2018 Abstracts

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Host Receptors and Microbes

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Micro Manipulator: Yersinia Pestis Actively Alters the Neutrophil Degranulation Response

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The greatest hurdle pathogens must overcome to cause disease in a host is the immune response actively working to eliminate the infection. Successful pathogens have evolved a variety of mechanisms to evade the immune system. *Yersinia pestis* causes the fatal disease known as plague and employs multiple strategies to subvert innate immune responses. Production of an LPS form not recognized by TLR4, inhibition of inflammasome recognition, blockage of phagocytosis by macrophages and neutrophils, and modulation of phagosome-mediated killing in the event of phagocytosis, are all known subversive mechanisms of *Y. pestis*. These events culminate in the production of an early non-inflammatory host environment allowing for *Y. pestis* colonization and proliferation. Eventually there is a switch to a pro-inflammatory response, but by this time it is too late for the host to overcome the infection. The most important virulence factor encoded by *Y. pestis* is a type three secretion system (T3SS). It is responsible for a vast array of immunomodulatory activity during *Y. pestis* infection. The T3SS is capable of transporting/injecting bacterial effector proteins directly into the cytosol of mammalian cells. Upon injection, these effector proteins interact with host proteins to inhibit, alter, or degrade targeted host proteins, resulting in inhibition of different signaling pathways. During infection, neutrophils and macrophages are the primary targets for *Y. pestis* T3SS-mediated injection. Previous work has demonstrated that T3SS effectors inhibit phagocytosis by both cell types, inflammasome activation and cytokine secretion in macrophages, and ROS and cytokine release in neutrophils. Unlike macrophages, neutrophils are also capable of releasing granules and vesicles containing signaling (e.g., chemokines, cytokines, chemotactic factors) and antimicrobial molecules in response to infection, a process referred to as degranulation. While limited studies with *Y. pseudotuberculosis* suggest T3SS-dependent inhibition of degranulation, neutrophil degranulation specifically in response to *Y. pestis* infection has not been examined. Here we describe the degranulation response of human neutrophils to infection with *Y. pestis* using a combination of flow cytometry and protein release assays, and define the role of T3SS effectors on this response. Specifically, we demonstrate T3SS-dependent inhibition of azurophilic and specific granules, and show that multiple T3SS effector proteins appear to contribute to this inhibition. While secretory vesicle release also appears to be inhibited by *Y. pestis*, surprisingly this occurs independent of the T3SS. Finally, while not released through degranulation, we also discovered that release of LTB4, a potent chemoattractant made by neutrophils, is also inhibited in a T3SS-dependent manner, but likely through the action of a different T3SS effector than required for inhibition of degranulation. This work provides a better understanding of how *Y. pestis* is able to subvert neutrophil function, likely contributing to the initial non-inflammatory host environment necessary for bacterial proliferation, dissemination, and eventual death of the host.

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A Novel Role of p38 MAPK in the Retinoic Acid-induced Expression of CD103 in Human Dendritic Cells

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Dendritic cell (DC) αE integrin (CD103) is an adhesion protein that is frequently used to categorize a subset of tolerogenic DCs within the human intestinal mucosa. It has been established that TGFβ1 is the driver of CD103 expression on T-cells. We and others have demonstrated that retinoic acid (RA), but not TGFβ1, drives the expression of CD103 on DCs. The goal of this project is to identify the pathways induced by RA that regulate CD103 expression within human dendritic cells, which is currently unknown. Interestingly, despite the availability of RA in the gastric and intestinal mucosa, there is a disparity between CD103 expression on DCs in these two immunological compartments. Our lab has previously shown that gastric DCs having significantly lower CD103 expression than intestinal DCs. This suggests that other mucosal-associated factors regulate CD103 expression on DCs. To explore the RA-induced molecular pathways of CD103 expression in human DCs, we used a protein array to identify kinase candidates. We found increased phosphorylation of p38 MAPK and TAK1 when RA was added to monocyte-derived DCs (MoDCs). We chose to focus on p38 MAPK as previous literature has found interactions between p38 MAPK and NFAT1c, a transcription factor shown in T-cells to bind the promoter region of *ITGAE* (CD103 gene). Using western blot analysis, we confirmed the increased phosphorylation of p38 MAPK upon MoDC culture with RA (P=0.04). We next tested the hypothesis that RA-induced p38 MAPK activation would enhance CD103 expression by using a p38 MAPK inhibitor (SB202190). FACS analysis revealed that MoDCs cultured with RA and SB202190 had CD103 expression of 18% (±10%), which was similar to untreated MoDCs CD103 expression of 19% (±6%). MoDCs cultured with RA had CD103 expression of 41% (±9%), significantly higher than the inhibitor and untreated MoDCs. RT-qPCR corroborated these findings with MoDCs cultured with RA and SB202190 having a more than 2-fold decrease in *ITGAE* mRNA expression compared with MoDCs cultured with RA alone. We next tested the hypothesis that NFAT1c is involved in CD103 expression of dendritic cells by using a peptide inhibitor of NFAT1c. FACS analysis was used to evaluate CD103 expression of MoDCs cultured with RA. We demonstrated a dose dependent decrease in CD103 expression of MoDCs cultured with increasing concentrations of the NFAT1c peptide inhibitor; 10mM-13% (±4%), 25mM-7% (±2%), and 50mM-5% (±2%). These data suggest that both p38 MAPK and NFAT1c are involved in the RA-induced molecular pathway of CD103 expression. Further research will include looking at the interactions between p38 MAPK and NFAT1c and investigating other molecular contributors of RA-driven CD103 expression in human MoDCs and freshly isolated human gastric DCs. Overall, our research demonstrates a novel contribution of p38 MAPK and NFAT1c in the molecular pathway of RA-induced human DC CD103 expression.

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Structural Basis for Lipopolysaccharide Capture and Extrusion by ABC Transporter $LptB_2FG$

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Lipopolysaccharide (LPS) is critical for the survival of Gram-negative bacteria. The synthesis of LPS starts on the cytoplasmic side of the inner membrane (IM), and the LPS transport is completed when it is inserted into the outer leaflet of the outer membrane (OM). Seven LPS transport (Lpt) proteins form a trans-envelope complex that is responsible for LPS extraction and transport. The $LptB_2FG$ complex functions as an ATP-binding cassette (ABC) transporter, and extracts the LPS out of the periplasmic leaflet of the IM. The extracted LPS is then subsequently delivered to LptC, LptA in the periplasm, and eventually the LptDE complex in the OM. Due to the lack of structural information, the mechanism of LPS extraction and transport is poorly understood. We have utilized single-particle cryo-electron microscopy (cryo-EM) to obtain the structures of $LptB_2FG$ in lipid nanodiscs without and with nucleotide binding at high resolution. Our results indicate that, after LPS capture, ATP binding causes large conformational changes of $LptB_2FG$ to extrude LPS, and suggest a mechanism whereby the conformational

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transition of $LptB_2FG$ couples ATP binding/hydrolysis with LPS extraction. Our studies proposed the structural details of LPS capture and extraction mechanisms.

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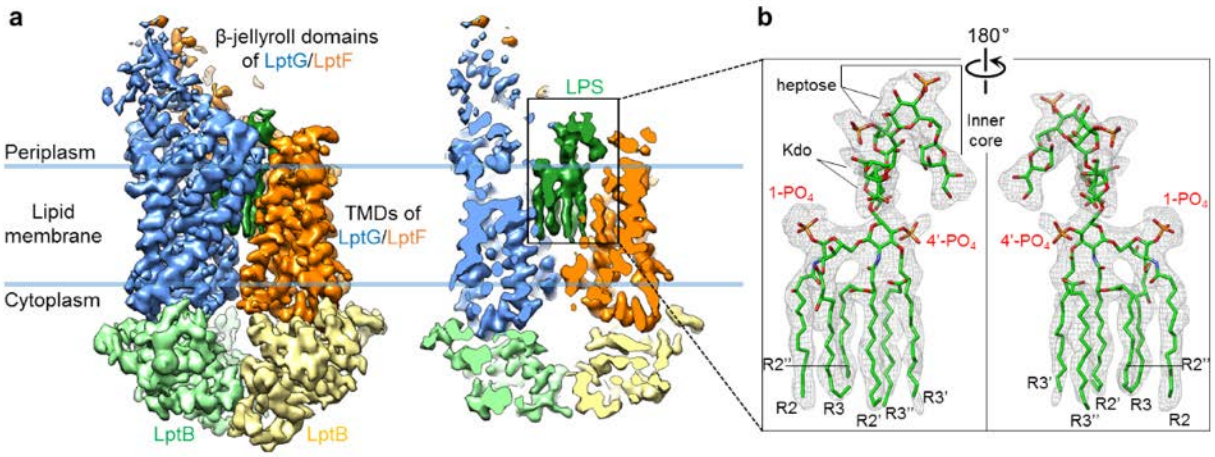

Differential Signaling Through TLR7 or TLR8 Determines the Phenotype of Human Monocytes During RNA Virus Infection

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Despite being a major cell population in blood and one of the major cellular targets of many RNA virus infections in peripheral blood, the molecular consequences of interactions between human monocytes and RNA viruses, and the signaling pathways responsible for the activation of these cells during infection are not well understood. Toll-like receptors ⟪TLR⟫ are a major family of pattern recognition sensors that trigger specific activation pathways in cells from both the innate and adaptive arms of the immune system. There are at least 10 TLRs in humans, from which TLR7 and TLR8 recognize single-stranded RNA. Despite both recognizing the same general ligand, we and others have demonstrated different phenotypic outcomes on cells stimulated through either TLR7 or TLR8. Our laboratory has recently observed fundamental differences in the phenotype and function of monocytes stimulated via either TLR7 or TLR8, in the context of RNA virus infections, and specifically, in terms of type I IFN responses and effector cytokines they produce, as well as differences in cell surface markers. We have defined the molecular mediators that are responsible for these differences in phenotype, performing *ex vivo* experiments with human monocytes isolated from blood and we have shown the relevance of these data in common RNA virus infections, demonstrating that TLR7 and/or TLR8 stimulation by RNA viruses in human monocytes accounts for much of the phenotype the cells acquire upon virus interaction

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Inflammasomes Confer Protection via IL-18 and Pyroptosis, and are Negatively Regulated by IFN-γ-dependent Nitric Oxide During *Brucella* Infection

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Brucellosis, caused by the intracellular bacterial pathogen *Brucella,* is a zoonotic disease for which arthritis is the most common focal complication in humans. Here we show caspase-1 and caspase-11 initiate *Brucella*-induced joint inflammation, but also restrict *Brucella* replication. In contrast, AIM2 and NLRP3 were dispensable for control of joint infection by *Brucella*. Both IL-1 and IL-18 promoted joint inflammation, however only IL-18 contributed to protection against *Brucella* infection of the joint. *In vitro* studies demonstrated caspase-1 and caspase-11 both induce pyroptosis which limited *Brucella* infection in macrophages. While early IFN-γ production required caspase-1/11 and IL-18, caspase-1/11-dependent clearance of *Brucella* was not entirely IFN-γ dependent. However, IFN-γ deficiency resulted in severe inflammation that was entirely inflammasome dependent and, in particular reliant on NLRP3. IFN-γ was vital for induction of the nitric oxide (NO) producing enzyme, iNOS, in infected joints, and NO inhibited caspase-1 activation in *Brucella*-infected macrophages *in vitro*. During *Brucella* infection *in vivo*, exacerbated joint inflammation was seen in iNOS deficient mice along with an increase in joint IL-1β compared to wild-type mice. Collectively these data demonstrate inflammasomes induce inflammation in an IL-18 and IL-1 dependent manner, and inflammasome-dependent IL-18 and pyroptosis restrict *Brucella* infection. Moreover, IFN-γ reduces inflammation by inhibiting excessive caspase-1 activation through production of NO.

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Novel Role of Gastric Releasing Peptide (GRP)-Mediated Signaling in the Host Response to Influenza Infection

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Gastrin Releasing Peptide (GRP) is an evolutionarily well-conserved neuropeptide that was originally recognized for its ability to mediate gastric acid secretion in the gut. More recently, however, GRP has been implicated in pulmonary lung inflammatory diseases including bronchopulmonary dysplasia, chronic obstructive pulmonary disease, emphysema, and others. Antagonizing GRP or its receptor (GRPR) mitigated lethality associated with the onset of viral pneumonia in a well-characterized mouse model of influenza. In mice treated therapeutically with the small molecule GRP inhibitor, NSC77427, increased survival was accompanied by decreased numbers of GRP-producing pulmonary neuroendocrine cells (PNEC), improved lung histopathology, and suppressed cytokine gene expression. In addition, *in vitro* studies in macrophages indicate that GRP synergizes with the prototype TLR4 agonist, LPS, to induce cytokine gene expression. Thus, these findings reveal that GRP is a previously unidentified mediator of influenza-induced inflammatory disease that is a potentially novel target for therapeutic intervention.

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Evaluating the Recruitment of Dendritic Cells to the Gastric Epithelium During *Helicobacter Pylori* Infection Using Human Gastric Organoids

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Dendritic cells (DCs) are important for communication between the innate and adaptive immune systems in response to infection. As professional antigen-presenting cells, DCs process foreign antigens and to present them to T cells. T cell activation, which is important for eliminating the pathogen, is accomplished by DCs that have processed bacterial antigens. We have found DCs in the human gastric mucosa, either in direct contact with the basolateral surface of the epithelial cells, or integrated into the epithelia. This close association might facilitate uptake of bacterial antigens by DCs; however, the precise mechanisms by which the DCs are recruited to the epithelial cells are unknown We hypothesize that close association of DCs with the gastric epithelium allows uptake of luminal *H. pylori* and promotes DCs' responses to epithelial-derived mediators. The aim of this study was to determine if the gastric epithelium actively recruits DCs under steady state conditions and upon *H. pylori* infection

To investigate DC recruitment by the gastric epithelium under steady state conditions, we established human gastric organoids from human gastric tissue samples and performed chemotaxis assays with monocyte-derived DCs (MoDCs). MoDCs were recruited by organoid supernatants in a transwell migration assay. Using a Human Chemokine Array, we identified, CXCL1, CXCL5, CXCL7, CXCL17, CCL20, CCL26 and IL-8 in the organoid supernatant fluids. CXCL1, CXCL5, CXCL16, CXCL17 and IL-8 were assessed for their ability to recruit MoDCs based on their receptor expression on MoDCs, which we have tested previously. Each of these chemokines, but not IL-8, recruited MoDCs. This indicates that these chemokines might be responsible for the active recruitment of DCs to resting gastric mucosa. We have seen similar results in the recruitment of MoDCs to organoid in our co-culture experiments. Live imaging and endpoint analyses by confocal microscopy revealed that MoDC added to gastric organoid cultures spontaneously migrated through the Matrigel matrix towards the basolateral side of the epithelium, where they formed tight interactions and extended dendrites between the epithelial cells.

To investigate DC recruitment by the gastric epithelium upon *H. pylori* infection, we injected organoids with *H. pylori* using a microinjection system. To confirm the infection of organoids we tested IL-8 expression in the supernatant fluids using ELISA. Higher levels of IL-8 in infected organoids, compared to mock injected organoid, confirmed successful infection. The chemotaxis assay using supernatant fluids from infected organoids will be assessed soon. We anticipate that there will be a different in chemotactic index of MoDC toward the infected and mock-infected organoids, consistent with an increased number of epithelial cell-associated DCs in *H. pylori*-infected gastric mucosa. We will determine which cytokines are present in supernatant fluids from infected organoids. In summary, we have identified four chemokines, CXCL1, CXCL5, CXCL16, CXCL17, secreted by resting gastric epithelium that actively recruit DCs. Our results from infected organoids will indicate whether these or other chemokines are responsible for recruitment of DCs upon *H. pylori* infection.

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The Role of Hyperuricemia in Modulating Autophagy Flux and Inflammasome Activation during Bacterial Infection in Macrophages

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Background: Autophagy is a homeostatic process that regulates and recycles intracellular structures. In addition, autophagy a host defense mechanism that help reduce the burden of many infections. The inflammasome is a multiprotein structure that results in the release of proinflammatory cytokines after the preceding activation by danger signals. There is an apparent interplay between autophagy and the inflammasome to regulate inflammatory responses. Uric acid in plasma acts as anti-oxidant and in certain conditions acts as inflammatory danger signal. Gouty arthritis is a commonly encountered inflammatory joint disease. The hallmark of this disease is hyperuricemia that results in the precipitation of monosodium urate crystals in the joints, leading to inflammation. A small subgroup of gout patients develops septic arthritis, which present a challenge during clinical diagnosis and is usually undetected.

Purpose: We aimed to investigate the effect of bacterial infection during hyperuricemia on autophagy and the inflammatory profile of macrophages. We hypothesized that during hyperuricemia the inflammatory response by macrophages will be aggravated.

Methods: To test our hypothesis, we monitored the effect of bacterial infection during hyperuricemia on autophagy and on inflammasome activation in macrophages. We used three different strains of *S. aureus* and two different strains of *K. pneumonia* to infect macrophages in presence and absence of uric acid. In order to eliminate the differences in virulence factors and effects of live bacterial infection in macrophages we used inactivated whole cell formalin-fixed bacteria. To this end, we measured the following different cellular responses: autophagy flux, IL-1β release, and nitric oxide release.

Results: We found that uric acid enhanced autophagy and nitric oxide release, which resulted in an overall reduction in inflammasome activation assessed as decreased IL-1β levels. Since autophagy induction in macrophages is considered an anti-inflammatory process, we investigated the impact of autophagy flux induction in infected macrophages on the levels of production of the pro-inflammatory cytokine IL-1β. We observed that IL-1β production was significantly higher when macrophages were infected with the Gram-negative bacterial strains of *K. pneumoniae* (*p* S. aureus which produced little to no detectable amounts of IL-1β. This difference can be attributed to the presence of endotoxin/LPS in Gram-negative bacteria which is a potent inducer of TLR4. Further, autophagy flux was enhanced by co-stimulation with bacteria and soluble uric acid, whereas IL-1β increased during bacterial infection but decreased when macrophages were co-stimulated with bacteria and uric acid. These results suggest that uric acid exerts modulatory effects on autophagy and reduced inflammation during bacterial infection in macrophages.

Conclusion: Uric acid enhances autophagy flux during bacterial infection consequently reducing inflammasome activation in macrophages. Understanding the effect of uric acid on the interplay between autophagy and inflammation will facilitate therapeutic discovery and design.

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MicroRNA-302/367 Cluster Activates Mitophagy Receptors to Impact Bacterial Clearance by Regulating Oxidative Stress and NF-κB

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Abstract: Mitophagy has recently been implicated in bacterial infection but the underlying mechanism remains largely unknown. Here, we uncover a novel role of microRNA-302/367 cluster in regulating mitophagy and its associated host response against bacterial infection. We demonstrate that miR-302/367 cluster expression was significantly increased after *Pseudomonasaeruginosa* infection. Overexpressing miR-302/367 cluster accelerated the mitophagic response in macrophages, thus promoting the induction of reactive oxygen species (ROS) and increasing clearance of invading *P. aeruginosa*, whereas inhibiting miR-302/367 decreased clearance of *P. aeruginosa*. It appears that phagocytosis of *P. aeruginosa* by alveolar macrophages was not altered by overexpression or inhibition of miR-302/367 cluster in macrophages. In particular, blocking mitophagy receptor prohibitin 2 (PHB2) as well as use of autophagy inhibitor 3-methyladenine (3-MA) and silencing autophagy-related gene 7 (Atg7) reduced the effect of miR-302/367 cluster on induction of mitophagy and subsequent *P. aeruginosa* clearance. Mechanistically, we illustrate that miR-302/367 cluster bound to the 3'-untranslated region of NF-κB, a negative regulator of mitophagy, accelerated the process of autophagy and subsequent killing of intracellular *P. aeruginosa* by manipulating ROS and NF-κB. Collectively, our findings elucidate that miR-302/367 cluster functions as powerful regulators in mitophagy-mediated *P. aeruginosa* elimination and pinpoint a previously unknown functional link between miRNAs and mitophagy.

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Characterization of the Innate Immune Response Following Listeria Infection in IL-15 Deficient Mice

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Interleukin-15 (IL-15) plays indispensable roles in lymphocyte development, homeostasis and immune responses. IL-15 is essential for the homeostasis of memory $CD8^+$ T cells, natural killer (NK), NKT, and type 1 innate lymphoid cells and intestinal epithelial lymphocytes (IEL). IL-15 promotes the survival of Mφ and DC and induces the expression of IL-12 and type 1 interferons. IFNγ as well as viral and bacterial Toll-Like Receptor (**TLR**) ligands (such as LPS and PolyI:C) are potent stimulators of IL-15 in different cell types. Not surprisingly, IL-15 is involved in chronic inflammation and is a pathogenic factor in autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and type 1 diabetes. NOD.*Il15*$^{-/-}$ mice remained diabetes-free at 7 months of age compared to only 30% in control mice. Furthermore, inhibiting IL-15 signaling during the insulitis stage (between 3 and 6 weeks of age) prevented the development of type 1 diabetes in NOD mice. These observations suggest that the pathogenic role of IL-15 may not be at the level of CD8+ T cells only. To better understand the role of IL-15 in myeloid cells, we used the model of low-dose *Listeria monocytogenes* infection where innate immune responses play a major role in clearing the infection. We observed that recruitment of monocytes/macrophages from circulation was not affected in *L. monocytogenes* infected IL-15 deficient mice. However, the activation state of these cells, as seen from CCR2 expression and upregulation of MHC class I molecules, was compromised in the absence of IL-15. These observations suggest that IL-15 plays an important role in regulating the monocyte infiltration in the spleen during systemic infections.

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Staphylococcus Aureus ATP Synthase Is Critical for Promoting the Anti-inflammatory Milieu during Biofilm-associated Infections

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Staphylococcus aureus (*S. aureus*) is a major cause of hospital-associated infections that result in thousands of deaths every year. Patients undergoing surgery for knee/hip arthroplasty or craniotomies for brain tumor treatment have increased risk of infection, where bacteria have a propensity to form biofilms on the implanted device or native skull bone, respectively. Biofilms are a heterogeneous population of bacterial cells contained in a matrix composed of extracellular DNA, proteins, and polysaccharides that establish chronic infections based on their recalcitrance to antibiotics and ability to evade immune-mediated clearance. Our laboratory has previously demonstrated that *S. aureus* biofilms skew the host immune response to an anti-inflammatory state, which is defined by alternatively activated anti-inflammatory macrophages (MΦs), increased recruitment of myeloid-derived suppressor cells (MDSCs), and minimal infiltration of neutrophils. *S. aureus* biofilm infections are extremely difficult to eradicate and generally require multiple surgeries, increased hospital stays, and prolonged recovery, creating a significant economic healthcare burden. Therefore, it is imperative to identify central mechanisms responsible for *S. aureus* biofilm immune evasion in an effort to uncover novel pathways to redirect the host immune system to facilitate biofilm clearance. To accomplish this goal, we screened the Nebraska Transposon Mutant Library for *S. aureus* mutants that elicit heightened pro-inflammatory responses from MDSCs and MΦs during biofilm co-culture compared to wild type (WT) biofilms that favor anti-inflammatory attributes. This approach identified three ATP synthase mutants, namely *ΔatpA, ΔatpD,* and *ΔatpG*, which induced significantly higher IL-12 and IL-6 levels compared to WT biofilms. Based on the heightened pro-inflammatory responses *in vitro*, we next examined the virulence of the ATP synthase mutants *in vivo* using two distinct mouse biofilm infection models, namely orthopedic implant and craniotomy that accurately depict the development and immune responses found in patients with these infections. All three *atp* mutants (*ΔatpA, ΔatpD,* and *ΔatpG*) displayed significant reductions in bacterial burdens in both orthopedic implant and craniotomy models concomitant with a shift to a more pro-inflammatory immune response, characterized by a significant decrease in MDSCs at the site of infection coupled with increased neutrophil infiltrates compared to WT *S. aureus*. Collectively, this work establishes the importance of *S. aureus* ATP production in polarizing anti-inflammatory cellular responses during biofilm infections.

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Mapping Cholesterol Metabolism and Statin-Mediated Protection from a Lethal Bacterial Infection Using Multimodal Mass Spectrometry Imaging

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Several studies implicate diverse roles for statins in protecting against both viral and bacterial infections. Statin drugs are inhibitors of the enzyme HMG-CoA reductase, a key step in cholesterol biosynthesis (mevalonate pathway). Statin use is linked to protection from lethal disease in a range of bacterial infection models, and in humans statin use is associated with reduced onset of acute bacterial infection. Flux in the mevalonate pathway underlies direct immunomodulation resulting in increased Type I Interferon production as well as production of the signaling metabolite 25-hydroxycholesterol. We sought to evaluate the protective potential of one of the most common statins (simvastatin) against lethal *Francisella novicida* (*Fn*)infection and understand the role of

controlling mevalonate flux during infection. Using a combination of SIMS-imaging and MALDI-2 MSI, we mapped cholesterol metabolism and statin treatment in the progression of a lethal infection with *Fn*. Mice were infected with *Fn* strain U112 (300 CFU - 10xLD_{100}) for 48 hours and spleens were collected for imaging. Simvastatin was administered at 50 mg/kg in 10% DMSO in either a pre-treatment (3 days prior), post-treatment (at and 1 day following infection) or 10 mg/kg in a 14 day low-dose pre-treatment regimen. Frozen sections were thaw mounted on ITO glass slides and used directly for SIMS-imaging or prepared for MALDI-2 MSI on a TM Sprayer with a solution of DHB in 2:1 (v:v) chloroform:methanol. Cholesterol and related metabolites were imaged in positive ion mode. Using MALDI-2 MSI, we identified an accumulation of cholesterol (ion signature *m/z* 369) at 48 hours post-infection (h.p.i.) in *Fn* infected mice compared to uninfected (sham) control spleens. Similarly, by SIMS-imaging, an increase in cholesterol-rich puncta was observed in *Fn* infected spleens. To test the role of attenuated mevalonate flux in the progression of *Fn* infection, we treated mice with simvastatin. Animals pretreated with simvastatin for 3 days prior to infection showed protection from a lethal *Fn* infection. Clinical presentation of signs and symptoms of the infection were also delayed by 12 hours. Animals treated with simvastatin at the time of infection and at 24 h.p.i. were not protected from infection and had normal time-to-onset of clinical symptoms or animals treated with a low-dose extended pre-treatment. The treatment effect was interpreted as a host-based response since the antimicrobial activity of simvastatin against *Fn* had no antibacterial activity (0.1 – 100 ug/mL). Total free cholesterol content of the spleen was higher in infected spleens than sham infected, regardless of statin treatment suggesting an off-target protective mechanism of simvastatin. These results point to a protective mechanism for simvastatin that is independent of total cholesterol level. In related studies, cholesterol metabolites have been shown to play a role in shifting the innate immune response to bacterial infection, specifically by the metabolite cholesterol-25OH (chol-25OH). Chol-25OH is implicated in switching between IL-1β- and IFN-β-driven inflammation. Therefore, we used MALDI-2 to detect and map the chol-25OH metabolite in *Fn* infected spleens. Chol-25OH was detected as the ion *m/z* 385.3465 [Chol-25OH-H_2O+H] in a similar distribution to cholesterol. Several uncharacterized cholesterol metabolites were detected in *Fn* infected spleens demonstrating strong spatial distribution biases. These uncharacterized metabolites will be the focus of future work along with mechanistic studies of chol-25OH. Here, we used advanced MSI techniques to map and characterize cholesterol metabolites to understand their immunomodulatory role in bacterial infection.

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Treatment of *S. Aureus* craniotomy-associated Biofilm Infections with 3D Printed Antibiotic Scaffolds Containing Viable Macrophages

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A craniotomy is the temporary removal of a skull fragment to access the brain, such as during tumor removal or epilepsy surgery, which is immediately replaced intra-operatively. The infection incidence after craniotomy ranges from 0.8-12%, with a high frequency caused by *Staphylococcus aureus* (*S. aureus*), which forms a biofilm on the native bone. Biofilms are bacterial communities encased in a self-produced matrix composed of extracellular DNA, polysaccharides, and proteins. Currently, there are no existing methods to prevent or treat craniotomy-associated biofilm infections without surgical interventions. In our mouse model, a craniotomy is performed and the excised bone flap is incubated with *S. aureus*, whereupon the bone is rinsed to remove any non-adherent bacteria and reinserted. This procedure results in *S. aureus* colonization of the bone flap, which ultimately leads to infection persistence in the subcutaneous galea and brain. We have engineered an innovative 3D bioprinted scaffold to harness the potent antibacterial activity of macrophages (MΦs) together with antibiotics to mitigate infectious complications following craniotomy. The theory behind this approach is to promote bacterial dispersal from the

biofilm through the action of inflammatory MΦs, whereupon bacteria regain their metabolic activity and become antibiotic-sensitive, since most currently available antibiotics rely on active cell division and protein synthesis, both of which are limited in intact biofilms. Our 3D scaffold contains rifampin and daptomycin printed in a PCL/Hap slurry, with viable macrophages incorporated into a HA/gelatin hydrogel, which we have tested for both the treatment and prevention of craniotomy-associated infections. For the treatment paradigm, the bone flap was removed at day 7 post-infection, a time at which a mature biofilm has formed, and replaced with a 3D printed antibiotic scaffold, with or without MΦs. The 3D antibiotic scaffold reduced bacterial burdens in the galea and brain by at least 100-fold at early time points, which was potentiated by bioprinting MΦs into the 3D antibiotic scaffold, indicating an additive effect. We also examined a prevention paradigm, where scaffolds were placed at the time of surgery and challenged with *S. aureus* one day later at the surgical site. Interestingly, unlike the treatment paradigm, the incorporation of viable MΦs into the 3D antibiotic scaffold did not enhance *S. aureus* clearance compared to antibiotic alone. In both paradigms, decreased bacterial burdens were associated with reduced myeloid-derived suppressor cell (MDSC) and neutrophil infiltrates. Taken together, our 3D bioprinted scaffold is an attractive treatment modality, since it delivers therapeutic antibiotic levels more rapidly than systemic administration because the scaffold is placed immediately adjacent to the infection site. In addition, the incorporation of viable MΦs into the 3D scaffold is an important advance, which we show offers improved therapeutic benefit for the treatment of established biofilms that are the most clinically challenging scenario.

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Dectin-1 Signaling in Polarizing Different Subsets of CD4+ T Cells

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Dendritic cells bridge innate and adaptive immunity by directing differentiation of naïve T-helper cell into specific lineages of effector T cells through presenting antigen, stimulating co-stimulatory molecules and creating an appropriate cytokine milieu. Activated dendritic cells can induce different subsets of T helper cells including Th1, Th2, Th17, Th9 lineages. Little is known about the factors contributing to Th9 polarization or whether commensal fungi regulate the Th9 response, and in this study, we have examined the mechanisms by which dendritic cells sense *C. albicans* and direct Th9 polarization. We have observed that DCs stimulated with the hyphal *C. albicans* induce stronger Th9 polarization than the yeast form, and that DCs stimulated with hyphae polarize Th9 responses without requiring commonly-used Th9 polarizing culture conditions (i.e. supplementing TGF-β and IL-4). Like previously-characterized ability of *C. albicans*-stimulated DCs to induce Th17 cells, the ability of yeast and hyphae to induce Th9 cells is dependent on activation of Dectin-1, a receptor for b-glucan in fungal cell walls that is essential for protection against many fungal infections. Polymorphisms in the genes for Dectin-1 and CARD9, an adaptor protein required for Dectin-1 signaling, are associated with increased susceptibility to fungal infection. We hypothesized that the size difference between hyphal and yeast forms of *Candida albicans* may contribute to their different capacities to polarize Th9 cells. To test this, we stimulated DCs with different sizes of beads (3, 6, 15, 25, and 45 µm) coated with β-glucan prior to co-culture with naïve T helper cells. DCs stimulated with larger beads (≥15 µm) stimulated significantly more Dectin-1-dependent Th9 T cell polarization compared to DCs stimulated with smaller beads. This result implies that DCs may recognize the sizes of microbes and that this might be an important factor in defining the nature of the immune response. In ongoing studies we are examining the mechanisms of Dectin-1 stimulated polarization of Th9 subtypes as well as the pathogenesis of Th9 driven disease. These studies may be helpful in developing novel therapies for fungal infections as well as for manipulating host responses to commensal fungi that may influence immune responses in allergy, inflammatory bowel disease, and cancer therapy.

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***Filifactor Alocis* Activates a MyD88-independent, TLR-NFκB Signaling Axis in Neutrophils.**

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Neutrophils are the primary innate immune cell recruited to the oral cavity, where they are linked with protection against periodontal disease. However, periodontal pathogens modulate neutrophil signaling pathways to subvert killing and to propagate inflammation, which affords a source of nutrients. Our laboratory has shown that the newly appreciated oral pathogen, *Filifactor alocis*, is able to manipulate neutrophil functional responses and 60% remains viable inside human neutrophils after 4 hours of challenge. However, the signaling cascades initiated by this oral pathogen are still unknown, so this project is aimed to characterize their signaling interactions and determine if *F. alocis* modulates TLR signaling to circumvent neutrophil functions. *In vitro* experiments testing ROS production suggest that neutrophils recognize *F. alocis* through the TLR 2/6 receptor, similar to the synthetic lipopeptide FSL-1. This was confirmed by testing degranulation after blocking TLR6 with neutralizing antibodies. Downstream of TLR2 ligation, we examined the involvement of Myeloid differentiation primary response protein 88 (MyD88) in *F. alocis* signaling. An RNA-seq screen of *F. alocis*-challenged human neutrophils showed a time-dependent decrease in transcriptional levels of MyD88 after 6 hours of infection with *F. alocis.* As observed by western blotting, after infection with *F. alocis,* MyD88 protein levels did not decrease in PLB-985 cells, a neutrophil-like cell line. To examine this further, neutrophils were isolated from the bone marrow of WT and *myD88*$^{-/-}$ mice, and their functional responses were tested against *F. alocis.* MyD88 deficiency did not affect the phagocytic capacity, intracellular ROS production, or microbicidal ability of murine bone marrow neutrophils, indicating that these neutrophil responses are MyD88 independent. Finally, NFκB activation was tested in neutrophils challenged with *F. alocis* by intracellular staining of IKBα, the NFκB inhibitor protein. Degradation of IKBα results in NFκB activation and a decrease in fluorescence, which we measured using flow cytometry. There was a downward shift in IKBα signal after 30 minutes of challenge with *F. alocis* and phosphorylation of the p65 subunit of NFkB was detectable at 1 hr by western blotting of PLB cell lysates. Together, these data suggest that *F. alocis* is recognized by neutrophils via TLR2/6 and activates NFκB. However, MyD88 does not appear to play a large role in bacteria killing, ROS production or phagocytosis.

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Identification of LMAN1 as a Receptor for House Dust Mite (HDM)

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The house dust mite (HDM) is one of the most common indoor allergens responsible for the development of allergic rhinitis, an important step in the so-called “atopic march” to extrinsic or allergic asthma. In fact, sensitization to mite allergens early in life has been shown to be associated with worsened overall respiratory health later in life. The most clinically relevant species of dust mites are Dermatophagoides farinae and Dermatophagoides pteronyssinus of which 60-80% of atopic individuals are reactive. Given this, much research has been dedicated to understanding the mechanisms by which house dust mites cause allergic sensitization. In this study, we have used an unbiased receptor capture approach (TriCEPs) to find receptors on dendritic cells which directly bind HDM. Using this method, we have identified LMAN1 (Lectin, Mannose Binding 1) as a novel receptor for HDM. LMAN1 is a mannose binding lectin which is primarily known to act as a cargo transporter between the ER, ERGIC, and Golgi compartments. The role played by LMAN1 in the recognition of and response to HDM, is currently unknown. This study aims to verify LMAN1 as a bona fide HDM receptor, elucidate the mechanisms of LMAN1-mediated HDM recognition, and identify the consequences of increased and decreased activity of this receptor on the cellular phenotype and type 2 polarizing ability of dendritic cells.

Inflammation and Cell Death in Host-pathogen Interactions

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Neutrophil-derived Microvesicles Influence the Anti-*Staphylococcal* activity of Phagocytes

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In addition to directly eliminating pathogens, neutrophils (PMN) indirectly influence antimicrobial immune activity by altering the inflammatory tone of other phagocytes. Microvesicles (MVs) are membrane-bound particles shed from intact cells, and are capable of transporting proteins and lipids from one cell to another. PMN generate and release MVs in response to stimuli, including formyl peptides and bacteria such as *S aureus* (SA). Different types of PMN MVs have been described as having pro- or anti-inflammatory effects, and the extent of their impact on phagocytes remains to be defined. PMN-derived MVs were isolated by centrifuging activated human PMN at 4,000 x g to remove intact cells, followed by ultracentrifugation of the supernatant at 100,000 x g to sediment the MVs. PMN activated by N-formylmethionyl-leucyl-phenylalanine (fMLF; 1 µM) for 20 min at 37^{o}C generated PMN-fMLF MVs. Separately, human PMN were fed opsonized *S aureus* (USA300 LAC) for 10 min at 10:1 MOI; SA-harboring PMN were centrifuged at 500 x g, resuspended, and incubated for 20 min at 37^{o}C to generate PMN-SA MVs. We discovered that a small number of viable SA were isolated together with PMN-SA MVs (0.08 ± 0.03% of original inoculum, n = 4); these newly identified PMN products are referred to as MV complexes.

We measured PMN-derived MV influence on phagocytic function of naïve PMN. PMN that had been incubated for 5 min at 37^{o}C with vehicle, PMN-fMLF MVs, or MV complexes (10 µg/mL) were fed opsonized green fluorescent protein-expressing SA [GFP-SA] at 1:1 MOI for 10 min at 37^{o}C. We centrifuged suspensions at 500 x g to isolate PMN and used flow cytometry to quantitate phagocytosis of the GFP-SA. MVs elicited higher levels of phagocytosis, as defined by the percentage of PMN that were GFP-positive. PMN-SA MV complexes (mean ± SEM relative to vehicle 106 ± 5%, *p*-value NS, n = 4) were less effective than an equal protein concentration of PMN-fMLF MVs (109 ± 2%, *p*-value 0.02, n = 4). These data suggest that PMN-derived MVs can relay signals to naïve PMN and thereby modulate phagocytic activity.

To assess the influence of PMN-derived MV complexes on the antimicrobial ability of human monocyte-derived macrophages (HMDM), we pretreated adherent HMDM with vehicle or MV complexes (2 µg/mL) prior to feeding of opsonized SA at 1:1 MOI for 30 min at 37^{o}C. CFU recovered from lysed HMDM were enumerated. At t = 90 min post-phagocytosis, compared to t = 0, a lower proportion of SA survived in MV complex-treated HMDM than in vehicle-treated HMDM (vehicle: 91 ± 5 %; MV complex: 74 ± 5 %; *p*-value 0.04, n = 5). These data demonstrate that MV complexes can augment the antimicrobial capacity of HMDM against SA, an organism not optimally killed by HMDM.

We also tested the impact of PMN-derived MVs on HMDM inflammatory cytokine release. As replicating bacteria would complicate interpretation, this set of experiments utilized sterile-filtered (0.45 µm) PMN-SA MVs, instead of MV complexes. We stimulated HMDM to secrete interleukin-1β (IL-1β) over a 20-h period using lipopolysaccharide (LPS; 50 ng/mL) at t = 0 and silica (500 µg/mL) at t = 4, in the presence or absence of PMN-SA or PMN-fMLF MVs (2 µg/mL added at either t = 0 or t = 4). As expected, HMDM stimulated with LPS and silica secreted IL-1β (4.5 ± 1.1 ng/0.5M HMDM, n = 9). Adding either type of PMN MVs together with LPS decreased IL-1β release (normalized to LPS/silica alone: PMN-SA MVs 82 ± 9%, *p*-value 0.09; PMN-fMLF MVs 66 ± 14%, *p*-value 0.04, n = 9). PMN MVs still inhibited IL-1β release when the MVs were added at t = 4h (PMN-SA MVs 84 ± 4%, *p*-value < 0.01; PMN-fMLF MVs 86 ± 8%, *p*-value 0.18; n = 5). Taken together, these data demonstrate the ability of PMN MVs, including those from SA-stimulated PMN, to act as anti-inflammatory mediators.

Overall, our studies illustrate that PMN-derived MVs modulate responsiveness of naïve phagocytes to SA, and thereby allow PMN to shape inflammation.

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Post-Surgical Inflammation Versus Infection in a Rat Model of Central Nervous System Catheter Infection

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Bacterial infection is a frequent and serious complication of CSF shunt placement for the treatment of hydrocephalus with the majority of infections occurring in the first 30 days after surgery. Traditionally diagnosis of shunt infection relies on culture; however, culture may not always be reliable in the setting of biofilm, slow growing or fastidious organisms and antibiotic pretreatment. When cultures are negative clinicians are forced to rely on CSF indices such as white blood cell count, glucose and protein which are neither sensitive nor specific. In the setting of recent shunt placement some literature supports post-surgical meningitis, as demonstrated by increased neutrophils in the CSF, which can confound the diagnosis of shunt infection in the case of negative culture. The presence of post-surgical meningitis needs to be clarified and newer methods for shunt infection diagnosis are needed. We hypothesized that *Staphylococcus epidermidis* CNS catheter infection has a distinct profile of inflammatory cell influx as well as chemokines and cytokines compared to post-surgical inflammation induced by sterile CNS catheters. To evaluate this hypothesis, we adapted a previously published murine CNS catheter infection model to generate infection with *S. epidermidis* in Lewis rats for CSF sampling. As expected there was an increase in neutrophils and macrophages in the brain tissue and cerebrospinal fluid in animals with *S. epidermidis* infected catheters compared to those with sterile catheters. This difference was most striking in the CSF, where animals with sterile catheters had minimal neutrophils and macrophages present in the CSF which is in contrast to previously published human literature. Additionally, there were elevated levels of the pro-inflammatory cytokines IL-1β, CCL2, CCL3 in animals with infection compared to the post-operative inflammatory state. While inflammatory cell influx and cytokines decreased over the course of infection the decrease was most significant in CSF suggesting that monitoring levels in CSF could provide a means to diagnose infection and tract infection course to determine length of antibiotic therapy.

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***Francisella Tularensis*-mediated Modulation of Metabolism and Delay of Human Neutrophil Apoptosis**

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F. tularensis is a Gram-negative, facultative intracellular organism and the etiologic agent of the disease tularemia. As few as ten organisms are sufficient to cause severe disease, with a mortality rate of up to 60% in untreated infections. *F. tularensis* infects many cell types, including polymorphonuclear leukocytes (PMNs; neutrophils), the most abundant leukocyte in circulation. Our lab has demonstrated that *F. tularensis* prolongs human PMN lifespan by interfering with the intrinsic, extrinsic and phagocytosis-induced apoptosis pathways. *F. tularensis* preserves mitochondrial integrity and inhibits caspase activity as a means to delay PMN apoptosis. We have also identified bacterial lipoproteins (BLPs) as relevant active factors in conditioned media. However, all of the factors that function to prolong neutrophil lifespan, and the mechanisms by which these factors are interfering with the major apoptosis pathway in neutrophils remain undefined. The ability of metabolism to influence inflammation is established and our data demonstrate that genes encoding glucose transporters and enzymes of the glycolysis pathways are significantly upregulated in PMNs within 3-6 hours of *F. tularensis* infection, and induction of hexokinase 2 (HK2) has been confirmed by immunoblotting. Based on these data, we hypothesize that metabolic reprogramming of *F. tularensis*-infected PMNs contributes to prolongation of cell lifespan. Consistent with this,

the HK2 inhibitor 2-Deoxy-D-glucose (2-DG) and the glucose transporter 1 (GLUT1) inhibitor WZB-117 both elicited dose-dependent apoptosis of *F. tularensis*-infected PMNs at 24 hpi. Furthermore, the phosphofructokinase (PFK) inhibitor 3PO induced rapid cell necrosis. Ongoing experiments are focused on further analysis of PMN metabolism and studies to determine if *F. tularensis* BLPs selectively affect glycolysis or other aspects of apoptosis inhibition.

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Helicobacter Pylori Infection Modulates Human Neutrophil Chemotaxis

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Helicobacter pylori is a Gram-negative, spiral-shaped bacterium that colonizes human gastric mucosa. A defining feature of this infection is a chronic, neutrophil-dominant inflammatory response that can progress from gastritis to peptic ulcer disease or gastric adenocarcinoma. Neutrophils navigate toward sites of infection or wounding via a process called chemotaxis. During this process of directed cell migration, receptors for chemoattractants, such as IL-8, C5a or fMLF are activated at the front of the cell. Subsequent signaling drives actin polymerization and extension of a leading edge. As the cell moves forward, the trailing edge of the cell (the uropod) retracts. It is generally believed that the multilobed nucleus of human neutrophils facilitates chemotaxis. As we have shown that H. pylori infection induces profound neutrophil nuclear hypersegementation, we predicted that chemotaxis of infected cells may be enhanced. We tested this hypothesis using an EZ-TAXIScan system and quantified migration of both control and infected cells toward C5a, IL-8, or fMLF, using buffer as a negative control. To our surprise, we found that H. pylori infection decreased neutrophil chemotaxis to all tested stimuli. Subsequent flow cytometry studies suggested a complex underlying mechanism as receptors for IL-8, CXCR1 and CXCR2, were downregulated after infection, whereas CD88 and FPR, which bind C5a and fMLF, were not. Further analyses of H. pylori-infected neutrophils suggested that these cells exhibit delayed uropod retraction during migration, which may in part account for the chemotaxis defect. Studies of Rho GTPase dynamics will be a focus of future studies. Taken together, our data suggest that H. pylori curtails neutrophil chemotaxis, which may contribute to cell accumulation in the gastric mucosa.

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Treponema Denticola Triggers Differential Oncostatin M Response from Innate Immune Leukocytes and During Infection

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Periodontitis is a serious condition affecting up to 47% of the US population, leading to destruction of soft tissue and alveolar bone that can lead to tooth loss. The spirochete *Treponema denticola* is one of the numerous bacteria present in the dental biofilm associated with periodontitis; complexed together with other pathogens such as *Porphyromonas gingivalis.* Neutrophils and macrophages, cells of the innate immune system, are a crucial part immune surveillance in the oral cavity and host response during periodontitis. Both neutrophils and macrophages play a role in regulation and development of the inflammatory response, through production and release of various cytokines. *T. denticola* induces a specific cytokine and chemokine signature compared to other oral commensal and pathogenic organisms. Oncostatin M (OSM) is a pleiotropic cytokine belonging to the IL-6 family with both pro- and anti-inflammatory effects. OSM may exacerbate disease conditions through modulation of secondary signaling networks and processes. OSM is elevated in several inflammatory conditions including periodontitis. The specific cell source(s) of OSM and how this cytokine is affected during periodontitis is not well understood. Human neutrophils (blood and oral wash) and macrophages (blood) were exposed *in vitro* to *T. denticola* and OSM protein (ELISA) and gene expression (RT-PCR) levels were measured. A murine airpouch model

was used to compare neutrophil recruitment and OSM response following exposure to *T. denticola* or another oral pathogen *P. gingivalis in vivo*. OSM levels and the presence of different oral spirochete species were examined in saliva samples and gingival tissue specimens from subjects with (n=4) or without (n=3) periodontitis. We observed an elevation in OSM protein release with prolonged synthesis from human peripheral macrophages and neutrophils when co-incubated with *T. denticol*a. Exposure of *T. denticola* to oral wash neutrophils also increased release of OSM. *T. denticola* induced neutrophil recruitment to the airpouch along with OSM production in the airpouch lavage fluid. When compared to *P. gingivalis*, *T. denticola* recruited less neutrophils together with more robust OSM levels in the airpouch. OSM levels were elevated in the saliva from subjects with periodontitis compared to healthy which correlated with the salivary presence of a number of oral *Treponema* species, including *T. denticola*. Immunohistochemistry analysis of gingival tissue sections from patients afflicted with periodontitis demonstrated strong OSM protein presence in the gingival epithelium and immune cell infiltrate, compared to healthy control gingival tissue. Our results indicate that *T. denticola* induces OSM release and synthesis from neutrophil and macrophages *in vitro*. *T. denticola* differentially induces OSM from neutrophils in vivo, compared to other oral pathogens. Increased OSM is clearly associated with periodontitis in both saliva and tissue specimens. The interaction of *T. denticola* with innate immune cells may drive differential OSM-mediated damage, characteristic of periodontitis.

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$CD4^+$T Cells Upregulate Immune Checkpoint VISTA in Response to Septic Challenge

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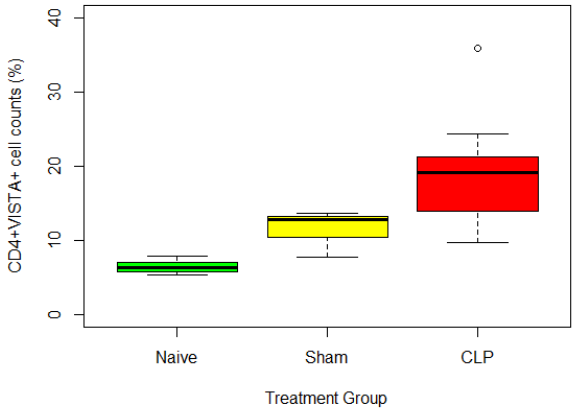
Sepsis is suggested to arise, in part, when the immune response is unable to quell an infectious challenge due to immune dysfunction. There are two immune phenotypes that are thought to contribute to sepsis and subsequent multiple organ failure: immune suppression and hyper pro-inflammatory response to infection. Critically ill patients and experimental animal models demonstrate these septic immune phenotypes, such as the elevated systemic immune response and immunosuppression, as well as a marked increase in expression of checkpoint regulatory proteins, such as CTLA-4, PD-1, BTLA, 2B4, etc. These regulators appear to be associated with the immune dysfunction phenotype and septic morbidity.

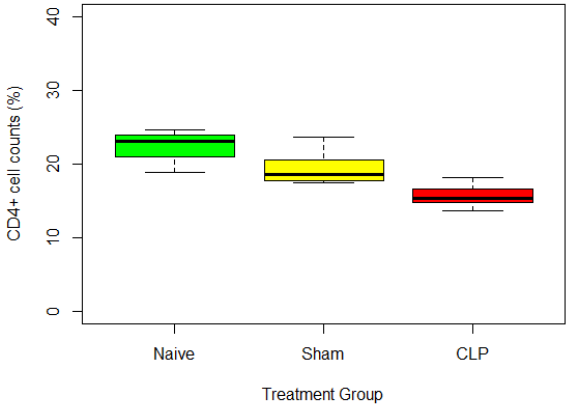
A majority of these checkpoint proteins comprise the B7 family of ligands and receptors. A novel immune checkpoint protein has recently been characterized, with respect to immune disease, which demonstrates both nonredundancy and synergism with checkpoint regulators within the B7 family, like PD-1. V-domain immunoglobulin suppressor of T-cell activation (VISTA) suppresses T-cell proliferation, activation, and cytokine production. VISTA is constitutively expressed in hematopoietic tissues and can function as a ligand in its soluble form or be expressed on the surface of APCs to limit T cell activation. It has also been suggested to act as a receptor on T cells to further suppress T cell activation via signaling cascades. The extracellular domain of VISTA has sequence homology with other B7 family ligands, including PD-L1 and PD-L2, but also contains a unique cysteine pattern in the ectodomain that may contribute to the nonredundant T cell regulation observed. Thus, VISTA could be a promising potential target to be manipulated in tandem with other checkpoint regulators to address specific immunological disorders.

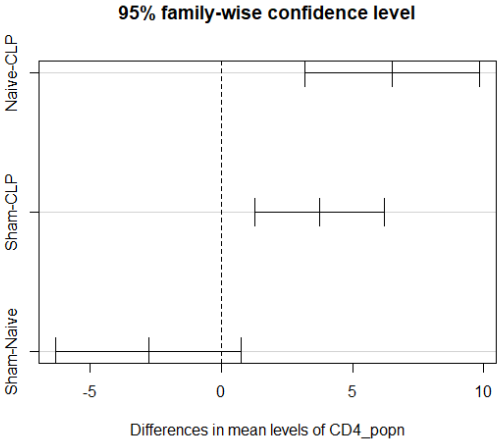
Since little is known about the expression, regulation and/or role of VISTA in immune response to sepsis, we queried whether the onset of experimental sepsis in mice alters the expression of VISTA on T-cells present in the spleen (a population known to exhibit significant immune suppression and/or activation-induced cell death). Inasmuch; cecal ligation and puncture (CLP) method was used as a proxy for intra-abdominal polymicrobial septic challenge in mice. As previous studies have shown 24 hours after CLP to be the optimal time point at which immune suppression is evident, but the mice have not yet succumb, we chose this time point at which to harvest

the spleen, thymus, and blood. These tissues were then processed for flow cytometric analysis of CD3, CD4 and/or VISTA expression. We have found that VISTA surface expression significantly increases on both splenic $CD4^+$ and $CD3^+$ T cell populations in CLP mice compared to sham and naïve mice. In addition, this increased surface expression appears to correlate with a significant loss in $CD3^+$ and $CD4^+$ T cell populations in the spleen. This loss in T cell populations could be due to decreased proliferation or apoptosis, which are both hallmarks of immune suppression observed following septic challenge in humans and mice.

Based on these data, it appears that VISTA expression is positively correlated with suppression of the immune response to septic challenge in our CLP murine model. This relationship may potentiate severe immune pathologies that result in multiple organ failure and death.







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Pathogen Blockade of TAK1 Triggers Capsase-8 Dependent Cleavage of Gasdermin D and Cell Death

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Gasdermin D (GSDMD) mediates inflammasome-controlled pyroptotic cell death by creating membrane pores. GSDMD is cleaved by caspases 1 and 11; but it is unclear whether other caspases can process GSDMD into a pore-forming N-terminal fragment that triggers cell death and cytokine release. The effector YopJ secreted by Yersinia

bacteria blocks TAK1 kinase activity in host cells, and triggers RIPK1-caspase-8 dependent apoptosis. We found that YopJ also triggered caspase-8 mediated cleavage of GSDMD, largely independent of caspase-1/11. GSDMD contributed to caspase-8 controlled release of inflammatory cytokines IL-1β and IL-18, and macrophage death. Thus, Yersinia-induced caspase-8 activation leads to cell death with aspects of both apoptosis and pyroptosis. We conclude that caspase-8 can direct GSDMD processing and that TAK1 activity prevents caspase-8 induced GSDMD cleavage and cytotoxicity.

Inflammatory Signaling in Leukocytes

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Toxoplasma Gondii Induces IL-1β Production and Pyroptosis-independent Release from Primary Human Monocytes Through the Syk Signaling Pathway

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Monocytes are critical mediators of host defense against *Toxoplasma gondii* infection and initiate a robust inflammatory response mediated by IL-1β release. However, the mechanisms by which IL-1β is produced by and released from *T. gondii*-infected human monocytes are only partially defined. Previously our lab has shown that *T. gondii* infection monocytes derived from multiple independent donors induced NLRP3 inflammasome activation and IL-1β release from cells. We now observe that *T. gondii* infection induced rapid spleen tyrosine kinase (Syk) phosphorylation in primary human monocytes and that pre-treatment with two Syk-specific inhibitors, R406 or Entospletinib, reduced IL-1β maturation and release from infected monocytes in a dose-dependent manner. mRNA analysis from infected monocytes demonstrated that Syk inhibitors decreased parasite-induced *IL-1β* and *NLRP3* transcripts, suggesting that Syk functions upstream of NF-kB-dependent transcript production in human monocytes. Pharmacological inhibitors of PKCd, the CARD9 complex, and IKK similarly reduced pro-IL-1β levels in cell lysates and IL-1β release from infected monocytes, indicating that *T. gondii* infection triggers IL-1β production via a Syk-CARD9-NF-kB signaling pathway. IL-1β is thought to be released primarily through an inflammatory form of cell death called pyroptosis, which is driven by caspase-1 activation. However, cell viability assays indicate that *T. gondii* induction of IL-1β release from infected monocytes was not associated with cell death. Moreover, extracellular glycine, a pyroptosis inhibitor, did not reduce IL-1β release from infected monocytes, and treatment with a caspase-1 inhibitor reduced *T. gondii*-induced IL-1β release without affecting cell death. Taken together, our data indicate that *T. gondii* induces inflammasome activation and IL-1β release from primary human monocytes via a pyroptosis-independent mechanism. This research expands our knowledge of how innate immune cells regulate inflammation during parasite infection of primary human monocytes.

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TAK1 Restricts Spontaneous Activation of the NLRP3 Inflammasome

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NLRP3, a cytoplasmic sensor, is a global sensor of infection and stress. Upon activation, NLRP3 assembles an inflammasome complex consisting of adaptor protein ASC and cysteine protease caspase-1; in this complex, caspase-1 gets activated and promotes proteolytic cleavage and activation of IL-1β and IL-18. As a global sensor,

NLRP3 is critical for providing protection against a host of pathogens. However, aberrant activation of the NLRP3 inflammasome has also been associated with inflammatory disorders in humans that include cryopyrin-associated periodic syndromes (CAPS), diabetes and arthritis. Thus, understanding the mechanisms that regulate and control activation of the NLRP3 inflammasome is crucial, which remain unknown. Using a genetic approach, we have identified TAK1 as a central kinase that inhibits spontaneous NLRP3 inflammasome activation. Activation of the NLRP3 inflammasome requires two signals – a priming signal often provided by TLR and an activating signal required for NLRP3 oligomerization and inflammasome assembly. However, macrophages lacking TAK1 spontaneously activated the NLRP3 inflammasome without requiring the priming and subsequent activating signals. To our knowledge, this is the first study to show spontaneous NLRP3 inflammasome activation. Thus, TAK1 plays a central role in maintaining NLRP3 quiescence. Mechanistically, we further showed that TAK1 suppressed homeostatic NFκB and ERK activation to limit spontaneous TNF production by macrophages. Furthermore, TNF autocrine signaling through RIPK1 kinase activity promoted spontaneous NLRP3 inflammasome activation. Thus, spontaneous NLRP3 activation in TAK1-deficient macrophages could be rescued by TNF neutralization or inhibition of RIPK1 kinase activity. In conclusion, our studies highlight a critical role for TAK1 in maintaining NLRP3 inflammasome quiescence and preserving cellular homeostasis.

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MAPK Regulation of the Resolution of Inflammation Following LPS-Induced Acute Lung Injury

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Macrophages have important functional roles in regulating inflammation and its resolution during lung injury and infection. However, the macrophage intracellular signaling pathways that facilitate the resolution of inflammation are not well understood. In Cystic Fibrosis (CF), over-activation of macrophage signaling pathways is thought to contribute to increased and detrimental inflammation in the lung. In human lung explants from individuals with CF, we have detected robust activation of the MEK1/2-ERK1/2 pathway in leukocytes in the airway and alveolar space. Several inhibitors of the MEK1/2 pathway have been developed as cancer therapeutics, however their potential effects as immunomodulators have not been extensively characterized. We identified that pharmacologic inhibition of macrophage MEK1/2 increased M(IL-4/IL-13) polarization and increased efferocytosis of apoptotic cells. In addition, therapeutic application of a MEK1/2 inhibitor (MEKi) 24 hours after initiation of LPS-induced acute lung injury (LPS-ALI) in mice decreased inflammation and injury. Further, MEKi therapeutic application leads to similar beneficial effects without impaired bacterial clearance in a *P. aeruginosa* murine pneumonia model. To determine the role of myeloid-MEK1 in regulating the resolution of LPS-ALI we generated $Mek1^{fl/fl}LysM^{cre-/-}$ and $Mek1^{fl/fl}LysM^{cre+/+}$ mice. Alveolar macrophages obtained from naïve $Mek1^{fl/fl}LysM^{cre+/+}$ mice have a greater than 95% reduction of MEK1 relative to $Mek1^{fl/fl}LysM^{cre-/-}$ controls while BMDM have ~50% reduction in MEK1. When stimulated in vitro with LPS, both cell types derived from $Mek1^{fl/fl}LysM^{cre+/+}$ mice have reduced inflammatory gene expression 4 hours after stimulation. In addition, at 24 hours after LPS-ALI, alveolar macrophages from $Mek1^{fl/fl}LysM^{cre+/+}$ mice have reduced inflammatory gene expression. Unexpectedly, however, $Mek1^{fl/fl}LysM^{cre+/+}$ mice display enhanced inflammation, as measured by weight loss, increased alveolar macrophage inflammatory gene expression, increased bronchoalveolar lavage fluid cytokines and chemokines, and increased leukocyte recruitment into the lung on days 2-4 after initiation of LPS-ALI. We are further characterizing the $Mek1^{fl/fl}LysM^{cre+/+}$ model to understand the role of specific cell functions of MEK1 in regulating the resolution of inflammation. Our goal is to leverage these models to better develop therapeutic approaches to reduce detrimental inflammation during lung injury and infection.

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LincRNA-EPS and lincRNA-Cox2 Regulate Expression of Inflammatory Cytokines in Macrophages and Mice Treated with TLR2 and TLR4 Agonists or During *Klebsiella Pneumoniae* Infection, but Are Dispensable for Endotoxin Tolerance

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Long non-coding RNAs (lncRNAs) are critical regulators of immune responses, but their role in sepsis is largely unknown. During sepsis, TLR sensing of microbes triggers the "cytokines storm", disseminated intravascular coagulation and organ damage. Patients surviving systemic inflammatory response syndrome develop profound immunosuppression that resembles TLR tolerance and often succumb to secondary infections. To study the sepsis-associated "cytokine storm", we used *in vivo* exposure of mice to LPS (endotoxemia) and Pam3Cys, while endotoxin tolerization of macrophages *in vitro* was employed to mimic features of sepsis-associated immunosuppression. This study showed that LPS-treated macrophages decreased expression of lincRNA-EPS while upregulating lincRNA-Cox2 levels. Using knockout mice, we demonstrated that lincRNA-EPS acts to restrain KC, IL6 and CCL5 expression in the serum, spleen, lungs and liver of LPS or Pam3Cys-treated mice. Compared to wild-type macrophages, lincRNA-EPS$^{-/-}$ cells infected with *Klebsiella pneumoniae* or stimulated with LPS showed changes in expression levels of several inflammatory mediators, e.g. up-regulation of *ccl2, ccl3, ccl4, ccl5, ccl9, cxcl9, il6, il15, peli1, tnfsf4* and *socs1* and down-regulation of *ccl7, cxcl3, cxcl5, cxcr4, fzd6* and *itgax*. *K. pneumoniae*-infected wild-type and lincRNA-EPS$^{-/-}$ macrophages exhibited similar levels of TNF-a and bacterial 16S RNA. Compared to wild-type animals, pulmonary *K. pneumoniae* infection of lincRNA-EPS$^{-/-}$ mice resulted in higher levels of serum and lung IL-6 and lung CCL5, whereas lung TNF-a levels were not affected. Finally, we showed that lincRNA-EPS and lincRNA-Cox2 are not required for LPS tolerance induction in macrophages. Thus, lincRNA-EPS and lincRNA-Cox2 shape macrophage-elicited cytokine induction upon exposure to microbial agonists and regulate host responses to *K. pneumoniae*.

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Pellino-1 Promotes Toll-like Receptor-dependent Responses to *Mycobacterium Smegmatis*

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The E3 ubiquitin ligase Pellino-1 elicits K48- or K63-linked ubiquitination of protein substrates, initiating proteasomal degradation or promoting signaling, respectively. While studies with LPS uncovered Pellino-1 as a positive TLR regulator, the role of Pellino-1 in controlling host responses to intracellular bacteria has been unknown. Using Pellino-1 knockout (KO) mice, we have examined cytokine and microbicidal responses of macrophages to *Mycobacterium smegmatis,* and determined the impact of Pellino-1 on manifestations of endotoxemia and infection with *M. smegmatis*. While control macrophages harboring "floxed" Pellino-1 (Pellino-1fl/fl) responded to *M. smegmatis* by robust induction of TNF-α, IL-6, IL-1β and CCL5, *M. smegmatis*-infected Pellino-1 KO macrophages exhibited impaired cytokine production, despite similar bacterial burdens at the start of infection. At later times post infection, Pellino-1 KO macrophages displayed reduced induction of NO and increased bacterial burdens. Furthermore, Pellino-1 KO macrophages stimulated with LPS or infected with *M. smegmatis* had impaired K63-linked ubiquitination and activation of IRAK1, TBK1, and reduced phosphorylation of MAPK and p65 NF-κB. When subjected to endotoxic shock, Pellino-1 KO mice had reduced serum TNF-α and IL-6 and showed increased survival. Systemic infection of Pellino-1 KO mice with *M. smegmatis* yielded decreased

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levels of CXCL1 and IL-6 in the serum, spleen and liver but led to increased bacterial burdens in these organs. Thus, Pellino-1 promotes MyD88- and TRIF-dependent cytokine and microbicidal responses to *M. smegmatis* by increasing K63-linked polyubiquitination of IRAK1 and TBK1, and regulates manifestations of endotoxemia and systemic infection with *M. smegmatis*.

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Toll-like Receptor-10 Is a Novel Regulator of Immune Responses in Human Plasmacytoid Dendritic Cells

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Plasmacytoid dendritic cells (pDCs) are the most potent producers of type-I interferons (IFNs) in humans. This property of pDCs renders them double-edged swords as they have protective effects as well as play major deleterious roles in the pathogenesis of IFN-dependent autoimmune diseases. Toll-like receptors (TLRs) are the most thoroughly studied group of pattern-recognition receptors (PRRs) that play a central role in innate immunity. TLRs help host immune cells to recognize pathogen-associated molecular patterns (PAMPs) and activate a plethora of innate responses including innate cell activation and cytokine production. 13 mammalian TLRs have been recognized thus far: 10 are expressed in humans and 12 in mice. Among them, TLR10 is the only TLR family member without a known ligand and clearly defined functions. One major impediment to studying TLR10 is its absence in mice. A recent study on TLR10 knock-in mice demonstrated its intrinsic inhibitory role in B cells, indicating that TLR10 is a potential drug target in autoimmune diseases. Here, we have interrogated the expression of TLR10 in human pDCs, how TLR10 expression is modulated, and how antibody-mediated engagement of TLR10 might affect the function of pDCs. We observed that primary human pDCs, B cells, and monocytes constitutively express TLR10. Treatment with different viral stimuli failed to alter TLR10 expression on pDCs. Upon pre-incubation with an anti-TLR10 antibody, production of cytokines in pDC including interferon-α, tumor necrosis factor-α, interleukin-6, and interferon-λ was downregulated in response to stimulation with DNA and RNA viruses. Similar effects were seen in monocytes. Expression of co-stimulatory markers on pDCs including CD40, CD80, CD83, and CD86 was not modulated by TLR10 engagement. TLR10 engagement did not inhibit uptake of viruses by pDCs, suggesting that the inhibitory effect is molecular rather than mechanistic. Upon further investigation into the possible mechanism, we documented phosphorylation of signal transducer and activator of transcription 3 (STAT3) upon antibody-mediated engagement of TLR10. This lead to induction of inhibitory molecule suppressor of cytokine signaling 3 (SOCS3) expression; in contrast SOCS1 and SOCS5 (which are not dependent on STAT3) were not induced. We also documented the inhibition of nuclear translocation of transcription factor interferon regulatory factor 7 (IRF7) in pDCs following TLR10 engagement. Our data provide the first evidence that TLR10 is constitutively expressed on the surface of human pDCs and works as a regulator of their innate response. Our findings suggest the potential of harnessing the function of pDCs by antibody-mediated targeting of TLR10 that may open a new therapeutic avenue for autoimmune disorders.

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TLR8-dependent Innate Recognition and Inflammasome Activation by Methanogenic Archaea in Human Phagocytes

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The importance of the microbiota on health and immune homeostasis is widely accepted and the interaction between the microbiota and our body is currently investigated. Although viruses, fungi and archaea are part of our microbiota, research is strongly biased towards bacteria. Recently, Archaea have been shown to be present at nearly every part of the body in high numbers, yet, their influence on health and disease is largely unknown. *Methanosphaera stadtmanae*, a methanogenic archaeon associated with the human gut, induces strong inflammatory responses in human phagocytes and some studies indicate that it may play a role in inflammatory bowel diseases and lung hyperresponsiveness. However, the mechanism how this archaeon is sensed by the immune system has not been evaluated until now. This study aims to elucidate the receptors, archaeal structures and signaling pathways that are engaged upon activation of human immune cells by *M. stadtmanae*.

To investigate the inflammatory response upon stimulation with *M. stadtmanae*, we used human monocytes and moDCs as well as the human monocytic cell line BLaER1. ELISA, qPCR, fluorescence microscopy and Western Blot were used to characterize cellular activation. Stable knockout cell lines were generated with CRISPR/Cas9 to specifically identify the receptors that recognize *M. stadtmanae*.

Our study clearly shows that *M. stadtmanae* not only induces secretion of pro-inflammatory cytokines but also highly increases the expression of type I and III interferons in primary human myeloid cells as well as in the monocytic cell line BLaER1. Using the CRISPR/Cas9 system to generate various knockout cell lines, we were able to identify TLR7 and TLR8 as main receptors for recognition of *M. stadtmanae,* in particular its RNA. Moreover, *M. stadtmanae* activates the NLRP3 inflammasome in a TLR8-dependent manner sharing features with the LPS-induced alternative pathway. This yet undescribed activation mechanism is independent of pyroptosomes formation and potassium efflux.

Our study describes for the first time a specific recognition mechanism of an archaeal species by human innate immune cells in detail. As *M. stadtmanae* is suggested to be involved in different inflammatory diseases, our findings might help to understand how archaea may contribute to disease pathogenesis.

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Platelets Influence Regulatory T Cell Proliferation and Activity During Sepsis

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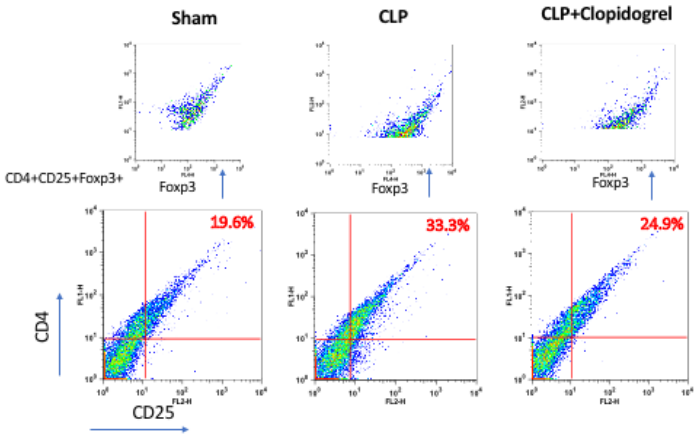
Sepsis, a complex clinical syndrome resulting from a serious infection, is a major healthcare problem associated with high morbidity and mortality. To begin developing an effective pharmacologic therapy for treating sepsis, we investigated the effect of an antiplatelet treatment on the proliferation of regulatory T cells (Tregs) in a murine model of sepsis. Platelets regulate immune responses through direct interactions with immune cells and secretion of inflammation modulators. Tregs are a subset of T lymphocytes that downregulate the body's immune response and promote the resolution of inflammation. Septic patients have been found to have elevated levels of circulating Tregs, and this increased prevalence has been directly associated with increased mortality. Previous studies have shown that platelets can influence the proliferation and activation of Tregs *in vitro*, but the influence of platelets on Tregs *in vivo* has not been fully investigated. We propose that suppression of platelet functions during sepsis can reduce Treg proliferation, possibly leading to a favorable reduction in immune suppression and restoration of immunological homeostasis. To investigate the influence of platelets on Treg proliferation in septic mice, we blocked the $P2Y_{12}$signaling pathway of platelets and measured the resulting population sizes of Tregs. $P2Y_{12}$ is a $G_i$protein-coupled purinergic receptor displayed on platelet surfaces. Stimulation of $P2Y_{12}$ by ADP binding leads to platelet aggregation and potentiation of platelet secretion. To block the $P2Y_{12}$ signaling pathway, we used clopidogrel, a $P2Y_{12}$ antagonist. To induce sepsis, we used a standard procedure of cecal ligation and puncture (CLP). We administered clopidogrel to mice orally one day before surgery using a loading dose of 30 mg/kg in PBS and again two hours before surgery using a maintenance dose of 10 mg/kg in PBS. We treated the mice in the negative control group with PBS only. We isolated splenetic cells from mice in three different groups: (1) sham, (2) CLP only, and (3) CLP with clopidogrel treatment. Using annexin V as a marker for apoptosis, we found that approximately 44% of the cells isolated from the septic mice treated with clopidogrel were apoptotic, which is greater than the results for the sham mice (~15%) but significantly decreased compared to the results for the untreated septic mice (~67%) (n = 3, p12signaling pathway alters aggregation of platelets and CD4+ T cells in whole blood. Platelet and CD4+ T cell aggregates will be discriminated by forward and side light scattering and identified by their positive staining for PE-CD41 or FITC-CD4, respectively. Events double positive for FITC and PE will be identified as aggregates of platelets and CD4+ T cells and expressed as a percentage of gated CD4+ T cells. We found that aggregation of platelets and CD4+ T cells was reduced in the samples from the septic mice treated with clopidogrel (15 ±5 %) compared with the samples from the untreated septic mice (38 ±6 %) (n= 3, *p<0.05 untreated CLP vs treated CLP).* Based on our results, we conclude that blockade of the $P2Y_{12}$signaling pathway limits spleen damage, restrains Treg proliferation, and reduces aggregation of platelets and CD4+ T cells in septic mice. Our study indicates that targeting platelets to control Treg proliferation and activity may be a promising strategy for treating sepsis.



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Involvement of RIP2 in the Production of Specialized Pro-resolution Lipid Mediators

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Inflammatory Bowel Diseases (IBD) are chronic, relapsing inflammatory disorders of the gastrointestinal tract which are generally classified into 2 types: Crohn's Disease (CD) and Ulcerative Colitis (UC). Nucleotide Oligomerization Domain 2 (*NOD2*) is the first identified bona fide susceptibility gene for Crohn's Disease (CD), and polymorphisms within *NOD2* continue to be the leading genetic risk-factor for the development of CD. NOD2 is a cytosolic receptor which recognizes muramyl dipeptide (MDP), a breakdown product of bacterial peptidoglycan. Receptor Interacting Serine/Threonine Kinase 2 (RIP2) mediates many of the downstream signals resulting from NOD2 engagement including activation of NF-kB, MAPK, IRF, and autophagy pathways which are important in mediating host defense. Dysregulation of NOD2:RIP2 signaling (decreased and increased) has been associated with the development of various inflammatory disorders, suggesting that, correction of such defects through manipulation of RIP2 activity, may be beneficial. Indeed, genetic loss or pharmacologic inhibition of RIP2 has been demonstrated to alleviate disease in murine models of inflammatory bowel disease, multiple sclerosis, and allergic airway inflammation. Conversely, multiple studies also demonstrate the efficacy of prophylactically promoting NOD2:RIP2 signaling to reduce experimental colitis, bacterial, and viral infections. These paradoxical findings may be reconciled if NOD2/RIP2 engagement induces both a host defense and an immunoregulatory response. In support of this dual role of NOD2:RIP2 signaling, we demonstrate the RIP2-dependent production of pro-resolving lipid mediators during the course of an MDP-induced peritonitis model. Specialized pro-resolving lipid mediators (SPMs) are lipid mediators which are enzymatically derived from polyunsaturated fatty acids (PUFAs). A large body of work has shown the protective effects of SPMs in various inflammatory diseases in both dampening inflammation and promoting resolution or wound healing. Interestingly, while in vivo MDP administration resulted in production of SPMs, in vitro stimulation of M2 polarized THP-1 cells with the same agonist resulted in a dose dependent *decrease* in the production of lipid intermediates in these pathways, which was mirrored by a dose dependent *decrease* in the levels of enzymes which catalyze these reactions. Both in vivo and in vitro effects of MDP stimulation on the levels of lipid mediators were reversed by the RIP2 inhibitor Gefitinib. These data suggest that RIP2 activity is important both in the production of, and in the regulation of SPMs, likely in a temporal and cell type -specific fashion.

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Outsmarting Inflammation: A Novel Role for Glutamate in Th2 Immune Responses

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Our laboratory recently discovered that glutamate signaling through the kainic acid receptor (KAR) leads to increased production of IgE in the human system. This finding is critical because it is one of the first to highlight the importance of this neurotransmitter receptor on immune function. Here we show *in vivo* relevance for this receptor as KAR deficient mice display a reduction in T_H2 immune responses upon challenge with ovalbumin or infection with *Nippostrongylus brasillensis*(*Nb*). Using the house dust mite (HDM) model of allergic airway disease, mice lacking KARs also had significantly abrogated disease as determined by a reduction in airway hyperresponsiveness, and production of mucus, cytokine, and IgE. Given the pronounced phenotype observed in the HDM model, we examined other cell types critical in T_H2 immune responses, namely the mast cell. KAR deficient mice had significantly reduced responses in passive systemic anaphylaxis. Interestingly, these mice have

normal T_H1 immune response upon challenge with ovalbumin in CFA or LCMV infection. Thus, KARs may serve as a novel target in the specific regulation of T_H2 immunity.

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Inhibition of TLR9 Signaling *in Vitro* and *in Vivo* by Blocking TIR Domain Interactions

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TLR TIR dimerization causes recruitment of TLR adapter proteins leading to the formation of a MyDDosome signaling complex that activates IRAKs and nuclear factor-κB. Overactivation of TLRs is implicated in the pathogenesis of inflammatory and autoimmune disorders. TLR9 inhibitors have a significant therapeutic potential due to the involvement of this receptor in the development of several inflammatory diseases. We have screened a library of TLR9-derived cell-permeating decoy peptide and identified new inhibitors of TLR9. The library comprised peptides that represent different surface patches of TLR9 TIR domain. Most effective TLR9 inhibitors, cell-permeating peptide 9R34 and its modification, 9R34-ΔN, were derived from AB loop, *β*-strand B, and N-terminal residues of BB loop of the TLR9 TIR domain. TLR9 signaling was also inhibited by peptides derived from *α*-helices C, D, and E (9R6, 9R9 and 9R11), however with lower efficacy. Cell-based peptide binding experiments revealed that 9R34-ΔN interacted stronger with TLR9 TIR and weaker with TIRAP TIR domains. When tested in mice, 9R34-ΔN inhibited systemic cytokine activation caused by TLR9 agonist ODN 1668 and protected the D-galactosamine pretreated mice against TLR9-induced lethality. Results of the study, considered together with results of previous screenings of TLR peptide libraries, suggest a common mode of TIR domain interactions in the primary TLR signaling complexes. In this mode, TIR domains of TLRs and TLR adapters interact through four structurally homologous regions, leading to the formation of a double-stranded filamentous structure that can elongate either from one or two ends. Individual TIRs within the strands are in a similar orientation rotationally and interact through regions located on nearly opposite surfaces, which are close to the *β*-strands that form two surface exposed edges of the b-sheet, *i.e. β*-strands B and E. Interactions between the strands are through two different sites, one of which is predominantly formed by *α*-helix D and another combines residues of helices B and C.

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Gasdermin D Dependent IL-1β Secretion by Neutrophils Occurs in the Absence of Pyroptosis

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Gasdermin D (GSDMD) in macrophages is cleaved by inflammasome-activated caspase-1 or caspase-11 to generate a 31 kDa N-terminal fragment (N-GSDMD), which oligomerizes in the plasma membrane (PM) to form pores that mediateIL-1β release and pyroptosis. In contrast, inflammasome activation in neutrophils results in caspase-1- dependent IL-1β release that is independent of pyroptotic lysis as measured by LDH release, and does not form PM pores as indicated by the absence of propidium iodide dye uptake. Here we show that under inflammasome activated conditions, neutrophil GSDMD is cleaved to 31 kDa N-terminal fragment by active caspase-1, but N-GSDMD neither localizes to the PM, nor forms functional PM pores. Moreover, GSDMD is required for efficient secretion of mature IL-1β from the neutrophils. In contrast to C57BL/6 neutrophils, $GSDMD^{-/-}$ neutrophils exhibit intracellular accumulation of active caspase-1 and mature IL-1β. These findings reveal that, despite their shared myeloid lineage and the requirement for GSDMD in IL-1β release, neutrophils and macrophages utilize GSDMD in fundamentally different ways and indicate an unconventional secretory

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mechanism for IL-1β in neutrophils which are independent of both non-lytic efflux through GSDMD pores and pyroptotic lysis.

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Pro-Resolving Mediators Produced During Coagulation Activate Human Phagocytes Intracellular Single Cell Signaling in Whole Blood 

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Phagocytes play a pivotal role during the acute inflammatory process by controlling host defense mechanisms, via temporal regulation of classical pro-inflammatory eicosanoids and the production specialized pro-resolving mediators (SPMs) that include resolvins, protecting and maresins. Coagulation is a host protective response to a barrier breach that can occur during infection. Here, we investigate the relationship between coagulation and the resolution of infectious inflammation through using state of the art metabololipidomic-based profiling approach in human whole blood during coagulation. We identified temporal clusters of endogenously produced pro-thrombotic and pro-inflammatory lipid mediators (eicosanoids) as well as specialized pro-resolving mediators (SPMs). In addition to classic eicosanoids, a specific SPM cluster was identified in human whole blood consisting of resolvin E1, resolvin D1, resolvin D5, lipoxin B_4, and maresin 1 each at bioactive [0.1-1.0 nM] concentrations. Using mass cytometry, we present evidence that SPM evoke intracellular signaling pathways during host defense and coagulation at a single cell level using state-of-the-art time of flight mass cytometry (CyTOF) in human leukocytes. Nineteen phenotypic surface makers and eight phosphoepitopes intracellular proteins were analyzed using CyTOF in human whole blood samples incubated with or without SPM (RvE1, RvD1, RvD5, LXB_4 and MaR1) cluster. High dimensional visualization t-Stochastic Neighbor Embedding (viSNE) unsupervised clustering analysis demonstrated that the SPM cluster produced in human whole blood targets leukocytes, directly activating signal transduction pathways at the single-cell level. In $CD15^+$ neutrophils and $CD14^+$ classical monocytes, we found that SPM cluster induces the abundances of phosphorylation of extracellular signal–regulated kinases 1 and 2 (pERK1/2) cyclic adenosine monophosphate response element– binding protein (pCREB), p38 mitogen-activated protein kinase (MAPK), ribosomal protein S6 and serine/threonine-specific protein kinase (AKT) at a single cell level. In addition, we found that RvE1 specifically induces pERK1/2 in $CD16^+$ non-classical monocytes and plasmacytoid dendritic cells (pDC). RvD5 and LXB_4 substantially increased the abundance of pS6 in $CD20^+$ B cells. The SPMs, RvE1, RvD1, RvD5, MaR1, and LXB_4, did not stimulate the phosphorylation of nuclear factor kB (NF-kB) or the signal transducer and activator of transcription (STAT) family members STAT3 and STAT5. In whole blood, this SPM cluster enhances containment and phagocytosis of *Escherichia coli* (*E. coli*) by neutrophils ($CD66b^+$ cells) at concentrations as low as 100 pM. The SPM cluster also enhanced the phagocytosis of by classical monocytes ($CD14^+$ cells) in whole blood at 1 nM as measured by flow cytometry. These studies identify a proresolving circuit, including endogenous molecular brakes and accelerators, which promoted host defense and control resolution mechanisms.

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The Actin-bundling Protein L-plastin Regulates Alveolar Macrophage Pro-inflammatory Signaling

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Alveolar macrophages (AMs), unique lung-resident phagocytes, maintain the balance between pro-inflammatory signals essential to pathogen clearance and anti-inflammatory signals key to maintaining gas exchange. Prior research shows that mechanical stretch induces macrophage production of the soluble pro-inflammatory cytokine IL-1β. However, the mechanisms by which mechanical forces stimulate IL-1β are unknown. Because lung stiffness varies in disease (*e.g.*, lungs stiffen in the highly inflammatory condition, acute respiratory distress syndrome (ARDS)), understanding how substrate stiffness regulates AM IL-1β production will support creation of immunomodulatory therapies to treat pulmonary inflammation. Here, we present preliminary data suggesting the actin-bundling protein, L-plastin (LPL), links AM mechanosensation to NLRP3 inflammasome activation. LPL is a hematopoietic-specific member of the a-actinin family, localizes to macrophage podosomes, and is essential for pulmonary immunity. Mice lacking LPL are highly susceptible to pulmonary infection with pneumococcus. Susceptibility to infection correlates with impaired development of AMs and with a failure to upregulate IL-1β production. Pneumococcus activates IL-1β production primarily through the NLRP3 inflammasome. We show that LPL is required for IL-1β production following NLRP3 inflammasome activation *in vitro*. LPL is also required for the formation of podosomes, integrin-mediated, mechanosignaling adhesive organelles that support macrophage adhesion and motility. In the absence of LPL, podosomes are poorly formed and macrophages fail to respond normally to changes in substrate stiffness, indicating defective mechanosensation. To test if mechanotransduction directly regulates NLRP3, we cultured macrophages upon collagen-coated, functionalized polyacrylamide gels, and found that the amount of IL-1β produced by wild-type macrophages is directly proportional to substrate stiffness. We found minimal NLRP3-induced IL-1β production by LPL-deficient macrophages on all substrates, regardless of stiffness. Preliminary data suggests that LPL regulates NLRP3 inflammasome assembly, not priming. We are now substantiating these findings by testing if LPL and/or mechanotransduction regulate the NLRC4 and AIM2 inflammasomes in addition to the NLRP3 inflammasome, and by determining if mechanotransduction regulates the first (prime), second (assembly), or both steps of NLRP3 inflammasome activation. Defining the role of LPL in alveolar macrophage mechanotransduction and NLRP3 inflammasome activation will provide insight into a potentially therapeutically targetable pathway to ameliorate pulmonary conditions such as ARDS.

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Extracellular Vesicles in Oral Squamous Carcinoma Carry Oncogenic miRNA Profile and Reprogramme Monocytes via NF-kB Pathway

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Extracellular vesicles (EVs) are carriers of different biomacromolecules that participate in cellular signaling and disease pathogenesis. Although it has been shown that EVs can play an active role in cellular communication and different stages of cancer progression, the role of EVs in oral squamous cell carcinoma (OSCC) cancer pathogenesis, especially in the cross-talk of cancer cells with immune cells is unknown. Here, we present a detailed analysis of findings regarding the profile of EVs in OSCC and the role of EVs and associated miRNAs in the cross-talk of malignant cells with monocytes. We demonstrate that EVs are detectable in significantly higher quantities in the plasma of patients with OSCC. Oncomir miRNAs (such as miR-21, miR-27) were detectable in high quantities in the circulating EVs and plasma of patients with OSCC. EVs isolated from the circulation of OSCC patients and

OSCC cell lines showed comparable miRNA signature, indicating the tumor origin of EVs in the circulation of patients with OSCC. Danger signals such as LPS and ethanol increased the production of EVs. EVs were taken up by monocytes after co-culture. Mechanistically, uptake of EVs derived from oral cancer cells by monocytes caused activation of the inflammatory pathway, NF-κB activation, and establishment of a pro-inflammatory and pro-tumorigenic milieu marked by increased levels of IL6, CCL2, PEG2 and MMP9 levels. Series of experiments involving the introduction of exogenous oncogenic miR-21 mimic induced a similar pro-inflammatory and pro-tumorigenic profile in monocytes, indicating the role of EV-associated miR-21 in modulating the immune response in monocytes.

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Epigenetic Underpinnings of Dysregulated Inflammatory Monocyte-Macrophage Responses Following Chronic Heavy Ethanol Consumption

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Chronic heavy drinking (HD) significantly increases the risks for infection and delays wound healing. This increased susceptibility is believed to be mediated by dysregulation in primarily innate immunity. While a large body of work documented ethanol-associated alterations in functional responses of innate immune cells such as monocytes and macrophages, the epigenetic mechanisms that underlie these changes are less understood. Using a rhesus macaque model of voluntary ethanol self-administration and RNA-Seq, we recently reported that heavy drinking (HD) exerts its most significant impact on innate immune cells, affecting coagulation and wound healing pathways. Furthermore, ethanol consumption is associated with exaggerated ex vivo lipopolysaccharide (LPS) responses. To better understand changes within this lineage, we isolated splenic macrophages and examined their response to LPS. Our analyses revealed that HD is associated with significant differences in Toll-like Receptor (TLR) signaling signatures, with elevated gene expression of *TLR2*, *TLR4*, and chemokine receptor *CCR1*. When challenged with LPS for 16 hours, these cells secreted higher levels of pro-inflammatory cytokines IL-6, IL-1b, and neutrophil chemoattractant IL-8. This hyper-inflammatory phenotype was further evident at the transcriptional level, where cells from heavy drinking group persisted in a pro-inflammatory phase when cells from control animals had transitioned to a more regulatory phase. To uncover the epigenetic changes that underlie these hyper-inflammatory responses, we measured HD associated alterations in chromatin accessibility in resting splenic macrophages using an assay for transposase-accessible chromatin followed by sequencing (ATAC-Seq). Our analyses suggest ethanol-associated differences in chromatin landscape of macrophages promoters overlapping pathways of innate immune responses such as positive regulation of NF-kB signaling and stress-activated MAPK cascade. Transcription factor footprinting analyses revealed ethanol-associated increases in open-chromatin regions in the macrophages harboring binding sites for HIF1a, a hypoxia-induced transcription factor. Interestingly, HIF-1a functions as a metabolic sensor, activating glycolytic genes essential for LPS responses and several studies have implicated HIF-1a in mediating ethanol-induced oxidative stress and liver injury. These data suggest the potential role of epigenetic priming particularly, at LPS responsive sites of the genome. To establish downstream mechanisms to test this hypothesis, we are currently characterizing the metabolic status of these cells and examining the link between oxidative stress and the ability of cells to undergo an early glycolytic switch. Additionally, we are investigating the genome binding profiles of HIF-1a and promoter-specific histone modifications such as H3K4me3 and H3K9Ac in order to gain a comprehensive understanding of the molecular mechanisms that explain innate immune dysregulation associated with chronic ethanol consumption.

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The Effect of Advanced Age and Alcohol Consumption on Alveolar Macrophage Response to *Streptococcus Pneumoniae*

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The world's population is aging: the global proportion of individuals aged > 65 years (termed "elderly") is expected to double by 2050. An increasingly common behavior in the elderly is alcohol consumption, with 40% consuming an average of 1-2 drinks/day 3 days a week. Even though the elderly typically drink less than younger individuals do, the negative health effects of alcohol may be more potent in older drinkers due to slower metabolism. The elderly also experience "inflamm-aging," or low-grade systemic inflammation present even in otherwise healthy individuals. Similarly, alcohol use correlates with heightened systemic inflammation and reduced cell-mediated immunity, which may contribute to the increased incidence and severity of infection with *Streptococcus pneumoniae* seen in the elderly. Surprisingly, the combined effect of alcohol and advanced age on the immune response to infection have yet to be investigated. To test this, we gave young and aged mice ethanol by oral gavage for 3 days (dose of 1.0-1.25 g/kg ethanol; designed to raise blood alcohol to 60-80 mg/dL at 30 min). One hour after the last gavage, we isolated alveolar macrophages by brochoalveolar lavage and stimulated the cells overnight with inactivated *S. pneumoniae* at a multiplicity of infection (MOI) of 20. Our results indicate that after stimulation, alveolar macrophages from aged vehicle-treated mice had significantly lower expression of proinflammatory cytokines *Tnf-a* and *Il-6* compared to young mice given vehicle (3.0-fold reduction [$p<0.0001$] and 3.5-fold reduction [$p=0.03$], respectively), and these levels were increased when comparing aged mice given vehicle vs. ethanol (2.1-fold increase [$p=0.0034$] and 5.1-fold increase [$p=0.0001$], respectively). In parallel, we observed the highest amount of secreted TNF-α from stimulated cells isolated from aged ethanol-treated animals (64% increase vs. young animals given ethanol [$p=0.02$] and 51% increase vs. young animals given vehicle [$p=0.09$]). A similar effect was seen with neutrophil chemokine gene expression in stimulated macrophages, where *Cxcl1* and *Cxcl2* were decreased in aged vs. young vehicle-treated mice (2.0- and 2.4-fold reduction, respectively; $p<0.0001$), but expression was significantly increased when comparing aged animals given vehicle and those given ethanol (2.8- and 3.6-fold increase, respectively; $p<0.0001$). Conversely, we found no significant difference in gene expression of monocyte chemoattractant *Ccl2* between macrophages isolated from vehicle-treated young and aged mice after *ex vivo* stimulation. However, *in vivo* ethanol exposure increased *Ccl2* expression in cells from young mice by 1.5-fold and from aged mice by 3.3-fold ($p<0.0001$). Previous studies in our laboratory found that macrophages from young mice given a single, higher dose of ethanol *in vivo* produced significantly lower levels of IL-6 and TNF-α following *ex vivo* stimulation compared to vehicle-treated mice; in contrast, the current studies did not reveal a similar effect, indicating that the duration and dosage of the ethanol treatment influence the immune response upon subsequent challenge. Despite the differences in cytokine and chemokine expression after *ex vivo* stimulation, we found no difference in lung bacterial burden between treatment groups after a 24-hour *in vivo* infection. Future experiments aim to investigate cytokine and chemokine variations at multiple time points post-infection and determine the mechanism(s) leading to dysregulated immune responses associated with alcohol and advanced age following infection. Supported in part by NIH R01 GM115257 (EJK), R21 AA023193 (EJK) and R01 AG018859 (EJK).

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JAK3 Restrains *Porphyromonas Gingivalis*-Induced Inflammatory Responses Through Ubiquitination-mediated Wnt3a Signaling

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Objectives: *P. gingivalis*-mediated disruption of innate homeostasis is considered a driving force of chronic periodontitis. Unlike pro-inflammatory events, anti-inflammatory processes that occur in response to *P. gingivalis* infection are poorly understood. Herein, we investigated the anti-inflammatory role of JAK3, a cytokine-activated cytosolic tyrosine kinase, in the regulation of *P. gingivalis*-induced oral inflammation.

Materials and Methods: *P. gingivalis* 33277 was used to infect C57BL/6 mice and stimulate human monocytes from healthy donors. Cytokine production was measured by ELISA. Phosphorylation and ubiquitination of signaling molecules were determined by western blotting and immunoprecipitation. The expression of Wnt3a and Dvl3 in gingival tissue was tested by immunohistochemistry. Pharmaceutical inhibition, siRNA, exogenous DNA constructs were employed to inhibit or promote the activity of target molecules. CEJ-ABC distance was measured to assess alveolar bone loss in mouse infection studies.

Results: *P. gingivalis* induced phospho-activation of JAK3, leading to the phospho-inactivation of Nedd4-2, an E3 ubiquitin ligase, and increased Wnt3a expression. Inhibition of JAK3 enhanced K48-linked ubiquitination of Wnt3a in MG-132 treated human monocytes. Moreover, inhibition of JAK3, Wnt3a or Nedd4-2 robustly enhanced the activity of NF-kB and the production of pro-inflammatory cytokines upon *P. gingivalis* stimulation. Downstream molecules of Wnt3a signaling including Dvl3 and GSK3-b were also modified by JAK3 and negatively regulated pro-inflammatory cytokine release upon *P. gingivalis* challenge. *In vivo*, inhibition of JAK3 enhanced the infiltration of inflammatory cells, reduced the expression of Wnt3a and Dvl3 in gingival tissues and aggravated the severity of bone resorption in mice orally infected with *P. gingivalis*.

Conclusions: JAK3 restrains the pro-inflammatory response to *P. gingivalis in vitro* and *in vivo* through ubiquitination-dependent expression of Wnt3a and its downstream Dvl3-GSK3-b signaling axis.

Clinical Significance: JAK3-mediated anti-inflammatory mechanisms facilitate innate homeostasis. Further elucidation of this anti-inflammatory network may identify novel therapeutic targets for the control of periodontitis and, perhaps, other chronic inflammatory diseases.

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Leukocyte Gene Expression of Dairy Cattle in Response to Flooring Type and Additional Substance P

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Cows on concrete flooring have classical immunological indicators of chronic pain compared with those housed on rubber flooring; increased peripheral blood mononuclear cell numbers, and more leukocyte RNA expression of IL-1β and less expression of IL-1Ra. Previous results showed that the leukocyte populations and their phenotypes that enable functions (CD18 and CD14) are altered by concrete flooring and that responses to additional substance

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P (a neurotransmitter associated with pain; SP) are altered in cows under those conditions. We then hypothesized that certain gene expressions may be modulated by the flooring, day of the study, and additional SP. The model was that 1st-calf heifers were assigned to rubber or concrete flooring. On day -3, 7, 21, 45 and 90 of the 2nd lactation, 5-part differential counts of whole blood and buffy coat leukocyte marker expressions were analyzed. Buffy coat cells were further stimulated with SP in vitro. We evaluated RNA expression of 2 cell populations (adherent or those in the supernatant) that were from cows on the concrete or rubber flooring that has been incubated with or without additional SP. Cells for RNA analysis were incubated in 6-well plates for 30 min at 37° C with 5% CO_2. Cells in the supernatant were removed and treated as lymphocytes. The remaining cells were scraped into additional RPMI and considered the monocyte/macrophage population. The 4 treatments were lymphocyte +/- SP and monocytes +/- SP; (L, LP, M, MP). We used a Trizol extraction protocol, amplified cDNA, and tested gene expression of GRK (known to be modified with chronic pain and an indicator of the switch to chronic pain), and Toll Like Receptors (TLR) 2, 4, and 5 (to determine if recognition of pathogens may be attenuated with chronic pain or additional SP) using Taqman gene expression assays with 18S as the internal standard. We only used RNA extracted from d 7 and 21 from the second lactation, since that is where we saw the greatest difference in cell phenotype and differential counts. Expression of TLR2 was not different on d 7 or between d 7 and 21, but on d 21 TLR2 was less ($P \leq 0.05$) for LP than for either of the monocyte treatments (M or MP). Additionally, TLR2 expression of L was less ($P = 0.03$) than MP on that day. No other treatment differences were detected for TLR4, TLR5, or GRK. However, a day effect ($P = 0.01$) was found for GRK, such that all treatments expressed less GRK on d 21 than on d 7. These data suggest that the older rubber flooring in this study may not be having as great an effect as we saw in the first study with the new flooring. But, the data still showed some modulation by SP for lymphocytes and by time after calving in the second lactation. These results suggest that rubber flooring may need to be monitored for resilience to maintain the advantages gained by its use.

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Chronic Super-low Dose lipopolysaccharide Exposure skews Macrophages to a Non-resolving Low-grade Inflammatory State Through Simultaneous Upregulation and Suppression of Pro-inflammatory and Homeostatic Mediators

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Chronic inflammation has been associated with a variety of highly prevalent diseases such as atherosclerosis, cancer, and neurodegenerative diseases. Recently, a phenomenon known as subclinical endotoxemia has been associated with many patients with these diseases and is defined as a low level (subclinical) of bacterial endotoxin or lipopolysaccharide (LPS) found in the circulation. Since LPS is a well-known immmunostimulatory agent with high doses in the circulation resulting in the severe disease sepsis, it stands to reason that this low level of LPS may be contributing to the propagation of these chronic inflammatory diseases. However, the underlying connection is still unclear. In the past, we have published data showing that chronic exposure to super-low levels of LPS (100 pg/mL) resulted in exacerbated atherosclerosis in $ApoE^{-/-}$ mice and impaired wound healing in a mouse model, and that this effect was predominantly mediated by polarized inflammatory macrophages. However, the underlying molecular mechanism behind these effects still remains elusive. In the present study, we found that chronic exposure to super-low dose endotoxin resulted in upregulation of inflammatory markers such as MCP-1 and p62 accompanied by the simultaneous downregulation of anti-inflammatory/homeostatic mediators such as Nrf2 and FPN. We also found that addition of the homeostatic modulator sodium phenylbutyrate (also known as 4-PBA) was able to restore the effects of chronic super-low dose LPS in mouse macrophages. This study presents a novel view of the polarization of macrophages by chronic super-low dose LPS exposure showing both the upregulation of an inflammatory arm and the simultaneous repression of an anti-inflammatory/homeostatic arm in one unified system. Further, we show a novel use of 4-PBA in restoring the targets Nrf2 and FPN, while reducing the expression of the inflammatory markers MCP-1 and p62.

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KIAA0599 Augments TRIF- and Myd88-dependent Toll-like Receptor Signaling

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Introduction: KIAA0599 (also known as PLEKHG3) is a Rho family small GTPase (Rho)-specific guanine nucleotide exchange factor (GEF). KIAA0599 has been reported to regulate cell polarity and cell motility via activation of Rho GTPases, Rac1 and Cdc42, under the control of PI3K signaling pathway. Although Rho GTPases, especially Rac1 and Cdc42, contribute to chemotaxis of immune cells, little is known about the crosstalk between signalings of TLRs and RhoGEFs/Rho GTPases. Here, we investigated the effect of KIAA0599 on TLR signaling and further revealed the mechanism of enhancement of the Toll/Interleukin-1 receptor domain-containing adaptor inducing interferon (IFN)-β (TRIF)-dependent pathway of TLR signaling.

Materials & Methods: HEK293 cells were transfected with nclear factor (NF)-κB-, IFN-β promoter- or PRDIII-I promoter-dependent luciferase reporter plasmid and the plasmids expressing KIAA0599 and/or other signaling molecules. TLR signal was activated by over-expression of Myeloid differentiation factor 88 (MyD88) or TRIF. After 24 h incubation, the cells were lysed and luciferase activities were determined.

Results: Expression of KIAA0599 enhanced TRIF-mediated IFN-β promoter activation and MyD88-mediated NF-κB activation, whereas KIAA0599 alone did not activate NF-κB or IFN-β promoter. KIAA0599 also enhanced TRIF-mediated PRDIII-I promoter activation, which is predominantly activated by IFN regulatory factor (IRF)-3, but not by NF-κB. KIAA0599 also enhanced IFN-β promoter activation induced by TBK1 (tumor necrosis factor receptor-associated factor family member-associated NF-κB activator (TANK)-binding kinase 1) and by IKKi (inhibitor of NF-κB kinase i). Moreover, the expression of KIAA0599 enhanced IFN-β promoter activation induced by over-expression of 5D-IRF-3, a constitutive active form of IRF-3. Since KIAA0599 is activates Rho GTPases, we further examined the effect of dominant-negative form of Rho GTPases. The enhancement of TRIF-dependent IFN-β promoter activation by KIAA0599 was not affected by the expression of dominant-negative form of Rac1, Cdc42 or RhoA.

Conclusion: We revealed that KIAA0599 upregulates both TRIF- and Myd88-dependent pathway of TLR signaling. Previously we have shown that ARHGEF15 enhances TRIF-mediated IFN-β promoter activation via increasing IRF-3 phosphorylation in dependent on RhoA activation. KIAA0599, however, enhanced it independent of Rac1, Cdc42 or RhoA small GTPases. Since KIAA0599 enhanced 5D-IRF-3-mediated promoter activity, KIAA0599 may not contribute to the IRF-3 phosphorylation. These data suggest that KIAA0599 up-regulates TLR signaling via novel mechanism other than that by ARHGEF15. KIAA0599 was reported to localize to the leading edge of migrating cells. Contribution of KIAA0599 to TLR-signaling may regulate spatio-temporal activity of TLR-signaling in innate immune cells.

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Mycobacterium Tuberculosis Lipoarabinomannan Activates Neutrophils via TLR2/1 and Is Distinct from Pam_3CSK_4

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Tuberculosis infects a quarter of the world's population and is the ninth leading cause of death with extensive global public health cost. Neutrophils (PMN) are the most numerous leukocytes in the peripheral blood and are often the first responders to sites of infection and trauma. PMNs play an important role in the early innate immune response to *Mycobacterium tuberculosis* infection in the lung. Interactions between PMNs and mycobacterial lipids impact the activation state of these migrated cells with consequences for the surrounding tissue in terms of resolution versus ongoing inflammation. PMNs sense pathogens via pattern recognition receptors (PRR) on the cell surface resulting in signal transduction and pro- and anti-inflammatory cellular responses. Ideally, these responses are well regulated and robust enough to extinguish the threat quickly without causing excessive damage to host tissues. Toll-like receptors (TLR) are PRRs that recognize pathogen associated and danger associated molecular patterns (PAMPs and DAMPs). TLR2 forms a heterodimer with either TLR1 or TLR6 and each complex recognizes distinct ligands. *M. tuberculosis* is known to engage TLR2 but the specificity for TLR2/1 versus TLR2/6 is not clearly defined as many studies have been performed in TLR2 knockout animals. We hypothesized that lipoarabinomannan from *M. tuberculosis* (*Mtb*.LAM), a known virulence factor, would prime PMNs in a TLR2/1-dependent manner, and investigated this with specific comparison to purified synthetic TLR2 agonists, FSL-1 and Pam_3CSK_4. In contrast to previous work, we found that stimulation with *Mtb*.LAM did not induce priming of the PMN respiratory burst or rapid mobilization of intracellular granules. However, exposure of PMNs to *Mtb*.LAM did elicit pro- and anti-inflammatory cytokine release that was profoundly impacted by a common SNP in TLR1. Moreover, *Mtb*.LAM did not elicit endocytosis, phosphorylation of p38 MAPK, priming of elastase secretion, L-selectin shedding, or secretory vesicle mobilization, although all processes occurred with Pam_3CSK_4 stimulation. Proteomic analysis confirmed that stimulation with Pam_3CSK_4 versus *Mtb*. LAM elicited distinct patterns of protein activation and mobilization. In summary, *Mtb.* LAM activates PMNs in a TLR2/1-dependent manner that is distinct from Pam_3CSK_4. We speculate that the failure of *Mtb*.LAM to prime PMNs allows for a delay in host detection and expansion of the granuloma.

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Elevated Circulating Serotonin (%HT) in Sickle Cell Anemia May Play a Role in Inducing $CXCR4^{HI}$ Effector Cell Neutrophil Generation

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Introduction: Sickle cell anemia (SCA), a hereditary disease of the red blood cells, is associated with a chronic inflammatory state, resulting from constant intravascular hemolysis and ischemia-reperfusion processes. Neutrophil activation and recruitment to the vascular endothelium plays a fundamental role in the vaso-occlusive processes that characterize the disease. However, neutrophil populations are not homogeneous and shifts in their effector functions suggest that peripheral instructor signals act may modulate the differentiation and activity of these cells. Immunomodulatory effects of the bioactive amine, serotonin (5-Hydroxytryptamine, 5-HT), have recently been described, due to its action on myeloid and lymphoid leukocytes. 5-HT is stored by platelets in their

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dense granules and secreted back into the plasma following platelet activation. In the present study, we investigated a role for platelet-derived serotonin in the modulation of the CXCR4^{hi} neutrophil population in SCA.

Methods: Peripheral blood samples were collected from steady-state SCA patients not on hydroxyurea (HU) therapy (SCA patients), SCA on HU (SCAHU) and healthy control (CON) individuals. Leukocyte morphology and cell surface expression were investigated by imaging flow cytometry (Amnis Imagestream Mark II), using IDEAS software analysis, using anti-CD41a for platelet identification and using anti-CD66b antibodies for the identification of the neutrophil population and anti-CXCR4. Plasma and serum 5-HT levels were determined by ELISA.

Results: Plasma serotonin levels were found to be significantly higher in SCA patients (n=9, 203.8±16.4ng/mL) than in CON and SCAHU individuals (n= 8, 129±8.7 ng/mL and n= 7, 145.4±55 ng/mL; respectively, P0.05). At the same time, the frequency of CXCR4^{hi} neutrophils was greater in the peripheral blood of SCA than in and SCAHU CON individuals (n=6, 9,9%±1.5, 6.9%±1.6 and 4.26%±0,37, respectively; p< 0.005) and a positive correlation was found between plasma levels of 5-HT and the percentage of neutrophils CXCR4^{hi} present in peripheral blood (Pearson r = 0.7220; Pin vitro with 5-HT for 4 hours, concentrations of 250ng/ml and 300ng/ml 5-HT significantly increased the percentages of CXCR4^{hi} neutrophils (30%, Phi neutrophils also presented greater aggregate formation with platelets, and displayed a higher density of platelets aggregated (MFI) to them (79.02%±4.0 and 58470±9274), when compared to the other neutrophils (60%±6.2 and 19840±1627). Furthermore, the addition of a non-selective inhibitor of 5HTRs was able to reverse such an effect.

Conclusion: Taken together, results indicate that elevated 5-HT release in SCA, due to platelet activation, may play a role in modulating the phenotype of the neutrophil population in these patients, with potentially important implications on leukocyte function. 5-HT receptor antagonists may also represent a novel drug target worthy of further investigation for SCA. **Financial support**: FAPESP

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The Contribution from interleukin-27 Towards Rheumatoid Inflammation

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Background/Purpose: Rheumatoid arthritis (RA) is characterized by autoimmune-mediated destruction of joint synovial tissue and the development of extra-articular manifestations, including subcutaneous rheumatoid nodules. In this study we completed a comprehensive analysis of rheumatoid tissues. The objectives of this study were to document and compare IL27 and IL27R gene expression in subcutaneous rheumatoid nodule tissue with that in RA and OA synovial tissues, identifying the extent of expression and the cells responsible and provide further insight into the mechanism of IL27 expression in the different forms of rheumatoid inflammation.

Methods: Synovial tissue was obtained from patients with RA (n=30) or Osteoarthritis (OA; n=10) receiving joint replacement surgery. Subcutaneous nodule tissue (n= 22) was obtained following elective surgery. Included were "paired" rheumatoid synovial and nodule tissues from the same patient, obtained at the same times or at different times. Duplex digital PCR assays (Applied Biosystems) were used to determine absolute gene expression, including for IL27A, IL27RA, IL27RB, within both synovial and nodule samples. Differences in gene expression between tissues were determined by Mann Whitney U tests and Spearman correlations were used to establish relationships between gene expression. RNAscope was used to assess the expression of IL27A and IL27R A *in situ* and association with inflammatory cells.

Results: From digital PCR, the expression of IL27A was significantly higher (~3-fold) in rheumatoid subcutaneous nodules when compared to RA synovia (p=0.005). Both rheumatoid nodules (p+monocyte/macrophages in the synovial lining and occasional T- and B-lymphocytes in sub-lining locations. Rare observations of IL27A and IL27RA expression occur in these sub-lining locations, associated with lymphocytes. In nodules IL27A expression is maintained within cells reaching the outer layers of the palisade layer. However, IL27RA expression is more obvious in perivascular areas.

Conclusions: Localized gene expression, sufficient for the production of biologically active, heterodimeric interleukin-27 cytokine (IL-27), occurs within affected rheumatoid joint synovium and extra-articular nodule tissues. The distribution of IL27A expression is consistent with production from monocyte/macrophages and dendritic cells. Expression of IL27RA suggests that infiltrating T- and B-lymphocytes remain capable of responding to localized IL-27 production. Interestingly, we find the expression of both genes persists within lymphoid aggregates in rheumatoid synovia. Significant differences in the expression of IL27A and IL27RA between RA and OA synovia reflect the low inflammatory activity in the latter tissues despite easily identified monocyte/macrophages. Our data raise the question of whether functions attributable to IL-30 -the IL27p28 subunit of IL-27 alone - are possible as part of rheumatoid inflammation.

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Mechanoregulation of Macrophage Lipid Accumulation

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Tissue mechanics vary dramatically amongst tissues and in different disease states. For example, in atherosclerosis the area overlying plaques become stiff while the necrotic core becomes soft. Macrophages are major mediators of plaque development and other studies show that changes in arterial stiffness precede visible plaque development. We previously showed that toll-like receptor (TLRs)-induced macrophage cytokine production was increased as growth surface stiffness decreased. Thus, we hypothesized that changes in stiffness may alter macrophage lipid uptake and be a potential pathogenic mechanism to therapeutically target. Here we show that growth mechanics regulate expression of key receptors for accumulation of low-density lipoproteins (LDLs). While expression of scavenger receptor B1 and CD36 increased with increasing growth surface stiffness, Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) decreased. Expression of LDL export proteins was unaffected. Uptake of both acetylated and oxidized LDL was enhanced in macrophages attached to stiffer surfaces. Unlike our previous findings that ROCK was critical for mechanoregulation of TLR-induced cytokine production, inhibitors of ROCK did not affect LDL accumulation. Instead, a non-traditional mechanosensitive protein/ion channel regulated macrophage LDL accumulation. Thus, important macrophage functions are precisely regulated by physical cues provided by through cell sensation of microenvironmental mechanics. These studies have implications in the regulation of macrophage function in diseased tissues, such as cancer, fibrosis, and atherosclerosis, and offer novel pharmacological targets for the manipulation of macrophage function in vivo.

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Leukocytes in Immune Privilege Sites

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The Early Expansion of Anergic NKG2A^{pos}/CD56^{dim}/CD16^{neg} NK Represents a Therapeutic Target in Haploidentical Haematopoietic Stem Cell Transplantation

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Natural Killer cells are the first lymphocyte population to reconstitute early after non myelo-ablative and T cell-replete haploidentical hematopoietic stem cell transplantation with post-transplant infusion of cyclophosphamide. The present study characterizes the transient and predominant expansion starting from the 2nd week after haploidentical hematopoietic stem cell transplantation of a donor-derived unconventional subset of NKp46$^{neg-low}$/CD56^{dim}/CD16^{neg} natural killer cells expressing remarkable high levels of CD94/NKG2A. Both transcription and phenotypic profiles indicated that unconventional NKp46$^{neg-low}$/CD56^{dim}/CD16^{neg} natural killer cells are a distinct natural killer cell subpopulation with features of late stage differentiation, yet retaining proliferative capability and functional plasticity to generate conventional NKp46^{pos}/CD56^{bright}/CD16^{pos} natural killer cells in response to interleukin-15 plus interleukin-18. While present at low frequency in healthy donors, unconventional NKp46$^{neg-low}$/CD56^{dim}/CD16^{neg} natural killer cells are greatly expanded in the following 7 weeks after haploidentical hematopoietic stem cell transplantation and express high levels of the activating receptors NKGD and NKp30 as well as of the lytic granules Granzyme-B and Perforin. Nonetheless, NKp46$^{neg-low}$/CD56^{dim}/CD16^{neg} natural killer cells displayed a markedly defective cytotoxicity that could be reversed by blocking the inhibitory receptor CD94/NKG2A. These data open new important perspectives to better understand the ontogenesis/homeostasis of human natural killer cells and to develop a novel immune-therapeutic approach that targets the inhibitory NKG2A check point, thus unleashing natural killer cell alloreactivity early after haploidentical hematopoietic stem cell transplantation.

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The Dark Side of a Commensal: A Commensal Bacterium Precipitates Ocular Surface Inflammation in a Model of Muckle-Wells Syndrome

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We recently demonstrated that the ocular surface, which is profoundly antibacterial, nevertheless harbors a resident commensal flora. *Corynebacterium mastitidis (C. mast),* a commensal which also colonizes humans, tunes mucosal immunity at the ocular surface by eliciting production of IL-17 from conjunctival γδ T cells, and confers resistance to pathogenic Candida and Staph infections (St. Leger et al, Immunity 2017). Muckle-Wells Syndrome (MWS) is one of several human autoinflammatory disorders known as Cryopyrin Associated Periodic Syndromes

(CAPS), in which a gain-of-function mutation of the *NLRP3* inflammasome gene results in inflammation of the skin, joints and conjunctiva of the eye. Using a knock-in mouse model expressing the mutated *NLRP3* gene cloned from a MWS patient, we show that, in this immunologically abnormal host, the commensal *C. mast* behaves as a pathobiont and induces ocular inflammation. Mechanistic studies suggested that the NLRP3 inflammasome acts within dendritic cells, and also intrinsically within γδ T cells, to enhance production of IL-1 and IL-17, leading to tissue pathology. Importantly, 3 patients with mutations affecting NLRP3 inflammasome function, responded robustly to *C. mast* lysate, whereas 5 healthy controls had a minimal response. We propose that an abnormal immune response to commensals may underlie the recurrent conjunctivitis that is characteristic of CAPS and similar autoinflammatory disorders affecting the NLRP3 inflammasome.

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Macrophage-derived Tgf-β Drives the Expansion of Tolerogenic CD103^{int} DCs in the Lungs of Neonatal Mice

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Pulmonary infections are one of the main causes of morbidity and mortality in newborns in both mice and humans. This susceptibility to infectious disease is caused, in part, by the altered functions of the neonatal immune system. Using the murine model, we tested whether lung conventional dendritic cells (cDCs) contributed to the limited immune responses of neonates. We found that under steady-state conditions, the lungs of adult mice contain two sets of cDCs, one characterized as CD11b^{+} SIRPa^{+} (cDC2) and the other characterized as CD103^{hi} XCR1^{+} (cDC1). In contrast, the lungs of neonatal mice have an additional population of cDC1 (XCR1^{+} CD103^{int}) that are homeostatically activated (CCR7^{+}, CD40^{+}, PDL1^{+}) independently of microbial colonization or TLR signaling. Despite their activated state, these CD103^{int} DCs have a tolerogenic gene expression program. Consistent with phenotype, the CD103^{int} DCs impair CD4 and CD8 T cell expansion and prevent their differentiation into functional effector T cells. Given that the expression of a_Eb_7 integrin is sensitive to the levels of Tgf- β, and that the tolerogenic DCs have low levels of CD103 (a_E integrin), we tested whether local production of Tgf- β might promote the tolerogenic phenotype of neonatal cDC1s. We found that mice with a macrophage-specific deletion of TGFb (LysM-cre x Tgf-β FL) failed to accumulate CD103^{int} DCs. Overall, our results show that the presence of the activated CD103^{int} DCs in newborn lungs prevents CD4 and CD8 T cells responses and that the accumulation of these tolerogenic DCs is dependent on macrophage-derived Tgf-β.

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Characterization of Regulatory T Cell-trafficking and Development During Experimental Autoimmune Uveitis

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Uveitis is a leading cause of blindness worldwide and it involves inflammation of the eye that can be damaging to the ocular tissues. An animal model of autoimmune uveitis (AU) known as experimental autoimmune uveitis (EAU), is widely utilized to gain a better understanding of the human disease. EAU resolves spontaneously without a relapse of uveitis. We and others have shown that the resolution of the disease is in part due to the emergence of ocular antigen-specific regulatory T cells (Post-EAU Tregs). These cells are identified in the spleen of mice that have recovered from EAU. However, the stability of these cells and the mechanism by which they function to

provide resistance to uveitis is not well understood. Therefore, the goal of this project is to characterize the post-EAU Tregs in terms of their stability and determine their tissue trafficking as a mechanism of their suppressive effect.

Forkhead Box Protein 3 (FoxP3) is a transcription factor that regulates the development and function of Tregs. The expression of FoxP3 can be unstable, therefore we incorporated a lineage tracing reporter mouse ($FoxP3^{GFP\text{-}Cre}$; $Rosa26^{stopfl/fl\text{-}Tom}$) into our EAU model. This mouse expresses fluorescent markers that allow for identification of Tregs that express FoxP3 ($FoxP3, GFP^{+}Tom^{+}$) or no longer express Foxp3 ($exFoxP3, GFP^{-}Tom^{+}$) to determine the stability of the post-EAU Tregs. These fluorescent markers also allow us to ask the tissue homing behavior of the post-EAU Tregs. We can also further determine when and where these Tregs emerge during the course of EAU.

To assess the emergence and tissue trafficking of Tregs during EAU, we induced uveitis by immunizing the lineage tracing reporter mice with emulsified retinal antigen, interphotoreceptor retinoid binding protein (IRBP) with Complete Freund Adjuvant and we monitored the disease course. We isolated T cells from the eyes and lymphoid tissues of immunized mice at 4 different time points during disease to quantify the relative abundance of Tregs in these tissues. We further assessed the suppressive function of IRBP-reactivated Tregs from recovered mice by the adoptive transfer of these Tregs into recipient EAU mice.

Early during EAU, we found that Tregs emerged in the eye and increased in frequency as EAU progressed to resolution. Also, the Tregs were found to be present in the lymphoid tissues (spleen, bone marrow, and cervical lymph node) during the course of the EAU. Specifically, both FoxP3 and exFoxP3 cells emerged in the eye and were also identified in the lymphoid tissues during the course of EAU. In contrast, the recovered mice, harbored both FoxP3 and exFoxP3 T cells in the lymphoid tissues but not the eye. Interestingly, the post-EAU Tregs capable of suppressing uveitis in the recipient mice had stable FoxP3 expression, but exFoxP3 cells did not suppress EAU, and the post-EAU Tregs trafficked to both the eye and lymphoid tissues at the onset of EAU.

Findings from this study elucidate a dynamic relationship of Treg migration between the eye and secondary lymphoid tissues as it contributes to the resolution of EAU. Also, this study highlights that stability of FoxP3 transcription factor is a requirement for the suppressive function of post-EAU Tregs. Interestingly, these Tregs traffic to the target tissue to prevent a uveitic relapse but migrate out after resolution. This work suggests that unstable FoxP3 expression or aberrant tissue trafficking may provide an explanation for chronic and relapsing autoimmune uveitis.

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IL-10 Protects Against neuron Death In *Staphylococcus Epidermidis* Biofilm and Microglia Co-cultures

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Background: Infants are at high risk of *S. epidermidis* ventriculoperitoneal shunt infection. Our mouse model of infant CNS catheter infection has shown an increase in parenchymal spread of infection in infant mice. We also observe a decrease in peripheral immune cells, such as monocytes and macrophages in infant mice compared with adult mice.

Significance: *Staphylococcus epidermidis*, the most common cause of CNS catheter infection, creates biofilms that aggregate upon the catheter and through various methods of self-preservation, cause persistent disease. This type of bacterial-derived catheter infection significantly and frequently impacts infants as a complication of the treatment of hydrocephalus. Approximately 2,400 hospital admissions each year are due to *S. epidermidis*

infection, which is greater than admissions for all patients with pneumococcal meningitis in the U.S. These infants are also at greater risk for poor neurologic outcomes following infection, through mechanisms that have yet to be defined.

Hypothesis: We hypothesized that IL-10 plays an important role in immune regulation and neuroprotection in the setting of CNS catheter infection. This may be particularly important in young hosts as a protective mechanism for the developing brain. Identifying mechanisms contributing to neuron death may help with therapies to improve neurologic outcomes.

Methods: To evaluate this hypothesis, we co-cultured 6-day old *S. epidermidis* biofilms with wild-type or IL-10 KO primary microglia for 6 hours creating a biofilm conditioned media (WT-BCM; IL10-BCM). Additional exposure groups include *S. epidermidis* biofilm conditioned media (BCM), IL-10 microglia conditioned media (IL10-MGCM), wild-type microglia conditioned media (WT-MGCM), neuron conditioned media (NCM) and control media (CM) was then added to wild-type primary neurons for 48 hours to measure neuron death via LDH release and MTT assay.

Results: Conditioned media from the co-culture of IL-10 KO microglia and *S. epidermidis* biofilm (IL10-BCM) resulted in 36% higher LDH release into supernatant than IL10-MGCM and 32% higher than either WT-MGCM or WT-BCM. There was no significant difference between LDH release between WT-MGCM and WT-BCM. MTT results were also consistent with neuron death after exposure to microglia and biofilm, with a greater degree of neuron death noted in the IL10-BCM group than either the WT-BCM or BCM alone groups when compared to CM exposure.

Conclusion: Neuron death, as suggested by decreased MTT absorbance is greatest after exposure to IL-10 KO microglia/biofilm co-culture conditioned media. Increased LDH release is consistent with MTT results and with increased neuron death when exposed to IL-10 KO microglia/biofilm conditioned media. Thus, IL10 appears to protect against neuron death in this *in vitro* co-culture system. Because this is mediated via conditioned media, this suggests that interaction between microglia and *S. epidermidis* biofilm produces a soluble factor that increases neuron death. The cellular viability of neurons is affected through the crosstalk of bacteria interacting with the inflammatory cells, such as microglia in the setting of CNS catheter 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.

Ligands of Innate Immunity: Structure & Function

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Role of Lipid a Hydroxylation in *Pseudomonas Aeruginosa* Persistence and Infection

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Bacterial pathogens have evolved adaptive responses to the environmental changes encountered when they enter a host from an external reservoir. Two-component regulatory systems often mediate these responses, which can include modifications of the bacterial cell envelope that enhance the ability to colonize, spread to different tissues, and avoid the hosts' normal defenses. Modifications to essential cell membrane components such as lipid A (the hydrophobic membrane anchor and bioactive component of lipopolysaccharide (LPS)) are important for pathogenesis. The two-component regulatory system PhoP/PhoQ has been implicated in adaptation in several

Gram-negative bacteria and many lipid A modification enzymes fall under its influence. The opportunist pathogen *Pseudomonas aeruginosa* (PA) has a unique PhoP/PhoQ system, possibly due to its large ecological niche and varied infection strategies. Patients with cystic fibrosis (CF) suffer from chronic airway infections with PA and experience worsening, irreversible lung injury that leads to premature death. This lung injury is mediated by an inflammatory process that results, at least in part, from stimulation of the innate immune system by PA lipid A. Lipid A structure can be altered by a number of modifying enzymes, such as acyltransferases, including HtrB1 (PA0011) and HtrB2 (PA3242) and the coordinating dioxygenases, LpxO1 (PA4512) andLpxO2 (PA0936). As such, PA lipid A structure can be penta- to hepta-acylated based on acyltransferase enzyme regulation and activity. Additionally, the secondary acyl chains can be modified with hydroxyl groups. In PA, LpxO1 and LpxO2 are responsible for the addition of a hydroxyl group to the 2 position of the myristate chains on lipid A and are at least partially under control of the PhoP/PhoQ system. Mass spectrometry fragmentation analysis of lipid A has revealed that LpxO1 preferentially acts on the 2-acyl-oxy-acyl chain (added by HtrB2) and LpxO2 acts on the 2'-acyl-oxy-acyl chain (added by HtrB1). The exact role of the 2-OH group in virulence and pathogenesis remains unknown. Additionally, it is unknown if the LpxO enzymes act on the fully-synthesized lipid A or during an earlier synthesis step, possibly in coordination with acyl-carrier protein or the HtrB enzymes. Here, we describe the role of 2-hydroxylation on membrane dynamics, antibiotic susceptibility, and general growth characteristics *in vitro*. Additionally, we use an intranasal murine model of infection to determine the level of hydroxylation lipid A undergoes during an *in vivo* infection and how the course of infection is altered in response to hydroxylation status.

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Synthesis and Function of Symbiotic Bacterial Lipopolysaccharide Partial Structures

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The structures of LPS and its active principle lipid A vary among bacterial species to adapt to their respective environments. Symbiotic bacterial LPS regulate the immune functions of hosts to construct a codependent relationship. In this study, we synthesized symbiotic bacterial lipopolysaccharide partial structures **1**-**8** including Kdo moiety (the peculiar acidic sugar that links between lipid A and polysaccharide part) and revealed the immunomodulating functions of symbiotic bacterial LPS/lipid A.

We have first focused on *Helicobacter pylori* and *Porphyromonas gingvalis* as representative parasitic bacteria. Synthesized *H. pylori* lipid As **1a**, **2a**, **3a** and *P. gingivalis* lipid A **4**, **5** showed IL-18 inducing activity while most of them inhibited IL-6 induction induced by *E. coli* LPS [1)]. Parasitic bacteria trigger chronic inflammation while avoiding bactericidal effect derived from acute inflammation. These results indicated that LPS/lipid A of parasitic bacteria are some of the regulatory factors for these characteristic inflammatory responses.

Alcaligenes faecalis was found to inhabit human Peyer's patches by Kiyono *et al* in 2010[2)]. Because Peyer's patches play an important role in the gut immunity, we expected that *A. faecalis* regulates immune responses via innate immune receptors. In fact, *A. faecalis* LPS showed characteristic immunomodulating activity, i.e., weaker cytokine inducing activity via TLR4 than *E. coli* LPS and no lethal toxicity. Interestingly, *A. faecalis* LPS promoted the production of IgA antibodies at the same level as *E. coli* LPS.[3)] We isolated the LOS as the major component and

determined the chemical structure of LOS by using NMR and MS. We then synthesized *A. faecalis* lipid A **8** via a new efficient synthetic route and evaluated its immunoregulating activity. Synthetic **8** showed weaker IL-6 and IL-10 inducing activity than *E. coli* LPS with bell-shaped concentration-dependency. Interestingly, in the presence of *E. coli* LPS, *A. faecalis* lipid A **8** promoted the production of anti-inflammatory IL-10 in a concentration-dependent manner. These results suggested that *A. faecalis* LPS/lipid A is associated with maintenance of homeostasis.

Reference
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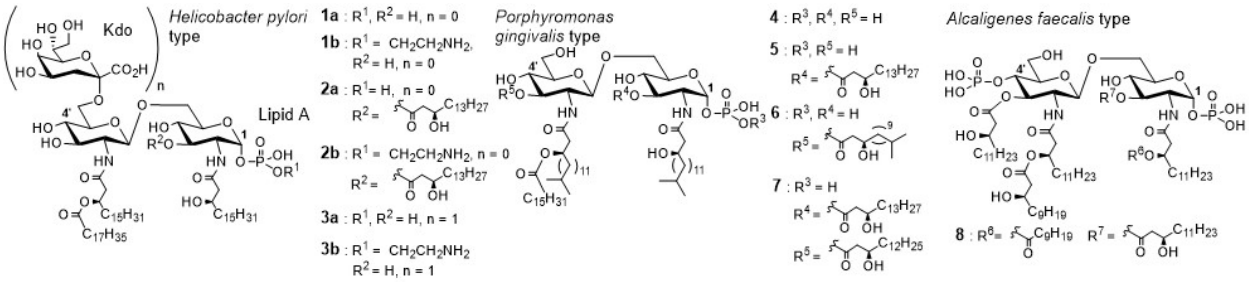

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The TLR4 Agonist Monophosphoryl Lipid a Induces Dynamic Reprogramming of Macrophage Metabolism for Enhancement of Antimicrobial Functions and Infection Resistance

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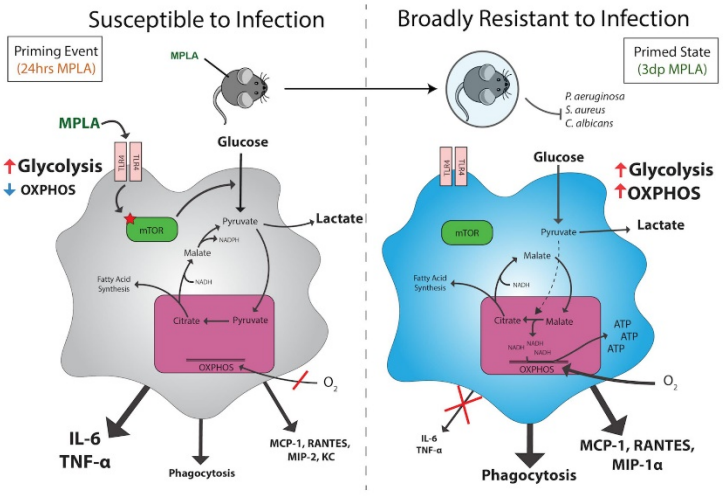
Due to the increasing global threat of nosocomial infections, there is a strong need for alternative immunomodulatory therapies to protect those patients at high risk from developing life-threatening infections. Our studies show that priming of mice with the clinically utilized TLR4 agonist monophosphoryl lipid A (MPLA) mediates protection against a variety of common nosocomial pathogens, including *P. aeruginosa*, *S. aureus*, and *C. albicans*. Augmented resistance against infections correlates with recruitment of innate leukocytes and improved pathogen clearance. This protection persists for at least 15 days after MPLA treatment and is independent of the adaptive immune system. We found that this protection is dependent on the presence of macrophages, and that MPLA induces sustained augmentation of macrophage glycolysis. In the current study, we hypothesize that MPLA induces broad-spectrum infection resistance by driving sustained metabolic reprogramming of macrophages that fuels enhanced cellular antimicrobial responses. To test this hypothesis, we primed bone marrow-derived macrophages (BMDMs) with MPLA for 24 hours, washed them and allowed them to rest



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for 3 days. Glycolysis and oxidative metabolism were assessed via glycolytic stress and mitochondrial stress tests, respectively. We found that MPLA induces persistently increased glycolysis and oxygen consumption in macrophages in conjunction with mitochondrial biogenesis, increased malate shuttling, and increased ATP production, a program that is dependent on mTOR activation. This enhanced energetic state fuels augmented phagocytosis and constitutive monocyte/macrophage-targeted chemokine secretion, while pro-inflammatory cytokine and neutrophil-targeted chemokine secretion is suppressed. Blockade of mTOR signaling with rapamycin inhibited the development of the metabolic and functional phenotype and blocked MPLA-induced resistance to infection. Our findings reveal that TLR4 agonist-induced metabolic reprogramming evolves to support a macrophage phenotype that is highly effective at mediating bacterial clearance, and supports the use of MPLA to facilitate broad-spectrum resistance to infection in vulnerable patients.

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A Distinct Late Acyl Transferase Contributes to Synthesis of KDO-containing Lipopolysaccharide in *Francisella*

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Francisella tularensis is the etiologic agent of tularemia, a highly infectious condition characterized by widespread bacterial dissemination within the host and a high mortality. The ability of the bacterium to frustrate immune defenses is likely achieved in part by unusual structural features of the bacterium's lipopolysaccharide (LPS). Unlike the LPS of many pathogens, the LPS of *Francisella* contains a lipid A that is both structurally heterogeneous and functionally inert, neither stimulating nor inhibiting innate immune detection. Our previous studies of the Live Vaccine Strain of *Francisella tularensis* revealed an additional aspect of the structural heterogeneity of *Francisella* LPS. Namely, the bacterium's high molecular-weight O-antigen capsule is associated with fatty acids unique to lipid A, yet distinct from the copious free lipid A that the Francisellae express, suggesting that the the capsule is a distinct form of LPS. We now report that the unusual lipid A-like structure we previously identified in the capsule is not unique to the O-antigen capsule, but is also found in all KDO-containing LPS structures in both LVS and in the closely related *Francisella novicida*, which lacks a capsule. These observations suggested that the synthesis of the free lipid A and of LPS differ and led us to hypothesize that two LPS late acyltransferase genes participated in the generation of these distinct structures. In support of this notion, knock-out of one of the two LPS late acyl transferases of *F. novicida* resulted in compositional changes in the KDO-associated lipid A (loss of myristate) while leaving the organism's copious free lipid A unaltered. The results suggest that variables in lipid A structure play a role in the regulation of the unique and diverse LPS structures generated by Francisellae.

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Identification of Potential Endogenous Ligands for CD14

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CD14 is a myeloid cell differentiation antigen expressed predominantly on the surface of monocytes and macrophages that plays a prominent role in innate immunity, particularly as a receptor for LPS and a component of the TLR4-MD2 receptor complex. Mice lacking CD14 are highly resistant to the lethal effects of LPS as well as to infection with Gram-negative bacteria. We hypothesize that CD14 may have additional functions and thus we screened a human brain cDNA library to identify potential endogenous ligands using the yeast-two-hybrid (Y2H) system, with CD14 as the “bait” protein. This screen resulted in 55 different potential endogenous ligands for CD14, several of which are known to be involved in different aspects of inflammation. One of these, PON2, is a member of the paraoxonase family that appears to act as an antioxidant, protecting cells from oxidative stress. Further Y2H analyses show that interaction of CD14 with PON2 is specific to a 3’ fragment of PON2 that includes

a region with polymorphisms that have been implicated in atherosclerosis and diabetes and that are associated with risk for both Alzheimer's and vascular dementia. Based on this information, we are studying the functional implications of CD14-PON2 interactions and the effects on oxidant activity as well as studying the functional implications of CD14 interactions with the other identified proteins.

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Gasdermin D Is Hepatoprotective in Models of Non-infectious Injury

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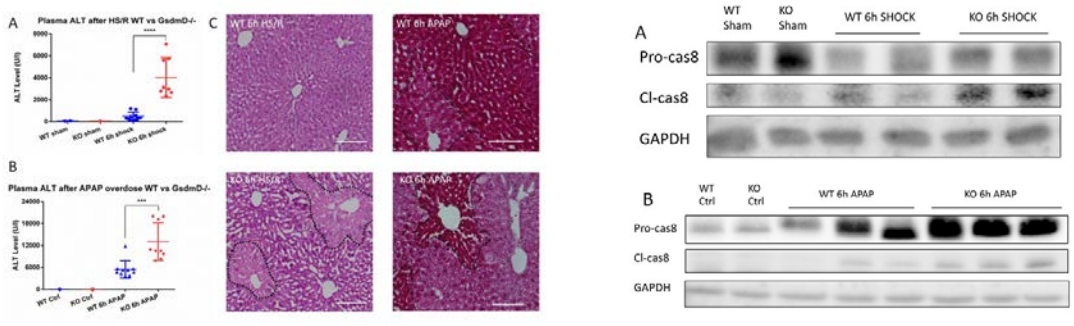
Introduction: Gasdermin D (GsdmD) is a cytosolic protein recently identified as the executioner of pyroptotic inflammatory cell death. Upon activation, caspase-1 and caspase-11 cleave GsdmD releasing its N-terminus domain, which binds to membrane lipids to form a pore leading to pyroptosis with release of cellular contents and membrane rupture. GsdmD amplifies inflammatory responses during infection improving clearance of intracellular bacteria, but less is known about its importance in non-infectious injuries. We assessed the role of GsdmD in two clinically relevant non-infectious injury models with different severity.

Methods: WT and GsdmD$^{-/-}$ mice were subjected to hemorrhagic shock for 1.5h followed by resuscitation for 4.5h (HS/R), or acetaminophen (APAP) overdose (400mg/kg) intraperitoneally for 6h. Liver and blood were harvested for ALT, histology and immunofluorescence, cytokines and immunoblot. Primary mouse hepatocytes (HC) were isolated from WT and GsdmD$^{-/-}$ mice for *in vitro* stimulation.

Results: GsdmD$^{-/-}$ mice had significantly increased liver damage after HS/R or APAP overdose compared with WT (ALT level at 6h after HS/R (485±119 vs 4162±725; WT vs GsdmD$^{-/-}$; n=7, p-/-; n=9, p=0.0006) (**Fig.1 A, B)**). Similarly, GsdmD$^{-/-}$ mice had increased circulating IL-6 and HMGB1 vs WT, indicating increased inflammatory responses. Furthermore, H&E showed extended necrotic areas in GsdmD$^{-/-}$ mice livers (**Fig.1 C**), and increased TUNEL positive cells compared with WT, confirming more severe liver injury. Western blot showed increased cleaved (activated) caspase-3 in GsdmD$^{-/-}$ mice compared with WT after HS/R or APAP overdose at 6h, indicating increased apoptosis. In the more severe APAP overdose model, but not HS/R, increased phosphorylated (activated) MLKL and RIP were also detected in GsdmD$^{-/-}$ liver compared with WT, suggesting increased necroptotic cell death. Caspase-8 is a mediator of inflammasome activation and also a key regulator of apoptosis and necroptosis pathways. Full-length pro-caspase-8 favors necroptosis and cleaved caspase-8 favors apoptosis. We found caspase-8 is upregulated in both sterile injury models in the absence of GsdmD, with increased cleaved caspase-8 in GsdmD$^{-/-}$ mice after 6h of both HS/R and APAP overdose. Interestingly, there was increased accumulation of pro-caspase-8 in GsdmD$^{-/-}$ mice after APAP overdose but not after HS/R, corresponding to increased necroptosis after APAP overdose (**Fig.2**). We used hydrogen peroxide (H_2O_2) to treat cultured HC to mimic different levels of redox stress between the two sterile injury models. Total caspase-8 was upregulated after H_2O_2 but cleavage of caspase-8 is inhibited by H_2O_2 at higher concentrations so increasing accumulation of pro-caspase-8. Treatment with antioxidant N-acetyl cysteine(NAC) increased caspase-8 cleavage.

Conclusion: Our study reveals a hepatoprotective role for GsdmD in non-infectious liver injuries through down-regulation of caspase-8-induced apoptosis and necroptosis. The specific mechanism of protection varies with severity of redox injury and is model specific. Our study also suggests fine tuning of cell death pathways through caspase-8 cleavage in liver.



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The TLR4 Agonist Monophosphoryl Lipid a Mediates Broad Protection Against Infection via Augmentation of Innate Antimicrobial Functions

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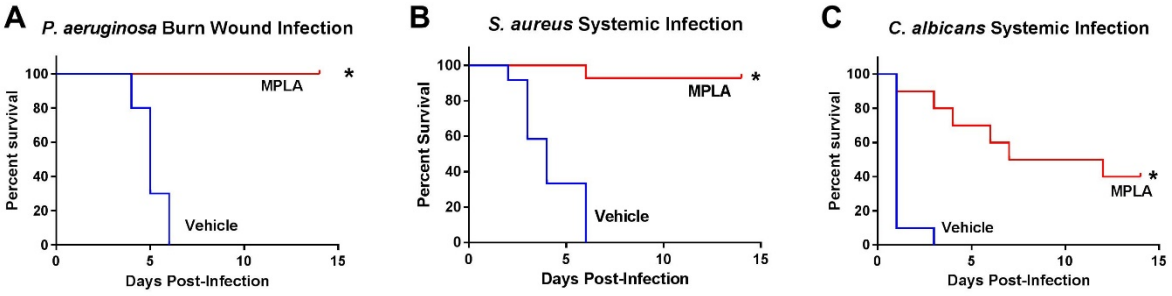
Infection with antibiotic resistant organisms is becoming one of the biggest healthcare threats, which leads to the need for alternative immunomodulatory therapies to protect patients at high risk from developing life-threatening infections. Our studies show that priming of mice with the TLR4 agonist monophosphoryl lipid A (MPLA) mediates protection against live Gram-negative bacterial infections. Previous studies have suggested that endotoxin tolerance is the mechanism by which TLR4 agonists can mediate protection against high dose LPS or live Gram-negative bacterial challenge. However, we recently showed that endotoxin tolerance induced by TLR agonist treatment actually does not predict the antimicrobial response to live infection. In this study, we hypothesized that MPLA would also be able to protect mice against a non-LPS-containing Gram-positive bacterial infection through augmentation of antimicrobial responses. To test this hypothesis, mice were primed with MPLA, followed by systemic challenge with *Staphylococcus aureus*, a common antibiotic resistant pathogen. We found that MPLA-primed mice had significantly improved resistance to *S. aureus*, leading to reduced mortality, improved bacterial clearance and reduced organ injury. MPLA-mediated protection was maintained in $RAG2^{-/-}$ mice as well as $CCR2^{-/-}$ mice, indicating protection was not dependent on lymphocytes or monocytes. However, the protection was lost when neutrophils or macrophages were depleted, indicating that neutrophils and mature macrophages are required for the MPLA-mediated protection in mice. Furthermore, neutrophils and macrophages, but not monocytes, from MPLA-primed mice had augmented phagocytic capacity. Finally, MPLA was also shown to mediate protection against severe *Candida albicans* systemic infection. This finding demonstrates the ability of MPLA to protect against a broad spectrum of pathogens, including Gram negative and Gram positive bacteria, as well as fungal pathogens. In conclusion, these data demonstrate that priming mice with the clinically available TLR4 agonist MPLA mediates resistance to a severe Gram-positive infection through augmentation of neutrophil

and macrophage antimicrobial functions. This work supports the use of MPLA as a clinical agent to reduce the incidence of broad nosocomial infections caused by a variety of organisms.



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Treatment with TLR Agonists Protect Against Severe Nosocomial Infections

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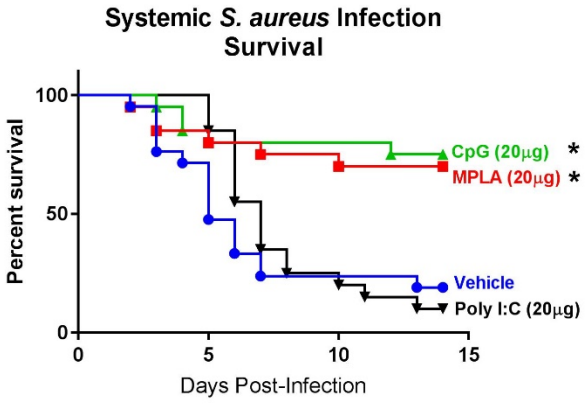
Infections by antibiotic-resistant organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* are the leading cause of death in severely burned patients. Thus, strategies aimed at augmenting the host response to infection may decrease the incidence and severity of infections in burn patients. We have shown that treatment of burn-injured mice with the clinically relevant TLR4 agonist monophosphoryl lipid A (MPLA) enhances bacterial clearance and survival in a *P. aeruginosa* burn wound infection model, as well as systemic *S. aureus* and *C. albicans* models by augmenting neutrophil recruitment, antimicrobial functions, and leukocyte metabolism. Recent studies reveal that the antimicrobial effects endowed by MPLA involve distinct contributions from both the MyD88- and TRIF-dependent arms of the TLR4 signaling pathway, but the relative contributions of each pathway remain unknown. To examine that question, the current study aimed to assess the efficacy of MyD88- and TRIF-selective agonists in our models of burn-associated infection. Based on our preliminary data, we hypothesize that agonists that activate MyD88 signaling will promote innate antimicrobial responses and induce protection against infection in burn-injured mice. To test this hypothesis, burn-injured mice were treated with the MyD88-selective TLR9 agonist CpG, the TRIF-selective TLR3 agonist Poly I:C, or the dual activating TLR4 agonist MPLA, followed by infection. Treatment of mice with CpG, but not Poly I:C, mediated protection against a both systemic *P. aeruginosa* infection and systemic *S. aureus* infection, similarly to MPLA. Both MPLA and CpG, but not Poly I:C, induced neutrophil recruitment and facilitated bacterial clearance resulting in preservation of core body temperature. In an *ex vivo* model of TLR agonist stimulation, it was found that both MPLA and CpG, but not Poly I:C, were able to augment leukocyte metabolism in a MyD88-dependent manner. These findings suggest that MyD88-dependent signaling plays an important role in the induction of TLR agonist-mediated antimicrobial responses, while TRIF dependent signaling seems to be less



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imperative for antimicrobial clearance. Further defining the distinct roles of MyD88- and TRIF-dependent signaling in generating protective antimicrobial responses against a variety of infectious pathogens in the burned host will likely reveal new insights that will advance current knowledge and can be harnessed to facilitate protection against infection in burn patients.

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In Vitro and *in Vivo* definition of the LOS-TLR4 Structure-activity Relationship Using Rationally Designed LOS Variants

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Toll-like receptor 4 complex (TLR4) is the major innate immune receptor responsible for surveillance of Gram-negative bacteria. TLR4 detects lipopolysaccharide and lipooligosaccharide (LPS/LOS) through the membrane anchor moiety lipid A. Lipid A exerts endotoxic activity via TLR4 in an NF-κB driven manner. The minimal structural criteria of LPS/LOS for modulating TLR4 activity is not well defined, despite decades of active research. Additionally, structural modification of LPS/LOS for TLR4 modulation is an attractive therapeutic strategy for diverse applications including immunomodulatory vaccine adjuvants and anti-sepsis treatments. One strategy for defining a cumulative LOS-TLR4 structure-activity relationship (SAR) is biosynthesis of a library of LOS mixtures with single structural modifications, followed by purification, structure elucidation, and systematic evaluation of inflammatory activity.

In order to define the inflammatory rheostat function of TLR4, we targeted specific lipid A moieties for structural modification that were previously implicated in attenuating TLR4 activity and identified as important for binding stability by predictive *in silico* molecular modeling. We have engineered, characterized, and tested 7 LOS structural variants using Bacterial Enzymatic Combinatorial Chemistry (BECC) in the *E. coli* strain D31m4 that bears a deep rough (Re) hexa-acylated LOS. Hexa-acylated, bis-phosphorylated lipid A of *E. coli* is the most potent agonist of TLR4 described to date. LOS was extracted using a modified petroleum ether extraction. LOS structural variants were confirmed by direct infusion of samples into the electrospray source of a linear ion trap mass spectrometer with multiple stage collision induced dissociation tandem mass spectrometry (MS^n). All molecules were screened for *in vitro* activity in TLR4-expressing tissue culture cell line, HEK-Blue NF-κB reporter cells. These included the 1- and 4'-phosphates (*Francisella novicida* LpxEand LpxF, respectively), the 3-O-acyl position (*Salmonella enterica* PagL), and the 3'-acyl-oxo-acyl position (*Salmonella enterica* LpxR), targeted alone, or in combination with at least one other structural modification. LOS from these mutants elicited attenuated NF-κB signaling, as predicted, when compared to the parent LOS. As expected, all mutants generated by this method showed differing degrees of activity, when compared to the parent strain

Bacterial lipid structural modifications influenced by the host are important for pathogenesis; current *in vivo* techniques are lacking that maintain the spatial distribution of host and bacterial signaling components. Mass spectrometry imaging (MSI) is a technique for mapping molecular signatures in tissue that has rapidly expanding applications, especially in three-dimensional studies. Tissue preparations that both preserve histology and are compatible with the intended mass-spectrometry endpoint are central to successful MSI studies. We have developed a method to hydrolyze lipid A from bacterial LOS within tissue using MSI. This advance required development of a lung inflation method compatible with both MSI and rodent models of bacterial infection. We now report here a robust method for lung preparation for MSI that is compatible with automated spatial segmentation and 3D molecular reconstructions to complement LOS/lipid A imaging on-tissue. By combining the

defined SAR mutants with a lung infection model and MSI, we can visualize the LOS/TLR4/SAR host response at the molecular level throughout the lung.

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Antibody-mediated β2 glycoprotein-1 Activation of Toll-Like Receptors

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Beta-2 Glycoprotein 1 (β2GP1), an abundant serum glycoprotein, was recently identified as a component of innate immunity and a potential Toll-like receptor (TLR) agonist. We developed and reported monoclonal antibodies (mAbs; 2aG4 and mch1N11) that bind to and dimerize β2GP1. These mAbs localize specifically to tumors and can activate innate immune anti-tumor activity and facilitate an adaptive anti-tumor immune response. However, the mechanism of immune activation induced by these mAbs is unclear. We propose that mAb-mediated β2GP1 dimerization induces TLR-mediated activation of innate immune cells in the tumor microenvironment. To this end, we demonstrate here that 2aG4 or mch1N11 mediated dimerization of β2GP1 stimulated pro-inflammatory (M1) polarization of bone marrow-derived macrophages (BMDMs). Further treatment of BMDMs with mAb dimerized β2GP1 was associated with TLR signaling determined by western blot and immunocytochemistry analysis. 2aG4 and mch1N11 bind to distinct epitopes on β2GP1 and while each mAb induces β2GP1-dependent activation of TLR signaling mch1N11 is more potent. Recent literature indicates that the conformation of β2GP1 is important for binding to TLR. Based on this we tested whether 2aG4 or mch1N11 altered β2GP1 conformation by circular dichroism (CD) and transmission electron microscopy (TEM). These studies demonstrated that 2aG4 and mch1N11 have distinct effects on β2GP1 conformation. Interestingly, the conformation induced by mch1N11 is an 'open' structure consistent with what has been shown to have the highest binding to TLR. Future studies are focused on demonstrating which TLR is critical for β2GP1-mediated innate immune cell activation and documenting in vivo efficacy of targeting β2GP1 as a method for enhancing immune therapy of cancer.

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Modulation of TLR4 Signaling with Synthetic Lipid a Mimetics

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Introduction: Gram-negative bacterial lipopolysaccharide (LPS)–induced Toll-like receptor 4 (TLR4)-mediated pro-inflammatory signaling plays a key role in the pathogenesis of numerous acute and chronic diseases which highlights the potential of addressing TLR4 as therapeutic target. Activation of TLR4-mediated innate immune response was demonstrated to be immunoprotective against infectious challenge, whereas down-regulation of TLR4 signaling was shown to be beneficial for management of sepsis and chronic inflammation. TLR4 is also shown to bridge the innate and adaptive immunity, which features stimulation of TLR4 as a feasible approach for development of novel vaccine adjuvants.

Objectives: The endotoxic amphiphilic portion of LPS, a glycophospholipid Lipid A, is composed of a conformationally flexible β(1→6) linked diglucosamine backbone which is bisphosphorylated and multiple acylated by the long chain (*R*)-3-acyloxyacyl- and (*R*)-3-hydroxyacyl residues. Although the mode of *E. coli* Lipid A interaction with TLR4/MD-2 complex is established, the structural basis for ligand discrimination by the TLR4 system is largely unknown. There is still a lack of evidence on how structurally different Lipid A/LPS molecules interact with TLR4/MD-2 and accessory proteins LBP and CD14 and why even minor variations in the structure of Lipid A often result in a dramatic change in activity. To address these basic questions, we developed a library of

artificial lipid A – like glycolipids (Lipid A Mimetics, LAMs) based on the intrinsically rigid glycosidically (1↔1)-linked disaccharides and investigated their TLR4-dependent immunomodulatory potential.

Methods: Several series of structurally different Lipid A mimetics (LAMs) were prepared via challenging chemical synthesis. Compounds were evaluated for the ability to either activate TLR4 complex or to inhibit LPS-induced release of cytokines through multiple tests in hTLR4/MD-2/CD14$^{+/-}$transfected HEK293 cells, immortalized mouse macrophages, human MNC and lung epithelial cells.

Results: We report on the crystal structure-based design, advanced chemical synthesis and immunobiological evaluation of novel sugar-based immunostimulants with picomolar affinity for TLR4 which induce highly potent and adjustable TLR4-mediated signaling in a species-independent manner. Synthetic Lipid A mimetics (LAMs) induced the release of various cytokines in human MNC, human epithelial cells, and immortalized murine macrophages, where the efficiency of signaling was effectively and predictably regulated by chemical modifications of LAM molecules.

We also developed a library of potent TLR4 antagonists with nanomolar affinity for TLR4 which compete with LPS for binding to the co-receptor protein MD-2 and inhibit LPS-triggered release of numerous inflammatory cytokines in a concentration-dependent manner. The unequivocal advantage of LAMs consists in their chemical and hydrolytic stability, rationalized by the presence of a stable secondary phosphate group in place of a labile glycosidic phosphate functionality of lipid A. In contrast to many natural lipid As which demonstrate pronounced species-specificity, LAMs display immune-stimulatory properties or anti-endotoxic activity in a species-independent way.

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Identification of S100A9 and Toll-Like Receptor 4 as Novel Interacting Partners of Cationic Antimicrobial Protein CAP37

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2. National Institute of Health

Cationic Antimicrobial Protein of 37 kDa (CAP37) is constitutively expressed in human neutrophils and can be induced under inflammatory conditions in other cell types. For example, this secreted multifunctional protein is induced in corneal epithelial cells in response to corneal infection. In addition, we recently showed that topical application of CAP37 promotes corneal re-epithelialization in a mouse model. Interestingly, several peptides derived from CAP37 can recapitulate the antimicrobial and/or wound healing effects of the full-length protein. The current study was designed to identify membrane receptor(s) mediating the wound-healing effects of CAP37 and CAP37-derived peptides in corneal epithelial cells.

To identify the receptor(s) that mediates corneal re-epithelialization induced by CAP37, we used an innovative ligand-based receptor capture method based on a TriCEPS reagent that features (1) an NHS group for coupling with the ligand protein, (2) a hydrazine cross-linker for coupling to the receptor, and (3) a biotin for purification of the complex. Direct interactions of CAP37 and CAP37-peptides with the candidate binding partner S100 calcium-binding protein A9 (S100A9) identified in this screen were tested in vitro using an enzyme-linked immunosorbent assay (ELISA). We also used ELISA to test the interactions of CAP37 and CAP37-peptides with Toll-like receptor 4 (TLR4), a receptor for S100A9. Activation of TLR4 was quantified in HEK-blue^{TM} hTLR4 cells, following incubation with increasing concentrations of ligands S100A9, CAP37, or CAP37-derived peptides. We also quantified TLR4 activation in these cells, treated with various combinations and ratios of ligands, either pre-incubated together or added simultaneously to the cells.

Our results show that CAP37 interacts with S100A9 and TLR4 in a dose-dependent and saturable manner, with K_D values of 9 and 98 nM respectively. Four peptides, covering two distinct domains of CAP37 were similarly able to bind S100A9 and TLR4, with K_D values ranging from 22 to 44 nM and from 19 to 270 nM, respectively. As expected, the TLR4 agonist, S100A9, was able to activate TLR4 on HEK-blue^{TM} hTLR4 cells, in a dose-dependent manner, at concentrations ranging from 1 to 100 nM. By contrast, neither CAP37 nor any of the tested CAP37 peptides were able to activate TLR4 when used within this range of concentrations. However, CAP37 and one CAP37 peptide ($CAP37_{20-44}$) partially activated TLR4 when used at higher concentrations (1 and 100 µM respectively) and thus appear to be partial agonists. The other three interacting CAP37 peptides did not activate the receptor at doses up to 100 µM and thus appeared to be antagonists of TLR4. Finally, CAP37 and all four binding peptides appeared to quench S100A9 and/or compete with S100A9, with the net effect of inhibiting S100A9's activation of TLR4.

We speculate that a physical interaction of CAP37 and certain CAP37-derived peptides with S100A9 and TLR4 could modulate the activation of TLR4 on corneal epithelial cells, thus promoting corneal wound healing. These findings will be key to optimization and translation of CAP37-derived peptides into innovative therapeutics with inhibitory effects of the pro-inflammatory receptor TLR4.

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S100A14 Can Stimulate Activation of Immune Cells and Crosstalk Between NK Cells and Monocytes.

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Despite risky behaviors and needle-sharing some HIV-1-exposed people who inject drugs remain sero-negative, suggesting mechanisms of resistance to HIV-1 are present in this population. We have observed in the proteome of NK cells from HIV-exposed sero-negative people who inject drugs (HESN-PWID) overexpression of the protein S100A14, when compared to control donors. In addition, S100A14 levels were increased in the plasma of HESN-PWID when compared to control donors. Here, we investigated the potential of S100A14 to induce innate immune activation *in vitro.*

We added recombinant S100A14 to PBMCs from healthy donors and measured activation/maturation markers on NK cells, dendritic cells and T cells after an overnight incubation and production of TNF-alpha in monocytes after 5 hours. To determine if NK activation in a PBMC mixture requires crosstalk with monocytes, we isolated monocytes and NK cells and analyzed the activation of NK cells after co-culture of both cell type. To further investigate the potential of S100A14 to promote an antiviral state, we collected supernatant and cell lysate from PBMCs to measure the secretion of IL-12 and the expression of Mx1, respectively. Using an antibody against His-Tag in PBMCs we verified if the extracellular S100A14 was being internalized by immune cells. To determine potential triggers of S100A14 overexpression in NK cells, we measured the intracellular expression of S100A14 in NK cells after exposure to several stimuli.

In this study we demonstrated that S100A14 exposure increases CD69 expression on NK cells and CD8+ T cells, CD83 expression on DCs, and TNF-alpha production in monocytes. S100A14-induced activation of NK cells is dependent on cell-to-cell contact with monocytes. S100A14 activation of PBMCs promotes the secretion of IL-12p40 and the expression of Mx1. Extracellular S100A14 can be uptake by monocytes and DCs but not by NK cells or CD4+ T cells. Finally, we found that S100A14 is normally expressed by NK cells, and that S100A14 can auto-stimulate its overexpression in NK cells. This data demonstrate that S100A14 can stimulate immune cells and amplify its response by promoting crosstalk and expression of Interferon-mediated proteins, suggesting that S100A14 may be part of the mechanism of resistance to HIV-1 observed in HESN-PWID.

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Biologic and Structural Properties of the Lipid A of *Granulibacter Bethesdensis*, an Emerging Pathogen in Patients with Chronic Granulomatous Disease

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Granulibacter bethesdensis is a Gram-negative bacterium that can infect patients with Chronic Granulomatous Disease, a primary immunodeficiency arising from defective phagocyte NADPH oxidase activity. Previous studies have demonstrated that despite efficient internalization by human neutrophils, *Granulibacter* was markedly hypostimulatory compared to *Escherichia coli* at inducing NADPH oxidase. Moreover, cytokine production by primary monocytes in response to *Granulibacter* was also significantly decreased compared to *E. coli*. Here, we also show that cytokine production in whole blood required 10-100 times more *G. bethesdensis* CFU/ml than *E. coli* suggesting that *Granulibacter* may possess an atypical endotoxin or other microbe-associated molecular pattern(s). We therefore purified *G. bethesdensis* LPS (*Gb*LPS) using hot phenol/water extraction for functional and structural analyses. *Gb*LPS partitioned to the aqueous phase and gel filtration chromatography resolved higher molecular mass rhamnose-rich carbohydrates from fractions with a laddering pattern typical of smooth LPS. Unlike typical LPS, hydrolysis of Lipid A from O- polysaccharide required strong acid. NMR and mass spectrometry studies demonstrated that the carbohydrate portion of lipid A consists of an α-Man-(1→4)-β-GlcN3N-(1→6)-α-GlcN-(1↔1)-α-GlcA tetrasaccharide substituted with five acyl chains. The hybrid type [-β-GlcN*p*3N-(1→6)-α-GlcN*p*-] amino disaccharide in the tetrasaccharide backbone is substituted by amide linked N-3'14:0(3-OH), N-2' 16:0[(3-O-16:0), N-2 18:0(3-OH) and ester linked O-3 14:0(3-OH), and 16:0. The identification of glycero-d-talo-oct-2-ulosonic acid (the first constituent of the core region of LPS) covalently attached to GlcN3N of lipid A, may account for the acid-resistance of *Gb*LPS. The presence of GlcN3Nis in agreement with identification of *GnnA* and *GnnB*homologues in *Granulibacter*. Purified Lipid A from *Granulibacter*had >10-fold lower proinflammatory potency compared to *E.coli*Lipid A as measured by cytokine induction in human blood. Structural properties of *Gb*LPS may contribute to immune evasion during pathogenesis and the ability of this organism to persist *in vitro* and *in vivo.*

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Synthesis and Immunological Evaluation of Self-adjuvanting Anticancer Vaccine Candidates Conjugated with TLR2 Ligand

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Tumor cells display aberrant self-antigens, called tumor-associated antigens (TAAs). Sialyl-Tn (STn) is one of TAAs composed of carbohydrates and richly expressed on a number of tumors, but it is rarely observed on normal

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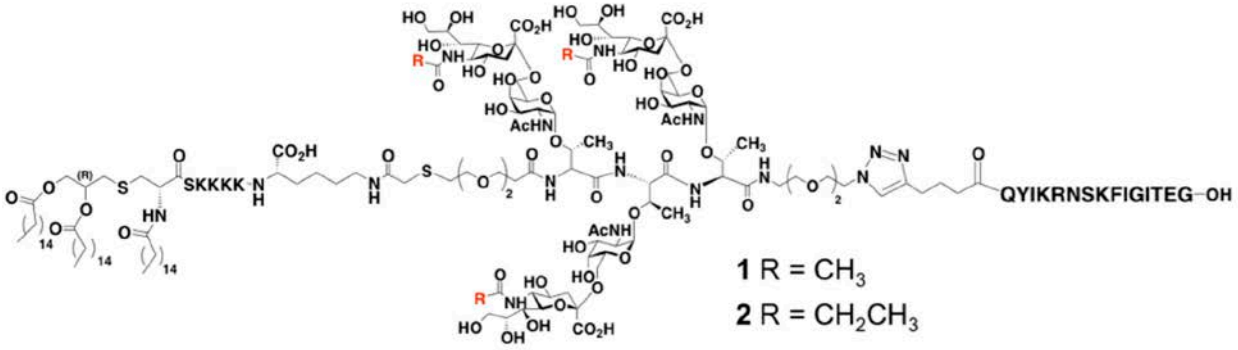
tissues. The STn-keyhole limpet hemocyanin (KLH) conjugate was developed as a therapeutic vaccine (Theratope®) for metastatic breast cancer. Unfortunately, Theratope® failed in phase III clinical trial in 2003.[1] Cancer patients immunized with Theratope® produced high level of anti-STn IgG but low level of anti-mucin IgG that should be responsible for anti-cancer response.

Here, we report the synthesis of the clustered STn **1** and **2** conjugated with the TLR2 ligand (lipopeptide ligand, Pam_3CSK_4) and the T cell epitope (Figure 1).[2] The native and *N*-propionyl triSTn were used as the tumor antigens that mimic small MUC1 fragment, since triSTn sequences in MUC1 are highly expressed on various tumors and *N*-propionyl sialic acid proved to enhance the immunogenicity of STn [3]. The conjugated triSTn **1** and **2** were constructed *via* copper click chemistry and thioether formation. Immunization of vaccine **1** and **2** to mice induced anti-triSTn IgGs, which recognized triSTn-expressing PANC-1 and HepG2. The IgGs stimulated by vaccine **2** have a higher specificity to triSTn against mono-STn compared with the one stimulated by vaccine **1**.

Anti-HER2 antibody trastuzumab has been used for the treatment of HER2 positive breast cancer. HER2 derived peptide vaccines are alternative choices for the treatment of HER2 positive cancers. A HER2 derived 9-mer epitope (9aa: DTILWKDIF) was predicted to possess a high immune potential as B cell epitope as well as T cell epitope.[3] We synthesized the conjugates of DTILWKDIF, $(DTILWKDIF)_2$, $(DTILWKDIF)_3$ with Pam_3CSK_4 and evaluated their immunogenicity. The results indicated 9aa- Pam_3CSK_4 induced robust humoral immune responses. The induced IgGs exhibited high specific affinity to HER2-overexpressing BT474 cells.



Metabolism & Physiology in Inflammation and Immunity

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Sirtuin 1 Regulates Mitochondrial Function and Redox Homeostasis in Respiratory Syncytial Virus Infected Dendritic Cells

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Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infection in children worldwide. Sirtuin 1 (SIRT1), an NAD^+-dependent deacetylase, has been associated with the induction of autophagy and the regulation of inflammatory mediators. Our laboratory has reported that SIRT1 promotes dendritic cells (DC) activation associated with autophagy-mediated processes during RSV infection and the absence of SIRT1 activity alters the antiviral immune response through the regulation of innate cytokine production. A primary

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consequence of dysregulated SIRT1 is the generation of ROS due to mitochondrial dysfunction and an inability to sequester damaged mitochondria in autophagosomes. In this study, we investigated if SIRT1 loss induces mitochondrial dysfunction and consequently leads to ER stress in the cell. *Sirt1*$^{fl/fl}$*CD11c-Cre* mice were generated and used in this study. DCs from *Sirt1 Cre+* (SIRT1 deficient) mice showed decreased basal and the maximal mitochondrial respiration (OCR) as compared to the wild type (WT) mice. Infection with RSV further decreased the respiration capacity of *Sirt1 Cre+* cells. The WT DCs showed an increased potential to utilize both glycolysis and mitochondrial respiration for their energy demands. However, *Sirt1 Cre+* DCs showed an altered metabolic profile, with glycolysis as a preferred pathway to respond to the energy demands of the cell during RSV infection. This alteration was evident in flow cytometry showing an accumulation of increased number of depolarized mitochondria signifying decreased mitochondrial membrane potential ($\triangle\Psi_m$) during RSV infection. As depolarization of mitochondrial membrane leads to reactive oxygen species (ROS) generation we also examined the mitochondrial-generated ROS in these cells. *Sirt1 Cre+* DCs generated elevated levels of ROS that were further elevated with RSV infection. Dysfunctional mitochondria and the associated ROS leads to oxidative stress and DNA damage. Reverse Phase Protein Array (RPPA) analysis of *Sirt1 Cre+* and WT DCs identified in a range of differentially phosphorylated protein pathways that play a critical role in energy metabolism, autophagy and oxidative stress, and DNA damage. Thus, SIRT1 regulates innate immune signaling in DCs by altering the metabolic processes during homeostasis and inflammatory stress.

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The Role of Iron Metabolism in Neonatal Susceptibility to E. Coli K1 Sepsis

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Background: Sepsis is one of the primary causes of death in the neonatal period. *Escherichia coli* which displays the K1 antigen is a major pathogen in neonatal sepsis and meningitis, but is not pathogenic in adults. The mechanism behind the unique susceptibility of neonates to this strain of *E. coli* is unknown.

Methods: We employed a murine model of neonatal *E. coli* K1 sepsis to study this differential susceptibility. Injection of *E. coli* C5 intraperitoneally (IP) results in 76% mortality in 5-day old neonatal C57BL/6 mouse pups compared to 0% mortality in 2-4 month old mice within 72 hours of injection ($p<0.0001$).

Results: *E. coli* K1 grew rapidly in the peritoneal cavity of neonatal mice in the first 12 hours of infection; in contrast, adult mice completely cleared the infection during this time($p=0.002$, Mann-Whitney test). Neonatal immune cells (splenic and peritoneal-resident macrophages and neutrophils) killed and phagocytosed *E. coli* K1 equivalently to those of the adults. Unexpectedly, *E. coli* K1 displayed enhanced growth in the cell-free peritoneal wash fluid of neonates compared to that of the adults, suggesting that a nutritional factor permitted differential growth. Peritoneal fluid from neonatal mouse pups contained significantly more iron than that of adult mice (284±58 vs 34±19 ng total iron/mg total protein, $p=0.02$), and adding this concentration of iron to adult wash fluid enhanced bacterial growth. Adult mice injected with 450 ug of ferric ammonium citrate IP 2 hours before *E. coli* K1 infection suffered 100% mortality 100% mortality within 24 hours compared to 0% mortality in the group injected with *E. coli* K1 or ferric ammonium citrate alone. Breeding females were placed on a chemically defined diet containing 45 ppm iron for 2 weeks before mating, and then switched to a diet containing either 4ppm, 45ppm, or 220 ppm iron on embryonic day 15. Pups born from mothers that were given the 4ppm iron diet were highly resistant to infection compared to pups born from mothers on the 45 ppm and 220 diets, with 75% of pups surviving infection compared to 10% and 0% survival respectively.

Conclusions: Taken together, these results suggest that neonatal susceptibility to *E. coli* K1 sepsis is influenced by an excess of iron in the peritoneal cavity that allows for rapid unchecked growth of the bacteria. This pool of excess iron is a potential therapeutic target in treating neonatal sepsis.

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Lactate Production by *S. Aureus* biofilms Promotes Myeloid-derived Suppressor Cell (MDSC) Activity and Bacterial Persistence

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Staphylococcus aureus (*S. aureus*) is a leading cause of infections involving indwelling medical devices, including prosthetic joints and catheters, where bacteria have the propensity to form biofilms. Our laboratory has identified preferential myeloid-derived suppressor cell (MDSC) recruitment as a critical mechanism for biofilm persistence, as MDSCs are the main source of IL-10, which skews biofilm-associated monocytes and macrophages toward an anti-inflammatory state. To assess biofilm-derived factors that actively drive MDSC expansion/activity, we designed a screen of the Nebraska Transposon Mutant Library (NTML) to identify *S. aureus* mutants impaired in their ability to trigger MDSC IL-10 production. Significant hits involved in lactate biosynthesis were identified, suggesting that biofilm-derived lactate is an important regulator of MDSC activity. Using a mouse model of orthopedic implant biofilm infection with wild type (WT) *S. aureus*, we have found that extracellular D-lactate levels progressively increase beginning at day 7 post-infection, the time at which a mature biofilm has formed, coinciding with a 77-fold increase in IL-10 expression in MDSCs isolated from infected joints. In addition, D-lactate is significantly elevated in the synovial fluid of patients with prosthetic joint infection (PJI) compared to aseptic controls and corresponds with significant MDSC recruitment in the former. Furthermore, we show that D- and L-lactate levels are reduced in *S. aureus* lactate mutants during orthopedic implant biofilm infection, and are associated with a significant decrease in MDSC infiltrates and reduced IL-10 production at later time points. These changes translated into enhanced monocyte recruitment, improved biofilm clearance, and less bone pathology. Histone acetylation classically modulates gene expression, whereby acetyl groups bound to lysine residues of histone protein tails lead to reduced interaction with DNA, promoting promoter accessibility and transcription. Lactate has been shown to inhibit histone deacetylases (HDACs), and promoters important for MDSC expansion, recruitment, and activity are regulated by histone acetylation. The IL-10 promoter is activated by acetylation and we show that treatment of mice with a histone deacetylase inhibitor (HDACi) augments *S. aureus* biofilm infection by increasing IL-10 levels. Furthermore, ChIP-Seq demonstrated that histone H3 promoter acetylation was dramatically increased genome-wide in MDSCs recovered from mice infected with WT *S. aureus* vs. lactate mutants, providing molecular evidence that biofilm-derived lactate functions as HDACi. Collectively, these studies demonstrate that *S. aureus* biofilm-derived lactate promotes MDSC inhibitory by inducing IL-10 production, which prevents immune-mediated clearance, and, by extension, biofilm persistence.

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Exercise Physiology and Sterile Inflammation: Elevated Post-marathon Mitochondrial Damage-associated Molecular Patterns (mtDAMPs)

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PURPOSE: The purpose of these studies was to generate preliminary data describing mitochondrial damage-associated molecular patterns (mtDAMPs, which influence the immune system) in human plasma samples, after the muscle injury evoked by extreme exercise, such as a marathon. We hypothesized that circulating concentrations of mtDAMPs—specifically, mtDNA—are elevated post-marathon relative to pre-marathon. Digital droplet PCR (ddPCR), an extremely sensitive assay, enables evaluation of the change in mtDAMP levels pre- vs. post-marathon.

METHODS: All procedures were IRB approved and all subjects (n=3) provided informed consent. Blood was obtained by antecubital venipuncture at baseline and within 48 hours post-race. Blood was centrifuged, plasma aliquoted, and stored at -80°C for further analyses. Total plasma DNA was isolated using a commercially available mini kit (Zymo Research). Cytochrome oxidase III (COX III) primers were used to query for evidence of mtDNA. Digital droplet PCR was performed using the Bio-Rad QX200 system and EvaGreen supermix.

RESULTS: Preliminary data generated from pre-marathon samples indicate that ddPCR can detect extremely small amounts of circulating mtDAMPs. Levels of mtDAMPs (reported as copies/microliter) consistently increase by up to 10-fold following a marathon, presumably because of skeletal muscle contraction-induced injury to the muscle cell membranes.

CONCLUSION: Mitochondrial DNA in the circulation increases following the marathon.

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BATF3 Deficiency Leads to Development of Obesity and Increased Susceptibility to Chronic Intestinal Inflammation

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Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract. The development of IBD is affected by several factors including genetic susceptibility, immune response, intestinal microbiome, and other environmental factors. Recent studies have suggested that obesity is associated with more severe IBD and that mesenteric fat is a source of pro-inflammatory cytokines. In obesity, pro-inflammatory macrophages accumulate in adipose tissue and their numbers correlate with inflammation and insulin resistance. BATF3 is a transcription factor implicated in the development of peripheral $CD8a^+$ and $CD103^+$ dendritic cells (DCs). Here, we show that aged *Batf3*$^{-/-}$ mice developed obesity as characterized by increased body weight, size of white adipocyte in abdominal fat deposits, and development of hepatosteatosis. High-fat diet further enhanced the metabolic phenotype in *Batf3*$^{-/-}$ mice compared to WT mice. We observed increased fasting glucose concentrations, increased adipocyte size, and more pronounced hepatosteatosis in *Batf3*$^{-/-}$ mice. Moreover, under high-fat diet *Batf3*$^{-/-}$ mice were more susceptible to dextran sulfate sodium (DSS) colitis with increased numbers of M1 macrophages ($CD11b^+CD11c^+F4/80^+$) in the intestine and mesenteric fat tissue. Our data suggest that the deficiency of the transcription factor BATF3 and lack of $CD103^+$ DCs promotes the infiltration of M1 macrophages into abdominal fat deposits and increases susceptibility to DSS. In conclusion, we demonstrated that BATF3-dependent $CD103^+$ DCs are protective for the development of obesity and intestinal inflammation.

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Nanoparticle-mediated Targeting of Macrophage Metabolism Promotes *S. Aureus* Biofilm Clearance

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Staphylococcus aureus (*S. aureus*) is a leading cause of community-acquired and nosocomial infections. The ability to form a biofilm allows *S. aureus* to colonize biotic/abiotic surfaces and contributes to antibiotic resistance, making these infections difficult to eradicate and associated with significant morbidity and economic burden. In the case of prosthetic joint infections (PJIs), patients undergo two surgeries, first for removal of the infected device and placement of an antibiotic impregnated spacer, followed by a second surgery for prosthesis re-implantation. This treatment burden necessitates the development of novel therapeutic approaches to manage biofilm-associated infections. Our laboratory has shown that *S. aureus* biofilms actively skew the immune response, as reflected by the preferential recruitment of myeloid derived suppressor cells (MDSCs) and anti-inflammatory macrophages (MΦs). Upon MDSC depletion or in IL-10 KO mice, where MDSCs are the primary source of IL-10, MΦ pro-inflammatory activity was augmented, which translated into reduced biofilm burdens. Furthermore, the adoptive transfer of pro-inflammatory MΦs significantly attenuated *S. aureus* biofilm growth. Therefore, the immune polarization state of MΦs plays a key role in dictating biofilm persistence. Recent metabolic studies have shown that alterations in MΦ metabolism shape their inflammatory phenotype and function. For example, anti-inflammatory MΦs are biased towards oxidative phosphorylation (OxPhos) and inflammatory MΦs favor aerobic glycolysis. Our findings support the hypothesis that *S. aureus* biofilm-associated MΦs experience a metabolic shift favoring OxPhos and dampening of aerobic glycolysis, which results in their polarization towards an anti-inflammatory state that promotes biofilm persistence. This study leveraged a novel nanoparticle-targeted approach to inhibit monocyte/MΦ OxPhos activity *in vivo* to re-program cells to a pro-inflammatory state. Nanoparticles were synthesized with the following features: 1) incorporation of Cy5 fluorochrome to monitor particle distribution in real-time by *in vivo* imaging (IVIS) as well as the specificity of nanoparticle uptake by monocytes/MΦs vs. other leukocytes by flow cytometry; 2) conjugation with tuftsin, a peptide derived from the IgG heavy chain Fc domain, to target FcR-mediated uptake in monocytes/MΦs; and 3) encapsulation of oligomycin, a naturally occurring ATP synthase inhibitor, to interfere with OxPhos and drive cells towards glycolytic metabolism. Using our mouse model of *S. aureus* orthopedic implant infection, >90% of nanoparticle uptake was specific for monocytes/MΦs. In addition, *S. aureus* biofilm burdens were significantly decreased in mice receiving Cy5/Tuftsin/oligomycin nanoparticles compared to Cy5/Tuftsin alone over a 28 day period, presumably by enhancing glycolysis and pro-inflammatory function of infiltrating monocytes/MΦs. This was supported by metabolomics and RT-qPCR analysis of monocytes recovered from infected mice receiving Cy5/Tuftsin/oligomycin but not control nanoparticles. Understanding the relationship between metabolism and inflammatory function of monocytes/MΦs during biofilm infections may be leveraged to advance therapeutic options for patients with PJIs.

Myeloid Cell Development, Differentiation, and Novel Functions

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Resveratrol Inhibits TCDD-induced Generation, Biological and Metabolic Functions of Myeloid-derived Suppressor Cells

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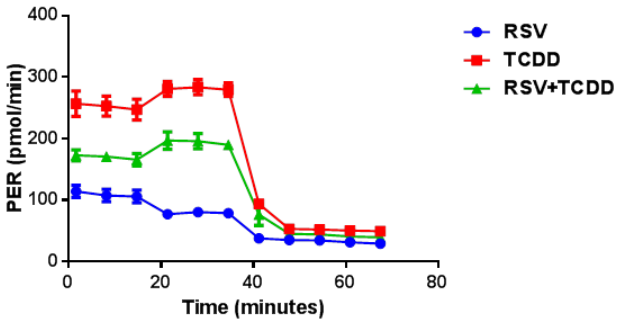
Myeloid-derived suppressor cells (MDSCs) are CD11b+Gr-1+ cells with immunosuppressive activity that are derived from the bone marrow (BM) progenitor cells. They include Ly6G-Ly6C+ monocytic (M-MDSCs) and Ly6G+ Ly6C+ Granulocytic (G-MDSCs) MDSCs. In the current study, we demonstrated that administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a highly toxic environmental organic pollutant induced MDSCs and this was reversed following treatment with 3,5,4'-trihydroxy-trans-stilbene (Resveratrol, RSV), a polyphenol derived from various edible plants such as grapes, peanut, berry, and veratrum. To this end, C57BL/6 mice were injected with TCDD (10µg/kg) i.p. in the presence or absence of oral administration of RSV (100mg/kg). MDSCs and its subsets were estimated from peritoneal exudates 24hrs later. We found that TCDD increased MDSCs, specifically granulocytic MDSCs, whereas resveratrol treatment led to their decrease. This may be due to TCDD-induced migration of the cells from the bone marrow toward the peritoneal site, while resveratrol treatment immobilized these cells in the bone marrow. Cell bioenergetic profile of MDSCs was evaluated in TCDD group with and without RSV treatment by real time metabolism analyzer. Data revealed significantly reduced glycolytic proton efflux rate (PER) in TCDD-induced MDSCs following RSV treatment when compared to untreated TCDD-induced MDSCs. Furthermore, we examined the suppressive activity of TCDD-induced MDSCs with or without RSV treatment on ConA-mediated T cell activation. We found that RSV impaired the suppressive activity of MDSCs on ConA-activated T cell proliferation in vitro. Together, our data revealed that RSV prevents immunosuppressive effects of TCDD via impairment of MDSC induction and functions in the peritoneal exudate. (Supported in part by NIH grants P01AT003961, R01AT006888, R01AI123947, R01AI129788, R01MH094755, P20GM103641 and MOHESR, Iraq).



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Adipose-Resident Myeloid-Derived Suppressor Cells Modulate Immune Cell Homeostasis in Healthy Mice

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Under steady-state conditions, adipose tissue (AT) is highly metabolic, using fatty acid oxidation reactions to maintain an appropriate energy balance within the body. Even at relatively low concentrations, reactive oxygen species (ROS) produced by these reactions can damage tissue. If left unchecked, this can develop into the chronic AT inflammation characteristic of obesity. In healthy AT, a network of resident anti-inflammatory immune cells

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modulates this endogenous inflammation, maintaining immune homeostasis within the tissue. Characterization of the non-adipocyte, stromal & vascular cells (SVF) in AT from lean, healthy mice identified a population of immunosuppressive $CD11b^{Hi}$ $Ly6C^{Hi}$ $Ly6G^{-}$ SSC^{Low} cells. This surface marker combination, together with the ability to attenuate activated T cell proliferation suggests that there is a population of myeloid-derived suppressor cells (MDSCs) resident in healthy (i.e. non-inflamed) AT. MDSCs are a heterogeneous collection of immunosuppressive cells associated with tumors and chronic inflammation. We hypothesized that these adipose-resident MDSCs (aMDSCs) could contribute to the anti-inflammatory AT microenvironment by preventing aberrant, ROS-induced inflammation, either directly with their own immunosuppressive effector functions or indirectly by promoting the suppressive phenotypes of other AT resident cells such as macrophages and T regulatory cells. We found that these cells produced IL-10, which plays a role in both T regulatory cell development and "M2" macrophage polarization. Inhibiting arginase and iNOS, *in vitro*, diminished, but did not completely abrogate the suppressive activity of aMDSCs, further demonstrating the redundant and multi-faceted approach these cells could be using to affect AT inflammation. *In vivo* depletion of aMDSCs from healthy mice incited changes in AT immune cell dynamics after only 1 week of treatment with the αGr-1 antibody, increasing the number and percentage of $CD4^{+}$ T cells and altering proportions of various adipose-resident macrophage populations. This shift in the composition of AT resident immune cells resembles what is seen in the early stages of obesity, where infiltrating pro-inflammatory macrophages and T cells promote the development of chronic AT inflammation. Although preliminary, our findings suggest that adipose resident MDSCs could be key regulators of the AT immune cell network, working to prevent degradation of the homeostatic, immunosuppressive microenvironment. These endogenous aMDSCs could represent a new therapeutic target for diabetes and obesity, as studies have already demonstrated that injections of culture-derived MDSCs into obese mice increases insulin sensitivity & delays the development of metabolic syndromes.

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Ramifications of a Shift in Myeloid Cell Phenotype Exacerbates Anemia of Critical Illness

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Introduction: Anemia, immune-suppression and monocytosis are immune consequences of a traumatic burn injury. Also, burn injury bifurcates the common myeloid progenitors (CMP) away from megakaryocyte erythrocyte progenitor (MEP) production and toward granulocyte monocyte progenitor (GMP) commitment via high myeloid transcription factor (MafB) expression. Resulting GMPs are preprogrammed to a macrophage bias with diminished MHC-II. It is known that monocytes that mature into tissue macrophages also serve a unique function of nurturing the newly developing erythroblasts during erythropoiesis. Besides the hematopoietic shortcoming of bone marrow commitment, it is not known whether myeloid cells stemming from burn-induced microenvironment also impede red blood cell development. As burn injury affects both the erythroid and myeloid arms of hematopoiesis, we utilized a mouse model of burn injury to study the phenotype of erythroblast island macrophages (EBI MØ). The concept of EBI MØ has been known for more than sixty years but poorly studied due to technical difficulties in identifying island MØ. It is conceivable that erythroblasts that are dislodged from the central MØ should still express remnants of the adhesions from the MØ of interest. Therefore, we elucidated the requirement of EBI MØ phenotype from the erythroblast perspective as well as the macrophage perspective to comprehend the interference of myeloid abnormality during erythroblast maturation after burn injury.

Method: Adult mice (B6D2F1) were subjected to 15% TBSA scald burn (B) or sham burn (S). Mice were sacrificed on post burn day 7 and bi lateral femurs were harvested. Total bone marrow cells (TBM) were eluted and probed for erythroid cells (CD71, Ter119 and Syto16) and EBI MØ (F4/80, ER-HR3, Vcam1, Siglec1 and Ly6G) by a nine color multi-parametric flow cytometry. TBM cells were gated as total erythrons ($CD71^{pos}$ $Ter119^{neg/pos}$), early

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erythroblasts ($CD71^{pos}Ter119^{neg}$) and late erythroblasts ($CD71^{pos}$ $Ter119^{pos}$). TBM cells with $F4/80^{pos}$ $ER\text{-}HR3^{pos}$ $Vcam1^{pos}$ $Siglec1^{pos}$ and Ly6G $^{pos/neg}$ phenotype were determined as EBI MØ.

Results: As expected, total erythrons per million TBM cells were decreased by 44% in burn group compared to sham (S= 539 ± 3.5 $x10^3$, B= 375 ± 14 $x10^3$; p<0.0001) Conversely, total number of EBI MØ per million TBM cells was marginally higher in burn group compared to sham (S= 225 ± 4.0 $x10^3$, B= 264 ± 5.4 $x10^3$; p<0.002). Between all adhesion molecules characteristic of EBI MØ, Siglec-1 expression (as determined by mean fluorescent intensity) was reduced by 40% in burn group compared to sham (S= 2977 ± 58, B= 1913 ± 71; p<0.0001). Amongst the erythroblast developmental stages, early and late erythroblast subsets were associated with EBI MØ at a ratio of 2:1 and 5:1 respectively in both groups (S versus B not significant). However, there were more early erythroblasts per million TBM cells in burn group (S= 61.2 ± 2.5 $x10^3$, B= 153± 10.7 $x10^3$; p+ EBI MØ (Siglec-1: S= 2497 ± 73, B= 1681 ± 102; p<0.0001. Ly6G: S= 407 ± 14, B= 323 ± 14; p<0.002). On the other hand, we noticed a 2.4 fold decrease in the number of late erythroblasts in burn group (S= 290 ± 7 $x10^3$, B= 121 ± 16 $x10^3$; p<0.0001).

Conclusion: Results imply that after burn injury, there is a significant stagnation of early erythroblasts in the bone marrow preventing their maturation into late erythroblasts and reticulocytes. This could be due to defective EBI MØ phenotype lacking predominantly in Siglec-1 expression. Defective red blood cell development resulting in anemia of critical illness is partly due to ramification of myeloid cell phenotypic alteration after burn injury.

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Enhanced Myelopoiesis and Aggravated Arthritis in *S100a8*-deficient Mice.

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Expressed strongly by myeloid cells, damage-associated molecular pattern (DAMP) proteins S100A8 and S100A9 are found in the serum of patients with infectious and autoimmune diseases. Compared to S100A9, the role of S100A8 is poorly understood. We investigated its biological activity in collagen-induced arthritis using the first known viable and fertile *S100a8*-deficient (*S100a8*$^{-/-}$) mouse. Although comparable to the wild type (WT) in terms of lymphocyte distribution in blood and in the primary and secondary lymphoid organs, *S100a8*$^{-/-}$ mice had increased numbers of neutrophils, monocytes and dendritic cells in the blood and bone marrow, and these all expressed myeloid markers CD11b, Ly6G and CD86 more strongly. Granulocyte-macrophage common precursors were increased in *S100a8*$^{-/-}$ bone marrow and yielded greater numbers of macrophages and dendritic cells in culture. The animals also developed more severe arthritic disease and oedema leading to aggravated osteoclast activity and bone destruction. These findings were correlated with increased inflammatory cell infiltration and cytokine secretion in the paws. This study suggests that S100A8 is an anti-inflammatory DAMP that regulates myeloid cell differentiation, thereby mitigating the development of experimental arthritis.

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Characterization of GM-CSF-derived Macrophages Response to Lymphocytic Choriomeningitis Virus Infection

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) can modulate the differentiation and functions of macrophages (MØ). Our main aim is to evaluate how these cytokines influence immunity to virus infection. To address this, we differentiated bone marrow derived macrophages (BMDMs) in either GM-CSF or M-CSF (referred to as GM-CSF MØ or M-CSF MØ respectively) and measured pro- and anti-inflammatory cytokine responses to virus infection. We used two strains of Lymphocytic Choriomeningitis Virus (LCMV): -CL13 or -ARM, that cause chronic or acute infections respectively. GM-CSF MØ infected with either LCMV-ARM or CL13 for 6 hours produced more IL-6 than M-CSFMØ. In contrast, infected M-CSF MØ generated more IL-10 than GM-CSF MØ at the same time point. Interestingly, in M-CSF MØ, the LCMV-ARM strain induced more IL-10 production than CL13. However, we could not detect any IL-12p70 or IL-23 after infection from both cell types. We also discovered that GM-CSF MØ were more efficient than M-CSF MØ in supporting antigen specific- CD8+ T cell proliferation. Taken together, our data demonstrate that GM-CSF and M-CSF MØ, differ in how they respond to viral infection by production of different levels of cytokines, and how they interact with CD8+ T cells.

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Characterization of Eosinophil Immune Phenotypes

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Background: Eosinophils are evolutionarily conserved granulocytes typically associated with parasite killing or allergic diseases. Although eosinophils are traditionally characterized as destructive and cytotoxic cells with a unilateral activity of degranulation releasing toxic proteins, we and others are identifying eosinophils as immune regulatory cells in health and disease. We hypothesize that there are many subtypes of immune polarized infiltrating eosinophils and that their type is disease/tissue specific and can be used as a diagnostic/prognostic indicator of health and disease.

Objective: To identify and characterize eosinophil immune subtypes based on microenvironment exposure to polarizing cytokines.

Methods: Blood-derived eosinophils were purified and cultured with cytokines IL-33/GM-CSF/IL-4 induce a Th2-polarized molecular microenvironment (E2) and eosinophils exposed to IFNγ/TNFα induce a Th1-polarized microenvironment (E1). RNAseq was completed with confirmation RT-PCR on “fingerprint genes” that are uniquely characteristic of each subtype. Protein array analysis and functional studies were completed for each subtype.

Results: RNAseq revealed E2 eosinophils have 414 genes uniquely upregulated to resting cells, and E1 have 100 genes uniquely upregulated of which we selected 5 “fingerprint genes for each subtype as identifiers”. Differentially expressed between E2 and E1 are 371 and 386 upregulated, respectively and 407 and

107downregulated, respectively. E2 eosinophils release Th2 mediators, IL-13 and CCL17 and degranulate, whereas E1 eosinophils release Th1 associated mediators CXCL10, CXCL9, CCL5, and do not degranulate. Flow cytometric analysis shows changes in cell surface expression of Ly6C, CD69 and ST2 as well.

Conclusion: Similar to the stratification of T cells: Th1, Th2, Th17; innate lymphoid cells: ILC1, ILC2, ILC3; and macrophage: M1, M2, etc., eosinophils have the potential for subtype/phenotype differentiation upon exposure to the cytokine microenvironment. These studies are early in this characterization process, but future findings will likely lead to a greater classification of eosinophil subtypes with specific activities in disease and health.

Neutrophils and Phagocyte Functions

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Neutrophil-specific Knockout Demonstrates a Role for Mitochondria in Regulating Neutrophil Motility in Zebrafish

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Neutrophils are fast moving cells essential for host immune functions. Although they primarily rely on glycolysis for ATP, isolated primary human neutrophils depend on mitochondrial membrane potential for chemotaxis. Whether mitochondria regulate neutrophil motility in vivo, however, and the underlying molecular mechanisms remain obscure. Here, we visualized mitochondria in an interconnected network that localizes to the front and rear of migrating neutrophils using a novel transgenic zebrafish line. To disrupt mitochondrial function genetically, we established a gateway system harboring the CRISPR/Cas9 elements for tissue-specific knockout. In a transgenic line, neutrophil-specific disruption of mitochondrial DNA polymerase, *polg*, significantly reduced the velocity of neutrophil interstitial migration. In addition, inhibiting the mitochondrial electron transport chain or the enzymes that reduce mitochondrial reactive oxygen species also inhibited neutrophil motility. In addition, the reduced cell motility resulted from neutrophil-specific knockout of *sod1* was rescued with *sod1* mRNA overexpression or treating with scavengers of reactive oxygen species. Together, our work has provided the first in vivo evidence that mitochondria regulate neutrophil motility, tools for the functional characterization of mitochondria related genes in neutrophils, and insights into immune deficiency seen in patients with primary mitochondrial disorders.

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Gene Expression Profiling of Intima-infiltrating Macrophages in Early Human Atherosclerosis

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Introduction: Atherosclerosis is a chronic inflammatory disease involving formation of lipoprotein-rich lesions in the arterial wall and infiltration of these lesions by macrophages, which drive disease progression. Animal models of atherosclerosis have identified pathological changes in lesion-resident macrophages, but reproducing these results in humans remains challenging. Our objective is to examine gene expression in macrophages isolated from patient coronary atherosclerotic lesions in order to better characterize macrophage dysfunction in human disease.

Methods: Aortic punch samples were obtained from patients undergoing coronary artery bypass graft surgery. Samples were sectioned and stained using an anti-CD163 antibody to identify lesion-resident macrophage populations. These cell populations were isolated through laser capture microdissection (LCM). Gene expression profiling was performed on LCM-dissected macrophage populations by human whole-exome microarray, with

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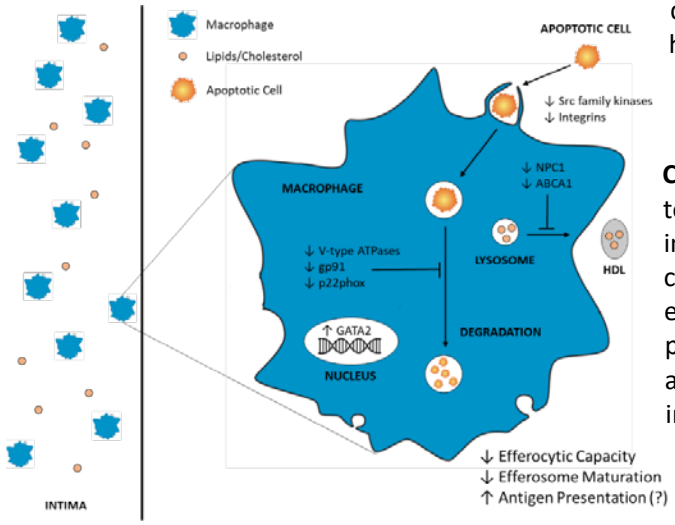
peripheral blood mononuclear cell-derived macrophages from healthy donors as a control. We established that our samples contained evidence of intimal thickening in the absence of plaque and necrotic core formation, indicating an early stage of atherosclerotic disease.

Results: Macrophage populations were isolated and qPCR analysis demonstrated enrichment of macrophage-specific CD14 compared to whole-section controls, demonstrating isolation of a pure macrophage cell population. Gene expression profiling revealed a total of differentially ~5,000 regulated genes and ncRNAs in patient lesion-resident macrophages, with particular enrichment in pathways involved intracellular transport, phagocytosis/efferocytosis and phagosomal/efferosomal cargo processing. Interestingly, we also identified significant upregulation of the hematopoietic transcription factor GATA2 (single nucleotide polymorphisms in GATA2 are associated with coronary artery disease) and several genes regulated by this transcription factor. We demonstrate that overexpression of GATA2 in human macrophage cell lines in vitro resulted in a decreased efferocytic capacity and diminished efferosomal maturation.



Conclusions: This study is to our knowledge the first to assess the transcriptional profile of intima-infiltration macrophages from the initial stages of coronary atherosclerosis in humans. We identify efferocytosis and cargo processing as dysregulated pathways in these intima-infiltrating macrophages, and are the first to identify a potential role for GATA2 in potentially driving defects in efferocytosis observed in macrophages in the setting of atherosclerosis.

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Immune Triage: Prioritization of the Innate Immune Response When Faced with Multiple Simultaneous Insults

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The innate immune system is responsible for a number of essential functions in the human body including tumour immunosurveillance, pathogen clearance, development, and wound healing. Many of these processes must happen simultaneously. We are interested in how the innate immune response prioritizes what it responds to when faced with multiple simultaneous insults. Our previous research has demonstrated how infection with influenza A virus impacts a secondary pulmonary infection with bacteria. We have expanded on this concept to show how viral or bacterial pulmonary infection alters the ability to heal wounds in a distal site. Retrospective analysis of clinical data showed that patients with pneumonia are at risk for poor wound healing. Using animal models of cutaneous wounds, we have demonstrated that lung infections suppress the infiltrate of leukocytes into the wound. As these cells are essential for the early stages of tissue repair there is a delay in the rate of wound healing. The innate immune response in the lung is not suppressed in the dual insult model, and pulmonary pathogens are cleared equivalently in wounded and unwounded mice. This demonstrates that lung infections are prioritized over cutaneous wounds, likely because maintaining a functioning lung is essential for survival. We are able to restore the rate of wound healing to that of uninfected mice by treating the wounds with chemokines that increase the amount of infiltrating leukocytes. Importantly, this study establishes new potential treatment possibilities for patients that are faced with complicated comorbidities. These data demonstrate a new biological

premise when the innate immune response is responding to both an infectious and non-infectious insult. The concept of immune triage will be broadly applicable to many diseases that involve the innate immune response.

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Extracellular Adenosine Enhances the Ability of Neutrophils to Kill *S. Pneumoniae* by Blunting IL-10 Production

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PMNs are crucial for initial control of *Streptococcus pneumoniae* (pneumococcus) early on during lung infection, however, as the infection progresses their persistence in the lungs is detrimental to the host. CD73 is surface enzyme that is required for production of extracellular adenosine (EAD), which we previously showed is required for host resistance against pneumococcal pneumonia in mice. It is now appreciated that PMNs in tissues can be heterogeneous, so we wanted to explore if the inability of PMNs to control pneumococci later in infection correlated with phenotypic differences, particularly in CD73 expression, over time. We found that 18 hours after intratracheal inoculation of C57BL/6 mice with *S. pneumoniae*, a time point at which PMN presence in the lungs no longer correlated with control of bacterial numbers, both the percentage of pulmonary PMNs expressing CD73 and the amount of CD73 expressed on PMNs decreased dramatically. PMNs from $CD73^{-/-}$ mice failed to kill pneumococci *ex vivo* and supplementation with exogenous EAD was sufficient to reverse this defect, indicating that EAD production was crucial for PMN anti-microbial activity. Adoptive transfer of PMNs from wild type mice prior to lung challenge was sufficient to boost the resistance of $CD73^{-/-}$ mice to infection. Further, EAD-mediated resistance could be largely attributed to its effects on IL-10 secretion. PMNs from $CD73^{-/-}$, but not wild type mice, up-regulated IL-10 production upon pneumococcal infection *ex vivo* and during lung challenge, responses that were blunted by exogenous EAD. Addition of recombinant IL-10 impaired the ability of PMNs from wild type mice to kill pneumococci *ex vivo*, and treatment with anti-IL-10 boosted the anti-bacterial activity of $CD73^{-/-}$ PMNs. Importantly, administering anti-IL-10 also significantly boosted the resistance of $CD73^{-/-}$mice to pneumococcal lung infection. This study demonstrates that EAD, produced by CD73, enhances PMN anti-microbial function by blunting IL-10 responses. These findings further suggest that there are changes in PMNs in the lungs over the course of bacterial pneumonia and that CD73 expression on PMNs may be indicative of a PMN phenotype that is associated with lower anti-microbial activity.

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Targeting ADAM17 with a mAb as a Potential Therapeutic Intervention in Sepsis

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Sepsis is a syndrome instigated by a disordered hyperinflammatory response against infection. It is a leading cause of mortality worldwide and one of the most expensive conditions treated in US hospitals. Neutrophil migration from the blood into tissue locations plays a critical role in the clearance of pathogens and preventing their spread. However, overwhelming inflammation impairs neutrophil migration and promotes other immune dysfunctions. ADAM17 (A disintegrin and metalloprotease domain 17) regulates cell surface density of various receptor on the surface of neutrophils. This includes CXCR2 and L-selectin, which are critical for neutrophil migration. In a murine-based polymicrobial sepsis model induced by cecal-ligation and puncture (CLP) we found that ADAM17-null mice have a significant survival advantage over wild-type mice. This was accompanied with the higher recruitment of neutrophils and improved bacterial clearance, implicating the critical role of ADAM17 in the pathogenesis of sepsis. Based on these findings, we evaluated the therapeutic significance of an ADAM17 function-blocking mAb

on the outcomes of CLP-induced sepsis. For clinical relevance, we also administered ertapenem, an antibiotic often employed for the management of sepsis. We examined the effects of ADAM17 mAb treatment before or after the induction of sepsis. C57BL/6 mice treated with MEDI3622 showed significantly longer survival and better sepsis outcomes in comparison to the groups treated with an isotype control antibody or ertapenem alone. These findings were consistent in male and female mice as well as BALB/c mice. Our results thus indicate the beneficial effects of targeting ADAM17 for sepsis treatment. Blocking ADAM17 with a mAb may provide a novel host-directed therapeutic approach for prophylactic and therapeutic sepsis treatment.

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The Nucleation Promoting Factor Wiskott-Aldrich Syndrome Protein and Scar Homolog (WASH) Differentially Regulates Granule Subsets Secretion and the Oxidative Response in Neutrophils

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The actin cytoskeleton regulates vesicular movement during exocytosis and a dual function for actin as both negative and positive regulator of exocytosis has been reported. In neutrophils, granule exocytosis is regulated by cortical actin which acts as a mechanical barrier, but a possible active role of actin in the regulation of neutrophil exocytosis is currently unknown. To increase our understanding of the function of actin modulators in the control of neutrophil functions, we examined the role of WASH in neutrophil exocytosis. Using neutrophils from WASH-knockout (WASH-KO) mice (Vav-Cre strain), we observed that WASH regulates differential exocytosis of neutrophil gelatinase and azurophilic granules. WASH-KO cells, showed increased basal azurophilic granule secretion as determined by myeloperoxidase exocytosis, measured by ELISA. Correspondingly, we observed an in vivo increase in plasma myeloperoxidase levels in the WASH-KO mice indicating that this model has a neutrophil-mediated pro-inflammatory phenotype. The plasma levels of pro-inflammatory cytokines including GM-CSF, IFN-γ and IL-1β were unaffected in the WASH-KO mice, suggesting that the pro-inflammatory, secretory, defective phenotype, is likely caused by a cell-intrinsic mechanism. An increase in the basal secretion of azurophilic granules was also manifested as an increase in basal plasma membrane levels of the azurophilic granule marker CD63. Surprisingly, secretion of gelatinase granules was impaired upon stimulation of WASH-KO neutrophils as detected by decreased MMP-9 secretion indicating differential regulation of granules subsets in WASH-KO neutrophils. The differential defective phenotype in azurophilic and gelatinase granule secretion was confirmed using human neutrophils and anti-WASH inhibitory antibodies. Impaired gelatinase granule secretion also correlated with reduced reactive oxygen species production in these neutrophils. In the absence of WASH, neutrophils display an increase in ERK-dependent signaling; however, inhibition of this pathway did not prevent increase azurophilic granule secretion. Immunofluorescence and super-resolution microscopy analysis showed pockets of azurophilic granules in areas clear of F-actin in the WASH-KO neutrophils, suggesting an active role for WASH-mediated actin assembly in regulating neutrophil exocytosis. Immunofluorescence analysis of endogenous proteins detected the colocalization of WASH with the small GTPase Rab27a, a key regulator of vesicular trafficking in neutrophils. Direct interaction between WASH and Rab27a was confirmed by immunoprecipitation of the binding proteins suggesting a cross-regulation of the secretory and the actin-remodeling machineries. Azurophilic granule exocytosis was inhibited by the Rab27a-JFC1 inhibitor Nexinhib20, thus supporting that active Rab27a is involved in the exacerbated secretion observed in WASH-deficiency. Our findings identify a unique role for the actin nucleation promoting factor WASH in differentially regulating neutrophil granule subset exocytosis and oxidative function, mediating actin remodeling associated with vesicular trafficking.

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HS1 Deficiency Protects Against Sepsis by Preventing Excessive Neutrophil Recruitment

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Sepsis remains an important health problem worldwide with high mortality rates as a consequence of inefficient treatments often resulting in multi-organ failure. Neutrophil recruitment is a critical event during sepsis as part of the innate immune response. However, the precise role of neutrophils in sepsis is still a matter of debate. While there is agreement that neutrophils are required to combat invading bacteria, there is also emerging evidence that excessive neutrophil recruitment leads to tissue damage thus further aggravating the disease. We recently showed that the actin-binding protein HS1 is indispensable for proper neutrophil extravasation. Thus, we hypothesized that HS1 also plays an important role during sepsis. Here, we evaluated the role of HS1 in a model of lethal sepsis induced by cecal-ligation and puncture (CLP) using HS1-deficient mice. Surprisingly, we found that HS1 deficiency has a protective effect during sepsis. This effect was associated with reduced numbers of infiltrating leukocytes in the lung, one of the most affected organs during sepsis. Histological analysis of lung tissues showed that HS1-deficient mice had less inflammation and tissue damage 24 h after sepsis induction. Additionally, cleavage of PARP was reduced in HS1-deficient mice suggesting that lungs were protected against apoptosis. Using IVM of the cremaster muscle in septic mice, we found that the systemic infection was unable to induce efficient neutrophil extravasation in the absence of HS1. Our results define HS1 as a critical regulator of sepsis development by supporting excessive neutrophil recruitment causing severe tissue damage, and thus contributing to organ failure.

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Plinabulin-Associated Neutrophil Demargination: Evidence for a Clinically Relevant Mechanism of Action for the Prevention of Chemotherapy-Induced-Neutropenia

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Introduction. Plinabulin (Plin) is a novel non-GCSF small molecule with anti-cancer activity, in development for prevention of Chemotherapy (Chemo)-Induced-Neutropenia (CIN) induced by Docetaxel (Doc), Adriamycin, Cyclophosphamide, Irinotecan, Gemcitabine, Carboplatin, Abraxane. Plin is administered on the same day, 30 minutes after Chemo as a single intravenous infusion, once per cycle. In a prospective phase (Ph) 2 clinical trial (Study 105, NCT03102606) comparing Plin head-to-head with Pegfilgrastim (Peg), Plin has at least equal efficacy as Peg for the prevention of grade 4 CIN, which was associated with a lower incidence of clinical sequelae from CIN (sepsis, severe infections, and Doc dose reduction) (Blayney, ASCO 2018; Mohanlal ASCO 2016). Plin maintained absolute neutrophil (N) count (ANC) within the normal range after Doc. In contrast, Peg showed an overshoot in ANC to levels approximately 3 times higher than upper limit of normal and was associated with more bone pain. The lack of ANC overshoot and bone pain with Plin suggests a mechanism of action (MoA) that is different between Peg and Plin for the prevention of CIN. Plin enhances Dendritic Cell maturation, leading to the release of IL-6 (Lloyd, AACR 2016). IL-6 has been reported to induce demargination of N and to shorten N transit

time from bone marrow (Suwa, Am J Physiol; 2000). Here, we evaluated the effects of Plin on N demargination. The data we analyzed was derived from Study 105, in which all NSCLC patients (pts) received Doc and dexamethasone (Dex) as premedication for Doc. Dex is known to induce N demargination from the bone marrow (Nakagawa, Circulation; 1989). Since Plin was given in a dose ranging from 0 to 20 mg/m2, in addition to the fixed dose of Dex, this analysis evaluated ANC increases due to Dex alone, and due to Dex with incremental Plin doses.

Method: The effect of Plin on Doc-induced CIN was evaluated with Plin doses at 0 (n=14), 5 (n=14), 10 (n=13) or 20 (n=14) mg/m2 in NSCLC pts from Study 105. All pts in all dose groups received dexamethasone (16 mg/day) premedication for Doc on Day (D) 0, D1 and D2. Doc was administered on D1, followed 30 min later by Plin infusion. ANC was taken at predose D1 (prior to Doc and Plin administration) and D2, D5,D6,D7,D8,D9,D10, and D15. Blood sampling for Plin pharmacokinetics (PK) was obtained before and at specified intervals after Plin dosing on D1. We analyzed the effect of Plin on ANC changes in two different ways: 1. By comparing the increase in ANC of incremental increases in Plin, against a fixed background of Dex. 2. By a nonlinear mixed effects PK/pharmacodynamic (PD) modeling approach to determine if Plin was additive to Dex in increasing the rate of production of ANC using a turnover (production and clearance) model for ANC. PK/PD modeling was performed on data that were available from 41 pts; 27 pts with Dex, Doc and Plin (at different dose levels), and 14 subjects with Dex and Doc, but without Plin. The Wilcoxon test was used to determine if the increased rate of production of ANC in Plin-treated pts (who also had received Dex) was significantly different from that of pts who had received Dex without Plin. The increase in ANC between D1 and D2 was considered as the extent of N demargination due to Plin, since the Dex dose was constant in all pts on those days, whereas the Plin dose was the only variable. N nadir occurred around D9.

Results: In the group that received Dex but no Plin, the increase in mean ANC on D2 vs D1 was 0.1 x10E9/L (p= 0.5). In the groups that received Dex and incremental doses of Plin, an increase in ANC on D2 vs D1 was observed with 1.8, 1.5, and 3.3 x10E9/L with Plin at doses of 5 (p< 0.02), 10 (p=0.09) and 20 (p< 0.004) mg/m2 Plin respectively. Mixed effect modeling showed that the addition of Plin significantly increased ANC (p= 0.038) and the effect was additive to that of dexamethasone. The ANC on D2 correlated positively with ANC at nadir: The higher D2 ANC, the higher was the ANC nadir (P<0.05).

Conclusion: We provide evidence of N demargination with Plin, that is consistent with Plin's increase in IL-6 release. N demargination was associated with a higher ANC value at nadir. Improved N-demargination by Plin may have clinical relevance in Plin's protection against CIN.

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Human Cathelicidin Peptide LL-37 Ameliorates Sepsis in a Mouse Model Through Induction of Microparticles with Antibacterial Potential from Neutrophils

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Sepsis is a systemic dysregulated inflammatory response to infection. Despite substantial advances in our knowledge regarding the pathophysiology of sepsis and subsequent improvements of clinical care, sepsis is still a major cause of mortality in the intensive care unit worldwide. Furthermore, a number of therapeutic trails concerning immune dysfunction have been disappointing outcomes. Neutrophils, the first line of host defense against invading microorganisms, release microparticles called ectosomes (Ect) upon stimulation. Ect (0.1-1 mm in diameter) contain neutrophil-derived proteins/peptides, and an elevated level of Ect in the blood of sepsis survivors suggests its pro-resolving potential. In an attempt to seek for therapeutic molecules for sepsis, we have

previously revealed that LL-37, a human cathelicidin antimicrobial peptide, improves survival of cecal ligation and puncture (CLP) septic model mice. In this study, we focused on the effect of LL-37 on the induction of Ect in CLP mice. First, we observed that Ect were produced in CLP mice, and the level of Ect in the peritoneal fluid and blood was augmented by intravenous administration of LL-37, which accompanied with lower bacterial burdens. Ect fractions isolated from LL-37- or control PBS-injected CLP mice (CLP-37-Ect or CLP-PBS-Ect) possessed antibacterial activity with higher potential of CLP-37-Ect. Interestingly, the antibacterial activity of CLP-37-Ect and CLP-PBS-Ect was partially inhibited by heparin (anionic proteoglycan) and anti-lactoferrin antibody, suggesting that cationic antibacterial proteins and lactoferrin incorporated into Ect play a role in the anti-bacterial activity. In fact, both CLP-37-Ect and CLP-PBS-Ect contained neutrophil granule-derived proteins with antibacterial activity such as CRAMP and lactoferrin. Furthermore, LL-37 induced the production of Ect from mouse bone marrow neutrophils *in vitro*, and the Ect possessed anti-bacterial activity. Together these observations suggest that LL-37 acts on neutrophils and induces the release of Ect, which exert antibacterial potential, thereby ameliorating sepsis. Our present findings regarding neutrophil Ect may provide a milestone for a novel sepsis therapeutic concept.

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Treatment of Borrelia Burgdorferi-infected Mice with Apoptotic Cells Alters Arthritis Severity via Activation of PPARγ And PGE_2

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Experimental Lyme arthritis is induced by the infection of C3H mice with the spirochete, *Borrelia Burgdorferi*, (Bb), and is a valuable model to study the regulation of inflammation during bacterial infection. We are interested in understanding the role that apoptotic cells (AC) play in inflammation resolution during murine Lyme arthritis. We show the number of AC within infected ankle joints significantly increases during arthritis resolution. Additionally, injection of AC into the tibiotarsal joints of Bb-infected mice reduces ankle swelling compared to control animals. *In vitro* studies using bone marrow derived macrophages (BMDM) co-cultured with Bb demonstrates the addition of AC decreases TNFα and KC production, while increasing IL-10 and PGE_2levels. The presence of AC in BMDM and also bone marrow neutrophils (BMN) cultures, decreases Bb phagocytosis, and also decreases BMN migration to LTB_4. To further explore this mechanism, we measured expression of PPAR-γ (peroxisome proliferator-activated receptor gamma), which is known to regulate cytokine expression. Addition of AC to BMDM/Bb co-cultures increased PPAR-γ transcription *in vitro*, and also within infected ankle joints compared to mock treated mice *in vivo*. We also found AC themselves can produce PGE_2, a bioactive lipid which activates PPAR-γ, setting up a positive feedback loop for the production PGE_2. These results suggest AC can alter macrophage and neutrophil effector functions and may play a role in downregulating inflammatory responses, perhaps via activation of PPAR-γ. In addition, injection of AC into infected joints may limit inflammatory responses or induce their resolution.

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Sweet, Bitter, Hot – "Chemosensory" Capabilities of Human Neutrophils

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In recent years, internal chemoreception within the human body, projecting beyond the external chemoreception of our classical chemical senses (such as smell and taste), has gained increasing attention. In particular evidence

that odorant and taste G-protein coupled receptors (GPCR) are functionally expressed in e.g. immune cells [1, 2] suggests new roles for these typical chemosensory receptors in the monitoring of our internal chemical environment. Based on findings that aroma/flavor-relevant food ingredients (i) are best agonists for our chemosensory receptors [3, 4], (ii) can enter the blood stream, and (iii) activate cell functions in isolated leukocytes [2, 5], we examined the impact of saccharin, the most widely utilized artificial sweetener, on basic cellular functions in neutrophils, in vitro and in vivo. In experiments with isolated neutrophils, derived from either buffy coats or a human intervention study, we measured saccharin-induced Ca^{2+} signaling, chemotaxis, saccharin-modulated phagocytosis, and, via RT-qPCR, monitored the effect of artificial sweeteners, among them saccharin, on the transcript levels of receptors and components of intracellular signaling pathways. We found that saccharin is capable of modulating cellular functions of isolated neutrophils by concentration-dependently and differentially activating a variety of biological target molecules, such as sweet-taste, bitter-taste, and chemesthetic receptors. This may suggest a chemoreceptive monitoring function of neutrophils within immunological homeostasis.

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Characterization of Low-Density Neutrophil Subpopulations in HIV-1-Infected Individuals

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Neutrophils represent the first innate immune cell population recruited to the sites of trauma and infection. Recent studies have demonstrated that neutrophils represent a heterogeneous population consisting of subpopulations with distinct phenotype and function. A subset of low-density neutrophils (LDNs) was identified co-purifying in the peripheral blood mononuclear cell (PBMC) layer following density gradient centrifugation in patients with inflammatory conditions. While the origin and function of LDNs are unclear, immunosuppressive functions have been ascribed to this population in multiple diseases including cancer and chronic viral and bacterial infections. Increased frequency of LDNs has been associated with the progression of vascular dysfunction. HIV-1-infection is associated with chronic damage to the gut endothelial lining which results in the translocation of microbes and microbial products. We hypothesize that chronic microbial translocation leads to an induction of specific subpopulations of neutrophils contributing to immune suppression and disease pathogenesis in HIV-1-infected individuals. Changes in neutrophil surface marker expression and an increase in the number of LDNs have been reported in HIV-1-infection; however, detailed characterization of specific neutrophil subpopulations in HIV-1-infected individuals has not yet been performed.

We have identified two distinct LDN subpopulations within the PBMC layer of HIV-1-infected individuals that display significant morphologic, phenotypic, and functional differences. The first subpopulation is characterized as $CD16^{+}CD14^{dim}CD64^{-}$ suggestive of a mature neutrophil phenotype (mLDN); the second population is

characterized as $CD16^{-}CD14^{-}CD64^{+}$ consistent with an immature neutrophil phenotype (imLDN). mLDNs exhibit elevated levels of CXCR2 and CD10 and a lower level of CD66b compared to imLDNs. We have optimized a method for the identification of imLDNs in whole blood using CD64 and CD16 surface markers without the need for density gradient centrifugation allowing for characterization of imLDNs close to their *in vivo* state. In HIV-1-infected individuals, sorted imLDNs have a banded morphology consistent with immature neutrophil maturation states. Sorted mLDNs have a similar morphology to whole blood neutrophils evidenced by multi-lobular nuclei. However, mLDNs display elevated levels of CD16, CXCR2, and a lower level of CD62L compared to the major population of circulating neutrophils. Importantly, we have observed relationships between mLDN surface marker expression levels and clinical parameters associated with liver disease progression in HIV-1-infected individuals. In vitro stimulation of whole blood with bacterial components, fMLF or LPS, induce significant expansion of mLDNs in the PBMC layer. Functional assessments demonstrate that mLDNs have higher capacities for phagocytosis and the production of reactive oxygen species compared to imLDNs, consistent with their immature phenotype. Determination of the characteristics of two newly identified LDN subpopulations will be instrumental in the elucidation of their role in disease progression and development of co-morbidities in HIV-1-infected individuals.

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Obese, Diabetic Mice Demonstrate Immune Dysfunction and Renal Injury Following Trauma

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Background: Traumatic injury results in 41 million emergency department visits and 2.3 million hospitalizations each year. While the in-hospital mortality rate after traumatic injury is less than 4%, long-term mortality rates escalate after hospital discharge to 16% at 3 years. Long-term trauma deaths are attributed to high comorbidity burden, physiologic frailty, immune senescence, persistent inflammation, infections / sepsis, hospital readmissions, and multisystem organ failure. Obese and type 2 diabetic patients are especially vulnerable to increased long-term mortality following traumatic injury given their propensity for recurrent infections and renal failure. A better understanding of the immune dysfunction associated with obesity and type 2 diabetes is necessary to develop therapeutic strategies to reduce the long-term mortality rates, resource consumption, and improve quality survival following traumatic injury. We hypothesize that obesity and diabetes act as an immune deficiency that delays wound healing, impedes tissue regeneration, and hinders bacterial surveillance, which increases long-term mortality following traumatic injury.

Methods: C57BL/6 (lean) and Diet Induced Obese (DIO, obese/diabetic) 30-week-old mice underwent traumatic injury (midline laparotomy). Naïve and trauma-injured mice were followed for weight loss. At serial time points, n=3 mice/group were euthanized. Neutrophil and monocyte numbers were evaluated in the bone marrow and the spleen. Neutrophil and monocyte reactive oxygen species (ROS) generation were assessed by flow cytometry. Urine was collected and BUN, creatinine, and NGAL determined for evaluation of kidney organ injury. Multiplex RT-PCR arrays were used to evaluate toll like receptors (TLR), complement, and immune signaling cascades. Cytokine analysis was evaluated with Luminex^{TM} technology.

Results: After traumatic injury, DIO mice exhibited greater weight loss compared to lean mice ($P<0.001$). Lean mice lost a maximum of 4% of their body weight, but by day 11 had started regaining weight and were near baseline by day 28. DIO mice lost a maximum of 16% of their body weight by day 7, and this significant weight loss persisted through day 28. DIO naïve mice had greater numbers of neutrophils ($P<0.05$) and monocytes ($P<0.001$) in the bone marrow compared to lean naïve mice. However, after traumatic injury DIO mice had decreased emergency granulopoiesis with less neutrophil numbers in the bone marrow compared to lean mice ($P<0.001$). Neutrophil and monocyte ROS generation in DIO mice was initially greater than lean mice ($P<0.05$); however, after 7 days post-injury, DIO mice neutrophil and monocyte ROS generation decreased and was much worse than that

of lean mice (P<0.001). DIO mice also had decreased urinary output after trauma, concerning for end organ injury. There were no significant differences in blood BUN, creatinine, or NGAL levels between DIO and lean mice; however, on light microscopy DIO mice had renal architectural damage compared to lean mice without any histologic indication of injury. Furthermore, DIO mice had significantly more C3 mRNA expression in the renal cortex, indicative of more renal injury. Finally, post traumatic injury, DIO mice had a delayed pro-inflammatory cytokine response, with peak levels of TNFa, IFNγ, and GM-CSF seen 7 days after trauma. DIO mice also had a decreased early anti-inflammatory phase, with an initial lack of IL-5, which recovered around day 7, with an observed increase in IL-13.

Conclusions: DIO mice have less physiologic reserve compared to lean mice. After traumatic injury, DIO mice have inadequate emergency granulopoiesis and demonstrate late defects in neutrophil and monocyte ROS generation. DIO mice also experience more renal injury following trauma. Finally, DIO mice exhibit defects in both the pro- and anti- inflammatory phases of the immune response following traumatic injury. The defects in DIO mice neutrophil and monocyte function, combined with renal injury and altered metabolism following trauma, may explain why obese, diabetics suffer increased long-term mortality following trauma.

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A High Content Microfluidic Assay for Leukocyte Activity

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Neutrophils play a central role during inflammation and infection, utilizing an arsenal of potent antimicrobial mechanisms that must be tightly orchestrated to avoid damage to host tissues. Dysregulation of neutrophil activity in some patient populations is associated with increased risk of infection, which can lead to sepsis. Recently, we demonstrated that measurement of unusual spontaneous neutrophil motility signatures can be used for accurate diagnostic for sepsis. Here, we hypothesized that the antimicrobial capacity of neutrophils from patients with sepsis might also be altered. We developed a new, high-content microfluidic assay that uses live-imaging of host-pathogen interactions *ex vivo* to evaluate neutrophil antimicrobial activity. We demonstrate use of the assay to test neutrophil chemotaxis, phagocytosis, production of reactive oxygen species, and suppression of microbial proliferation. As well as providing predictive potential in the clinic, this platform will be used for experimental modulation of host or pathogen activity, and as a screening platform for drugs that aim to modulate the neutrophil-microbe interface.

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Inhibition of Human Neutrophil Inflammatory and Apoptotic Caspases Regulate IL-1β Production and Neutrophil Lifespan During *Toxoplasma Gondii* Infection

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Neutrophils are a major player in host immunity to infection; however, the mechanisms by which human neutrophils respond to the intracellular protozoan parasite *Toxoplasma gondii* are still poorly understood. We investigated the activation of apoptotic and inflammatory caspases during *T. gondii* infection of human peripheral blood neutrophils from multiple healthy donors. Circulating neutrophils have a short half-life and apoptosis is the predominant cell death pathway in these cells. *T. gondii* infection inhibited the spontaneous, the starvation-induced, and the TNF-α-induced cleavage of caspase-8 and caspase-3. The effect of *T. gondii* infection on apoptotic caspases was associated with a significant reduction in the percentage of annexin V positive cells, a hallmark of the early stage of apoptosis. Moreover, *T. gondii* infection of human neutrophils reduced cell mortality over time,

as determined by 7-AAD staining, suggesting an increase in human neutrophil lifespan by inhibiting apoptosis. In addition to an effect on the apoptotic caspases, *T. gondii* infection inhibited the cleavage and activity of the inflammatory caspase-1 in human neutrophils, which contributed to the suppression of lipopolysaccharide (LPS)-induced IL-1β synthesis. *T. gondii* inhibition of caspase-1 required active parasite invasion, since heat-killed or mycalolide B-treated parasites did not prevent caspase-1 cleavage. *T. gondii* infection of human neutrophils treated with LPS also resulted in reduced transcript levels of *IL-1β* and *NLRP3* and reduced protein levels of pro-IL-1β, mature IL-1β, and the inflammasome sensor NLRP3. In *T. gondii*-infected neutrophils stimulated with LPS, the levels of MyD88, TRAF6, IKKα, IKKβ, and phosphorylated IKKα/β were not affected. However, LPS-induced IκBα degradation and p65 phosphorylation were reduced in *T. gondii*-infected neutrophils, and degradation of IκBα was reversed by treatment with the proteasome inhibitor MG-132. These results indicate that *T. gondii* suppression of IL-1β involves a two-pronged strategy whereby *T. gondii* inhibits both activation of caspase-1 and NF-κB signaling. Together, these findings represent novel mechanisms of *T. gondii* evasion of human neutrophil-mediated host defense by targeting the production of IL-1β and neutrophil lifespan, which allow parasite replication and survival.

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Extracellular Acidification During Ischemia-reperfusion Injury Promote Transcellular Extravasation of Neutrophil Which May Alter Vascular Permeability and Inflammatory Outcome

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Inflammatory conditions such as ischemia reperfusion injury or atherosclerosis are known to be associated with excessive neutrophil infiltration into tissue that drives disease pathogenesis. However, the correlation between neutrophil transmigration and inflammation is remains elusive. Neutrophil extravasation through underlying vascular bed can occur either between two endothelial cells (paracellular) or directly through an endothelial cell body (transcellular). We previously reported that deficiency in Rap1b, a member of Ras superfamily of GTPase, enhanced neutrophil transcellular migration via activation of AKT pathway (Kumar et al, JEM, 2014). Further, Rap1-deficiency increased neutrophil recruitment to inflamed lungs and enhanced susceptibility to endotoxin shock, suggesting mode of neutrophil migration may influence inflammatory outcome. Recent investigations suggest, increased neutrophil migration and expression of pAKT in when treated with acidic media. Since ischemia reperfusion (I/R) injury is known to be associated with increased tissue acidification, we hypothesized that endothelial acidification during IR injury could favor transcellular route of migration affecting vascular permeability and inflammatory outcome. Using a non-invasive in vivo model of IR injury, gold-plated, N42-grade neodymium magnets were placed over two-third surface of ear to induce ischemia for two hours. Following incubation, magnets were carefully removed to allow reperfusion to take place for another four hours. Endothelial junctions of blood vessels were visualized by fluorescent tagged PECAM antibody, injected intradermaly into the ear. Vascular leakage and neutrophil transmigration were detected via retro-orbital injection of fixable rhodamine labeled Dextran and fluorescent tagged Ly6g antibody respectively. Ears were formalin fixed and visualized using Z-stack confocal microscopy. FMLP stimulation of ear vasculature was used as control. Compared to FMLP stimulated group, IR injury triggered massive rhodamine dextran leakage in the ear. Increased vascular leakage correlated with colocalization of Ly6G positive neutrophils and decrease in PECAM expression. Importantly, compared to WT mice, increased amount of vascular leakage was observed in Rap1b KO mice suggesting important role of route of neutrophil transmigration in IR injury. Under in-vitro condition, FMLP stimulation of WT neutrophils pre-treated at acidic pH of 6.5 showed increased expression of Cd11b, F-actin and Vinculin on chambered glass slides. Importantly, it increased formation of invasive protrusions in 1μM transwell chambers under FMLP chemotaxis. Moreover, pre-treatment of WT neutrophils under acidic condition, increased frequency

of migration away from junction (transcellular), when cocultured over LPS activated BEND3 endothelial monolayer. Together, these results suggest that under conditions of extracellular acidification, WT neutrophil could adopt the phenotype of more inflammatory Rap1b KO neutrophil, favoring the transcellular route of migration which may alter endothelial permeability and influence inflammatory outcome. Since milieu acidification plays a major role in ischemic damage, our findings may be clinically important for our understanding of Ischemia reperfusion injury and development of approaches for effective treatment.

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Burn Injury Induces the Expansion of Granulocytes with a Suppressor Phenotype via IL-6 *trans*-signaling

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Severe tissue damage caused by trauma or burn injuries can induce a state of immune suppression. As a result, infections and sepsis are among the leading causes of complications and mortality in critically ill patients. In our well-established murine model of burn injury, we sought to investigate the phenotype and function of myeloid cells found in the peripheral blood after burn injury, hypothesizing that dysregulation of myeloid cell populations plays a central role in the immune dysfunction seen post-burn.

We observed an expansion of a $CD11b^{+}Gr\text{-}1^{+}(Ly6G^{hi}Ly6C^{hi})$ cell population in the peripheral blood of burned mice 24 hours after injury, increasing to 71±10% of $CD45^{+}$ peripheral blood cells compared to 13±2% in sham animals ($p<0.001$). This cell population also expressed arginase-1, CD31, and myeloid DAP12-associating lectin-1 (MDL-1), surface markers putatively associated with a myeloid-derived suppressor cell-like phenotype during critical illness. When purified by magnetic-activated cell sorting, such burn-induced $Gr\text{-}1^{+}Ly6G^{hi}$ cells were found to express much higher levels of genes associated with an immunosuppressive phenotype, including arginase-1 (*arg1*, 182±0.9-fold increase relative to sham, $p<0.01$), IL-10 (*il10*, 10±0.1-fold increase relative to sham, $p<0.01$), CCL2 (*ccl2*, 16±1.6-fold increase relative to sham, $p<0.01$), and inducible nitric oxide synthase (iNOS) (*nos2*, 25±0.8-fold increase relative to sham, $p<0.01$). Furthermore, microscopic examination of cytospin preparations of these cells revealed a morphologically homogenous population of cells with granulocytic morphology.

To elucidate the mechanisms regulating the expansion of this cell population, we employed antibodies blocking both membrane-bound and soluble IL-6 receptors (i.e. global IL-6 signaling) or isotype IgG controls, as well as a recombinant soluble gp130Fc peptide that selectively inhibits IL-6 signaling through the soluble receptor (i.e. IL-6 *trans*-signaling). Blockade of both global and *trans*-signaling after burn injury reduced the number of circulating $CD11b^{+}Gr\text{-}1^{+}$ cells to 30±9% and 37±5%, respectively, ($p<0.01$ compared to burn given isotype control antibodies), indicating that signaling through the IL-6 soluble receptor plays an important role in induction of this cell population and phenotype.

In summary, our results demonstrate that burn injury induces the expansion of a granulocytic cell population in peripheral blood that exhibits a phenotype associated with immune suppression, in part due to the actions of IL-6 *trans*-signaling through the soluble IL-6 receptor. Ongoing studies focus on the role of these cells in the dysfunctional immune responses to infection after burn injury, and the mechanisms by which such cells may exert an immunosuppressive function.

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Characterization of Newly Identified Human Neutrophil Subsets in HIV-1-infected Patients

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Despite successful viral load suppression using antiretroviral therapy (ART), patients infected with the human immunodeficiency virus (HIV) fail to fully recover their immune regulation and responsiveness. HIV-1 infection is associated with a breakdown of the gut mucosal barrier and subsequent microbial translocation to systemic circulation causing the immune system to be in a chronic inflammatory state. This chronic inflammatory state eventually puts HIV-infected patients at a higher risk for development of comorbidities including cardiovascular disease and liver disease. Despite comprehensive studies analyzing HIV-1-infection and its effect on the adaptive immune system, the role of innate immune system, specifically neutrophils, in mediating the effect of microbial translocation on disease pathogenesis remains unknown.

Neutrophils are the most abundant circulating population of leukocytes (50-70%) and the first innate immune responders to invading pathogens. A significant alteration in their functional capabilities is likely to exert a critical effect on the immune system. Recently, it has become accepted that neutrophils consist of multiple subpopulations with a variety of functional capabilities to influence both the adaptive and innate immune system. We hypothesize that changes in the phenotype and activation status of circulating neutrophils and the frequencies of specific neutrophil subpopulations may be associated with disease progression and development of comorbidities in HIV-1-infected patients.

We have identified phenotypic changes of total neutrophil population in HIV-1-infected individuals including changes in surface level expressions of CD16, myeloperoxidase (MPO), CD31, and CD64, indicating an immature neutrophil phenotype. We show that these changes in surface marker expression correlate with clinical parameters of liver and cardiac disease progression. Previous studies have shown that a population of low-density neutrophils (LDNs) co-purify in the peripheral blood mononuclear cell (PBMC) layer after density centrifugation and are expanded during inflammatory conditions. We demonstrate there are two distinct LDN subpopulations in the PBMC layer each at different stages of maturation which we define as mature low-density neutrophils (mLDN) and immature low-density neutrophils (imLDN). We establish that upon stimulation of whole blood with lipopolysaccharide (LPS) and N-formylmethionine-leucyl-phenylalanine (fMLF) leads to an expansion of the mLDN population but not the imLDN population. We developed a method for monitoring the imLDN subpopulation in whole blood without the need for centrifugation and allows us to characterize the imLDN population closer to its *in vivo* state. By using flow-cytometry and high dimension reduction analysis, we identified several novel neutrophil subpopulations with as of yet defined functions. Detailed characterization of neutrophil subpopulations will elucidate the role of neutrophils in disease progression and the development comorbidities in HIV-1-infected individuals and facilitate the pharmacological targeting of neutrophils or their products in HIV-1-infected individuals.

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Chronic Alcohol Increases Neutrophil Extracellular Traps (NETs) in the Liver and Neutrophil Depletion Attenuates Liver Injury in Alcoholic Hepatitis in Mice

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Background: Neutrophils, the most abundant circulating phagocyte in humans, play a crucial role as the first line of host defense against invading pathogens. As an anti-microbial strategy, neutrophils release mesh-like decondensed chromatin associated with histones and various cytoplasmic molecules, called neutrophil extracellular traps (NETs), in response to pathogen- and damage-associated molecules. Uncontrolled NETs formation is related to excessive inflammation and disease development. Sustained inflammation and

macrophage activation are characteristics of alcoholic liver disease. In addition, neutrophils are considered to be one of the major contributing cell types to liver injury and correlate with increased mortality in alcoholic hepatitis (AH). Here we hypothesized that neutrophil recruitment to the liver and abnormal NETs formation contribute to liver injury in in mice with alcoholic liver disease. In this study, we tested the effect of neutrophil depletion on alcohol-induced liver damage and inflammation and the role of macrophage-neutrophil interactions in alcoholic liver disease in mice.

Method: For chronic alcohol treatment, mice were fed with 5% alcohol contained Lieber Di Carlie diet for 4 weeks. *In vivo* NETs formation was accessed by double immunofluorescence staining (Neutrophil elastase and histone H3) with mouse liver specimens. Anti-Ly6G (1A8) or isotype antibody was used for neutrophil depletion *in vivo*. Some mice received LPS (0.5mg/kg, *i.p.*) at the end of 4 weeks alcohol feeding to mimic acute alcoholic hepatitis. Experimental outputs were assessed by ELISA, RT-qPCR, western blotting and flow cytometry.

Results: In the liver of alcoholic hepatitis mouse, we found a significant increase in neutrophil count compared to the one in the calorie matched liquid diet-fed control mice. Neutrophil elastase was increased in livers of alcohol-fed mice and it co-localized with histone H3 indicating NETs formation compared to mice with control, pair-fed diet. Unlike in pair-fed mice, NETs clearance by macrophages was delayed in the liver of alcohol-fed mice and this was associated with sustained increase in pro-inflammatory cytokines including MCP-1 and IL-6. Neutrophil depletion with the anti-Ly6G antibody in alcohol-fed mice attenuated alcohol-induced liver damage indicated by reduced serum levels of alanine aminotransferase (ALT) compared to control-ab treated alcohol-fed mice. Liver and serum levels of MCP-1 and IL-6 were elevated in alcohol-fed mice and this was attenuated by administration of the anti-Ly6G antibody compared to treatment with isotype control antibody. Bcl-xL and Cyclin D1, involved in hepatocyte survival, were reduced by alcohol treatment, which was prevented by neutrophil depletion. Neutrophil depletion also decreased the TUNEL positive cells in the liver compared to control antibody in alcohol-fed mice. LPS challenge in the chronic alcohol-fed mice increased ALT and IL-6 levels despite the absence of neutrophils. The substantial increase of systemic and hepatic IL-6 was associated with pSTAT3 increase and SOCS3 up-regulation in the liver. Moreover, neutrophil depletion increased the number of Ly6C low, repair, monocytes while the frequency of Ly6C high, inflammatory, monocytes was reduced in the liver even in alcohol-fed mice.

Conclusion: In a mouse model of alcoholic hepatitis, alcohol-induced increase in NETs formation contributes to liver damage and neutrophil depletion in chronic alcohol-fed mice reduces hepatocyte damage. Increased IL-6 and pSTAT3-SOCS3 up-regulation as well as increase of anti-inflammatory monocytes are associated with attenuation of hepatocyte damage after depletion of neutrophil leukocytes in alcoholic liver disease.

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Neutrophil Swarming Releases Nets and Contains Microbial Growth

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Neutrophil swarming seals off infection and protects healthy tissue. However, the details of the novel biological process of swarming are just emerging. Current *in vivo* models are qualitative, low throughput, and have limited access to mediators. Here, we leverage our microscale technologies to directly quantify the containment of microbes by neutrophil swarms.

Methods: We developed an assay to study thousands of synchronized swarming processes at once. Neutrophil swarming is triggered on large arrays of live microbe clusters, for which we control geometric features such as size and spacing. Time-lapse fluorescence imaging helps monitor the microbe-neutrophil interactions.

Results: We tested the swarming of human neutrophils against live fungi like *Candida albicans* and *Aspergillus fumigatus* and live bacteria like *Staphylococcus aureus*. While live microbes incubated alone grew well on the arrays, neutrophil swarms significantly delayed the growth of all microbes tested. Swarms contained fungal hyphae for up to 16 hours and disruption of swarming mediators compromised the ability to contain *C. albicans*. NETs were formed during swarming and disruption of NETs and ROS production compromised swarming control of fungi.

Conclusions: Neutrophil swarming occurs robustly against live microbe clusters and restricts their growth. These results establish swarming as a mechanism of fungal control that warrants further investigation. Our technology provides exquisite control over conditions during swarming against live microbes. These new tools provide direct access for quantification of cellular dynamics and molecular mediators during neutrophil-microbe interactions and overcome the limitations of current *in vivo* models of neutrophil swarming.

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Mechanisms of *H. Pylori*-Induced Neutrophil Nuclear Hypersegmentation

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Helicobacter pylori is a human pathogen that resides in the gastric mucosa, eliciting a neutrophil-dominant inflammatory response that can progress to peptic ulcer disease or gastric cancer. Neutrophils can exhibit significant functional and phenotypic plasticity, and we recently demonstrated that these cells undergo N1-like subtype differentiation *in vitro* following *H. pylori* infection. This phenotype is notable for profound nuclear hypersegmentation and a proinflammatory and cytotoxic phenotype. Our objective is to define the underlying mechanisms with a focus on nuclear hypersegmentation. We have shown that direct infection and both host and bacterial transcription and protein synthesis are essential. Recent studies using nocodazole and taxol identified an additional requirement for microtubule (MT) dynamics. Moreover, confocal analysis suggested that MTs are more robust and abundant following infection and may interact with the nuclear membrane as well as with *H. pylori* phagosomes. Inhibition by Brefeldin A suggested an additional role for anterograde transport, and to our surprise revealed unexpected effects of this drug on PMN microtubule dynamics. Finally, we used antibodies to lamin B receptor and STED super-resolution microscopy imagining to analyze neutrophil nuclei in unprecedented detail. These data suggest that the volume of the *H. pylori* infected PMN nucleus is significantly increased. Future studies include assessment of MT post-translational modifications, nuclear pore components, Golgi/ER proteins and cholesterol by immunoblotting and confocal and/or STED microscopy. Altogether, our data provide fundamental insight into the mechanisms that regulate neutrophil nuclear morphology as well as the consequences of *H. pylori* infection.

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Inflammatory Activation Alters Macrophage-commensal Dynamics

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Under healthy conditions, resident homeostatic macrophages reside in tissues, including in the oral environment. However, the chronic inflammatory disease periodontitis results in the infiltration of a large number of classically activated inflammatory macrophages. *Streptococcus gordonii* is a normally non-pathogenic commensal oral microorganism. While not causative, recent evidence indicates that the commensal oral microbiome is required for the full development of periodontal disease. We have recently reported that *S. gordonii* is better able to survive within inflammatory macrophages than non-activated or alternatively activated macrophages, and that this

survival depends on changes in macrophage phagosomal reactive oxygen species (ROS) production and acidity. With this study, we have begun to examine microbial synergistic effects on the innate immune system with *S. gordonii* and *Porphyromonas gingivalis,* a well-studied oral pathogen that promotes microbiome dysbiosis and immune dysregulation resulting in periodontitis. We find the survival of *S. gordonii* is significantly increased in macrophages activated in the presence of *P. gingivalis* as compared to unstimulated or cytokine-activated macrophages. We also find an additive stimulation of macrophages, as measured by pro-inflammatory cytokine release, when *S. gordonii* and *P. gingivalis* are co-incubated with these innate immune cells. We have also begun to delve into the consequences of *S. gordonii* interactions with *in vivo* macrophages of various activation states using an air pouch model of infection. Overall, our results suggest *S. gordonii* is capable of evading immune destruction and increasing inflammatory mediators, especially under conditions of existing inflammation initiated by a pathogenic community member such as *P. gingivalis*. Together these results begin to give us mechanistic insight into how a minor pathogenic community member such as *P. gingivalis* can influence the immune system sufficiently so that members of the commensal oral microbiome, including *S. gordonii*, can be driven to act as accessory pathogens and help promote disease.

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Proposed *in Vitro* model of Neutrophil Swarming in a Chronic, Low-level Inflammatory State

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Chronic, low-grade inflammation is an underlying condition across a globally increasing number of debilitating diseases. These diseases include obesity, atherosclerosis, and diabetes and their resultant low-grade inflammation can be effectivity modeled with low dose stimulants such as lipopolysaccharide (LPS). While the innate immunity plays a significant role in fighting infectious disease, an initial exposure to low dose LPS hinders secondary infection clearance and pre-disposes murine models for fatal sepsis. Neutrophils are the most prevalent circulating innate immune cell and their homotypic aggregation, or swarming, is a key mechanism in clearing pathogens greater than 20 µm in size. We hypothesize that neutrophil swarming ability is altered when in a low dose LPS primed state; potentially leading to an overall altered innate immune response in the face of infection. However, an *in vitro* model does not currently exist to reliably quantify and compare neutrophil swarms across treatment groups. Here we propose a novel model utilizing fungal zymosan coated beads as a uniform target to which neutrophils may swarm.

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Importance of Neutrophil NADPH Oxidase in Regulating Innate Responses to *Aspergillus Fumigatus* and Fungal Cell Walls

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Invasive pulmonary Aspergillosis is a severe life-threatening infection by the opportunistic fungal pathogen *Aspergillus fumigatus* (AF) in immunocompromised patients, including the primary immunodeficiency chronic granulomatous disease (CGD). CGD results from inactivating X-linked or autosomal recessive mutations in genes encoding subunits of the phagocyte NADPH oxidase that generates superoxide (O_2^-). Lack of reactive oxygen species (ROS) leads to recurrent bacterial and fungal infections and also dysregulated inflammation due to loss of incompletely understood redox mechanisms. This includes the response to fungal cell wall pathogen-associated molecular patterns (PAMPs), which is typically neutrophilic. However, it is still elusive whether deficient neutrophil ROS are directly linked with hyperinflammation. The objective of this study was to examine whether NADPH oxidase-derived ROS produced by neutrophils play a non-redundant role both in controlling pulmonary AF

infection and in limiting inflammation induced by fungal PAMPs. Neutrophils purified from NADPH oxidase-null mice produced substantially higher levels of proinflammatory cytokines such as CXCL2, TNF-α and IL-1β compared to WT neutrophils upon challenge with the sterile yeast particle, zymosan, or with sterile AF hyphae cell walls. To interrogate the relative role of the neutrophil NADPH oxidase in vivo, we developed mice with neutrophil–restricted deletion of *Ncf2*, which encodes the p67^{phox} NADPH oxidase subunit, using S100A8-Cre recombinase to target a floxed *Ncf2* allele. NCF2DS100A8-Cre neutrophils exhibited markedly reduced expression of p67^{phox} and only 10-20% of wild-type NADPH oxidase activity. Monocyte and macrophage p67^{phox} expression and NADPH oxidase activity were intact. NCF2DS100A8-Cre mice had increased neutrophilic inflammation and fungal burden when challenged with AF, and instillation of the sterile fungal particle zymosan into lungs induced significantly increased acute neutrophilic inflammation. Thus, deficient NADPH oxidase activity only in neutrophils resulted in impaired control of pulmonary AF and excessive fungal cell wall-induced inflammation. These results demonstrate that the neutrophil NAPDH oxidase has non-redundant roles in innate responses to AF and fungal cell walls.

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NAPDH Oxidase Modulates Neutrophil Effector Responses in the Oral Mucosa

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Neutrophils are actively recruited to the oral mucosa under homeostatic conditions where they mediate localized immune responses against symbiotic polymicrobial oral biofilms. Aberrant recruitment or hyper-activation of neutrophils is central in the immunopathology of periodontitis, a chronic inflammatory condition that results in the progressive destruction of periodontal tissues. However, under homeostatic conditions, neutrophils constantly interact with oral bacteria, but their activation is tightly regulated via incompletely understood mechanisms that limit excessive activation of neutrophil functional responses, thus preventing dysregulated inflammation and tissue damage. Here we demonstrate a novel role for NADPH oxidase and derivative reactive oxygen species (ROS) in modulating neutrophil effector responses to oral pathogens. Wild type neutrophils when challenged with the periodontal pathogen *Porphyromonas gingivalis*, rapidly activated NADPH oxidase generating ROS at the plasma membrane and within phagosomal compartments. Neutrophils lacking NADPH oxidase activity due to deletion of CYBB (gp91^{phox}) subunit of the NADPH oxidase complex demonstrated profound dysregulation of inflammatory pathways characterized by significantly elevated generation of proinflammatory cytokines and chemokines (TNF-a, CXCL8 and CXCL2). Further, oxidase deficiency resulted in early exocytosis of primary and tertiary granules and the subsequent release of granular proteolytic enzymes such as MMP-9. Excessive degranulation and cytokine responses were in part driven by prolonged phosphorylation of p38 mitogen associated protein kinase (MAPK) in oxidase null neutrophils. In contrast to the severe dysregulation of inflammatory pathways, we did not observe any defects in intracellular killing capacity of oxidase-null neutrophils. Hence, although in the established paradigm oxidants are associated with adverse outcomes in periodontal diseases, our studies demonstrate that in fact low-level generation of oxidants might be essential in the regulation of neutrophilic responses within the oral mucosa.

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An Engineer's Adventures in the Neutrophil World

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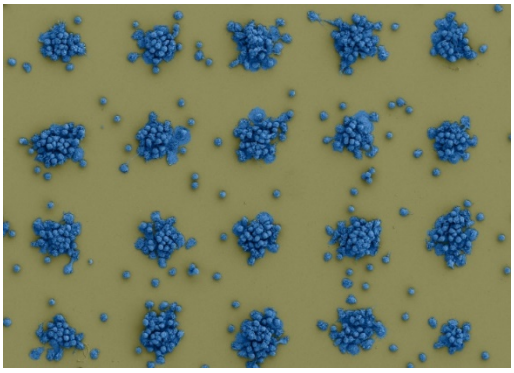
The absolute neutrophil count (ANC) is today a ubiquitous test prescribed by clinicians. However, besides ANC no other neutrophil tests are performed routinely in the clinic. Consequently, our knowledge about the human neutrophil phenotype changes in diseases is somewhat limited. At the same time, technological advances of *in vivo* microscopy and new tools to fluorescently label neutrophils have brought forward several new neutrophil functions in zebrafish and mice. These new findings, like the reversed migration, swarming, and NETosis, are reviving the interest in neutrophils among clinicians. To sustain this interest, new tools are needed that could probe neutrophils from patients in the context of the emerging biology. Here, we will discuss several microfluidic devices that measure reversed migration, swarming, spontaneous migration, and plexus vulnerability to neutrophils ex vivo, using human and animal neutrophils. Enabled by these devices, new tests could complement the ANC and will eventually guide clinical diagnosis, treatment, and monitoring of inflammation, infections, and sepsis.



The Microbiome in Inflammation and Immunity

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HIV-infected, ART-suppressed Intravenous Opioid Users Receiving Naltrexone Have Lower Immune Activation Compared to Similar Patients Receiving Methadone.

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Background: Intravenous drug (IVD) use is a primary route of HIV transmission in a number of countries (10% of cases worldwide). Opiates may accelerate the progression of untreated HIV infection through a variety of mechanisms including modulation of viral co-receptors CXCR4 and CCR5. Chronic opiate use results in a range of adverse effects, including increased susceptibility to infections and systemic immune activation. Medication-Assisted Treatment (MAT) is based on substitution with a μ opioid receptor (MOR) agonists such as methadone (MET) or buprenorphine, or abstinence support with MOR antagonists such as extended-release Naltrexone (XR-NTX). The effect of MET on microbial translocation, immune reconstitution and HIV persistence in individuals receiving suppressive antiretroviral treatment (ART) remains unclear.

Methods: We conducted a pilot study comparing levels of cell and plasma markers of immune activation markers, soluble microbial translocation markers and HIV persistence markers in a cross-sectional convenience cohort consistent of three groups: HIV-1-infected people who inject drugs (PWIDs) receiving suppressive ART and MET-based (group 1, N= 10) or XR-NTX-based (group B, N=6) MAT. As control, we assessed HIV-infected, non-OAD patients receiving suppressive ART (group C, N=10). Our assessments included plasma levels of sCD14 (an indicator of myeloid activation and microbial translocation) using ELISA, CD4+ and CD8+ T-cell and myeloid activation using multicolor flow cytometry on fresh blood specimens. All subjects were recruited at the Jonathan Lax Clinic/Philadelphia FIGHT (Philadelphia, PA) under the supervision of the Philadelphia FIGHT and Wistar Institute IRBs.

Results: Age, race and gender distribution, as well as current and nadir CD4 count and time on ART were similar in all three groups. The time on MAT was significantly longer for PWIDs receiving MET (43 ± 21months vs. 13 ± 7 for NTX), consistent with XR-NTX's use for support of abstinence vs. long-term harm reduction. sCD14 was significantly higher in PWIDS receiving MET-based MAT, as compared to both non-OAD controls and PWIDs (p= 0.0058) on XR-MTX-based MAT (p= 0.0262; overall Kruskal Wallis ANOVA: p= 0.0091). Using a mixed effect model, we show that MET-based MAT had a significant positive effect on sCD14 (p= 0.0005), which was maintained even after introducing time on MAT (p= 0.0007) or ART (p= 0.0005) in the model, indicating that PWIDs receiving MET have greater microbial translocation than PWIDs receiving XR-NTX. Similarly, MET treatment had a significant effect on T cell activation (% of $CD38^+$/HLA-DR^+ $CD8^+$ T cells, p= 0.0055) when modeled with ART and MET duration.

Conclusion: Altogether our results indicate that continued engagement of MOR by MET-based MAT results in higher immune activation and microbial translocation, compared to abstinence support with MOR antagonists.

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The Role of the Microbiome in the Development of Environmental Enteric Disease

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Diarrheal diseases kill approximately 2 million people every year, including 760,000 children under the age of 5. Along with high mortality rates, enteric diseases are a large contributor to global growth stunting and malnutrition. Repeated or unresolved enteric infections damage the intestinal tract, resulting in a condition commonly referred to as environmental enteric disease (EED). The etiology of EED is poorly understood due to the lack of robust animals models and non-invasive diagnostics. Infant rhesus macaques are susceptible to diarrheal diseases during the first year of life and develop symptoms consistent with EED that result in ~4% mortality. Therefore, in this study we used this animal model to interrogate the role of the microbiome in the development of EED. We first investigated longitudinal changes in gut microbiome composition and diversity in 80 infant Rhesus macaque over the first 8 months of life using sequencing of the V4 region of the 16s rRNA gene. We found that the Rhesus gut microbiome develops in a similar manner to humans but at more rapid pace, and shares many taxonomic similarity to humans from the developing world. Over the course of the study, 15 infants developed diarrhea. To uncover potential biomarkers of susceptibility to EED, functional potential of the gut microbial community was assessed using shotgun metagenomic sequencing using samples obtained at 1 (prior to development of disease) and 8 months of age (after the onset of diarrhea). This analysis revealed that the microbiome of infants that would later develop diarrhea were distinct from those of infants that never developed disease. The microbiome of these individual were enriched in pathways involved in the production of immunomodulatory products such as palmitoleic acid and methylerithrol phosphate. At 8 months of age, the microbiomes of infants that experienced

diarrhea were enriched in pathways for aerobic respiration and sulfur metabolism reflective of long term dysbiosis in the gut community. Lamina Propria Lymphocytes (LPLs) were characterized using flow cytometry and luminex assays. Colonic LPLs of infants that experienced diarrhea showed lower production of cytokines, chemokines, and growth factors both at rest and in response to PMA and Ionomysin stimulation. While sick individual showed a more muted response to stimulation, possibly due to exhaustion caused by the higher abundance of damage causing invasive pathogens such as *Campylobacter spp*. in individual with EED. These data suggest that the gut microbiome plays a key role in the development of EED, potentially through the modulation of the mucosal immune system altering host susceptibility.

Miscellaneous

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Chorioamnionitis Exposure Remodels the Neonatal Monocyte H3K4me3 Landscape and Alters Gene Transcription

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Background: Chorioamnionitis is a condition involving infection and inflammation of the chorion, amnion and placenta and is the leading cause of preterm birth. It leads to a fetal systemic inflammatory response that can change neonatal immune transcription and increases the risk of developing early onset neonatal sepsis. We have previously shown that neonatal monocytes gain the activating histone tail modification H3K4me3 at promoters of immunologically important genes as development progresses from preterm neonate to adult and that this gain is associated with more mature immune responses. It is unknown how chorioamnionitis exposure impacts this developmental gain in monocyte H3K4me3.

Study Aim: To evaluate the impact of chorioamnionitis exposure on the neonatal monocyte H3K4me3 histone modification landscape over the course of fetal and neonatal immune system development and to determine how this impacts monocyte gene transcription and function.

Methods: H3K4me3 ChIP-seq was performed on umbilical cord blood purified CD14+ monocytes from healthy and chorioamnionitis-exposed under 30 weeks extremely preterm neonates, 30-36 weeks late preterm neonates, and term neonates. Chorioamnionitis exposure was determined by histopathological review of the placenta. Raw ChIP-seq reads were mapped to the *Homo sapiens* genome hg19 using Bowtie 2, H3K4me3 peaks were called against input using MACS2, and DiffBind was used to identify differentially bound H3K4me3 peaks. The false discovery rate for differentially bound peaks was set at < 0.1. RNA-seq was then performed on term healthy and chorioamnionitis-exposed CD14+ monocytes, as this was the gestational age where the largest difference in differentially bound H3K4me3 peaks was observed between exposure groups. RNA-seq of unstimulated and LPS stimulated monocytes was performed to determine if chorioamnionitis exposure impacted gene transcription in a resting state and if it altered gene transcription after a secondary pathogenic stimulus. Raw RNA-seq reads were mapped to the *Homo sapiens* genome hg19 using HISAT2, reads were aligned and transcript abundance was determined using Subread, and reads were normalized and differentially expressed transcripts were determined using edgeR. The false discovery rate for differentially expressed transcripts was set at < 0.1.

Results: Chorioamnionitis exposure in neonatal monocytes of all gestational ages resulted in both removal and deposition of H3K4me3, with a net increase in total monocyte H3K4me3 in late preterm and term neonates. This increase was primarily noted in introns and intergenic regions, which is interesting because H3K4me3 is primarily known to be located at promoter regions of actively transcribed genes without a well described function in introns and intergenic regions. Chorioamnionitis exposure also decreased monocyte immune gene expression, both at

baseline and upon a secondary pathogenic exposure, resulting in a dampened monocyte immune response. The majority of enriched transcripts in the chorioamnionitis-exposed monocytes were in non-immune pathways. Additionally, H3K4me3 peak presence anywhere in the genome (promoter, exon, intron and intergenic regions) predicted mRNA expression over 50% of the time in both chorioamnionitis-exposed and unexposed monocytes.

Conclusions: This study revealed that chorioamnionitis exposure remodeled the neonatal monocyte H3K4me3 landscape, with age-inappropriate H3K4me3 patterning. Chorioamnionitis exposure also altered term neonatal monocyte transcription, with a less robust inflammatory response upon a secondary pathogenic exposure. Additionally, H3K4me3 peak presence near a gene was predictive of mRNA expression, irrespective of the genomic location of the H3K4me3. Together these finding demonstrate that a pathogenic inflammatory exposure in the neonatal period has a profound effect on monocyte transcription and transcriptional regulation that may result in long-term immune dysfunction.

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Supplementation of Female Mice with *Lactobacillus Johnsonii* protects Offspring from RSV-induced Pathogenesis

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Respiratory Syncytial Virus (RSV) is a ubiquitous pathogen that infects nearly all children before two years of age. Severe infection requiring hospitalization during the first year of life results in increased risk for the development of wheezing and childhood asthma in subsequent years. The role of the microbiome has become increasingly understood in protection from RSV-induced pathology and the development of allergic disease, including changes in metabolites driven by supplementation with various probiotics. In this study, we supplemented female mice with *Lactobacillus johnsonii* prior to mating and throughout pregnancy, and infected offspring at one week of age with RSV. Following this acute infection, we found that mice born to supplemented females had less mucus in the lungs, as well as lower Th2 cytokine production from draining lymph nodes. This correlated with changes in the bacterial populations in the cecum, with enrichment of *Akkermansi*a species in pups born to PBS-treated mice. To determine whether the protection was conferred *in utero*or by factors passed through milk, we cross-fostered pups from *L. johnsonii* supplemented or PBS-treated females. Pups born to PBS-treated females but raised by supplemented females had decreased mucus in the lungs, whereas mice born to supplemented females but raised by PBS-treated females had decreased Th2 cytokine production from the lymph nodes, indicating that both *in utero*exposure and consuming milk from supplemented females contribute to protection. We observed changes in cecal colonies that reflected altered pathology. In particular, those mice that were protected tended toward enrichment of *Lachnospiraceae* and *Bacteroidales*, while these were lacking in mice with higher Th2 cytokine production. Finally, those pups with increased Th2 cytokines had increased levels of plasma DiHOMES, lipokines that have previously been found correlate with the development of allergic disease. Together, these results suggest that alterations in the maternal microbiome affect the RSV-driven Th2 pathology in offspring.

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Suppressive Capability of Regulatory T Cells after Alcohol Intoxication and Burn Injury

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Regulatory T cells (Treg) are key components of intestinal immunity as they help prevent inflammatory responses against commensal bacteria in the intestine through direct cell-cell contact inhibition and the production of anti-inflammatory cytokines. Alcohol and burn injury disrupts the intestinal barrier function, leading to dysregulation of intestinal T cell responses. Previous studies from our laboratory have shown suppression of Th1 and Th17 responses following alcohol and burn injury, which in part may be mediated by anti-inflammatory Tregs. The goal of this study was to characterize the Treg population and determine whether alcohol intoxication combined with burn injury will alter their suppressive capability as measured by effector T cell proliferation. Briefly, male mice were gavaged with ethanol (~3 mg/kg) 4 hours before receiving a ~12.5% total body surface area full thickness burn injury using 85°C water for ~7 seconds, followed by resuscitation with 1ml normal saline. One day following injury, Peyer's patches and lamina propria cells from the small intestine were collected for analysis by flow cytometry, RT-PCR, and ELISA. We observed a 1.5-fold increase in $Foxp3^+$ Tregs in the Peyer's patches and a 2-fold increase in the lamina propria following ethanol and burn injury compared to sham mice. IL-10 producing Tregs were also increased in both tissues of injured mice compared to sham mice. Analysis by RT-PCR of small intestinal tissue did not show any difference in expression of Foxp3, CTLA-4, or IL-10. To further determine Treg suppressive activity, $CD11c^+$ antigen presenting cells (APC), $CD4^+CD25^+$ cells, and $CD4^+CD25^-$ cells were magnetically isolated from pooled spleen, mesenteric lymph nodes, and peripheral lymph nodes. $CD4^+CD25^-$ effector T cells were labeled with CellTrace Violet prior to CD3 stimulation and 72h co-culture with irradiated APCs and $CD4^+CD25^+$ cells. Following 72h of co-culture, cells were collected for flow cytometric analysis. We observed that Tregs isolated from ethanol and burn injured mice suppressed proliferation of effector T cells to a greater degree compared to those obtained from sham mice. These findings suggest that ethanol and burn injury results in increased Treg populations and their ability to suppress proliferation of effector T cells, which may contribute to dysregulation of T cell responses in this model. Additional studies to further characterize these Tregs are in progress. (Supported by R01AA015731, T32AA013527)

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Alcohol Increases Nitric Oxide Synthase 2 Expression in a Mouse Model of Colitis

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Ulcerative colitis (UC) produces a continuous mucosal lesion that is localized to the rectum and colon. These patients have periodic episodes of disease reactivation, known as flares, characterized by abdominal discomfort and bloody diarrhea. Studies show that alcohol consumption can induce UC flares and reactivation, however, the mechanism for this is not known. In this study, we assessed whether alcohol increases the expression of nitric oxide synthase 2 (Nos2) in a mouse model of colitis. Nos2 expression in the intestine has been linked with the severity of UC. Male C57BL/6 mice received either 2% DSS or normal drinking water ad libitum for 5 days. On days 5, 6, and 7, mice were gavaged with either alcohol (~3g/kg) or water and subsequently euthanized three hours after the last gavage. This led to the formation of four experimental groups [Control+Vehicle (Ctrl+V), Ctrl+Ethanol (Ctrl+E), DSS+V, DSS+E]. The large intestine was harvested and intestinal epithelial cells (IECs) were isolated, followed by RNA and protein extraction. Portions of the large intestine were also fixed for histological analysis. When compared to DSS+V, DSS+E had a 1.7-fold increase in Nos2 mRNA ($p<0.05$) and a 3-fold increase in Nos2 protein ($p<0.05$, as assessed by western blot densitometry). Histological analysis of colon sections revealed a trend towards increased colonic PMN infiltration in DSS+E compared to DSS+V. However, these neutrophils were not found to be positive for Nos2. We observed that Nos2 was primarily restricted to the apical surface of the intestinal epithelium. Moreover, when compared to the DSS+V intestine, the DSS+E intestine appeared to have increased Nos2 staining in terms of intensity, and distribution along the length of the intestine as well as the intestinal crypt. Together, these findings suggest that alcohol exposure primarily induces Nos2 expression in epithelial cells within the intestine following colitis. Furthermore, this increase in Nos2 may contribute to the flare of symptoms

associated with UC as observed in mice exposed to DSS and alcohol. (Support: R21AA022324, T32AA013527 and F31AA025536).

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MrgX2-mediated Internalization of LL-37 and Degranulation of Human LAD2 Mast Cells

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LL-37 is the sole antimicrobial peptide of human cathelicidin comprising of 37 amino acids, which is expressed mainly in epithelial cells and neutrophils, and activates mast cells. In the present study, to elucidate the mechanism of mast cell activation by LL-37, the relationship between the internalization of LL-37 and MrgX2-mediated mast cell activation (degranulation) was investigated, using a human mast cell line LAD2. LL-37 rapidly internalized into the cells, and induced the degranulation as assessed by the extracellular release of b-hexosaminidase. Importantly, pertussis toxin, a G-protein inhibitor, significantly suppressed both the internalization of LL-37 and degranulation of LAD2 cells. Furthermore, siRNA-mediated knockdown of MrgX2, a putative G protein-coupled receptor for LL-37, inhibited both the internalization of LL-37 and degranulation of LAD2 cells. Interestingly, LL-37 internalization was enhanced by the stable expression of MrgX2 in HMC-1 cells and HEK293 cells. In addition, internalized LL-37 was colocalized with MrgX2 in the perinuclear region of LAD2 cells. Finally, neuraminidase treatment, which removes negatively charged sialic acid from the cell surface, markedly reduced the internalization of LL-37 and degranulation of LAD2 cells, and the clathrin-mediated endocytosis inhibitor (dynasore and chlorpromazine) inhibited both the internalization and degranulation of LAD2 cells. Together these observations suggest that LL-37 binds with the negatively charged cell surface molecules, rapidly internalizes into the cells via the clathrin-mediated endocytosis and interacts (colocalizes) with MrgX2 for mast cell (LAD2 cell) activation (degranulation).

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Class IIa Histone Deacetylases Are Molecular Links Between Regulated Cell Metabolism and Inflammatory Outputs of TLR-activated Macrophages

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During toll-like Receptor (TLR)-mediated activation of macrophages, aerobic glycolysis is upregulated and the TCA cycle is disrupted. This metabolic shift results in skewing of cytokine production towards a proinflammatory phenotype, with IL-1beta production being amplified and IL-10 expression being repressed. Although inducible glycolysis is a key molecular switch for macrophage activation, the molecular mechanisms responsible are still poorly understood. In this study, we employed metabolomic, pharmacological, genetic and proteomic approaches to reveal class IIa histone deacetylases (HDACs) as essential molecular components of TLR-inducible glycolysis and associated inflammatory responses. We show that an enzymatic inhibitor of class IIa HDACs attenuates lipopolysaccharide (LPS)-inducible production of lactate and other glycolytic intermediates in activated macrophages. This is accompanied by inhibition of specific proinflammatory mediators including IL-1beta, as well as enhanced production of immunoregulatory IL-10. HDAC7, a proinflammatory class IIa HDAC that is induced during human macrophage differentiation, is required for immunometabolism-linked inflammatory outputs in these cells. In macrophages from Mac-HDAC7 mice that constitutively over-express HDAC7 in the myeloid compartment, LPS-inducible lactate production is increased and metabolism-associated inflammatory mediators

are correspondingly skewed. These effects are dependent on glucose uptake and the HIF1alpha transcription factor that bridges metabolic and inflammatory responses in macrophages. A proteomic screen identified the glycolytic enzyme pyruvate kinase isoform 2 (PKM2) as a macrophage-expressed HDAC7-interacting partner, with this interaction being validated in cells and with recombinant proteins. Class IIa HDAC inhibition reduced the formation of the HDAC7-PKM2 complex, skewed PKM2 complex formation towards glycolytic tetramers instead of proinflammatory monomers/dimers and abrogated the proinflammatory effects of PKM2 in macrophages. Accordingly, proinflammatory mediators were markedly enhanced in the sera of LPS-challenged Mac-HDAC7 mice, and conversely, a class IIa HDAC inhibitor reduced inflammatory responses and associated pathology in LPS-challenged mice. Our study has thus defined an HDAC7-PKM2 signaling axis as an essential component of regulated immunometabolism in macrophages, a molecular pathway that is amenable to pharmacological targeting for anti-inflammatory applications.

Inflammatory Signaling in Leukocytes

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Infectious Models Reveal a Unique Role for caspase-7 After Cytotoxic Lymphocyte Attack

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Bacteria often invade host cells to avoid extracellular immune defenses. This strategy can be counteracted by cytotoxic lymphocytes, such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), which hunt down and execute host cells harboring intracellular pathogens1,2. NK cells and CTLs use perforin to deliver granzyme B, which cleaves and activates the executioner caspases3. Among these, caspase-3 is considered to be the most critical, while the physiologic importance of caspase-7 has remained nebulous4. Here, we find a unique role for caspase-7 during perforin attack by NK cells and CTLs against hepatocyte-invasive Chromobacterium violaceum and Listeria monocytogenes. NK cells and CTLs triggered the activation of both caspase-3 and -7 in hepatocytes. Remarkably, Casp7–/– mice phenocopied Prf1–/– mice in their susceptibility to these infections; caspase-3 could not compensate for the loss of caspase-7. In contrast, Casp7–/– mice remained competent for perforin-mediated defense against actA mutant L. monocytogenes, which cannot move directly from one infected hepatocyte to the next. Neither was caspase-7 required during NK cell or CTL attack against murine cytomegalovirus or lymphocytic choriomeningitis virus. Our results suggest that caspase-7 is not specific for defense against bacteria per se, but rather that caspase-7 targets an aspect of cellular physiology that counteracts cell to cell spread.

Enabling Technologies for Leukocyte Research

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Labelfree Acoustofluidic Separation of Mononuclear Cells from Blood

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Ultrasonic manipulation of cells and particles in microfluidic system has in recent years gained much attention offering new means to perform unit operations such as cell separation, enrichment, buffer exchange and trapping, enabling integrated cell handling functions in lab-on-a-chip systems [1, 2].

In general, acoustic standing wave forces lend themselves well to manipulate particles and cells ≥3 um in microfluidic systems since the primary acoustic radiation force, F^{rad} dominates in the frequency range of 1-10 MHz that are commonly used in microfluidic systems.

E_{ac} is the

acoustic energy density, is the acoustic contrast factor, k_0, r_0, $k_p$andr_p are the compressibility and density of the fluid and particle, respectively, k is the wave number (2p/l), z is the position of the particle along the wave propagation axis, p_a is the pressure amplitude, c_0 is the speed of sound in the medium.

By operating a microfluidic channel in l/2 resonance mode, cells will be focused into the pressure node, in the center of the channel, provided that the acoustic contrast factor, F, is positive. The rate of migration into the pressure node is defined by the acoustic fingerprint (size, density and compressibility) of each cell type and thus offers a means to separate different cell types at the outlet of an acoustophoresis channel.

In spite of the significant sized and density differences between erythrocytes (RBC) and leukocytes (WBC) these will not lend themselves to acoustophoretic separation as the acoustophoretic mobility of the two cell types are essentially equal. This can however be circumvented by modifying the acoustophysical properties of the buffer used to dilute the whole blood sample. Using Stock Isotonic Percoll (SIP) as buffer generating a 1:10-1:20 diluted whole blood we have demonstrated that acoustophoretic isolation of mononuclear cells can be accomplished with a log 3 – log 3.4 depletion of the RBC fraction and at an MNC recovery of >80%. Being a continuous flow process without manual intervention this MNC isolation process offers several benefits as compared to batch operated density gradient centrifugation [3].

Based on the acoustophoresis principle we have also developed a system that enables multiplex separation of leukocytes into three subpopulations, lymphocytes, monocytes and granulocytes at three different outlets from the chip. Unfixed leukocytes were derived from a whole blood lysate after pelleting and resuspension in PBS. At sample flow rates of 100 uL/min (10^6 cells/mL) 99± 0.7% of the granulocytes were collected from the center outlet, whereas 66.8 ± 3.2% of monocytes were directed towards the side outlet 1 and 96.8 ± 3.3% of lymphocytes towards the side outlet 2. Purities of 98.8 ± 0.5%, 71.8 ± 10.1% and 96.6 ± 1.6% were obtained for the lymphocytes, monocytes and granulocytes, respectively. Fixed cells displayed a slightly lower separation performance with granulocyte, monocyte and lymphocyte recoveries of 76%, 56%, 85% respectively and the corresponding purities of 98%, 74% and 85%. At elevated flow rates, 300 uL/min, the separation performance is slightly lower yet providing purities of 98%, 21% and 93%.

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Microfluidic Devices for CD64+ Neutrophils Counting and Sepsis Diagnostic

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Abstract: Sepsis is the leading cause of mortality around the world. In the United States more than 1 million patients are diagnosed with severe sepsis every year, and 30-50 % of them die. Instead of relying on the current diagnostic parameters including patient's vitals (heart rate, temperature, respiratory rate, and oxygen saturation) we developed a biochip that can quantify molecular biomarkers from whole blood which are associated with the proinflammatory response of the patients at the onset of infection and have shown improved sepsis diagnostic accuracy.

Methods: We have developed a microfluidic biochip that can quantify total leukocytes, its differential (lymphocytes and granulocytes + monocytes) and antigen expression level of CD64 on neutrophils (nCD64) from a drop of whole blood. 10 microliters of blood is infused in the biochip along with lysing buffer to effectively lyse all erythrocytes. The leukocytes are preserved, and lysing process is halted by infusing the quenching buffer in the biochip. Each leukocyte gets counted as it passes through the microfluidic channel aligned with the coulter electrical counter. The leukocytes then pass through the immuno-affinity capture chamber, where anti-CD64 antibody is immobilized initially. CD64 expressing cells gets captured, and the percent capture of cells is linearly dependent on the CD64 expression levels on the cells [1]. The remaining non-captured cells pass through the second electrical counter, and the percent capture of cells is calculated comparing cell counts from both counters. The percent capture is compared against the calibration curve (nCD64 vs. percent capture) and biochip nCD64 level is calculated [1]. Furthermore, we developed an artificial neural network (ANN) based multivariate computational model and have used rate of cell capture as an additional parameter in the model and have shown higher accuracy of nCD64 level determination from the biochip as compared to using univariate regression techniques [2]. The model is trained with Bayesian Regularization optimization algorithm [2].

Results: We ran blood samples (n = 181) collected from sepsis suspected patients at Carle Foundation Hospital, Urbana and have shown high correlation coefficient (R^2 = 0.89) in between biochip total leukocytes versus control leukocyte counts obtained from haematology analyzer and correlation coefficient (R^2 = 0.87) in between biochip nCD64 versus control nCD64 levels (n = 102) obtained from flow cytometry [1]. Furthermore, in longitudinal clinical studies, we collected patients' blood samples at different times during their stay in hospital and have shown that at the time of admission the nCD64 levels were higher and cell counts were lower, however as they got recovered their cell counts and nCD64 level went to the healthy ranges [1].

References:

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Late Breaking Research

LB1

A Novel Infectivity Gene in the Lyme Disease Agent *Borrelia Burgdorferi* Is Important for Survival in the Skin During the Earliest Phase of Infection

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Lyme Disease is the most prevalent arthropod-borne bacterial infection in the United States. The causative agent of Lyme Disease is the spirochete *Borrelia burgdorferi* (Bb), transmitted via the bite of an infected *Ixodes scapularis* tick. Initial symptoms can include fever, fatigue, and a bull's-eye rash at the tick bite site. Bb enters the skin via the tick saliva, disseminates via the bloodstream, and colonizes various distal tissues. Left untreated, Lyme Disease can cause arthritis, carditis, or encephalitis, depending on the infected tissue. Bb overcomes multiple barriers to infection to colonize distal tissues and cause Lyme Disease. In particular, the ability of Bb to survive the innate immune response at the skin site of inoculation paves the way for its dissemination and tissue colonization. Through a mutagenesis screen, we identified a novel infectivity gene in Bb, *bbk13*, that is important for efficient mammalian infection. Gene *bbk13* encodes for a predicted transmembrane protein with a conserved SIMPL protein domain but the molecular function remains unknown. Loss of *bbk13* results in reduced bacterial loads in distal tissues in a mouse model of infection. By tracking the kinetics of Bb infection, we discovered that *bbk13* is important for Bb proliferation in the skin prior to bloodstream dissemination. Consistent with this finding, a Bb mutant lacking *bbk13* demonstrated reduced numbers in the bloodstream. Together, our data suggest that *bbk13* contributes to Bb infectivity by promoting survival in the earliest phase of infection. Elucidation of the molecular mechanism of *bbk13* requirement for Bb survival in the skin will aid in our understanding of how Bb evades the innate immune response at the earliest phase of infection.

LB2

Analysis of Plasma TLR2 in Standardbred Racehorses via a Novel Validated ELISA

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Toll-like receptor 2 (TLR2) was analyzed in plasma collected from Standardbred racehorses prior to racing using a validated enzyme-linked immunosorbent assay (ELISA). The method was developed using human TLR2-specific polyclonal antibody (PAb) and biotin-streptavidin chemistry to enhance sensitivity. Goat anti-human TLR2 PAb was employed as the capture antibody and its conjugate with biotin was used as the detection Ab. Recombinant TLR2 protein was utilized as the calibrator to generate a standard curve for quantification. The method was validated for specificity, sensitivity, precision and accuracy using both recombinant and endogenous TLR2 proteins. The method did not exhibit cross-reactivity with other proteins tested. When plasma samples were serially diluted, the reactivity proportionally decreased, indicating high specificity of the assay. When biotin-conjugated Ab was partially replaced with unconjugated Ab assay signals decreased proportionally. No signal was detected with analyte-negative plasma, when the capture Ab was absent or when an irrelevant antibody was applied. Concentration of TLR2 increased when whole blood samples were treated with lipopolysaccharide. Bovine serum albumin was the best suited standard diluent for recovery of standard protein while Tween 20 was the best suited sample diluent for detection of endogenous TLR2 in plasma. Inter-assay precision was £ 11.5 % and intra-assay precision was £ 7.1 %. Inter-assay accuracy was -11.7% ~ 12.8% while the intra-assay accuracy was -11.0% ~ 10.4%. In summary, the method is suitable for quantification of TLR2 in equine plasma. Finally, plasma

TLR2 concentration varied greatly among horses (n = 313). Approximately 21% had no or very low concentration, 49% fell within the detection range (0.125 ~ 8 ng/mL), 20% had concentrations from 8 to 50 ng/mL, while a few (< 10%) had extremely high concentrations (50 ~ 4000 ng/mL). Further investigation of possible physiological causes underlying the high individual variation in equine TLR2 plasma levels are warranted.

LB3

Use of an ADAM17 mAb as a Checkpoint Inhibitor to Enhance Human NK Cell Anti-tumor Effector Functions

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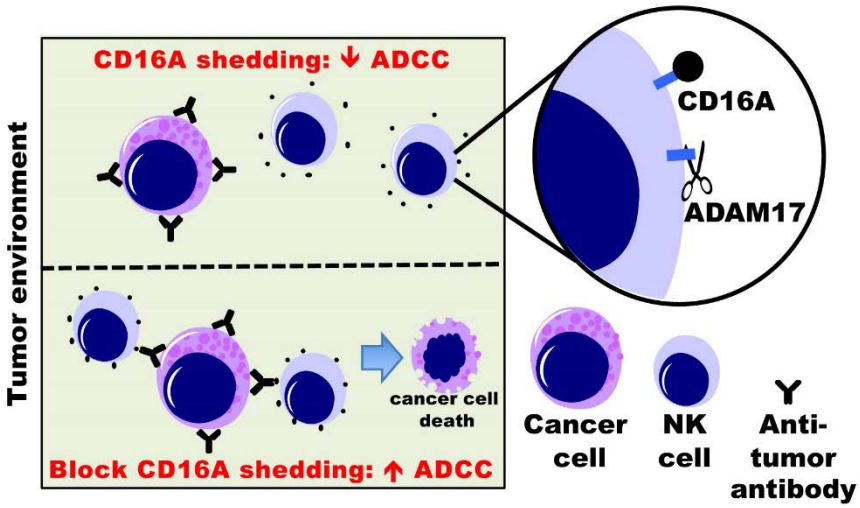
The IgG Fc receptor CD16A (FcγRIIIA) is expressed by circulating human NK cells and has an exclusive role in antibody-dependent cell-mediated cytotoxicity. CD16A is expressed at high levels on the surface of NK cells and, unlike other activating receptors, it undergoes a rapid downregulation in expression upon NK cell activation by diverse stimuli. This process involves the proteolytic release of CD16A by A Disintegrin And Metalloproteinase-17 (ADAM17). CD16A downregulation on NK cells can occur in the tumor environment and contribute to NK cell dysfunction. We examined the effects of MEDI3622, a unique human anti-ADAM17 function blocking mAb, on NK cell activation by tumor cells treated with various tumor targeting therapeutic mAbs. MEDI3622 effectively blocked CD16A downregulation in NK cells and caused a marked increase in their production of IFNγ, a cytokine with broad anti-tumor activity. The augmented release of IFNγ by NK cells was reversed by a function-blocking CD16A mAb. Moreover, NK92 cells, a human NK cell line that lacks endogenous FcγRs, expressing a noncleavable version of CD16A released significantly higher levels of IFNγ compared to NK92 cells expressing equivalent levels of wildtype CD16A. Our findings demonstrate that blocking CD16A cleavage is the primary means by which MEDI3622 enhanced the production of IFNγ by NK cells engaging antibody-bound tumor cells. Therapeutic antibodies have been generated against a number of tumor antigens for various malignancies, and several clinically successful antibodies induce NK cell effector functions. Targeting ADAM17 in NK cells with MEDI3622 in combination with anti-tumor mAbs may potentially increase their therapeutic efficacy.



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LB4

The Human Macrophage Scavenger Receptor Class a (CD204) Differentially Recognizes Fungal Cell Wall Pathogen Associated Molecular Patterns

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The innate immune system recognizes fungal pathogens through evolutionarily conserved pattern recognition receptors (PRRs) that interact with fungal cell wall pathogen associated molecular patterns (PAMPs). Scavenger receptors are a family of cell surface glycoproteins that have been implicated in the recognition of fungal pathogens. The human macrophage scavenger receptor class A (SRA aka CD204) is known to play a role in innate immune responses. However, the role of SRA in the recognition of fungal PAMPs is unclear. We examined the binding and structure activity relationships of fungal PAMPs by recombinant human SRA (rhSRA). Biolayer interferometry (BLI) was employed to examine the binding of mannans and glucans that differed in primary structure, Mw, polymer size, solution conformation and polymer charge. rhSRA has a higher affinity for *C. albicans* yeast mannan (K_D = 8.31 nM) than for (1→3, 1→6)-ß linked glucans. However, rhSRA does recognize a number of (1→3)-ß linked glucans. We found that a (1→3)-ß linked hexasaccharide glucan was the minimum binding subunit for rhSRA. The presence of a single (1→6)-ß side chain increased rhSRA affinity for the hexaose by >77 fold (K_D = 21.90 vs 0.282 uM), indicating that structure is an important determinant in glucan recognition. While hexasaccharides are the minimum binding subunit for SRA they are recognized with much lower affinity than higher Mw glucans (K_D = 282 vs 23 nM). Excluding the low molecular weight and low affinity hexa- and hepta-saccharides, a linear regression of either mg/mL or molar affinity vs molecular weight did not yield a slope significantly different from zero. However, the (1→6)-ß linked glucans derived from pustulan and *M. sympodialis* had molar affinities that were lower than expected (K_D = 185 and 123 nM). This suggests that SRA shows limited recognition of (1→6)-ß-glucans. We also observed that rhSRA bound neutral and slightly anionic carbohydrate PAMPs. These data demonstrate that human SRA differentially recognizes and interacts with fungal cell wall PAMPs. We speculate that SRA may be an important PRR for a variety of fungal pathogens.

LB5

Neutrophils from Septic Patients Have Higher Expression of Toll-Like Receptor 2 (TLR2) and Increased Uptake of *Candida Albicans*

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Invasive infections caused by commensal or opportunistic fungi have increased significantly over the past decade. Patients in the surgical ICU have one of the highest incidences of fungal sepsis. The majority of these life threatening infections are caused by *Candida albicans*. *C. albicans* sepsis in the surgical ICU patient has a mortality rate as high as 30 to 49%. It has been thought that sepsis-induced immune suppression increases susceptibility to fungal infections; however, the mechanisms underlying this remain unknown. Neutrophils are the first line of defense against *C. albicans* infection. The first step in the response to *C. albicans* by neutrophils is the recognition and interaction of membrane-bound receptors with fungal PAMPs. Dectin-1 is the key anti-fungal pattern recognition receptor in anti-fungal innate immune host defenses, but optimal Dectin-1 signaling is in cooperation with TLR2. The goal of the present study was to evaluate Dectin-1 and TLR2 expression on healthy control and septic patient neutrophils and to compare their ability to internalize *C. albicans*. After informed consent, blood samples were acquired from septic patients from our Infectious Disease service. Control blood was obtained from

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a commercial source. Blood leukocytes were isolated and stained with antibodies against Dectin-1 and TLR2 and analyzed by flow cytometry. Isolated neutrophils were incubated with pHrodo green labelled *C. albicans*, and uptake was analyzed by flow cytometry and imaging flow cytometry. Dectin-1, TLR2, and pHrodo green *C. albicans* mean fluorescence data were analyzed by patient group. We found that while Dectin-1 expression was not significantly affected, sepsis significantly increased expression of TLR2 on neutrophils compared to healthy controls ($p<0.01$) C. albicans than control neutrophils ($p<0.05$) C. albicans did not correlate with TLR2 expression in septic patient neutrophils, but uptake negatively correlated with TLR2 expression in neutrophils from healthy controls. From these data we can conclude that the increased incidence of fungal infection in critically ill septic patients is not due to loss of blood neutrophil Dectin-1 or TLR2, nor is it related to decreased uptake of *C. albicans*.

LB6

The NAD^+ Precursor Nicotinamide Riboside Facilitates Migration and Dampens Phagocytosis in Human Macrophages Through Upregulation of Prostaglandin E2

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Fasting and caloric restriction (CR) confer benefit against inflammation-linked diseases. The NAD+ precursor Nicotinamide riboside (NR) functions a CR mimetic by increasing NAD+ levels. Emerging evidence suggest immunomodulatory effects of NR, although the underlying mechanisms remain elusive. Using flow cytometry to screen for cell surface receptors in control and NR exposed human monocyte-derived M1 macrophages (HMDM) we found that NR increased the expression of CC-chemokine receptor 7 (CCR7) and decreased CD64 expression. Consistent with the canonical functions of CCR7 (chemotaxis) and CD64 (phagocytosis), we also found that chemokine CCL19 (ligand for CCR7) - induced macrophages migration was enhanced and that phagocytosis was diminished in response to NR administration. Prostaglandin PGE2 has previously been identified to coordinately promote CCR7 surface expression and migration of monocyte-derived dendritic cells and limits phagocytosis by alveolar macrophages. We assessed PGE2 levels in cultured HMDMs and found that NR increased PGE2 levels by about 70%. We also measured PGE2 levels in serum of normal volunteers that were treated with NR or placebo in a crossover study. We found PGE2 level was elevated in a cohort of 35 subjects with daily administration of NR for 7 days, as compared to placebo control. Our study suggests NR may modulates various functions of human macrophages during inflammation including phagocytosis and migratory behavior through upregulation of PGE2. Further investigation will be required to evaluate how NR modulates PGE2 pathway and to determine if PGE2 depletion or repletion in HMDMs can block or recapitulate the effects of NR.

LB7

BLT1-mediated N-glycosylation of NOX2 Mediates Its Surface Translocation and ROS-dependent Exocytosis in LTB_4-stimulated Human Mast Cells

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Exocytosis-mediated degranulation in human mast cells contributes to allergic inflammation. LTB_4 is a pro-inflammatory lipid mediator to elicit eosinophil exocytosis. However, detailed signaling mechanisms of eosinophil exocytosis induced by LTB_4 are poorly understood. Herein, we report that N-glycosylation-dependent surface trafficking of NOX2 play an important role in ROS-dependent surface upregulation of high affinity LTB_4 receptor BLT1 leading to exocytosis in human mast cells induced by LTB_4. Stimulation of mast cells with LTB_4 induced

production of intracellular ROS, surface upregulation of exocytosis marker protein CD63 via BLT1. LTB_4 induced phosphorylation of $p47^{phox}$, and inhibition of ROS generation with NOX2 inhibitors prevented LTB_4-stimulated exocytosis. LTB_4 increased expression of BLT1 and NOX2 at the cell surface, which peaked at 30 min after stimulation. LTB_4-triggered surface upregulation of BLT1 and NOX2 was blocked by pretreatment with NOX2 inhibitors and BLT1 antagonist, respectively. Interestingly, glycosylated NOX2 at 91 kDa was highly expressed at 30 min after LTB_4 stimulation. Inhibition of LTB_4-induced NOX2 glycosylation with N-glycosylation inhibitors, tunicamycin led to disability of NOX2 to travel to the cell surface, which resulted in marked reduction of LTB_4-induced ROS generation, surface up-regulation of BLT1 and exocytosis. Moreover, we confirmed that BLT1 interacted with glycosylated NOX2 in LTB_4-stimulated HMC-1 cells by immunoprecipitation. Furthermore, we found that BLT1 bind to N-terminus of NOX2 in HMC-1 cells induced by LTB_4 using NOX2 mutation study. These results suggest that BLT1-mediated N-glycosylation of NOX2 play an important role in its surface trafficking, which can regulate ROS-dependent exocytosis via interaction of BLT1 with N-terminus of NOX2 in LTB_4-stimulated mast cells.

LB8

Inactive Rhomboid Protein 2 Protects *Interleukin 10*-deficient Mice from Colitis by Limiting Colonization of the Gastrointestinal Tract by Pathogens

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Inflammatory bowel disease (IBD) represents a heterogeneous group of idiopathic inflammatory conditions of the colon and small intestine, affecting about 0.3% of the population. It arises from complex interactions of intestinal immune system with the gut microbiome. The key pathogenic role of Tumor necrosis factor (TNF) in chronic intestinal inflammation made it a major therapeutic target for IBD. Anti-TNF agents markedly reduced the progression of disease which was limited to 30% of patients and also developed long term intolerance. We analyzed the pathophysiological role of iRhom2, a crucial regulator of the maturation and function of ADAM17 (also known as TNF Convertase) in immune cells to identify a molecular target that involves in the activation of TNF. IRhom2-deficient mice were crossed and bred with *interleukin 10*-deficient ($Il10^{-/-}$) mice to generate $irhom2^{-/-}/Il10^{-/-}$ mice and offspring were observed for signs of colitis for a year. Homozygous $Rhbdf2^{-/-}/Il10^{-/-}$ mice developed spontaneous colitis and exhibited severe weight loss compared to $Il10^{-/-}$ mice within the first 16 weeks of age. This might be attributed due to alterations in the gut microbiome in $Rhbdf2^{-/-}/Il10^{-/-}$mice with higher abundance of *Enterobacteriaceae, EHEC* and *B.acidifaciens*. The dysbiosis led to an enhanced Th1cell-driven inflammation in $Rhbdf2^{-/-}/Il10^{-/-}$ mice. Our data provide critical insights into how iRhom2 maintains intestinal homeostasis and establish a role for iRhom2/ADAM17 in driving dysbiosis as well as activation and regulation of protective immunity and inflammation.

LB9

Induction and Regulation of LPS-induced Inflammatory Responses in Murine Bone Marrow-Derived Macrophages

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A well-controlled innate immune response is characterized by the rapid initiation of an inflammatory response. However, this response is sustained only until the immune insult or injury is contained; at that point, the inflammatory response is terminated or resolved. Thus, the controlled and appropriate resolution of inflammation is an essential feature of the innate immune response. Our lab is interested in deciphering how the immune system is able to differentially regulate the immune response to specific stimuli. We have previously shown that the metalloprotease tumor necrosis factor (TNF) converting enzyme (TACE) controls the release of soluble TNF and activation of epidermal growth factor receptor signaling in a rhomboid 5 homolog 2 (iRhom2)-dependent manner. We demonstrate that the inhibition of TACE via metalloprotease inhibitor DPC333 can reduce lipopolysaccharide (LPS)-induced transcription of early pro-inflammatory genes such as TNF and interleukin (IL) 1 beta (B) in human monocytes as well as in murine bone marrow-derived macrophages (BMDMs). Further, CL264-induced transcription of TNF is differentially regulated at low and high doses in *iRhom2*$^{-/-}$ BMDMs. Finally, we show that low dose-induced transcription of TNF and IL1B is reduced in *Tnf1/2*$^{-/-}$ and *Tnf*$^{-/-}$ BMDMs. These findings suggest that LPS-induced transcription of TNF and IL1B is differentially regulated at high and low doses of LPS, invoking the activation of a currently unknown regulatory pathway.

LB10

Hypoxia Inducible Factor (HIF) Is Involved in NOX4-dependent Jurkat T Cell Death Induced by *Entamoeba Histolytica*

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Entamoeba histolytica is anenteric tissue-invasion protozoan parasite that causes amoebic colitis and occasionally liver abscess in humans. *E. histolytica* can induce host cell death by the induction of various intracellular signal mechanisms. These modulations triggered by *E. histolytica* are closely associated with tissue pathogenesis and parasitic immune evasion mechanism. Hypoxia-inducible factor (HIF) is a transcriptional regulator that controls various cellular responses including immune response. NADPH oxidase (NOX) 4 is one of the major enzymes responsible for intracellular ROS production. However, it is not known whether NOX4-derived ROS and HIF-1 can be involved in T cell death induced by *E. histolytica.* In this study, we investigated the signaling role of NOX4-derived ROS in Jurkat T cell death induced by *Entamoeba histolytica.* NOX4 is strongly expressed in resting state of Jurkat T cells. When Jurkat T cells were co-incubated with live trophozoites of *E. histolytica*, *E. histolytica* strongly induced NOX-derived ROS production and HIF-1α degradation in Jurkat T cells. Pretreatment of Jurkat T cells with NOX inhibitor (DPI) effectively blocked ROS production induced by *E. histolytica.* However, mitochondria or 5-Lipoxygenase did not affect ROS generation in Jurkat T cells. Knockdown of NOX4 protein expression in Jurkat T cells by specific siRNA prevent *E. histolytica*-induced ROS generation and DNA fragmentation. In addition, HIF-1α overexpression of Jurkat T cells also effectively reduced NOX4-derived ROS generation induced by *E. histolytica*. The overexpression of HIF-1α was markedly retarded *Entamoeba*-induced DNA fragmentation and LDH release in Jurkat T cells. These results suggest that NOX4 and HIF-1α plays a signaling role in the ROS-dependent cell death in Jurkat T cells induced by *E. histolytica*.

LB11

Monocytes Sequentially Rewire Immunometabolism During an Acute Inflammatory Response

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2. Metabolon

Complex changes in metabolism drive monocyte cell fate and function during an acute inflammatory response by sequentially activating and deactivating immune effector responses before resolving. How this metabolic rewiring evolves is unclear. Here, we used lipopolysaccharide-stimulated THP-1 human monocytes and unbiased metabolomics to track metabolic rewiring during phenotype shifts. We find that early changes in carbohydrate metabolism are rapidly followed by striking increases in catabolism of lipids, proteins, nucleotides, and amino acids. Specific metabolic signatures emerged during wide-spread catabolism, including selective increases in polyunsaturated fatty acids (PUFA) and fatty acid acyl carnitines, tryptophan catabolism to kynurenine and nicotinamide adenine dinucleotide (NAD), homocysteine and cysteine transsulfuration cycling that supports hydrogen sulfide (H2S) production, proline and glutathione synthesis, and catabolism of branched chain amino acids. Most notably, increases in succinate and aconitate-derived itaconate and tricarboxylic acid cycle (TCA) fragmenting at isocitrate and succinate aligned with deactivation, and itaconate levels fell during resolution. These data support that monocytes coordinately repurpose metabolic substrate selection, redox poise, and succinate and itaconate coupling to mitochondrial physiology during an acute inflammatory response.

LB12

Fibroblast Growth Factor Receptor Family Members Are Processed by Differently Activated Metalloproteases

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3. Weill Cornell Medical College

Fibroblast growth factor receptors (FGFRs) constitute a family of four structurally related high-affinity cell surface receptors. FGFRs are involved in a variety of cellular processes, including cell growth, migration, differentiation, survival, and regulation of endothelial and hematopoietic development by modulating levels of angiogenic cytokines and are essential for adult tissue homoeostasis. Recently, soluble FGFRs have been identified in the extracellular matrix of vascular endothelial cells as well as in multiple biological fluids, including blood. Whether soluble FGFRs are produced by the translation of alternatively spliced transcripts or released by ectodomain shedding of the transmembrane receptors is not completely understood. In order to determine whether membrane-bound FGFRs are proteolytically cleaved from the cell surface in a metalloprotease-dependent manner, we generated alkaline phosphatase (AP)-tagged FGFRs and overexpressed them in COS7 cells. We found that the release of soluble FGFRs was inhibited by the hydroxamate-based metalloprotease inhibitor DPC333. Furthermore, overexpression of various full-length membrane-anchored ADAMs, or their catalytically inactive mutants serving as negative controls, together with AP-FGFRs resulted in increased release of the extracellular domains of AP-FGFRs into the culture supernatants. Additionally, we found that metalloprotease-dependent shedding of AP-FGFRs was stimulated by two commonly employed activators of ADAM-dependent ectodomain shedding: phorbol esters or calcium ionophores. In contrast to FGFRs 1, 3, and 4 shedding, which was activated by phorbol esters or calcium ionophores, FGFR2 shedding was only induced by phorbol ester stimulation, suggesting differential regulation mechanisms of FGFR-mediated functions. Taken together, we present conclusive evidence in favor of the proteolytic mechanism of soluble FGFR generation. The identification of metalloproteases as

proteases responsible for constitutive and regulated FGFR shedding may therefore provide new insight into the regulation of FGFR functions.

LB13

Induction of Neutrophil Granule Exocytosis by the Oral Pathogen, *Aggregatibacter Actinomycetemcomitans*, Is Independent of Leukotoxin a Expression

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Background: *Aggregatibacter actinomycetemcomitans* (*Aa*) is a Gram-negative facultative bacterium associated with juvenile aggressive periodontitis. During infection at the subgingival pocket, polymorphonuclear leukocytes (PMNs) are the first to arrive and elicit an immune response. *Aa* secretes leukotoxin A (LtxA), a toxin considered as the primary virulence factor; and the main mechanism used by the bacteria to resist phagocytosis and killing by PMNs. LtxA creates pores in the PMNs membrane, ultimately leading to cell death. The hypothesis that LtxA may contribute to human neutrophil granule exocytosis was tested.

Methods: The expression of LtxA varies among *Aa* strains. For our studies, we challenged human PMNs with the following strains: *Aa652* mildly leukotoxic wild type strain (WT), highly leukotoxic strain *JP2* and leukotoxic-defective mutant, *JP2 LtxA-*, at a multiplicity of infection of 50 bacteria per neutrophil. PMNs were challenged with the three *Aa* strains for different time points (5-15-30-60-90 min) and the mean fluorescent intensity (MFI) of membrane markers of secretory vesicles (CD35) and specific granules (CD66b) was measured by flow cytometry. Further, the release of lactate dehydrogenase (LDH) upon bacterial challenge was measured by spectrophotometry. The significance of quantified results were analyzed using a One-way ANOVA with Bonferroni post-test and an interval of 95% confidence.

Results: All of the *Aa* strains induced secretory vesicles exocytosis starting at 5 min, with maximal degranulation being achieved by 15 min post challenge. Time dependent increase of specific granule exocytosis was observed with all the *Aa* strains up to 90 min post bacterial challenge. The induction of granule exocytosis by the three *Aa* strains was not related to a cytotoxic effect on neutrophils since low LDH levels were detected across all the time points tested.

Conclusion: In conclusion, these results indicate that stimulation of neutrophil granule exocytosis by the oral pathogen, *Aa*, is independent of LtxA. Additionally, the low levels of LDH release compared to rapid degranulation at 5 minutes, suggests that degranulation is a rapid process compared to the assembly of LtxA pore complexes in PMNs membranes. Potentially, providing a window for PMNs to exert their immune response against *Aa*.

LB14

Time-dependent Changes in Macrophage Phenotype in the Lung Following Exposure of Mice to Nitrogen Mustard

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Nitrogen mustard (NM) is a cytotoxic vesicant known to target the lung, causing acute injury which progresses to fibrosis. In these studies we developed a murine model of NM induced lung injury with the goal of assessing the role of macrophages in the pathogenic response. C57Bl6/J mice were treated with NM (0.08 mg/kg) or PBS control

intratracheally. Bronchoalveolar lavage (BAL), alveolar macrophages and lung tissue were collected 1-28 d later. NM exposure induced time-related histopathologic changes in the lung including alveolar thickening, perivascular inflammation and bronchiolar epithelium hyperplasia, along with interstitial fibroplasia and fibrosis which were most notable at 14 d. NM-induced structural and inflammatory changes were accompanied by increases in total lung resistance, tissue damping and elastance and decreases in compliance and static compliance. Time related increases in enlarged foamy macrophages were also observed in the lung. This was associated with a rapid and persistent increase in total BAL cell, percentage of neutrophils, protein and phospholipid content, and surfactant protein (SP)-D levels. Marked increases in TNFα, RAGE and decreases in HMGB1 were also evident. At 3 d and 14 d post NM, expression of iNOS and TGFβ was also upregulated in lung macrophages. Flow cytometric analysis showed that macrophages accumulating in the lung 3 d and 14 d post NM were $CD11b^{+}Ly6G^{-}F4/80^{+}Ly6C^{hi}$ pro-inflammatory macrophages, while at 1-28 d, macrophages were $CD11b^{+}Ly6G^{-}F4/80^{+}Ly6C^{lo}$ anti-inflammatory. At 1-3 d post NM, increases in $F4/80^{+}$ monocytic and $F4/80^{-}$ granulocytic $CD11b^{+}Ly6G^{+}Ly6C^{+}$ myeloid derived suppressor cells (MDSC) were also observed in the lung. These data demonstrate that NM induces structural and functional changes in the respiratory tract of mice. Moreover, these changes are associated with a sequential accumulation of proinflammatory/cytotoxic and anti-inflammatory/wound repair macrophages in the lung. Support: NIH AR055073, ES004738 and ES005022.

LB15

The Effect of Retinoic Acid on the Phagocytosis of Helicobacter pylori by Human Dendritic Cells

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Infection of the stomach with Helicobacter pylori bacteria is present in approximately 50 percent of the world's population. H. pylori infection can lead to diseases such as chronic gastritis, duodenal ulcers, and gastric adenocarcinoma, the third leading cause of cancer-related mortality worldwide. The goal of our research project was to determine whether retinoic acid (RA) alters the phagocytosis of H. pylori bacteria by human dendritic cells (DCs). Retinoic acid, a metabolite of Vitamin A, is attributed with driving a tolerogenic phenotype in dendritic cells. Previous work in our lab has shown RA production by both epithelial cells and DCs in the stomach. Moreover, RA increases the expression of CD103, a marker associated with tolerance, and decreases the expression of CD86, a marker associated with activated DCs. We hypothesized that RA in the stomach may affect the uptake of H. pylori by DCs. To test our hypothesis, we developed an imaging cytometry protocol to measure phagocytosis of GFP-expressing H. pylori. First, we used U937 cells, a monocytic human cell line, to optimize the protocol for ImageStream analysis. Using the U937 cell line, we tested a series of antibody surface markers and found that an anti-CD13 PE antibody resulted in bright and defined surface staining with equal distribution. Time course analysis of the uptake of H. pylori by the U937 cells revealed the optimum bacterial uptake after 60 minutes. We also found that synchronization of phagocytosis by spinning down cells with the bacteria prior to incubation showed an increased bacterial uptake. To analyze the role of RA on DC uptake of H. pylori, monocyte-derived DCs (MoDCs) were incubated for three days in culture media containing RA. H. pylori bacteria were then added to the DCs at an MOI 20 for 60 minutes followed by surface staining with anti-CD13 PE and nuclear staining with DRAQ5. In a preliminary experiment, 70.5% of the untreated DCs showed green fluorescence staining indicative of the presence of GFP-H. pylori. However, ImageStream analysis using a cell surface mask and the Internalization analysis wizard revealed that only 56.0% of the GFP-H. pylori positive DCs contained internalized bacteria, whereas the remaining 44.0% of DC only had surface-associated bacteria. In RA treated cells, 76.2% of the DCs showed green fluorescence staining indicative of the presence of GFP-H. pylori. However, using the same analysis revealed that 76.16% of the GFP-H. pylori positive DCs contained internalized bacteria, whereas the remaining 28.9% of DC

only had surface-associated bacteria. These results suggest that RA may increase the ability of DCs to phagocytose H. pylori bacteria.

LB16

Alterations in Leukocyte Recruitment into Mouse Skin Following Exposure to Sulfur Mustard

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Sulfur mustard (SM) is a chemical weapon first employed during World War I and it remains a significant military and civilian threat. The characteristic responses of human skin to SM involve erythema of delayed onset, followed by edema and the appearance of large blisters in affected areas. SM toxicity is also associated with a prolonged healing period. Increasing evidence suggests that leukocytes and mediators that they generate are important in the pathogenic responses to SM. We used a vapor cup model with SKH1-hr mice to study the early stages of leukocyte infiltration into skin following exposure to SM. The dorsal skin of mice was exposed to SM at MRIGlobal (Kansas City, MO) using thirteen mm diameter vapor cups mounted on the dorsal skin. After 1-14 days, skin sections were analyzed for inflammation and tissue injury. One-three days post SM exposure, we observed epidermal thickening, stratum corneum shedding, and basal cell karyolysis. Increased numbers of degranulated mast cells as well as Ly6g+ neutrophils and F4/80+ macrophages accumulated in the dermis and hypodermis. CCR2, a type 2 C-C chemokine receptor important in macrophage and mast cell trafficking to sites of injury, was expressed in inflammatory cells within the hypodermis, in dermal fibroblasts, and in cells surrounding hair root sheaths. After 3 days, a 150% increase in wound thickness when compared to controls was apparent; along with the loss of epidermal structures and the formation of an eschar. This was associated with a marked increase in the number of Ly6g+ cells within the eschar and F4/80+ macrophages as well as degranulated mast cells beneath the eschar. At this time, CCR2+ dermal fibroblasts and mast cells were scattered throughout the dermis. After 5 days, wound thickness increased to over 250% of control with epidermal hyperplasia, pyknotic and birds-eye nuclei in the basal layer and inflammatory cells in an edematous dermis. Seven days post–SM, a neo-epidermis was evident along with a decrease in Ly6g+ cells, while a decrease in F4/80+ macrophages and CCR2+ epithelial cells was not observed until day 14. After 14 days, epidermal regeneration was associated with decreased wound thickness, Ly6g+ cells, F4/80+ cells and CCR2 expression returned to basal levels while mast cell levels were still elevated. These data indicate that SM-induced toxicity is associated with an early infiltration of neutrophils and mast cells into the tissue. Subsequent macrophage migration into the skin was associated with the wound healing process. Decreasing neutrophil infiltration into skin and enhancing the accumulation of macrophages and mast cells may be an effective mechanism to mitigate skin toxicity induced by SM. Supported by NIH AR055073.

LB17

Innate Defense Against *Candida Auris*, an Emerging Fungal Pathogen.

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Candida spp. are common fungal colonizers of mucosal surfaces in healthy adults; yet, pathological infections can occur with perturbations to the commensal microbiota or host immunity allowing overgrowth and clinical symptoms. Normally this is limited to mucosal surfaces; however, bloodstream infections due to *Candida* spp. are common in healthcare settings, and are associated with a high rate of mortality. Recently, the MDR strain *Candida auris* has emerged as an important healthcare-associated pathogen that has rapidly disseminated to multiple countries, including now in the United States. This pathogen is particularly concerning because it is often resistant

to commonly used antifungal agents, with some strains exhibiting resistance to all currently available classes of antifungals (i.e., azoles, amphotericin B, and echinocandins). As of July 2018, a total of 340 clinical cases of *C. auris* have been reported in the United States. As nosocomial transmission has been reported, better understanding of the risk factors for colonization and gastrointestinal shedding of this emerging pathogen are of upmost importance. To date, few animal models of *C. auris* have been developed and no mucosal models have been described. We have developed a novel non-immunosuppressed mouse model in which dose-depedent *C. auris* oral mucosal colonization is observed as well as shedding of the pathogen in stool. We show that susceptibility to infection is dependent on innate host responses including the antimicrobial peptide, beta-defensin-1 (defB1).

LB18

Iron Homeostasis in Erythroblastic Island Macrophages

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Iron is essential for all life, but because it is toxic its availability must be tightly controlled. Macrophages (MFs) maintain systemic iron levels by both forming 'erythroid islands' supporting development of iron-rich erythroid cells and recycling these iron-rich red blood cells via erythrophagocytosis. Erythroblastic island (EBI) MFs, characterized as "nurse cells" because they nurture developing erythrocytes, ingest the extruded nuclei of developing red cells, though their basic biology of iron homeostasis is poorly understood. Multiple populations of bone marrow MFs have been identified based on their expression of CD11b, F4/80, Ly6C, Ly6G, CD169, CD68, and VCAM-1, amongst others. $CD11b^{lo/-}$ $Ly6C^{-}$ $Ly6G^{-}$ $F4/80^{+}$ $CD115^{int.}$ ($CD11b^{lo}$ MFs) were found to express intermediate to very high levels of CD71/Transferrin receptor (TfR), and upon further analysis, the $CD71^{hi}$ cells corresponded to Ter119+ erythroid progenitors, demonstrating that $CD11b^{lo}$ MFs associate with developing erythroblasts. $CD11b^{lo}$ MFs preferentially express Mer tyrosine kinase (MerTK), a receptor critical for uptake of pyrenocytes (extruded erythroblast nuclei). In contrast $CD11b^{+}$ MFs express little to no CD71 and MerTK and were not found to be associated with developing erythroblasts. $CD11b^{lo}$ MFs separated from erythroid progenitors exhibited significantly higher CD71 expression and displayed a distinct population of ferroportin-positive cells, suggesting the capacity to recycle iron. We next measured the labile iron pool using a fluorescent iron sensor (phen green SK diacetate; PGSK) where fluorescence is quenched upon binding iron and found that $CD11b^{lo}$ MFs exhibit an increased labile iron pool (LIP; indicated by low PGSK staining), relative to $CD11b^{+}$ M□s and monocytes. Sort-purified $CD11b^{+}$ and $CD11b^{lo}$ MFs were analyzed by Airyscan confocal microscopy, and revealed distinct localization of TfR/CD71 and unique endosome morphology. Our results suggest that $CD11b^{+}$ MFs exhibit CD71 in endosomal structures, consistent with its ability to undergo constitutive clathrin-mediated internalization, whereas CD71 localizes predominantly at the plasma membrane in $CD11b^{lo}$ MFs. Thus, $CD11b^{lo}$ MFs are functionally distinct EBI MFs and possess distinct localization of iron uptake proteins, such as CD71, suggesting that EBI MFs display unique iron transport and metabolism compared to other bone marrow MFs.

LB19

LPS Induces Development of Innate Immunity by Changing the Subunits of the Cellular Proteasome

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Proteasomes (>400,000) are present in all nucleated cells, but the function of its subunits have not been established. We have studied the role of the subunits in different cells and in macrophages from LMP knockout animals. We have observed differences in induction of cytokines and nitric oxide in response to LPS that can be

correlated with proteasome subunits and function. Review of our results suggest a novel model that macrophages/ monocytes in **early activated mode 1** (XYZ, low CT-like/PA ratio) phase in response to LPS release cytokines, TNF-α or IFN-γ, and growth factors that lead to activation of cells by acquiring LMP7, LMP2 and LMP10 proteasome subunits. Cells in **attack mode 2** acquire the capacity to induce NO, IFN-β, IFN-γ and STAT-1P in response to LPS. Such MΦ respond robustly to LPS and/or IFN-γ (from T cells), thereby promoting NO release for attack of bacteria in the host. After the attack Mode 2 has been accomplished, the cells naturally progress to a **tolerant mode 3,** where the cells become relatively refractory to LPS stimulation (thereby, allowing the healing process to begin). There are no new proteasome subunits being induced at this point, in large part, because of low expression of NF-κB. Generation of IFN-γ, followed by renewed LPS stimulation, serves to reverse the state of tolerance/refractoriness and renders the cells functionally active again, **active mode 4**. Alternatively, the cells can proceed towards a state of autophagy (self-eating, to conserve nutrients) in the absence of an external agonist.

These changes correspond to cells actually switching their proteasome proteolytic activities from predominantly low CT-like/PA activity (XYZ) to high CT-like/PA activity and T-like activities (LMP7/LMP10) in differentiated cells. It appears that these changes are also likely to be important for degradation of regulatory signaling proteins involved in LPS-induced (TLR4) and other signaling pathways. Thus, these protein complexes have the capacity to control signaling pathways induced by hormones, rate-limiting enzymes, and other agonists through the process of changing their subunits and corresponding types of proteolytic activities. Dysregulation of this pathway has the potential to lead to serious defects in the development and implementation of the host inflammatory processes. *Thus emerges the fundamental concept that the actual compositional structure of the proteasomes in cells, in large part, will dictate that cell's function and innate immunity. (This work was supported from grants provided by NIH to NQ)*

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