPV1 in human endothelial cells, or genetic deletion of *Trpv1* in mouse lung endothelial cells, increases both baseline and LPS-induced IL-6 and IL-8 secretion. Intriguingly, TRPV1 inhibition decreases inflammatory cytokine production in the presence of NADA.

Conclusion: Our results indicate that TRPV1 antagonizes the endothelial inflammatory response to TLR4 activation. Paradoxically, in the presence of exogenous NADA, TRPV1 inhibition further decreases LPS-induced inflammatory cytokine production, suggesting a pro-inflammatory role for TRPV1 in the presence of endocannabinoids. We hope that elucidating the role of TRPV1 in endothelial cell inflammation will allow us to better understand the therapeutic potential of endocannabinoids and other TRP channel modulators in modulating the vascular inflammatory response to bacterial infections.

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Role of PTPN2 in Platelet-Activating Factor-Induced PAFR Internalization and IL-6 Expression.

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Atherosclerosis is one of the most widespread inflammatory diseases, affecting millions individuals around the world. Platelet-activating factor (PAF) is an important mediator in the initiation and progression of this disease by modulating, among others mechanisms, cytokine production. Among these cytokines, interleukin 6 (IL-6) is very involved in atherosclerotic progression. IL-6 mRNA expression is triggered by PAF binding to its cognate receptor, PAFR, which, in addition to activating G-protein-dependent pathways, also activates the JAK/STAT pathways. As the JAK/STAT pathways are regulated by protein tyrosine phosphatases (PTP) PTP1B (PTPN1) and TC-PTP (PTPN2), this lead us to investigate if these phosphatases could be involved in PAFR-induced IL-6 production. In this report we concentrated on PTPN2, which has two splice variants, PTPN2.1 (48Kd) and PTPN2.2 (45KD), and these differ in their localization and function. Preliminary results obtained with human monocyte-derived dendritics cells (DCs) transfected with isoform-specific PTPN2 siRNAs showed that a decrease in PTPN2.1 but not PTPN2.2, protein expression, decreased PAF-stimulated IL-6 mRNA levels. Using HEK-293 cells stably transfected with PAFR (HEK-PAFR), PAFR-induced IL-6 promoter activation was investigated more in depth using IL-6 promoter-luciferase assays. Results obtained suggest that effects of PTPN2 on PAF-induced IL-6 transcription are, at least partially, G-protein independent. Using mutant PAFRs, we also determined that PTPN2.1 action is dependent on the internalization capacity of the receptor. With pharmacological inhibitors, we found that the PI3K pathway is necessary for PAFR internalization and preliminary results (Western blot) suggest that over-expression of PTPN2.1 increased Akt phosphorylation, indicating that this phosphatase may modulate the PI3K pathway. Internalization assays of PAFR, showed that PTPN2.1, but not PTPN2.2, could decrease PAFR internalization and this decrease was dependent on PI3K activation. In conclusion, PTPN2.1, but not PTPN2.2, modulates PAF-induced PAFR internalization, IL-6 promoter activity and mRNA levels via, at least partially, G-protein-independent mechanisms.

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Stabilin-1 Supports Growth of Mouse Mammary Adenocarcinoma and is Overexpressed on Early Stages of Human Breast Cancer

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Tumor-associated macrophages (TAM) is a subtype of M2-polarized macrophages with immunosuppressive properties which support tumor growth and metastasis. Previously we have identified stabilin-1, a multifunctional scavenger/sorting receptor, as a marker of M2 macrophages. Further studies demonstrated that stabilin-1 is expressed by TAM in several murine tumor models. However, its role in tumor progression was not defined.

In this study, tumor samples from two cohorts of female patients with breast carcinoma of different stages were analyzed for stabilin-1 expression using immunohistochemistry and immunofuorescent staining. The role of stabilin-1 in tumor growth was studied using subcutaneous model of mammary adenocarcinoma (TS/A) in BALB/c mice with stabilin-1 knockout

Stabilin-1 was expressed on significant part of TAM in human breast cancer. Three types of TAM were identified by co-staining with anti-stabilin-1 RS1 and anti-CD68 antibody: CD68+stabilin-1-, CD68+stabilin-1+ and CD68-stabilin-1+. The highest number of stabilin-1+ TAM were found on stages I and IIa of disease. To identify the role of stabilin-1 in breast cancer progression the growth of TS/A adenocarcinoma tumors was compared in wild type and stabilin-1 knockout mice. The results demonstrated that knockout of stabilin-1 gene resulted in inhibition of tumor growth by 36%. Flow cytometry and confocal microscopy analysis revealed that adhesion/internalisation of SPARC and its transport into the endocytic pathway was significantly impaired in stabilin-1 ko TAM. Since SPARC is known to inhibit the growth of solid tumors including breast cancer our results suggest that knockout of stabilin-1 in TAM induces accumulation of extracellular SPARC resulting in suppression of tumor growth. We have also examined whether the absence of stabilin-1 affects expression profile in TAM using Affymetrix microarray assay. The results revealed that stabilin-1 expression in TAM affected the expression of only few genes with the strongest fold change observed for PKCbeta. To assess if stabilin-1 induces activation of PKCbeta gene, HEK293 cells stably expressing stabilin-1 were transfected with PKCbeta promoter construct. Promoter activity was measured in 48h with or without addition of stabilin-1 ligand, acetylated low density lipoprotein (acLDL). The results demonstrated that stabilin-1 did not activate PKCbeta promoter either in the absence or presence of acLDL indicating that binding and internalization of endocytic ligands by stabilin-1 is not associated with activation of intracellular signalling.

Our data indicate that stabilin-1 expression on TAM is necessary on the early stages of tumor growth in human cancer. Genetic knockout of stabilin-1 inhibits growth of mouse mammary adenocarcinoma tumor. Stabilin-1 deficient TAM demonstrate significantly decreased ability for the endocytic clearance of SPARC. In addition, the clearance function of stabilin-1 is not associated with signalling induction.

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The Role of Transcription Factor FoxQ1 in the Transmigration of Monocytes in Inflammatory Conditions

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Monocyte polarization in the circulation is a result of the interplay between circulating and tissue-derived molecules. However, the molecular mechanism of the specific activation of monocytes in blood stream is still poorly understood. Recent studies showed that the levels of cytokine IL-4 are increased in the circulation during Th2-associated inflammation. Using microarray assay we found that monocytes respond to IL-4 by strong induction of FOXQ1 gene expression and observed its

upregulation in monocytes of patients with acute atopic dermatitis. However function of FOXQ1 in monocytes was unknown. In the presented study we investigated the regulation of FOXQ1 gene expression in human monocyte-derived macrophages and analyzed effect of FOXQ1 on monocyte transmigration.

Analysis of FOXQ1 expression in human monocyte–derived macrophages by qRT-PCR confirmed that IL-4 induces FOXQ1. We showed that TGF-β1 in combination with dexamethasone amplifies the effect of IL-4. In order to identify FOXQ1-induced genes and analyse function of FOXQ1 in macrophages murine macrophage-like RAW264.7 cells were stably transfected with mFOXQ1 or empty vector. A microarray analysis revealed that FOXQ1 target genes can be involved in the monocytes motility. In fact, the macrophage-like cells showed increased migration activity during FOXQ1 overexpression. The stimulatory effect of FOXQ1 on monocyte migration correlated with its ability to suppress expression of receptor PLXNC1 known to inhibit migration of monocytes and dendritic cells. In parallel with FOXQ1, PLXNC1 was downregulated in human monocytes upon IL-4 stimulation and in monocytes of patients with acute atopic dermatitis.

Our data indicate that FOXQ1 is upregulated by IL-4 and TGF-β1 and stimulates monocyte migration in response to inflammatory stimuli by suppression of PLXNC1. We hypothesise that FOXQ1 supports increased monocytes extravasation through the activated endothelium during chronic inflammation.

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Gamma Interferon Enhances Innate Immune Response to Neonatal Sepsis

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Background and aims
Sepsis represents the leading cause of mortality in the first few weeks of life. Newborns, particularly after preterm birth, present a severe defect in innate immune response to infection that can be reversed ex vivo with gamma interferon. We hypothesize that gamma interferon could enhance innate immune response in an *in vivo* murine model of neonatal *Escherichia coli*(*E.coli*) sepsis.
Methods
On day 5 of life, newborn rats received 0.2 microgram of gamma interferon (IFN) by subcutaneous injection. One day after, they were infected with a pathogenic *E. coli* strain. Levels of bacteraemia were monitored, and time of death was noted. Systemic inflammatory response was evaluated by cytokine plasmatic measurements. Qualitative and quantitative evaluation of innate immune response was based on measurements of transcripts profile of the main genes involved in the Gram-negative bacteria recognition.
Results
Thirty rats were infected, including 15 IFN stimulated rats, and 20 rats were either injected with IFN or saline, without infection. Twenty-seven hours after infection, survival rate was higher in infected IFN-stimulated group (4/10) than in infected sham-stimulated (1/10). Level of *E.coli* bacteraemia was lower 12 hours after inoculation in the IFN group. Cytokine levels were modulated primarily by infection. The infection-induced decrease of expression of recognition complex was reversed by IFN stimulation.
Conclusions
Gamma interferon enhances bacterial clearance and survival in a model of neonatal *E.coli* infection. It doesn't modify inflammatory response profile, but enhances the bacterial recognition.

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Inhibition of Tumor Growth and Metastasis in LLC-Tumor-Bearing Mice by Novel C-Jun N-Terminal Kinase Inhibitor

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The c-Jun N-terminal kinase (JNK) is an important regulator of apoptosis, expression of matrix metalloproteinases, and cancer cell migration, and targeting JNK has been reported to be beneficial in a variety of experimental tumor/metastasis models. Recently, we synthesized the sodium salt of 11*H*-indeno[1,2-b]quinoxalin-11-one (**IQ-1S**) and demonstrated it was a high-affinity JNK inhibitor. Treatment with **IQ-1S** also inhibited NF-κB and AP-1 activation in tumor cells. In this study, we investigated the effect of **IQ-1S** on tumor growth, metastasis, and survival in a mouse Lewis lung carcinoma (LLC) model. C57Bl/6 mice (25 per group) were injected with LLC cells s.c. into the right hind limb of each animal (Day 0). Treatment with **IQ-1S** or saline vehicle alone was performed daily (i.p., 20 mg/kg) starting 5 days before tumor inoculation and for 33 consecutive days after, and tumor volume was measured. The number of lung metastasis nodules was counted postmortem. We found that continuous treatment with JNK inhibitor significantly inhibited tumor growth by >60% at the second week after tumor inoculation. At day 8 after tumor inoculation, the average tumor size in the control group was 0.430 ± 0.021 cm^3 compared with only 0.152 ± 0.080 cm^3 in the **IQ-1S**-treated group ($p < 0.01$). Likewise, mice treated with **IQ-1S** had 52 ± 2.0 in comparison with 87 ± 0.2 metastatic nodules per mouse in the control group ($p < 0.01$). We also found that **IQ-1S** significantly prolonged survival of the LLC tumor-bearing mice from 30 (control) to 40 days in the treated group after tumor inoculation ($p < 0.0002$). To assess direct immunomodulatory effects of **IQ-1S**, a serum profile of 24 cytokines at 2, 4, and 6 hr after a single injection of the JNK inhibitor(i.p., 100 mg/kg) in normal mice was evaluated. Levels of interleukin 5 (IL-5) and granulocyte colony stimulating factor (G-CSF) were increased > 5-fold at 4 and 6 hr after **IQ-1S** administration. Molecular docking studies showed that the *syn*-isomer of **IQ-1S** fit well into the JNK1 binding site, which corresponded well with the position reported for co-crystallized JNK inhibitor SP600125. Taken together, our results suggest that the synthetic JNK inhibitor **IQ-1S** could be a novel therapeutic with immunomodulatory properties for treatment of lung cancer. Supported by Institutional Development Award (IDeA) Center of Biomedical Research Excellence grant GM103500.

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SVV Dissemination in a Rhesus Macaque Animal Model

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Primary infection with varicella zoster virus (VZV), a neurotropic alpha herpesvirus, results in varicella, also known as chickenpox. Following primary infection, VZV establishes a latent infection in the sensory ganglia, and can reactivate to cause herpes zoster (HZ), more commonly known as shingles. The primary mode of VZV transmission is through inhalation of virus-laden saliva droplets, however the mechanisms of viral dissemination from the lungs to the skin and ganglia remain poorly understood. Studies investigating VZV trafficking using in vitro cultures or the humanized SCID mouse model suggest that memory CD4 T cells can support viral replication and are responsible for transport to the skin. Moreover, it is unclear whether VZV transport to the ganglia occurs via the hematogenous route (carried by T cells) or by retrograde transport from the skin. However, these hypotheses have not been confirmed in vivo. We have recently shown that intrabronchial inoculation of young rhesus macaques with simian varicella virus (SVV), a homolog of VZV, recapitulates the hallmarks of acute and latent VZV infection in humans. In this study, we used this model to answer the following questions: 1) how and what immune cells carry and/or support viral replication 2) when does SVV reach the ganglia and 3) what are the characteristics of the immune response in the ganglia. We report that the highest SVV viral DNA load and infectious virus are found in CD4 and CD8 T cells, suggesting that T cells are indeed the primary host cells for transporting SVV. Moreover, SVV viral DNA can be detected as early as day 3 post infection in the sensory ganglia which supports the hematogenous theory for viral dissemination given the fact that vesicular skin rash in rhesus macaques with SVV infection does not develop until day 10 post infection. Indeed, memory T cells are also detected at day 3 post-infection in the ganglia. Additional analysis using the Ion AmpliSeq technology

revealed that SVV replicates briefly once it reaches the ganglia day 3 post-infection; however, it goes latent by day 7-post infection. In summary, our studies demonstrate that SVV reaches the ganglia primarily via the hematogenous route within memory phenotype T cells where it replicates briefly before establishing latency. Current efforts are aimed at characterizing the host response in the ganglia during acute infection and determining whether T cells serve only a transport vehicle or whether they also support viral replication. Collectively, these data will guide efforts to design targeted therapeutics to prevent viral dissemination, as well as guide efforts to improve vaccines against herpes zoster and therapeutics to reduce pain associated with shingles

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Restoring Ang-1/Ang-2 Homeostatic Balance Decreases Loss of Barrier Function in Lung Microvascular Endothelial Cell Monolayrers Co-Incubated with Hemorrhage-Primed Neutrophils.

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Development of effective therapies for acute respiratory distress syndrome (ARDS) has proved challenging. Multiple systemic factors such as extrapulmonary sepsis or multi-system trauma further complicate ARDS of indirect origin (iARDS). Characteristic of ARDS is an increase in lung micro-vascular permeability associated with endothelial cell (EC) loss of function. EC growth factors, Angiopoietin (Ang)-1&2, modulate EC activation/function through competitive binding to a shared receptor, Tie2, constitutively expressed on ECs. Ang-1, released by pericytes, promotes anti-inflammatory/pro-survival when binding to Tie2, while Ang-2, stored preformed and released from activated ECs, promotes decreased barrier function and increase lung vascular permeability. We have recently shown, in our mouse model of hemorrhage (Hem) priming for the development of iARDS following subsequent septic challenge, that depletion of peripheral blood neutrophils (PMN), prior to Hem, reduces pulmonary edema/tissue injury, and alters the ratio of Ang-1/Ang-2. To further assess the response of ECs to stimulated (Hem-primed) neutrophils in the development of ARDS, primary lung micro-vascular ECs were isolated and grown to confluence on Electric Cell-substrate Impedance Sensing (ECIS) arrays. In these arrays, confluent EC monolayers producean increase in electrical resistance. EC monolayers were co-incubated with Hem-primed or naive neutrophils with/without recombinant mouse Ang-1. Wells with untreated EC monolayers or EC monolayers treated with thrombin alone were used for controls. Barrier function was measured as normalized resistance change as a function of time. Ang-2 was measured in supernatants following aspiration and centrifugation. As expected, cell monolayers treated with thrombin exhibited the greatest decrease in resistance/loss of barrier function as compared with untreated cells; a significant decrease in resistance was measure for monolayers co-incubated with Hem-primed neutrophils. While Ang-2 in supernatant was similar in all monolayer wells co-incubated with Hem-primed neutrophils, the addition of Ang-1 to these monolayers restored resistance levels/barrier function to near that of untreated monolayers. Our data shows that EC interaction with activated/primed neutrophils contributes to loss of barrier function; this loss can be rescued through an increased concentration of Ang-1. These findings suggest that restoring the ratio of Ang-1/Ang-2 associated with vascular homeostasis may provide a potential therapeutic in the treatment of ARDS. Funding: GM103652/8257 (J.L.N)

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The Effect of Maternal Obesity on the Neonatal Immune System

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The development of the neonatal immune system is modulated by several key factors including the mother's nutritional status. Several studies have shown that a high maternal BMI combined with poor diet during pregnancy leads to adverse outcomes for both mother and infant. The risks to the mother include placental rupture, cesarean delivery, and increased rates of stillbirth. In addition, risks to children born to obese mothers include increased risk for developing obesity, type-2 diabetes, and cardiovascular disease. In the United States, more than 1 in every 2 women of reproductive age is overweight or obese, making maternal obesity potentially the biggest threat to the health of the developing neonate.

More recent observations suggest that children born to obese mothers are also at increased risk of developing asthma. These epidemiological observations are supported by data from murine studies that demonstrated worse outcomes in models of autoimmunity, and allergic sensitization in offspring of obese dams. This suggests, a dysregulation of immune function in the offspring of obese mothers, but the mechanisms remain poorly understood. The aim of this study was to further understand the impact of maternal obesity during pregnancy on the developing neonatal immune system. Specifically, we characterized the effect of maternal BMI on 1) frequency of major innate and adaptive immune cell populations, 2) cytokine production in response to toll-like receptor ligands and T cell polyclonal stimulation, and 3) concentration of circulating cytokines, chemokines, growth factors and metabolic hormones. These studies were carried out using cord blood samples collected from babies born to obese, overweight and lean mothers.

Our analysis revealed decreased numbers of eosinophils, basophils and CD4 T helper cells in cord blood samples obtained from babies born to obese mothers compared to lean mothers. Furthermore, CD4 T cell cytokine production was also altered as maternal BMI increases with decreased IL-4 and increased IL-17 production. In addition, monocyte and DC responses to TLR ligands were reduced in cord blood samples of babies born to obese mothers. Finally in fetal plasma, we also detected increased fetal insulin adipsin, etc; increases in inflammatory cytokines, chemokines, and growth hormones such as IFNg, IL-6, VEGF etc. The results of our pilot study displayed a trend towards overall immune system dysregulation at the innate and adaptive levels as well as in the periphery.

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A River Runs Through it: How Autophagy, Senescence and Phagocytosis Could Be Linked to Phospholipase D by Wnt Signaling

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Neutrophils and macrophages are professional phagocytic cells, extremely efficient at the process of engulfing and killing bacteria. Autophagy is a similar process by which phagosomes recycle internal cell structures during nutrient shortages. Some pathogens are able to subvert the autophagy process, funneling nutrients for their own use and for the host's detriment. Additionally, a failure to mount an efficient autophagy is a deviation on the cell's part from normal cellular function into cell senescence and cessation of the cell cycle. In spite of these reasons, the mechanism of autophagy and senescence in leukocytes has been understudied. We advance here the concept of a common thread underlying both autophagy and senescence, that implicates Phospholipase D (PLD). Such a PLD-based autophagy mechanism would involve two positive inputs: the generation of phosphatidic acid (PA) to help the initiation of the autophagosome and a protein-protein interaction between PLD and PKC that leads to enhanced PA. One negative input is also involved in this process: downregulation of PLD gene expression by mTOR. Additionally, a dual positive/negative input plays a role in PLD-mediated autophagy, β-catenin increase of autophagy through PLD upregulation and a subsequent feedback termination by Dvl degradation in case of excessive autophagy. An abnormal PLD-mTOR-PKC-β-catenin/Wnt network function could lead to faulty autophagy and a means for opportunistic pathogens to survive inside the cell.

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Interactivity between innate inflammatory Signal 3 molecules CD70, IFN-1 and IL-12 and the generation of primary and memory CD8+ T cell responses.

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A significant proportion of the influence that CD4+ T cells have on CD8+ T cell responses is mediated by the ability of CD4+ T cells to activate dendritic cells. This can be mediated by the CD40L-dependent induction of the TNF-superfamily member CD70 and the subsequent stimulation of its receptor, CD27. CD27 stimulation supports the expansion and survival of CD8+ T cells, much as described for signal 3 molecules such as type-1 interferon (IFN-1) and IL-12. However, it is unclear whether CD27-mediated stimulation is sufficient to serve as an independent signal 3 molecule, and how signals that emanate from other signal 3 pathways are intercalated with CD27 signals. We find that while stimulation of CD27 in isolation drives minimal CD8+ T cell responses to protein immunization with chicken egg ovalbumin (OVA), profound synergistic expansion is achieved by co-targeting TLR stimulation. Compared to CD27 stimulation alone, concomitant stimulation of CD27 and TLR substantially increases the proportion of short-lived effector cells, yet also promotes the ability to form long-lived memory without substantially increasing memory precursor frequencies. Notably, while IFN-1 contributes to the expression of CD70 on dendritic cells, the synergy between CD27 and TLR stimulation is dependent upon IFN-1 stimulation directly on CD8+ T cells, and is associated with the increased expression of CD25 and T-bet. Interestingly, in contrast to IFN-1, we find that IL-12 fails to synergize with CD27 stimulation to promote CD8+ T cell expansion, despite its capacity in driving effector differentiation via induction of T-bet and KLRG1. Together these data identify complex interactions between signal3 signaling pathways; how innate responsiveness contributes to adaptive immunity; and identify opportunities to influence the differentiation of CD8+ T cell responses.

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Induction of lipoxin A4 by Francisella tularensis: a mechanism for regulation of pro-inflammatory responses during early-phase tularemia.

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During respiratory tularemia, caused by inhalation of Francisella tularensis (Ft), we postulate that bacteria establish a principally anti-inflammatory environment and subvert host cell death programs to facilitate their unfettered replication within various cell types. Here we have explored the role of lipid mediators such as leukotrienes [i.e., lipoxin A4 (LXA4)] and prostanoids [i.e., prostaglandin E2 (PGE2)] in host inflammatory responses manifested early during infection with Ft.

LXA4 has potent anti-inflammatory properties and also is involved in down-regulation of PGE2. Addition of exogenous LXA4 during in vitro infection with Ft inhibits apoptosis as well as ablates PGE2 release by murine monocytes.
During infection, an inverse relationship was observed between the production of LXA4 and PGE2 in a murine model of respiratory tularemia suggesting that Ft induces the generation of these eicosanoids to modulate cell death and enhance its own survival. This notion is supported by our findings that Ft facilitates its survival and exponential replication by triggering the release of LXA4, which inhibits apoptosis of macrophages and other cells within the first ~3 days of infection. We also have elucidated the course of Ft infection in 5-lipoxygenase deficient mice and observed elevated levels of PGE2, increased apoptosis and increased cytokines and chemokine levels as compared to the wild-type. Our findings demonstrate that Ft infection induces the formation of lipid bodies, which serve as distinct intracellular sites for lipid mediator synthesis. Rapid induction of lipid bodies and subsequent generation of eicosanoids (such as PGE2 and LXA4) during the course of infection potentially has significant implications to the pathogenesis of this bacterium insofar as the initiation of anti-inflammatory milieu early during tularemic infection that allows unfettered replication and survival of Ft. Targeting such lipid mediators may have therapeutic benefit by limiting bacterial growth in tularemic individuals and perhaps those infected with other highly virulent respiratory pathogens.

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Vitamin B6 prevents IL-1ß production through inhibition of NLRP3 inflammasome activation

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Vitamin B6 is a water soluble vitamin and the active form, pyridoxal-5'-phosphate (PLP), acts as a necessary cofactor for more than 100 enzymatic reactions. There is growing epidemiological evidence that vitamin B6 has potential anti-inflammatory activity in a variety of inflammatory diseases, and patients with inflammation had significantly lower blood levels of PLP, compared with the control subjects. It has been previously observed that vitamin B6 inhibited lipopolysaccharide (LPS)‐induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in mouse macrophage RAW264.7 cells via suppression of nuclear factor-κB (NF-κB) activation. However, the effect of vitamin B6 on NLRP3 inflammasome activation has not been explored. Here we identify that incubation of macrophages with vitamin B6, including pyridoxal (PL) and PLP, abolished ASC specks formation and NLRP3 inflammasome activation, blocked subsequent caspase-1 activation and IL-1β maturation stimulated by NLRP3 inflammasome agonists. But other B6-vitamers, including pyridoxamine (PM) and pyridoxine (PN) had no suppression effect on IL-1β production. In addition, PL and PLP suppressed IL-1β production in transfected HEK293T cells. Importantly, in a mouse model of LPS+ATP-induced IL-1β production, we show that PL and PLP reduced IL-1β levels in both serum and peritoneal lavage. Moreover, PL and PLP also inhibited IL-1β production which stimulated by high dose of LPS in mice. Collectively, these findings reveal a mechanism through which PL and PLP inhibit NLRP3 inflammasome activation and reduce IL-1β production and suggest a possible role of vitamin B6 in preventing NLRP3 inflammasome-driven inflammatory diseases.

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Liver sinusoidal endothelial cells clear LPS from circulation.

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In addition to responding to the bacterial cell wall component lipopolysaccharide (LPS) by signaling and starting a cascade of inflammatory processes, immune cells can eliminate the bioactivity of LPS. The LPS molecules that are not inactivated are the ones that are thought to be involved in signaling and inflammation during sepsis. Understanding the molecular mechanism of LPS inactivation will help us treat sepsis. Liver, specifically Kupffer cells (KC), is thought to detoxify LPS from the circulation, mainly by deacylation and dephosphorylation of the lipid-A portion of LPS. Testing that the liver sinusoidal endothelial cell (LSEC) rather is the active scavenger cell for this function, we studied FITC labelled LPS (FITC-LPS) localization in mouse liver after intravenous infusion.

LSECs were most reliably identified by immunofluorescence microscopy (IF) of mouse liver sections using the rabbit anti-mannose receptor (MR) antibody. KCs were identified with macrophage marker anti-F4/80. Localizing FITC-LPS in mouse liver by (IF) at 1 minute after intravenous infusion, we found most of the FITC-LPS to be associated with LSEC. Image analysis and quantification of 100 optical sections from 3 different mouse livers suggested binding of FITC-LPS to LSEC and KC at a ratio of about 3:1. These results suggest that LSEC is the more vigorously endocytosing cell in the liver. The biological activity of LPS was investigated in vivo over the course of 45 min after intravenous infusion of FITC-LPS and by microscopic analysis of liver sections using a modified limulus amebocyte lysate assay with recombinant factor C and rabbit polyclonal anti-factor C antibody. Interestingly, factor C binding was not detectable in liver sections at early time points and was not apparent until 10 min, when the factor C signal appeared, colocalizing with FITC-LPS. It would appear that there could be a competitive inhibition of FITC-LPS with serum lipoproteins, especially HDL, and factor C; i.e., when LPS is already bound to lipoproteins, factor C may not be able bind to LPS. These in vivo biochemical interactions indicate the possible involvement of lipoproteins like HDL in promoting the clearance of LPS by LSEC.

The future direction involves understanding of the molecular mechanism of LPS detoxification by LSEC, including the role of serum lipoproteins, receptors and the enzymes involved.

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Proteasomes and Immunoproteasomes: Pivotal Regulators of Inflammation

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We have recently shown that proteasomes/immunoproteasomes affect multiple signaling pathways during LPS-induced inflammation. We showed that TLR signaling in the murine RAW 264.7 macrophage cell line results in replacement of basal macrophage proteasomal subunits (X, Y, and Z) with the inducible "immunoproteasome" subunits (LMP7, LMP2, LMP10) with distinct chymotrypsin-like(CT), post-acidic (PA), and trypsin-like (T) activities, respectively. This replacement results in a significant increase in the ratio of CT-like to PA activity and a minor increase in trypsin-like activity. We have also demonstrated that LPS stimulation of mouse macrophages triggers a selective increase inlevels of gene and protein expression of immunoproteasomes, resulting in modulation of specific functional activities of proteasome that causes an increase in nitric oxide (NO) production. Surprisingly, in human monocytes, an increase in gene expression of iNOS, and NO, was not observed upon LPS treatment. Based on this finding, we hypothesized that expression of proteasomal subunits varies in cell lines, and this contributes to differences in agonist-induced gene expression. To test this hypothesis, we compared the expression of proteasomal subunits and the effect of LPS on its activity by comparing RAW (mouse macrophages) and THP1 (human monocytes) cell lines. We found that phorbol 12-myristate 13-acetate (PMA) differentiated THP1 cells express inducible proteasome subunits, predominantly, LMP7, LMP2 and LMP10, while RAW cells express X, Y, Z, subunits. Furthermore, in THP1 cells, there was no change in the ratio of chymotrypsin (CT) and post-acidic (PA) activity after stimulation with LPS, which decreased after second LPS-challenge. In contrast, in RAW cells, there was a decrease in CT/PA activity after first LPS-challenge, that increased after second-challenge. Finally, we used resveratrol as proteasome activity modulator, to explore its effect on human monocytes in response to LPS. After 4h of treatment of cells with resveratrol and LPS, a down regulation in expression of select genes such as *Vcam1*, *resistin* and *Lmp7* was displayed as quantified by real-time PCR.In contrast, the expression of *iNos* was upregulated and we did not notice any change in expression of *Sirt1* and *Icam1*. Moreover, resveratrol treatment also decreased the expression of LMP7 protein in human monocytes, displaying its role in reducing inflammation. Thus our findings support a novel concept that different cells modulate gene expression differentially in response to agonists and drugs, because they comprise of different proteasome subunits.

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Isoprenoid Depletion Alters CD14 Expression in Murine Macrophages and Human Monocytes

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The mevalonate pathway is responsible for the production of non-sterol isoprenoid compounds such as farnesyl- and geranylgeranylpyrophosphate (FPP and GGPP). FPP and GGPP are lipid moieties that are critical for the proper function of cellular molecules including the Ras, Rho, and Rab families of small GTP-binding proteins. Genetic (mutations in mevalonate kinase) or pharmacological (statins/aminobisphosphonates) inhibition of the mevalonate pathway leads to alterations in the inflammatory response. In particular, patients with mevalonate kinase deficiency (MKD) exhibit a systemic inflammatory phenotype marked by recurrent episodes of disturbed cytokine production and fever attacks, phenotypes that have been linked to GGPP depletion in monocytes and macrophages. CD14 is the primary binding site on myeloid cells for bacterial lipopolysaccharide (LPS) and therefore plays a key role in cytokine production. CD14 can be found as two protein isoforms: a glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) and a soluble serum protein (sCD14). In order to examine the effect of isoprenoid depletion on the monocyte and macrophage response to LPS, cellular production of isoprenoid compounds was blocked with statins, followed by analysis of CD14 expression. Lovastatin treatment of RAW 264.7 macrophages leads to increased levels of cell-associated CD14 on both the RNA and protein levels, but decreased release of sCD14 following LPS stimulation. In order to further characterize these effects, we performed immunostaining for CD14 on RAW 264.7 macrophages following LPS stimulation. In control cells, CD14 is primarily localized to the plasma membrane and in a concentrated perinuclear region that partially co-localizes with the Golgi apparatus marker GM-130. However, while CD14 is still localized to the plasma membrane in lovastatin treated cells, the percentage of cells exhibiting a concentrated perinuclear staining pattern is decreased indicating a potential block in Golgi-apparatus trafficking. Co-incubation of lovastatin treated macrophages with either mevalonate or GGPP blocks alterations in CD14 expression and release indicating that these effects are dependent on inhibition of isoprenoid biosynthesis. Moreover, inhibition of Rho GTPases with either GGTI-298 or Toxin B results in increased cell-associated CD14, but does not block the release of sCD14 from these cells. In contrast to the findings in RAW 264.7 macrophages, lovastatin and GGTI-298 treatment both lead to increased expression of cell-associated CD14 and increased release of sCD14 in a human monocyte cell line (Mono-Mac 6) following LPS stimulation indicating that the mechanism of sCD14 release is cell-type dependent. Therefore, immune cell response to isoprenoid depletion may vary and a more mechanistic understanding of these differences has the potential to impact treatment of inflammatory diseases.

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Antimicrobial peptide resistance of Vibrio cholerae results from an LPS modification pathway related to nonribosomal peptide synthetases

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Current pandemic El Tor biotype of O1 Vibrio cholerae is resistant to cationic antimicrobial peptides (CAMPs), whereas the previous pandemic strain, of the classical biotype, is sensitive to CAMPs. The almEFG operon found in El Tor V. cholerae confers >100-fold resistance to polymyxins through glycylation of lipopolysaccharide. Here, we present the mechanistic determination of initial steps in the AlmEFG pathway. We verify that AlmF is an aminoacyl carrier protein and identified AlmE as the enzyme required to activate AlmF as a functional carrier protein. A combination of structural information and activity assays were used to identify a pair of active site residues that are important for mediating AlmE glycine specificity. Overall, the structure of AlmE in complex with its glycyl-adenylate intermediate reveals that AlmE is related to Gram-positive D-alanine:D-alanyl carrier protein ligase, while the trio of proteins in the AlmEFG system forms a chemical pathway that resembles the division of labor in non-ribosomal peptide synthetases.

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Human Pathogenic Francisella tularensis Lipid A Mutant for Immunization and Treatment

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Francisella tularensis subspecies *tularensis* (*Ftt*) and *Francisella tularensis* subspecies *holarctica* (*Fth*) are Gram-negative, intracellular bacteria. They are classified by the Centers for Disease Control and Prevention as Tier 1 select agents due to their high virulence, ease of aerosol generation, and historical weaponization. The endotoxic portion of *Francisella* lipopolysaccharide, lipid A, has a unique tetra-acylated structure that helps the bacterium evade cytosolic detection during mammalian infection. Previous work in our laboratory has shown that *Francisella* mutants lacking functional LpxF, a 4'-phosphatase in the lipid A synthesis pathway, produce lipid A with an extra phosphate group, as expected, as well as a retained fifth acyl chain, resulting in an avirulent and protective mutant. Immunization with *lpxF*-null mutants created in the mouse pathogens *Francisella novicida* (*Fn*) and *Francisella tularensis* subspecies *holarctica* Live Vaccine Strain (LVS) protected mice against a lethal, wild-type *Francisella* infection. In addition, treatment with serum from immunized mice is fully protective for up to 36 hours after a lethal wild-type *Francisella* challenge. We are currently generating unmarked *lpxF*-null mutants in the human pathogenic organism, *Ftt* and *Fth*, to test the protective potential. To accomplish this, we are using an allelic exchange procedure with a SacB suicide vector targeted against *lpxF*. These *lpxF*-null mutants could serve as defined strains for human immunization, which is desired over the many random mutations of LVS. In addition, further analyzing the protective immunoglobulins and immunogenic *Francisella* proteins could lead to the development of a monoclonal antibody for prophylaxis and treatment of *Francisella* infections.

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Nicotine exposure down-regulates pulmonary GABABR expression and promotes recruitment of quiescent neutrophil in the lungs

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Pulmonary accumulation of neutrophils has been documented in smokers that are predisposed to various inflammatory and infectious lung diseases. We have demonstrated that nicotine, an ingredient of cigarette smoke, stimulates human neutrophil chemotaxis in an ERK-dependent manner. Nicotine/cigarette smoke (N/CS) exposure decreases GABAB receptor expression in the hippocampus, but the changes in GABAB receptor expression in non-neuronal tissues is unknown. Therefore, we hypothesized that nicotine exposure in mice would down-regulate pulmonary GABABR expression, modulate release of pro-inflammatory mediators, and recruit neutrophils in the lungs. Nicotine-treated C57/BL animals were exposed to nicotine (100 μg/ml) in drinking water and nicotine water was changed twice a week for up to 3 months. Control mice were exposed to regular drinking water. Nicotine exposure did not induce apoptosis in the lung tissue nor did lung histology differ between the control and nicotine-exposed mice. However, nicotine exposure stimulated fibronectin expression in lung tissue, and this was associated with upregulation of ERK and Akt phosphorylation in lung cells. Nicotine exposure decreased intact GABABR expression and increased the 30 kDa fragment of the GABABR in lung tissue; this is interesting considering that a separate proteomic study identified this fragment as an Akt-binding protein. However, the role of this 30 kDa fragment of GABABR in the lungs is currently unknown. Nicotine also stimulated the release of pro-inflammatory mediators including interleukin-1 receptor accessory protein (IL-1RAcP), thrombospondin-1 (TSP-1), and nuclear factor-erythroid derived protein 2 (NF-E2) in the bronchoalveolar lavage fluid (BALF) of experimental mice, but not in control mice. Myeloperoxidase, a neutrophil specific enzyme, was detected in the BALF cell lysate of nicotine-treated mice, but not in control mice suggesting the presence of neutrophils. However, increased ERK activity was not detected in BAL cells suggesting a quiescent status for the recruited neutrophils. In summary, we identified alterations in GABABR expression in the lungs of nicotine-fed mice with concurrent increases in pro-inflammatory mediators and influx of neutrophils without lung damage. These results reveal that nicotine may prime recruited neutrophils to be activated in the lung in case of a secondary insult, suggesting a new paradigm of recruiting quiescent neutrophils in the lungs.

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Evidence Suggesting that Francisella tularensis Capsule is Anchored to a Unique Lipid A Structure

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Francisella tularensis (Ft), the Gram negative bacterium that causes tularemia, produces a capsule that is immunologically distinct from Ft lipopolysaccharide but contains the same O-antigen tetrasaccharide. To pursue the possibility that the capsule of Ft has a structurally unique lipid anchor, we have metabolically labeled *Francisella* with ^{14}C acetate during several generations of growth to facilitate highly sensitive structural analysis of capsule-associated lipids. The high molecular weight capsule was isolated from bacteria by SDS-proteinase K digestion and ethanol precipitation followed by gel sieving chromatography in 0.25% deoxycholate. Autoradiographic and immunologic analysis confirmed that this purified material was devoid of low molecular weight LPS and of the copious amounts of free lipid A that Francisellae express. Purified capsule was subjected to acid-base hydrolysis to release ester- and amide-linked fatty acids followed by lipid extraction. High-performance liquid chromatography analysis revealed the presence of the major 3-OH fatty acids of *Francisella* lipid A but with a different molar ratio of 3-OH C18:0 to 3-OH C16:0 (~2:1 in capsule-associated lipids vs. ~6:1 in free lipid A-rich samples). Moreover, the capsule-associated lipids contained C14:0 as the predominant non-hydroxylated fatty acid in contrast to C16:0 in free lipid A and LPS. These findings raise the possibility that *Francisella* selectively utilizes distinct lipid A species as anchors for its high molecular weight polysaccharide.

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Mapping of Francisella Lipid A and Host Lipid Response in a Mouse Infection Model Using Mass Spectrometry Imaging

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Francisella novicida, a model species for the Tier 1 Select Agent *Francisella tularensis*, is a facultative intracellular, Gram-negative, acutely lethal pathogen. Using mass spectrometry imaging (MSI) we have mapped both host- and pathogen-borne lipids within *Francisella novicida* infected tissue in a mouse model of infection. We report control of membrane lipid-processing components suggesting an important role for the arachidonic acid (AA)-dependent pathways in *Francisella* infection.

We have mapped the dynamic response of specific AA-bearing host membrane lipids in mouse spleen following infection with *Francisella novicida*. Phosphatidylinositol (*m/z* 885.7) was detected in abundance in the white pulp of the mouse spleen in the early-phase of the infection (twelve to twenty-four hours). Interestingly, ion mapping to the periphery of the white pulp suggests a cell-specific origin. We demonstrated that the majority of this signal is organized away from the immediate center of the white pulp following the activated response expected of the spleen. Tandem mass spectrometry confirmed the identity of the ion as phosphatidylinositol 18:0/20:4 (PI 18:0/20:4, SAPI), bearing an arachidonyl group. Abundance of PI 18:0/20:4 decreases in the late phase of the infection, possibly due to cell death, but *in vitro* reports suggest the specific release of AA from PI 18:0/20:4 in activated inflammatory cells, potentially explaining the observed decrease in SAPI parent signal at forty eight and sixty hours post-infection.

Additionally, we have used the same infected tissue to identify and map an integral bacterial component, lipid A in the infected mouse spleen. Lipid A is the membrane-associated component of lipopolysaccharide (LPS) in Gram-negative bacteria. *Francisella* lipid A (m/z 1665.1) ion signatures mapped primarily to the red pulp of the spleen, with signal first appearing between 24 and 36 hours post-infection, corresponding to the expected transition to sepsis.

Here, we use MSI to link pathogen-controlled, specific innate inflammatory mechanisms to the temporal dynamics of specific host lipids. Finally, mapping of bacterial infections using lipid A ion signatures presents a new approach to the study of host-pathogen interactions.

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Anaerobic Lipid A Palmitoylation in Pseudomonas aeruginosa is Regulated by Nitric Oxide Production

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Pseudomonas aeruginosa (PA) is a free-living Gram-negative opportunistic pathogen associated with long-

term airway infections of patients with cystic fibrosis (CF). One early adaptation to the CF lung is production of a pro-inflammatory lipid A containing palmitate. Increasing inflammation leads to an increase in tissue destruction and lung function, ultimately resulting in premature death of the patient.

PagP, the biosynthetic enzyme responsible for this CF-specific modification of PA lipid A, had not yet been identified in PA. We identified PA1343 as a candidate gene with palmitoyltransferase activity. A deletion mutant was constructed and analyzed for changes in lipid A structure. The ΔPA1343 mutant strain did not synthesize palmitoylated lipid A species. Immunological activity studies confirmed pro-inflammatory properties of palmitoylated lipid A, as well as showed protection against specific cationic antimicrobial peptides.

Subsequent experiments focused on elucidating the mechanism by which PA PagP expression and activity through the two-component system, PhoP/Q were regulated. PA is known to replicate in the oxygen limited mucus plugs in patients' lungs. Growth of PA under oxygen limitation resulted in the synthesis of palmitoylated lipid A species. Further, deletion of *phoP*, *phoQ*, and *pagP* all resulted in a loss of palmitoylation under anaerobic growth conditions.

Anaerobic respiration in PA requires the involvement of the enzymes of the denitrification pathway. To determine if components of these pathways were stressors of PhoP/Q, palmitoyltransferase activity was determined after growth in media containing nitrate and nitrite. PA specifically lacking the nitrite reductase, NirS, was unable to synthesize palmitoylated lipid A structures. The importance of the NirS component was believed to be due to enzyme activity and not merely structural interaction. Targeting of the active site of Nir, the heme d1 component confirmed the active enzyme was necessary. Growth of the *nirS* mutant, a NO deficient strain, was grown with the NO over-producing *norC* mutant. This restored the lipid A phenotype confirming the production of NO leads to anaerobic lipid A palmitoylation. Collectively, this data indicates production of subinhibitory concentrations of NO within the CF lung contributes to disease progression by increasing signals leading to modification but not bacterial clearance.

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The SaeR/S Gene Regulatory System Regulates Pro-Inflammatory Cytokine TNF- a during Staphylococcus aureus Infection

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Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that was once predominantly isolated from hospitalized patients but is now prevalent in communities. Once infected with *S. aureus* it can induce symptoms ranging from mild skin infections to fatal sepsis syndromes. Although our understating regarding the pathogenesis of this pathogen is advancing there is still a necessity to understand the relationship between *S. aureus* and the host immune system. This study primarily focuses on the production of tumor necrosis factor-alpha (TNF-α) in response to *S. aureus* infection. TNF-α is a pro-inflammatory cytokine produced by monocytes, T lymphocytes, and to a lesser extent neutrophils. Preliminary data suggests that there is an influential role for the SaeR/S two-component gene regulatory system of *S. aureus* in modulating TNF-α production. *In vitro* studies using isolated human monocytes synchronized with either wild-type (wt) or an isogenic *saeR/S* deletion mutant (Δ*saeR/S*) of S. aureus demonstrated a significant difference in TNF-α production, with Δ*saeR/S* having at least a 5-fold increase in TNF-α production by monocytes. Furthermore, the difference in TNF-α production between wt and Δ*saeR/S* was sustained for a pro-longed period of time; this suggests that the two-component SaeR/S gene regulatory system has an influential regulatory role in the production of TNF-α by monocytes. Other in vitro studies including: human whole blood assays, and isolated neutrophil assays, indicate that the regulation of TNF-α is an essential characteristic of *S. aureus* pathogenesis. These preliminary data suggest that recombinant TNF-α could be used as a therapeutic agent to help resolve the virulence of *S. aureus* ininfected patients. Taken together, this study provides insights into specific mechanisms used by S. *aureus* during staphylococcal disease and characterizes a relationship between a bacterial global regulator of virulence, SaeR/S, and the production of the pro-inflammatory cytokine TNF- α by the host immune system.

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Azithromycin treatment alters neutrophil influx in Pseudomonas aeruginosa infection

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Pulmonary infection with *Pseudomonas aeruginosa* in the later stages of Cystic Fibrosis (CF) is one of the leading causes of increased pathology and decline in lung function in patients. CF patients infected with *P. aeruginosa* are commonly treated with Azithromycin (AZM) for its immunomodulatory properties. Previously, our lab has shown that AZM can shift macrophage (MΦ) phenotype in vitro from inflammatory (M1) towards the alternatively activated (M2) phenotype. During *P. aeruginosa* infection, mice treated with AZM have increased percentage of M2-like MΦs with a decreased number of neutrophils (PMNs) by day 7 post infection and decreased production of IL-6 and TNFα in the lung without any differences in clearance of *P. aeruginosa*. Here, we examined the effect of AZM on PMNs during the more acute phase of *P. aeruginosa* infection. Mice were infected intratracheally with $1X10^5$ CFU *P.aeruginosa* embedded in agarose beads. Lungs were harvested 4 to 96 hours post infection and separated into digest and lavage portions to analyze interstitial and alveolar cell populations. Results show that PMN influx into the lung tissue and alveolar space peaked at 24-hours post infection. At 24-hour time point, there was decreased number of PMNs in lung lavage of AZM mice compared to the control mice without any changes in the numbers of $CD11b^+$ monocytes and $CD11c^+$ MΦs/DCs. However activation of PMNs, measured by the intensity of CD11b expression, did not differ between control and AZM treated mice. Our data suggest that treatment of mice with AZM dampens the early influx of PMNs into the lung, however might not play a role in activation status of these cells in vivo. Further studies are in progress investigating the PMN apoptosis as a possible difference between AZM and control treatment groups.

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A Humanized Mice Model for HIV Disease: The Role of Macrophages

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Mice are resistant to infection with human immunodeficiency virus type 1 (HIV-1). Genetic differences in the viral receptors and in the cofactor required for efficient HIV transcription between the two species, explain much of the resistance. Since the 1990s several different and useful mouse models have been developed. However, HIV pathogenesis and persistent infection of tissue macrophages has been comparatively understudied. A major limitation is the localization of macrophages to tissues that must be sampled in humans using invasive procedures. *In vitro* human monocyte-derived macrophage culture is the gold standard model for studying the molecular mechanisms of host-pathogen interplay. However, this model is also limited, as not all tissue macrophage are derived from cells of the bone marrow.

To begin to address these issues, we used NOD/scid IL-2Rgamma null (NSG) mice transplanted with human fetal CD34+ (hCD34) hematopoietic stem cells, which have recently been reported as a viable model to study numerous aspects of HIV infection, including virological, immunological and central nervous system components, to determine the kinetics and extent of HIV infection of tissue macrophage. NSG-hCD34 mice (Jackson Labs), 24 weeks post-engraftment were infected with 7000 TCID of HIV_{SF162}R3-GFP, a replication competent macrophage-tropic reporter virus or sham-infected and plasma viral load, hCD45, hCD4+ and hCD3+ T-cells, and hCD13 myeloid cells were measured at regular intervals in peripheral blood. To determine HIV burden in tissue macrophage, mice were sacrificed, perfused with saline buffer after 4 weeks post-infection and tissues rich in macrophages were harvested for protein lysates to detect viral antigen by Western blot analyses. In addition, tissues were prepared for frozen and paraffin sections and analyzed by immunocytochemistry and immunofluorescence for HIV-infected human T-cells and macrophages. Findings from this pilot study will be used to design larger longitudinal studies to establish whether tissue macrophage can serve as a persistent and latent reservoir for HIV.

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Inflammatory Caspases Are Innate Immune Receptors for Intracellular LPS

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The murine caspase-11 non-canonical inflammasome responds to various bacterial infections. Caspase-11 activation-induced pyroptosis, in response to cytoplasmic lipopolysaccharide (LPS), is critical for

endotoxic shock in mice. The mechanism underlying cytosolic LPS sensing and the responsible pattern recognition receptor are unknown. Here we show that human monocytes, epithelial cells and keratinocytes undergo necrosis upon cytoplasmic delivery of LPS. LPS-induced cytotoxicity was mediated by human caspase-4 that could functionally complement murine caspase-11. Human caspase-4 and the mouse homologue caspase-11 (hereafter referred to as caspase-4/11) and also human caspase-5, directly bound to LPS and lipid A with high specificity and affinity. LPS associated with endogenous caspase-11 in pyroptotic cells. Insect-cell purified caspase-4/11 underwent oligomerization upon LPS binding, resulting in activation of the caspases. Underacylated lipid IVa and lipopolysaccharide from Rhodobacter sphaeroides (LPS-RS) could bind to caspase-4/11 but failed to induce their oligomerization and activation. LPS binding was mediated by the CARD domain of the caspase. Binding-deficient CARD-domain point mutants did not respond to LPS with oligomerization or activation and failed to induce pyroptosis upon LPS electroporation or bacterial infections. The function of caspase-4/5/11 represents a new mode of pattern recognition in immunity and also an unprecedented means of caspase activation.

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