2019 Abstracts

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Acquired Immune Response to Infection

1

Immune Checkpoint Regulator, VISTA, Improves Survival in Murine Sepsis by Enhancing T-cell Crosstalk and Minimizing Inflammatory Tissue Injury

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Background: Despite exhaustive research on sepsis for over the last 50 years there remains neither an effective molecular biological/pharmacological treatment options nor methods of diagnosis. A recent focus in sepsis research has been on the role(s) of immune checkpoint regulators. The expression of these checkpoint receptors and ligand proteins serve to balance the immune response by initiating signaling cascades, resulting in either activating or suppressive immune cell changes. However, the complex network of immune checkpoint proteins has made finding an effective therapeutic target or biomarker specifically for sepsis difficult.

The B7/CD28 superfamily of immune checkpoint proteins is comprised of several regulators that have been implicated in pathologies of immune suppression, such as auto-immunity, some forms of cancer, chronic viral/pathogenic infection as well as sepsis. However, the therapeutic efficacy of these family members while quite robust in specifics forms of cancer, it has been less clear how this translates to other conditions, like sepsis where they are expressed. A recently discovered therapeutic candidate is V domain Immunoglobulin Suppressor of T-cell Activation (VISTA). VISTA also belongs to the B7/CD28 superfamily, however, it has unique structural/ signaling motifs and functional qualities that suggest it should act in a non-redundant fashion to B7/CD28 superfamily members like Programmed Cell Death Receptor (PD-1) in sepsis.

With the above background in mind, we will examine the hypothesis that immune checkpoint protein, VISTA, is upregulated on specific T-cell subsets to play a protective immunosuppressive role in early stage sepsis that reduces organ injury and overall mortality.

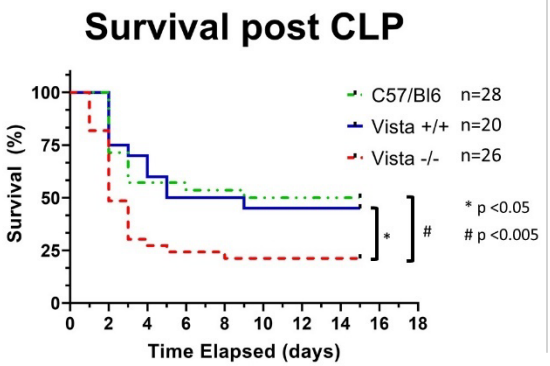
Methods: Cecal ligation and puncture (CLP) and sham (technical control) procedures were performed on wildtype C57B/L6 male mice aged 6-8 weeks old. Following CLP/sham, the spleen was harvested from mice at 24 and 48 hours post-CLP. Splenocytes were isolated and stained with anti-VISTA, anti-CD4, and anti-Foxp3 to delineate VISTA expressing effector T-cells (T_{eff}; $CD4^+Foxp3^-$) and T regulatory cells (T_{reg}; $CD4^+Foxp3^+$). Splenocytes were also separated into T_{eff} and T_{reg} populations using magnetic bead labeling, stained with fluorescent dye CFSE (a readout of proliferation), co-cultured for 5 days, and proliferation of T_{effs} was determined via flow cytometry. VISTA gene deficient mice ($VISTA^{-/-}$) were produced using CRISPR technology, subjected to CLP/sham, and survival was monitored every 24 hours for 14 days to produce a

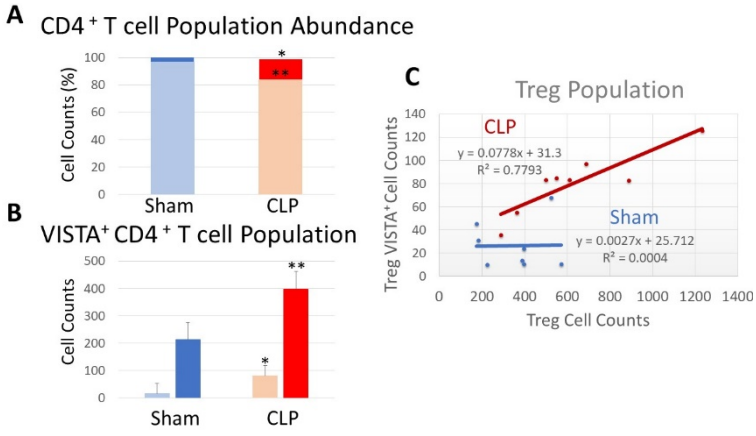
Kaplan-Meier survival curve. VISTA$^{-/-}$ mice also underwent the CLP/sham procedure and spleen and thymus samples were collected 24 hours later. Splenocytes were isolated, stained, and analyzed via flow cytometry using the same experimental design described previously for wildtype mice. Kidney NGAL levels were also assessed via western blot as an index of septic organ damage.

Results: In wildtype mice, splenic T_{reg} and T_{eff} CD4 subsets exhibited a significant upregulation of VISTA which correlated with an increase in T_{reg} abundance and a decrease in T_{eff} abundance. Using the *ex vivo* CFSE proliferation assay, we observed a significantly lower proliferative capacity of T_{effs} when cocultured with varying concentrations of T_{regs} following CLP. This suggests that splenic T_{regs} have increased suppressive function, VISTA expression, and abundance in septic wildtype mice. These immune cell phenotypes suggest that VISTA could mediate T-cell: T-cell crosstalk during early sepsis progression (within 24 hours).

Subsequently we found that the septic VISTA$^{-/-}$ mice had significantly increased septic organ injury (as indicated by increase kidney NGAL), and reduced survival when compared to littermate or wildtype controls. In the VISTA$^{-/-}$ mice, we also observed a more profound increase in T_{reg} abundance compared to wildtype mice. The absence of VISTA may decrease the potency/suppressive capacity of T_{regs}, thus, more T_{regs} must be mobilized to compensate for this loss of function in response to sepsis. This is in contrast with wildtype T_{regs} which appear to have high functionality at low concentrations.

Conclusion: Taken together, the wildtype and VISTA$^{-/-}$ results support a protective role for VISTA by which inflammation induced tissue injury is suppressed during early murine sepsis progression by mediating T-cell crosstalk. Thus, enhancing VISTA expression in early stage sepsis may provide a novel therapeutic option to prevent inflammation-induced death.





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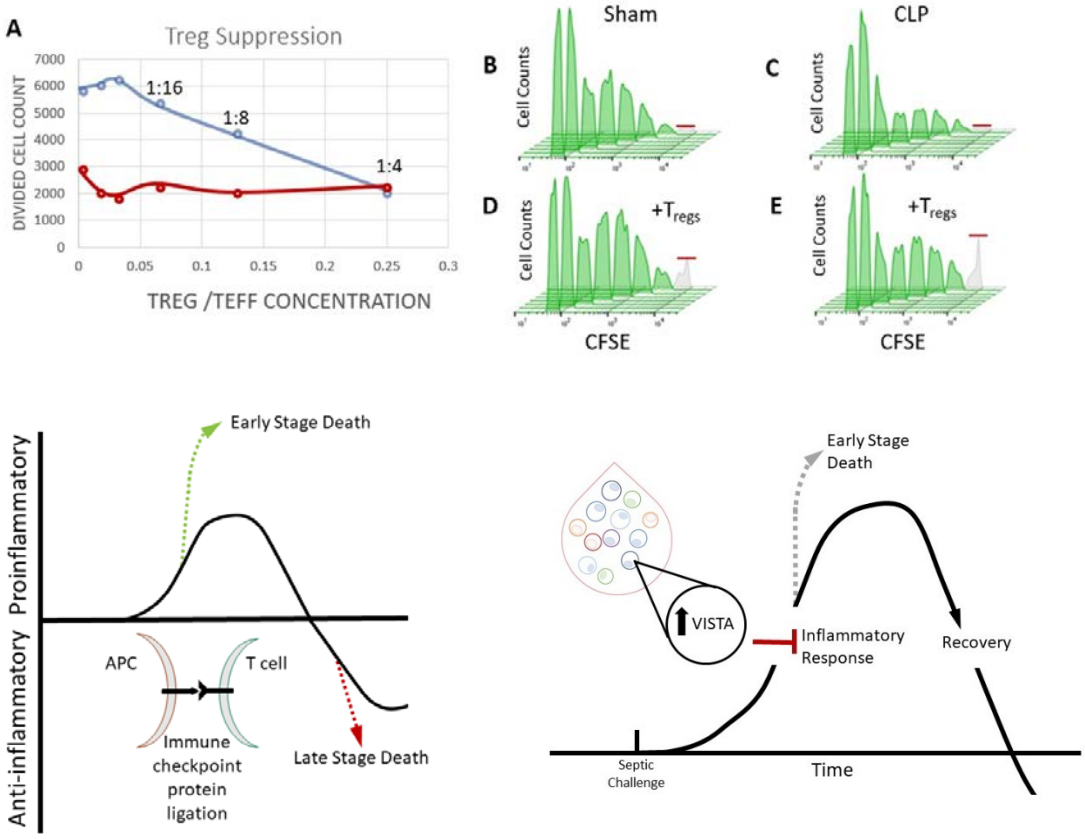

2

B Cells Inhibit CD4+ T Cell-mediated Protection Against *Brucella* infection

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Brucella spp., are facultative intracellular bacteria notorious for their ability to induce chronic, and often life-long infections. To date, no licensed vaccine exists for prevention of this zoonotic disease, and mechanisms underlying chronic illness remain elusive. We and others have observed B cell deficient mice challenged with *Brucella* display reduced bacterial burden following infection, but the underlying mechanism has not been clearly defined. Here we demonstrated neither Marginal Zone nor B-1a B cells are responsible for B cell-mediated susceptibility to infection. We did find a significant proportion of recoverable, intracellular *Brucella* within B cells during infection; however, the susceptibility to *Brucella* conferred by B cells could not be attributed solely to their capacity as a reservoir of infection. While B cell deficiency alone enhanced resistance to infection, combined B and T cell deficiency ($Rag1^{-/-}$) did not impact bacterial burden, suggesting T cells can mediate protection in the absence of B cells. Surprisingly, alpha beta T cell deficiency did not enhance bacterial burden indicating that while T cells can mediate protection, B cells may mitigate this ability. Using $Rag1^{-/-}$ animals to further investigate this relationship, we demonstrated adoptive transfer of $CD4^{+}$ T cells conferred protection against

Brucella infection. However, co-transfer of $CD4^+$ T cells with B cells abrogated this effect. In addition, we found B cells modulate $CD4^+$ T cell effector responses during infection. Collectively, our findings indicate susceptibility to *Brucella* infection is due in part to B cell inhibition of an otherwise protective $CD4^+$ T cell response.

3

Circulating and Local Biomarkers for Diagnosis of Tuberculous Lymphadenitis

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Background: The extrapulmonary tuberculosis (ETB) mainly Tuberculous Lymphadenitis (TL) represents currently a major public health problem in Tunisia. Cervical lymph node localization accounts for 75% of ETB notified cases annually. This form mimics other forms of non-tuberculous lymphadenitis (NTL) whether it is cancer or other infectious etiology. Diagnosis is challenging due to the paucibacillary nature of specimens. The identification of biosignatures specific to TL will certainly improve the management of patients. In the frame of an ongoing project we are collecting sera, circulating lymphocytes and lymph nodes-infiltrating lymphocytes in order to identify host markers for diagnosis of TL.

Aims and Methods: Herein we aimed to investigate the relative expression level of a set of inflammatory cytokines by the RT-PCR technique in a group of patients with TL *versus* a group with a NTL. We have also assessed by the ELISA technique the secretion of a set of cytokines after stimulation of PBMCs (circulating *versus* lymph node-infiltrating) by Mtb antigens.

Results: Our preliminary results indicate a higher expression level of IL-1b, IL-27 and IL-35 in the TL group as compared to the NTL group. In contrast, a lower level of IL-22 in the TL group was observed, suggesting a protective role of IL22 in this context. ELISA assays show that produced amounts of granzyme B and TNFa, mainly after stimulation with HBHA, were significantly higher in the TL group as compared to NTL.

Conclusion: These preliminary results pointed out the usefulness of IL-1b, IL-27, IL22 and IL-35 as potential markers for the discrimination between TL and NTL at transcriptional level. Moreover, HBHA-induced granzyme B and TNFa could be useful for the diagnosis of TL. In perspective we are looking for RNA-Seq and Mutiplex analysis of cytokines.

4

LPS Exposure in Mice Changes Immune Responses in Offspring

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We are constantly exposed to various environmental stimuli that shape our immune system and susceptibility to diseases. However, the impact of these changes on the host and their offspring have not been studied. Here, we have used LPS as an environmental trigger to understand the long-term consequences on the parents and their progeny. We hypothesize that LPS-induced immune changes are heritable. Our preliminary data demonstrate that a single dose of LPS challenge can promote long lasting immune changes in mice *in vivo*. Furthermore, F1 offspring born to LPS-exposed parents showed dramatically different PBL composition, with significantly increased neutrophil frequency. Finally, F1 offspring from LPS-exposed parents were found to be slightly resistant compared to control F1 offspring during lethal LPS challenge. These findings suggest that LPS-exposure in parents not only change their immune composition but also of their F1 progeny.

5

Exploring the Role of Regulatory T Cells in a Model of Ethanol-Accelerated Liver Fibrosis

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Objective: The immune response is a primary contributor to the development of alcohol-related liver disease (ALD), including alcoholic hepatitis (AH). Moreover, patients with severe AH often exhibit stage 3-4 fibrosis. During the initiation of fibrosis, hepatic stellate cells (HSCs) become activated and deposit extracellular matrix, hepatocytes are damaged, and hepatic nonparenchymal cells (NPC) promote tissue injury. Both the progression and resolution of fibrosis depends on interactions of NPCs, including resident and infiltrating leukocytes. T lymphocytes, including CD4+ and CD8+ T cells, are elevated in the portal and sinusoidal regions of the liver from patients with AH and cirrhosis. Interleukin-17 producing Th17 cells, a subtype of pro-inflammatory CD4+ T cell, are increased in biopsies from alcohol-related cirrhotic livers while peripheral regulatory T cells (Tregs) are decreased in AH patients. Collectively, the imbalance of Th17:Treg is thought to contribute to liver fibrosis in AH, however the mechanisms behind their interaction is not well understood. Moreover, the role of Tregs in resolving hepatic fibrosis in ALD has not been investigated. Therefore, we evaluated the immune phenotype of infiltrating Tregs in a model of ethanol-accelerated liver fibrosis.

Methods: Wild-type C57BL/6J female mice were acclimated to a complete liquid diet for 2 days then randomly assigned to an ethanol-containing diet (2% v/v, 11%kcal) or pair-fed control diet

for an additional 2 days. Mice then received a single injection of olive oil or CCl4 (1uL/g body weight) and tissue and liver NPCs were isolated up to 72hrs later.

Results: CCl4-treated animals displayed elevated indices of hepatocyte injury, including plasma alanine and aspartate aminotransferase (ALT, AST) activities; peak injury occurred 24hrs following CCl4 challenge with almost complete resolution by 72hrs; this response was independent of low-dose ethanol in the diet. Markers of liver fibrosis were elevated in CCl4-treated animals, with ethanol feeding in combination with CCl4 accelerating the fibrotic response. Expression of pro-fibrotic genes alpha-smooth muscle actin (a-SMA) and Col1A1 were increased 72hrs after CCl4 challenge as well as enhanced picrosirius red staining in the tissue. Similarly, CCl4 treatment increased the expression of pro-inflammatory and pro-fibrogenic mediators *IL-23a*, *CXCL1*, *TNFa*, *MCP-1*, *IL-6* and *TGFb* in liver, with peak elevations at 24hrs; the combination of ethanol feeding and CCl4 treatment further increased liver expression of these mediators. Moreover, the expression of *IL-17RA* mRNA and IL-17A protein was increased in the liver from the combined treatment groups at 24hrs. Treg dynamics were also influenced by both ethanol and CCl4, with peak infiltration at 24hrs and a second increase at 72hrs post-CCl4 treatment as measured by *FoxP3* mRNA. To better characterize the functional phenotype of these infiltrating Tregs, liver NPCs were isolated 24hrs after a single challenge of CCl4 or OO and stimulated for 4hrs with PMA/ionomycin in the presence of brefeldin A. Following stimulation, CD4+ and CD4+FoxP3+ T cells showed greater capacity to produce IL-17A/F following CCl4 challenge compared to OO by FACs analysis, suggesting that these infiltrating Tregs may be reverting their suppressive phenotype toward a “Th17-like” phenotype.

Conclusions: These data indicate that in our model of ethanol-accelerated fibrosis, infiltration of pro-inflammatory CD4+ T cells may be an important contributor to tissue injury and propagation of liver fibrosis via enhanced plasticity of infiltrating Tregs to “Th17-like” T cells. Targeting Tregs, via their suppressive function or via cell-based therapies, may prove to be a promising therapeutic approach for individuals with ALD.

6

Regulatory T Cells Suppress Trauma-Induced Inflammation and Control Adaptive Immune Cell Expansion

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Objective: Traumatic injury is the leading cause of death in the United States for people under 44 years old. This statistic does not include deaths caused by complications of trauma, which include the development of opportunistic infections due to a dysregulated immune system. To effectively control infections, the innate immune system initiates beneficial anti-microbial immune responses that may also cause destructive systemic inflammation in trauma patients. Conversely, the adaptive immune response reacts to injury in a counter-inflammatory manner to

help restore immune homeostasis to control excessive inflammatory reactivity to infections or danger signals (DAMPs). Unfortunately, this anti-inflammatory response to systemic inflammation and the release of previously sequestered DAMPs can also suppress anti-microbial immune function in trauma patients.

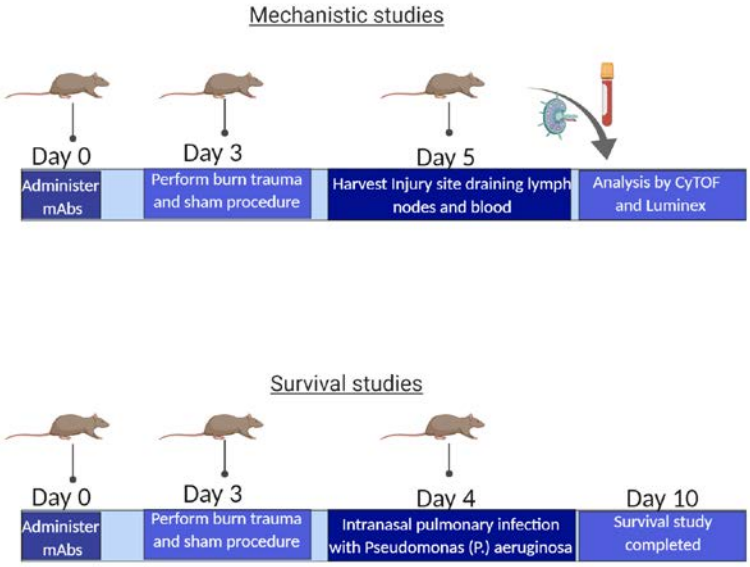
We previously reported that injury triggered a significant reduction in Th1-dependent IgG2a antibody formation and interferon-gamma production by antigen-stimulated T cells. Interestingly, we and others reported that promoting Th1-type immunity and blocking IL-10 improved polymicrobial sepsis survival responses in burn trauma mice. Mechanistic studies performed in Rag1-/-, CD4-/- and CD8-/- mice showed that burn injured $CD4^+$ T cell deficient mice developed higher inflammatory reactivity to innate stimulation than normal burn injured mice. Interestingly, it was shown that a type of lymphocyte known as Tregs were responsible for controlling the trauma induced inflammation and play a unique role in the *resolution of immune responses* in trauma. In this study, we tested the hypothesis that Tregs play an active role in controlling the host inflammatory response to traumatic injury in a Treg depletion mouse model.

Methods: Groups of C57BL/6 male mice were treated with anti-CD25 or isotype control mAb (1 mg/Kg) at 3 days prior to sham or burn trauma injury. Treg cell depletion was confirmed to last for 10 days post antibody treatment. The burn trauma model was performed on anesthetized mice by exposing the shaved dorsum (20% TBSA) to 90°C water for 9 seconds. At 2 days after injury, blood was collected to profile 23 different cytokines by Luminex technology and cell suspensions from injury-site draining lymph nodes were prepared for immunophenotyping by CyTOF mass cytometry (33 marker panel). To test the effects of Treg depletion on anti-microbial immune function, survival studies were performed on experimental groups of mice infected in lungs with *S. pneumoniae* bacteria 1 day after burn.

Results: To test the effects of Treg depletion on the systemic inflammatory response to trauma, we screened serum harvested from sham and burn trauma mice at 2 days' post trauma for multiple cytokines. We found that both sham and burn Treg depleted mice displayed enhanced inflammatory systemic phenotypes as compared to normal sham and burn mice. Specifically, we observed significantly higher levels of IL-1α and IL-33 in burn-injured Treg depleted mice compared with control mice. Moreover, we analyzed CyTOF staining data using machine learning clustering algorithms, ViSNE and SPADE. We found that burn-injured Treg depleted mice showed marked increases in activated $CD86^+$ MHC II^+ B cells suggesting that in the absence of Tregs, there is an increase in B cells capable of antigen presentation. We also identified a decrease in the abundance of a $CD4^+$ T cell population that expressed GITR and I-A/I-E proteins. Furthermore, we observed that Treg depletion delays death in mice from infection after burn trauma and secondary lung infection with *S. pneumoniae*. This suggests that the absence of Tregs augments inflammatory immune responses in injured mice which are primed to combat the pathogen, but may not be able to resolve this uncontrolled heightened inflammation.

Conclusions: Here we report that; 1) systemic levels of IL-1α and IL-33 were significantly increased in burn-injured Treg depleted mice compared with control mice, 2) Treg depletion

enhanced B cell expansion and caused a reduction in counter-inflammatory type $CD4^+$ T cells in burn trauma mice, and 3) Treg depletion altered the survival of sham and burn mice given a secondary *S. pneumoniae* lung infection. Taken together, these findings suggest that $CD4^+$ Tregs play a role in suppressing inflammatory and adaptive immune cell responses to trauma. The clinical significance of this hypothesis is that people with normal Treg levels may be better able to control trauma responses, while people with lower than normal Treg levels or functional responses may be at higher risk of trauma-induced inflammatory complications. Ongoing studies are addressing the unique subsets of $CD4^+$ Tregs that are activated by traumatic injury.



Autoimmune Disease

7

Therapeutic Effects of Tryptanthrin Oxime in Mouse Models of Rheumatoid Arthritis

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c-Jun N-terminal kinases (JNKs) participate in many physiological and pathological processes, including autoimmune inflammatory diseases. Recently, we synthesized oxime derivative of the plant-derived compound tryptanthrin (Tpr-Ox) and demonstrated that Trp-Ox is a high-affinity JNK inhibitor. Tpr-Ox inhibited secretion of matrix metalloproteinase (MMP)-3 by interleukin (IL)-1-stimulated human umbilical vein endothelial cells (HUVEC) and SW982 synovial sarcoma cells and the production of IL-6 by lipopolysaccharide (LPS)-stimulated MonoMac-6 monocytic cells. Evaluation of the therapeutic potential of Tpr-Ox *in vivo* showed that the compound significantly attenuated development of murine collagen-induced arthritis (CIA) and collagen-antibody-induced arthritis (CAIA). Severe cartilage erosion, synovial hyperplasia, and infiltration of

inflammatory cells were seen in the joints of saline-treated CIA and CAIA mice. In contrast, there was little cartilage erosion, synovial hyperplasia, and cellular infiltration in Trp-Ox–treated CIA or CAIA mice. Furthermore, histologic scoring showed significant differences between Trp-Ox–treated and control groups. Treatment with Tpr-Ox after induction of CAIA also resulted in decreased average clinical scores, and joint sections from Tpr-Ox-treated CAIA mice exhibited only mild signs of inflammation and minimal cartilage loss compared to those from control mice. Moreover, we found that the titers of IgG, IgG1, IgG2a, and IgG3 in the Tpr-Ox–treated group (dose 30 mg/kg) were significantly lower than those in the saline-treated group of CIA mice. Thus, Tpr-Ox reduced inflammation and cartilage loss associated with CIA or CAIA and can serve as a small-molecule modulator for mechanistic studies of JNK function in rheumatoid arthritis. This work was supported in part by National Institutes of Health IDeA Program COBRE Grant GM110732; USDA National Institute of Food and Agriculture Hatch project 1009546; and the Montana State University Agricultural Experiment Station.

8

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=11) 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: 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. This may play a role in mediating sterile inflammation after extreme exercise.

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9

Impact of Liver-resident and Auto Reactive NK Cells in the Pathogenesis of Primary Biliary Cholangitis

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The discovery of unconventional human Natural Killer (NK) cells in tissue endowed with adaptive traits is changing the conventional paradigm stating that they serve only as effectors against “non-self” targets. We recently described a unique subset of liver-resident NK (lr-NK) cells located within the hepatic sinusoids where they naturally interact with Kuppfer cells (i.e. liver macrophages) to provide an optimal immune-surveillance while keeping a certain threshold of immune tolerance. Our preliminary data showed that a significant fraction of this lr-NK cells become autoreactive and pathogenic relevant for killing autologous Biliary Epithelial Cells (BEC) in patients affected by primary biliary cholangitis (PBC). This NK cell-mediated destruction of intrahepatic biliary ducts correlated with the progression of this autoimmune disease and is also associated with higher frequencies of auto-reactive NK cells both in the liver and in the blood of PBC patients. We also observed that the decreased surface expression of several inhibitory checkpoints correlates with the expansion of auto-reactive NK cells both in liver and blood, thus suggesting that the lack of control by this immune checkpoint can unleash NK cell autoreactivity against autologous BEC.

10

Novel Functions of Inactive Rhomboid Proteins in Immunity and Disease

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iRhoms are closely related to a family of intra-membrane serine proteinases called rhomboids, but are lacking catalytic activity. In mammals, there are two iRhoms, iRhom1 and iRhom2, which have a similar domain structure and overlapping specificities as well as distinctive functions. These proteins are essential regulators for maturation and trafficking of a disintegrin metalloprotease ADAM17 from the endoplasmic reticulum to the cell surface, and are required for the processing of the proinflammatory cytokine TNF and several ligands of the epidermal growth factor receptor. iRhom2-dependent TNF and HBEGF-induced epidermal growth factor receptor activation have been recently implicated in the development and progression of several autoimmune diseases including, rheumatoid arthritis, lupus, and hemophilic arthropathy. This presentation focuses on recent advances and discuss our current understanding of iRhom biology, their implications in autoimmune pathologies, and their clinical applications for these disorders.

11

iRhom2 Is a Crucial Regulator of Adipose Tissue Inflammation and Insulin Metabolism in a Murine Model of Diet-induced Obesity

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Adipose tissue inflammation and metabolic syndrome are two comorbidities that are highly associated with obesity. Despite the increasing incidence of obesity in the United States, the relationship between inflammation and insulin resistance in obesity remains unclear. Previous studies have shown that the reduction of inflammatory cell types including M1 macrophage populations can ameliorate insulin insensitivity in murine models of obesity. While the inhibition of TNF was initially an attractive strategy, TNF inhibition alone has failed to mitigate metabolic dysregulation in patients with obesity or diabetes, suggesting that other molecules play a role in the onset of metabolic syndrome. TNF convertase enzyme (TACE) also known as ADAM17 is a cell surface metalloproteinase that mediates the maturation and release of soluble TNF and heparin-binding epidermal growth factor (HB-EGF). Recent studies have shown that the trafficking and cell surface expression of ADAM17 is tightly regulated by iRhom2 in inflammatory cell populations. Our findings suggest that iRhom2-deficient mice display decreased markers of adipose tissue inflammation, despite accelerated weight gain when fed a high fat diet. Therefore, we tested the hypothesis that low-grade inflammation triggers an iRhom2-dependent regulation of adipose tissue inflammation and insulin resistance in diet-induced obesity. The outcome of this study elucidates a novel role for iRhom2 as a metabolic regulator by directly establishing a link between iRhom2-mediated inflammation and insulin metabolism for the first time.

12

Lack of SATB1 Leads to Sjögren's Syndrome like Autoimmune Manifestations in Mice.

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Sjögren's syndrome (SS) is a systemic autoimmune disorder characterized by immune cell infiltration and progressive injury to salivary and lacrimal glands. A dominant clinical manifestation of SS is exocrinopathy, which leads xerostomia (dry mouth) and keratoconjunctivitis sicca (dry eye). Since the etiology of SS in human remains unclear, animal models are useful to investigate the pathogenesis of SS. Although several SS model mice share some characteristics with human SS patients, not all diagnostic criteria are fulfilled in any known mouse model of SS, even though dry mouth or dry eye is observed.

Special AT-rich sequence binding protein-1 (SATB1) is a genome organizer that regulates chromatin structure and gene expression. Previously, we reported that SATB1 conditional knockout (SATB1cKO) mice, in which the SATB1 gene is specifically deleted from hematopoietic

cells, are autoimmune prone, at least in part by defect of thymic central tolerance. In this study we found that SATB1cKO mice develop an SS-like disease.

Saliva and tear production were significantly decreased in SATB1cKO mice as early as 4 weeks of age. At this age, loss of saliva production was more prominent in female than in male SATB1cKO mice. Inflammatory cells infiltration and destruction of the gland structure were observed in salivary glands and lachrymal glands of SATB1cKO mice after 4 weeks old. At this age, major infiltrates were $CD4^{+}$T cells. The number of infiltrating B cells gradually increased in SATB1cKO mice with age and these cells dominated in older mice. In addition, transfer of T cells from SATB1cKO mice reproduced SS-like symptoms in recombination activating gene 2 (Rag2) knockout mice. These results indicate that T cells play a significant role in the pathogenesis of SS in SATB1cKO mice at least during the early phase of the disease. Therefore, SATB1cKO mice are useful SS model which fulfills almost all criteria necessary for diagnosis of SS in humans.

Changes in Tissue Immunity with Aging

13

Senescence-Associated β-Galactosidase Activity and Other Markers of Cellular Senescence Are Present in Human Peripheral Blood Mononuclear Cells During Healthy Aging and HIV Infection

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Introduction: Aging is associated with a decline in immune system performance termed “immunosenescence” which leads to less robust immune responses including decreased cytokine production and simulation of the adaptive immune response. Cellular senescence is characterized by a persistent cellular growth arrest, a failure to respond to stimuli, and an overall loss of cellular function. Although it is well-established that cells of the immune system can have decreased numbers and/or function with age, and that this occurs early in the context of HIV infection (so-called premature aging), whether immune cells undergo cellular senescence *in vivo* and whether cellular senescence plays a role in immunosenesence remains controversial due to the lack of specific markers that can reliably identify these cells in peripheral blood. Moreover, the differences between immune exhaustion and immunosenescence are not fully understood.

Methods and Results: To begin to address these questions, freshly isolated human peripheral blood mononuclear cells from healthy younger (22-26 y.o.) and older (56-65 y.o.) donors were fluorescently labeled for senescence-associated β-Galactosidase (SA-βGal) activity, anti-CD3, and anti-CD8 and were sorted using fluorescence-activated cell sorting. In addition, blood was collected from HIV-infected donors and labeled for SA-βGal activity. Flow cytometric analysis was utilized to identify CD14+ monocytes, CD3+ CD4+ T cells, and CD123+ BDCA2+ plasmacytoid

dendritic cells (pDC). qRT-PCR was used to quantify $p16^{INK4a}$ and cytokine production and immunofluorescence microscopy was used to measure DNA damage response foci.

We detected a significantly higher percentage of CD8+ T cells with high SA-βGal activity in the older vs. younger cohort of healthy donors. CD8+ T cells that were sorted for high SA-βGal activity displayed characteristics of senescent cells, including increased levels of transcripts for GLB1, p21, $p16^{INK4a}$, and inflammatory cytokines. Additionally, analysis of sorted SA-βGal positive and negative CD8+ T cells by immunofluorescence microscopy revealed that SA-bGal positive cells displayed significantly greater numbers of DNA damage response foci and $p16^{INK4a}$ protein levels. SA-βGal activity was also increased in CD8+ T cells, CD4+ T cells, monocytes, and pDC from older, HIV-infected donors. In addition, we observed shortened telomeres in pDC of older healthy adults and in HIV-infected individuals, as compared to younger HIV-uninfected donors.

Conclusions: We observed that individuals display increases in SA-βGal activity in several blood immune cell populations, and most consistently in CD8+ T cells. Additionally, we observed increase SA-βGal activity in CD8+ T cells, CD4+ T cells, monocytes, and pDC from an HIV- infected donor, as well as shortened telomeres in older healthy and HIV-infected subjects, similar to what has been shown in the literature for CD8+ T cells. These results indicate that HIV infection can induce cellular senescence in several immune cell populations and that decreased function is older HIV-infected and uninfected subjects is not simply a function of immune exhaustion. Sorting cells based on high SA-βGal activity allowed us to further characterize these cells for other senescence markers. The presence of $p16^{INK4a}$ and nuclear DNA damage response foci, which indicate double stranded DNA breaks or telomere dysfunction, along with the production of inflammatory cytokines clearly demonstrate that SA-βGal activity is a strong indication of cellular senescence in blood immune cells. SA-βGal activity can be leveraged to further identify senescent immune cells and elucidate cellular senescence's role in immunosenesence. Understanding this connection and how it contributes to the decline in immune response seen in healthy aging will further our understanding of premature aging and early onset aging-associated co-morbidities seen in HIV infection.

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Malt1 Deficient Mice Develop Osteoporosis Independent of Osteoclast-intrinsic Effects of Malt1 Deficiency

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This study tested the hypothesis that mucosa associated lymphoid tissue 1 (Malt1) deficiency causes osteoporosis in mice by increasing osteoclastogenesis and osteoclast activity. A patient with combined immunodeficiency (CID) caused by MALT1 deficiency had low bone mineral density resulting in multiple low impact fractures that was corrected by hematopoietic stem cell

transplant (HSCT). We have reported that Malt1 deficient macrophages, another myeloid cell type, are hyper-responsive to inflammatory stimuli. Our objectives were to determine whether Malt1 deficient mice develop an osteoporosis-like phenotype and whether it was caused by Malt1 deficiency in osteoclasts. We found that Malt1 deficient mice had low bone volume by 12 weeks of age, which was primarily associated with reduced trabecular bone. Malt1 protein is expressed and active in osteoclasts and is induced by receptor activator of nuclear factor κB ligand (RANKL) in preosteoclasts. Malt1 deficiency did not impact osteoclast differentiation or activity *in vitro*. However, Malt1 deficient (*Malt1*$^{-/-}$) mice had more osteoclasts *in vivo* and had lower levels of serum osteoprotegerin (OPG), an endogenous inhibitor of osteoclastogenesis. Inhibition of Malt1 activity in macrophages induced macrophage colony-stimulating factor (MCSF) production, required for osteoclastogenesis, and decreased OPG production in response to inflammatory stimuli. *In vitro*, MCSF increased and OPG inhibited osteoclastogenesis, but effects were not enhanced in Malt1 deficient osteoclasts. These data support the hypothesis that Malt1 deficient mice develop an osteoporotic phenotype with increased osteoclastogenesis *in vivo*, but suggest that this is caused by inflammation rather than an effect of Malt1 deficiency in osteoclasts.

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Extracellular Adenosine Signaling Reverses the Age-driven Decline in the Ability of Neutrophils to Kill *S. Pneumoniae*

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Despite the availability of vaccines and antibiotics *Streptococcus pneumoniae* (pneumococcus) infections such as pneumonia, bacteremia and meningitis remain a serious cause of mortality and morbidity, particularly in the elderly. This calls for a better understanding of the underlying pathways driving immunesenescence in aged hosts rendering them susceptible to infections. We previously found that polymorphonuclear cells (PMNs) are crucial for host defense against *S. pneumoniae* lung infection and that extracellular adenosine (EAD) production controlled the antibacterial function of these cells. EAD is produced by two exonucleosidases, CD39 and CD73, and signals via four G-protein coupled receptors, A1, A2A, A2B and A3. The objective of this study was to explore the age-driven changes in EAD pathway and its impact on PMN responses to *S. pneumoniae* infection. In comparison to young mice (2 months), bone-marrow-derived PMNs from old mice (18-22 months) exhibited elevated basal levels of the adenosine-degrading enzyme adenosine deaminase but similar levels of CD39, CD73 and all four adenosine receptors. Upon *S. pneumoniae* infection, the levels of CD39, CD73 as well as all four adenosine receptors were upregulated on PMNs from both young and old mice. PMNs from old mice failed to efficiently kill bacteria *ex vivo*, however, supplementation with exogenous adenosine rescued this defect. To identify which adenosine receptor(s) play a role in the anti-pneumococcal function of PMNs, we used specific receptor agonists and inhibitors. We found that A1 receptor signaling was crucial for the antimicrobial function of PMNs as inhibition of A1 signaling abrogated the ability of PMNs

from young mice to kill *S. pneumoniae ex vivo*. Importantly, activation of A1 receptors rescued the ability of PMNs from old mice to kill *S. pneumoniae.* Strikingly, triggering A1 signaling *in vivo* boosted the ability of old mice to control *S. pneumoniae* pulmonary infection. A1 agonist-treated old mice displayed lower bacterial loads, reduced clinical signs of disease and prolonged survival. Collectively, our findings demonstrate that with age, there are changes in the expression of EAD pathway components on PMNs. Further, targeting this pathway reverses the age-driven decline in PMN antimicrobial function which might have implications for incorporating clinically available adenosine-based drugs to combat pneumococcal pneumonia in the elderly.

16

Dissection of the Inflammatory Status of Hearts in Aging Mice with Invasive Pneumococcal Disease

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The elderly are at increased risk for dying from pneumonia. In addition, pneumonia survivors are at enhanced risk for disability and mortality well after the infection is cleared. In particular, Cardiovascular complications following pneumonia are a problem. One-in-four adults hospitalized for Streptococcus pneumoniae, the leading cause of community-acquired pneumonia, experiences an adverse cardiac event during time spent in the hospital. Survivors of pneumococcal pneumonia are also at increased risk for adverse cardiac events and cardiac-related death for up to 10 years following hospitalization. Because the elderly have the greatest risk for severe S. pneumoniae infection, these adverse cardiac events occur most frequently within the elderly. One potential reason why the elderly have increased susceptibility to infection during advanced age is inflamm-aging, a low but chronic increase in inflammatory mediators that occurs in tissues and blood during aging. Previously, inflamm-aging was shown to enhance susceptibility to pneumonia by increasing the expression of bacterial ligands on lung epithelial cells and inhibiting toll-like receptor activation of cells via A20-mediated blocking of NFκB and MAPK activation. Importantly, the mechanisms that impact age-related susceptibility to heart infection and damage remain unknown. To understand the age-related inflammatory changes in context of the heart, aged (18 months and older) and young (3-6 month) hearts from uninfected and infected C57Bl/6J mice were examined for differences in the activation status MAPK and NFκB via immuno-blot, cytokine levels via ELISA, bacterial burden, and cardiac damage by histology. We determined that aged mice have higher baseline levels of NFκB and MAPK activation and thickening of vascular walls within the heart suggestive of endothelial cell dysfunction. Importantly, aged mice failed to respond as efficiently to S. pneumoniae infection. This was demonstrated by aged mice having drastically increased bacterial burden (>100-fold) within the blood, and a dampened cytokine response within heart tissue compared to young mice. Ongoing studies are focused on revealing alterations in immune cell populations in the heart via flow cytometry. The results of these studies will help serve as the beginning of understanding how inflammation in hearts during aging impacts susceptibility to bacterial infections, such as invasive pneumococcal cardiac infections.

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Tissue-resident Alveolar Macrophages from Young and Aged Mice Respond Differently to Distal Injury

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Background: The systemic inflammatory response to injuries such as burns or scalds can have profound effects on the immune system, including tissue-resident leukocytes in uninjured organs. Little is known about how such changes affect the dysregulated immune system of the elderly, a population particularly vulnerable to injury-induced immune dysfunction and resulting infections such as sepsis and pneumonias. We hypothesized that "inflammaging" of the immune system alters the transcriptional response of tissue-resident alveolar macrophages in the lung to injury in aged animals, and sought to characterize such responses in young and aged animals.

Methods: To test our hypothesis, we subjected young (4 months) and aged (21 months) BALB/c mice to our nonlethal model of dorsal scald injury (15% total body surface area). 24 hours after injury, mice were euthanized and alveolar macrophages (AMs) were harvested by bronchoalveolar lavage. RNA was extracted from AM, converted into a cDNA library, and sequenced on an Illumina sequencing platform. Transcriptomic data was then analyzed for differential gene expression and pathway enrichment by gene set enrichment analyses (GSEA).

Results: AMs from young mice exhibited a greater diversity and magnitude of transcriptional changes after injury (964 differentially expressed genes), relative to macrophages from aged mice (216 genes). Among such changes were transcriptional hallmarks of glucocorticoid (GC)-mediated signaling in macrophages, including upregulation of anti-inflammatory GC response genes (*fkbp5, jdp2, socs2, klf4, irf2*) and downregulation of select components of inflammatory signaling pathways, including IL-1β (*il1b*), AP-1 complex (*fosb, junb, atf3*), NFκB complex (*nfkb1, nfkb2, relb, nfkbie, bcl3, ikbke*), and Nur77 (*nr4a1*). Of particular interest was FK506 binding protein 51 (*fkbp5*), a HSP90 co-chaperone and negative regulator of GC receptor activity that was elevated in AMs from both uninjured and injured aged mice. GSEA revealed downregulation of pathways related to Toll-like receptor, cytokine, and chemokine signaling, as well as cellular growth and cell-cycle pathways, in AMs from young mice after injury. While several of these genes and pathways were also differentially expressed in AMs from aged, uninjured mice, injury barely induced further transcriptional changes in cells from this age group. Furthermore, age by itself modestly upregulated markers of alternatively activated phenotypes in AMs regardless of injury, including MARCO (*marco*), Chil3/Ym1 (*chil3*), MerTK (*mertk*), PPARγ (*pparg*), IL-4rα (*il4ra*), and PAI-1 (*serpine1*). FDR < 0.01 and p < 0.01 for all comparisons listed above.

Conclusion: Tissue-resident AMs from aged mice are resistant to injury-induced GC signaling, failing to show the transcriptional changes induced by injury in AMs from young animals but exhibiting markers of an alternatively activated phenotype even without injury. These data suggest that AMs from aged mice may already be desensitized to GC-mediated suppression of inflammatory responses in resident tissue macrophages after injury.

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Alcohol Exposure Differentially Alters Intestinal Barrier Integrity, Liver Inflammation and Fecal Microbiome Composition in Young and Aged Mice

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Background: Alcohol consumption has been on the rise among the elderly and advanced age is a risk factor for poor outcomes in alcoholic liver disease (ALD) including increased hepatic complications. Microbiome dysbiosis and decreased gut barrier function have been shown to be involved in alcoholic liver disease with alterations in the gut-liver axis leading to increased inflammation in the liver. In addition, disruptions in the gut-liver axis have been shown occur in advanced age, along with an elevated basal inflammatory state called “inflamm-aging”. These factors may contribute to increased hepatic effects of alcohol in older subjects. Our goal was to determine if advanced age alters the murine response to more moderate alcohol consumption. Specifically, we wanted to determine the effects of aging on alcohol-induced alterations in the gut-liver axis by evaluating changes in fecal microbiome composition, gut permeability, intestinal AMPs and liver inflammation.

Methods: Young (4-6 months) and aged (18-22 month) Balb/cBy female mice were given an ethanol dose of 1.25 g/kg daily (BAC 80-100 mg/dl at 30 minutes after gavage) for 3 days or 5-6 weeks. At 60 minute after the final dose, feces were collected for bacterial sequencing along with harvest of ileum and liver for assessment of AMPs and inflammation. Intestinal permeability was measured by serum quantification of orally gavaged FITC-labeled dextran and bacterial colony forming units (CFU) from cultured mesenteric lymph nodes (MLN). Fecal microbiota were profiled using high-throughput bacterial 16S-rRNA gene sequencing. Ileum and hepatic expression of AMPs and inflammatory genes was measured by Real-Time PCR.

Results: Variability in overall microbiota composition, assessed by permutation-based multiple analysis of variation tests using the Morista-Horn dissimilarity index, revealed significant differences after ethanol consumption and age. Interestingly, ethanol consumption in aged mice led to unique alterations in the microbiome compared to consumption in young mice. In addition, in aged, but not young mice, ethanol exposure triggered a reduction in ileal expression of the microbiome regulating AMPs and increased intestinal permeability and translocation of bacteria to the MLN. Finally, advanced age yielded a number of inflammatory changes in the hepatic response to ethanol including increased expression of pro-inflammatory genes associated with “inflamm-aging”.

Conclusions: Our results reveal that in aged mice ethanol intake leads to unique microbiome alterations, impaired intestinal AMP production, intestinal barrier disruption and increased liver inflammation compared to responses in young mice. Future studies will explore whether shifting intestinal microbiota composition in aged mice, either through nutritional supplementation or modulation of AMP production, reduces post-ethanol liver inflammation and may ultimately lead to new treatment interventions for elderly patients with alcoholic liver diseases. (Supported by VA Merit 1 I01 BX004335 (RHM, KJN, EJK), NIH AG018859, GM115257, AA026295 (EJK) & AA027687 (HJH)

Immunity in the Lung

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Sirtuin 1 Regulates Mitochondrial Function and Immune 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), a NAD^+-dependent deacetylase, has been associated with induction of autophagy, reprogramming cellular metabolism, and regulation of immune mediators. In this study, we investigated the role of SIRT1 in mitochondrial function and immune homeostasis during RSV infection using Bone marrow derived Dendritic cells (BMDC) from C57BL/6J wild type littermates (WT) and *Sirt1*$^{f/f}$ *CD11c-Cre* (SIRT1-deficient, SIRT1-/-) mice. SIRT1-/- BMDC showed accumulation of an increased number of depolarized mitochondria, and an elevated level of mitochondrial reactive oxygen species (mROS) as compared to WT BMDC. The defect in mitochondrial membrane resulted in a remarkably decreased mitochondrial respiration (oxygen consumption rate, OCR), coupled with lower levels of ATP. RSV infection has further aggravated mitochondrial dysfunction in SIRT1-/- BMDC. It appears that the absence of SIRT1 induces baseline dysfunction to mitochondria that continues during RSV infection. This SIRT1 dependent mitochondrial dysfunction induced a pathological Th2 and Th17 immune response leading to inflammation. Reverse Phase Protein Array (RPPA) of SIRT1-/- BMDC identified a range of differentially regulated proteins involved in pathways that play a critical role in mitochondrial metabolism, autophagy, oxidative and ER stress, and DNA damage. We were able to identify an essential protein, acetyl CoA carboxylase (ACC1), downstream of SIRT1, that has a central role in fatty acid synthesis, and its differential expression leads to pathogenic innate immune responses, including cytokine and APC function for activation of pathogenic T cells. Blockade of ACC1 lead to metabolic reprogramming of DC that ameliorated the altered pathologic Th2 and Th17 immunity to the anti-viral Th1 response. This study identify pathways that are critical for appropriate DC function dependent upon cellular metabolic processes that dictate immune phenotypes. Collectively, these studies expand our understanding of the mitochondrial mediated innate immune response during RSV infection and may contribute to therapeutic strategies to prevent chronic lung pathology.

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The Coinfected Pulmonary Environment: How Influenza a Virus, Bacterial Pathogens, and the Host Interact and Adapt

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While influenza A virus (IAV) kills an estimated 500,000 people worldwide annually, bacterial infections, which are secondary to IAV, cause an estimated 50% of the deaths. In order to survive an infection, the host must be able to both clear the pathogen (resistance) and survive direct damage caused by the pathogen and indirect damage caused by the host immune response (tolerance). Most research to date has focused on alterations of the host immune response (resistance) during coinfection. My previous work discovered that IAV/bacterial coinfection can also alter host tolerance mechanisms. Our current work addresses all three sides of coinfection by answering: **How does coinfection alter IAV virulence, bacterial virulence, and lung epithelial tissue tolerance?** The lung epithelium is the primary site of infection initial by influenza A virus, and IAV/bacterial coinfection results in increased damage to this essential part of the lung. We focus specifically on changes to the lung epithelium caused by viral and bacterial pathogens (independent of infiltrating immune cells). Our data shows that infection with IAV primarily triggers apoptosis of the airway epithelium. *S. pneumoniae* infection alone does not increase cell death at early time points, but when IAV infected epithelial cells are infected with *S. pneumoniae* there is a rapid shift towards a necrotic/inflammatory type of cell death. We also demonstrate striking transcriptional changes in both the bacterial pathogen and IAV during coinfection. IAV rapidly adapts to increase the expression of genes that are involved in the manipulation of the immune response. Bacterial gene expression during coinfection demonstrate clear metabolic adaptations to the coinfected lung epithelial environment. **Our data shows that cell death and other rapid changes triggered by viral/bacterial coinfection in the lung epithelium alters both pathogen virulence and host tolerance.**

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Pulmonary Nuclear Factor-Erythroid-2-Related Factor (NRF2)-insufficiency After Burn and Inhalation Injury

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Introduction: The American Burn Association estimates that there are ~3,500 deaths each year from burns. There are multiple influences on morbidity and mortality in burn patients, with inhalation injury among the most significant. Combined burn and inhalation (B+I) injury occurs in 5-30% of all burn patients and leads to hyper inflammatory responses, increased acute lung injury (ALI) and subsequent bacterial infection susceptibility. B+I injury has an acute two-fold effect in the lungs: local tissue hypoxia and local tissue neutrophil-mediated damage resulting in further inflammation. Clear delineation of the mechanisms is needed to break this cycle of immune dysfunction in order to define appropriate treatments. Immune homeostasis at the cellular level

is restored by the transcription factor Nuclear Factor-Erythroid-2-Related Factor (NRF2) which induces anti-oxidant, superoxide de-toxifying and anti-inflammatory immune genes. *We hypothesized that NRF2-activation occurs in the lung after B+I injury, but is insufficient to reduce the acute oxidative damage and hyper-inflammation and tested this hypothesis using a mouse model of burn and burn patient samples.*

Methods: We firstly utilized our animal model of burn and inhalation injury, which accurately recapitulates hypoxia, immune dysfunction and susceptibility to bacterial infection. Briefly, a 100°C heated copper rod is applied to the skin of anesthetized shaved mice to generate a 20% Total Body Surface Area (TBSA) full thickness cutaneous burn, followed by a 6-minute woodsmoke exposure. Sham controls undergo all procedures except injury. Mice are then resuscitated with Lactated Ringers solution and maintained on oral morphine *ad lib*. 4hrs after injury, whole lung lysates were probed for NRF2 and HO-1 expression by Western Blot analysis. BAL harvested at 4 and 8hrs after injury was lysed and Nrf2 mRNA quantified by qtRT-PCR, normalized to GADPH and analyzed relative to sham levels. We also exposed peripheral blood mononuclear cells (PBMC) collected from B+I patients with moderate/severe (≥15% TBSA) and pulmonary dysfunction (< 357 SpO2/FiO2 ratio), enrolled into our IRB-approved patient Repository at the North Carolina Jaycee Burn Center, to Bardoxolone methyl (CDDO-Me) which is a NRF2 agonist. We measured cytokine levels after 24hrs of LPS stimulation *in vitro* and induction of various NRF2-target genes using qtRT-PCR.

Results: Western Blot analysis of mouse whole lung 4hrs after injury demonstrated increased expression of NRF2 protein in lungs from B+I animals. Conversely, there was no significant increase in the protein expression of Heme Oxygenase (HO-1), the protein product of an ARE downstream of NRF2. As this data suggested dysfunction in the transcriptional activity of NRF2 which requires its translocation to the nucleus, we performed confocal immunohistochemistry on BAL cells to ascertain the subcellular localization of NRF2. Concurrent nuclear DAPI staining confirmed that while NRF2 was expression was increased, its expression was restricted to the cell cytosol. Similarly, as NRF2 is known to induce its own gene expression after translocation and activation of ARE, we performed quantitative real-time PCR on the BAL cells demonstrating significantly reduced Nrf2 mRNA in BAL from B+I injury mice. These data suggest a potential mechanism for the associated NRF2-insufficiency to restore immune homeostasis following burn injury. To test this, we utilized CDDO-Me which drives NRF2 protein translocation to and accumulation in the nucleus. A hallmark of burn injury is a dysfunctional hyper-immune response to TLR ligands. Indeed, PBMC from patients with moderate/severe B+I injury produced elevated amounts of MCP-1 when stimulated with LPS *ex vivo* compared to healthy patient PBMC. *Ex vivo* treatment of patient PBMC with CDDO-Me significantly 1) reduced the LPS-induced MCP-1 production and 2) induced the NRF2-target gene NQO1.

Conclusion: Together these data indicate that 1) NRF2-mediated homeostasis is essential for but not sufficiently induced post-burn injury for controlling the morbidity and mortality associated with burn injury, 2) boosted activation of NRF2 is a viable approach to break the cycle of inflammation *in vivo*.

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Post-traumatic Pulmonary Infection: Chemokine-directed Innate Immune Responses Along the Lung-skin Axis Prioritize Pulmonary Defense over the Healing Wound

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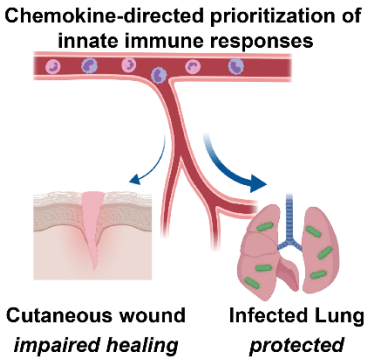
Trauma patients are at an increased risk of developing secondary infections, particularly in the lung. For these patients, the acute response to injury and infection relies on the rapid and coordinated activity of innate leukocytes including neutrophils and monocytes. We hypothesized that competing pulmonary and cutaneous inflammatory insults would negatively affect the innate immune response, leading to worse outcomes at both sites. To address this, we performed a retrospective analysis of 89,608 patients with midline abdominal incisions from the ACS National Surgical Quality Improvement Program. Pneumonia was correlated with a two-fold increase in the rate of wound dehiscence compared to patients without pneumonia, suggesting that pulmonary infection interferes with the ability to heal a distal wound. To understand this at the cellular level, we developed a murine model of post-traumatic pneumonia. Mice were wounded by the dorsal subcutaneous implantation of polyvinyl alcohol sponges, a model that recapitulates the acute stages of wound repair. Wounded mice remained uninfected or were infected intranasally with the opportunistic bacterium *Klebsiella oxytoca* five days later, and subsequent innate cellular and cytokine responses were assessed in the lung and the wound. Control groups were uninfected and unwounded, or infected alone. The presence of a dorsal wound did not alter the early control of bacterial infection compared to infected mice alone, nor did it affect the recruitment of neutrophils and monocytes or the concentration of cytokines and chemokines in the bronchoalveolar lavage fluid compared to infected mice alone. In contrast, pulmonary infection suppressed cutaneous wound healing. Infected mice had decreased monocyte and neutrophil trafficking to the wound as well as lower concentrations of proinflammatory cytokines and chemokines in the wound fluid (Figure 1). Exogenous delivery of CCL2 and CXCL1 to the wounds of infected mice increased the number of wound neutrophils and accelerated healing; however, this led to increased bacterial burden in the lungs of infected mice. These data suggest that innate immune responses along the lung-skin axis are delicately balanced to protect the lung, a vital organ, in this model of post-traumatic pneumonia. Ongoing work is examining the systemic signals that direct innate immune responses to protect the lung at the expense of a distal healing wound. This work aims to elucidate mechanisms by which the innate immune system is equipped to handle multiple insults, which may be broadly applicable to inflammatory conditions.



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The Role of the Actin-binding Protein Cortactin and Its Homologue Hematopoietic Cell-specific Lyn Substrate 1 (HS1) in the Onset and Progression of Sepsis

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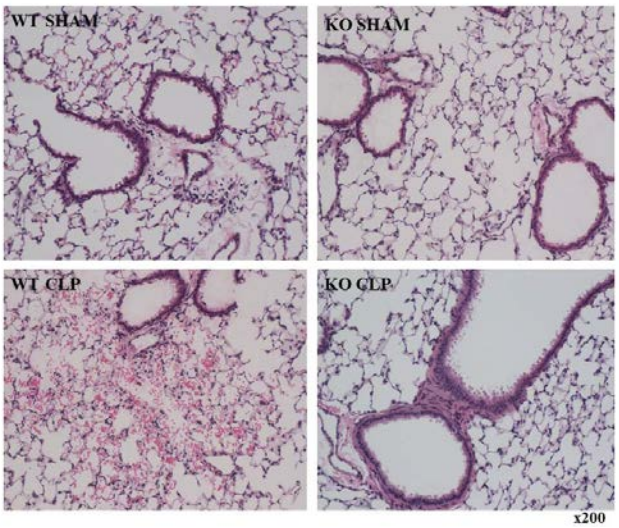
Introduction: Sepsis is a devastating disease, which occurs as a result of an exacerbated immune response to systemic infection. Major hallmarks of sepsis include increased vascular permeability and leukocyte recruitment to secondary organs such as lung and kidney, often leading to organ dysfunction and, in severe cases, organ failure. Cortactin is an actin-binding protein, expressed ubiquitously with the exception of neutrophils, that regulates vascular permeability and neutrophil recruitment. Cortactin-deficient mice showed increased vascular permeability but decreased neutrophil recruitment. HS-1 is a homologue of cortactin found exclusively in hematopoietic cells, which is indispensable for appropriate neutrophil extravasation. Therefore, endothelial cortactin and neutrophil HS-1 could play an important role during sepsis pathogenesis.

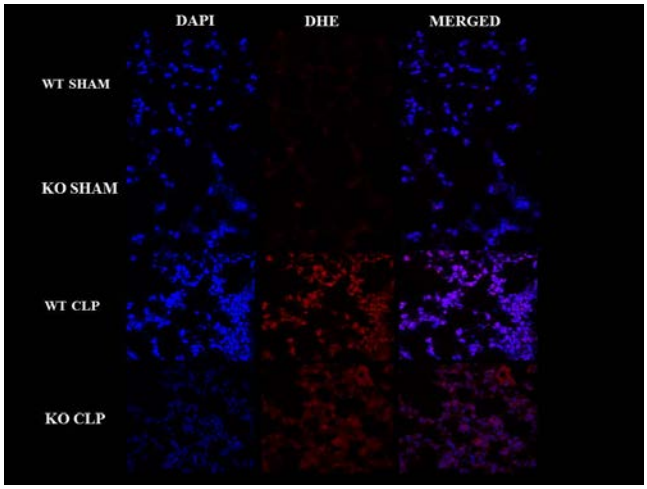
Aim/Objective: To elucidate the effects of cortactin and HS-1 deficiency on the onset and progression of sepsis.

Experimental Strategy: Sepsis was induced by cecal ligation and puncture in age-matched male cortactin-deficient (CTTN KO), HS-1 deficient (HS-1 KO) and littermate wild-type (WT) mice; and five day survival was monitored. Blood and lung tissue samples were collected 24 hours after CLP for protein and gene expression analysis. Lung histology was analyzed after hematoxylin/eosin staining of paraffin-cross sections of lung tissue. Oxidative stress was assessed in lung cross-sections using the dihydroethidium assay. Neutrophil numbers and phenotype were determined in blood, peritoneum and lung tissue by flow cytometry. Finally, hemodynamic parameters of septic cremaster venules and neutrophil recruitment were determined by intravital microscopy.

Results and Conclusions: Both cortactin and HS-1 deficiency improved survival of mice subjected to CLP. Of note, lung tissue of septic KO animals showed better preserved architecture of the alveoli, and absence of oedema, hemorrhage and mucus deposition. Gene expression of the inflammatory mediators tumor necrosis factor-α (TNFα) and interleukin-1β in the KO mice were down-regulated in comparison to septic WT mice. Neutrophil numbers in the peritoneum of both septic KO and WT mice were high but not significantly different implying that recruitment to site of infection was independent of HS-1. Moreover, we observed less oxidative stress and apoptosis in the lungs of both septic KO mice in comparison to septic WT mice. HS-1 deficiency reduced neutrophil numbers in the lung with the recruited neutrophils showing significantly higher levels of Gr-1, CD45 and ICAM-1 meaning the recruited neutrophil were more matured and effective in combating the infection. Also, systemic neutrophil extravasation was reduced in HS-1 KO mice. In summary, cortactin and HS-1 deficiency attenuates the systemic inflammatory response during sepsis, inhibits excessive neutrophil recruitment into secondary organs, and protects from tissue

damage. Thus, both cortactin and HS1 could be interesting candidates to develop novel treatment strategies for sepsis.





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Lung ALOX15 Impacts Pseudomonas Induced Transepithelial Neutrophil Migration

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Acute and chronic bacterial lung infection elicit neutrophil-driven inflammation in diseases such as pneumonia and cystic fibrosis (CF). Excessive recruitment and/or poor resolution of neutrophil responses contribute to tissue destruction and lung failure associated with pneumonia and CF. Neutrophil recruitment into the airspace is driven by infection-induced eicosanoid production of hepoxilin A3 (HxA3), by the airway epithelium. This process is further amplified through leukotriene B4 (LTB4) production by migrated neutrophils. Previous studies have suggested potential roles for ALOX15, a lipoxygenase, in promoting hepoxilin mediated neutrophil accumulation in the airspace after infection. In this study, we aim to characterize the role of ALOX15 in neutrophil trans-epithelial migration *in vitro* and *in vivo.* Our hypothesis is that *alox15* is responsible for HxA3 synthesis induced by *P. aeruginosa*, which drives neutrophil trans-epithelial migration. Employing an in vivo acute model of *P. aeruginosa* infection, neutrophil influx into the airspace after infection was significantly reduced in alox15-/- mice compared with C57BL6 controls. Markers of neutrophil influx into the airspace measured included myeloperoxidase by ELISA, elastase by ELISA, as well as the percentage and number of Ly6G+ cells. In parallel, a novel manipulatable *in vitro* mouse co-culture system was developed, to examine *the respective contributions of epithelial-expressed and neutrophil-expressed alox15* in driving *P. aeruginosa*-induced neutrophil transepithelial migration. Understanding molecular and cellular mechanisms that promote neutrophil infiltration of the airspace will present novel opportunities to develop therapeutic strategies to dampen neutrophil mediated damage in airway disease.

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Components of Pulmonary Surfactant Environment Suppress *Staphylococcus Aureus* Virulence

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S. aureus commonly colonizes the anterior nares and is frequently trafficked from the upper respiratory tract into the lungs via inhalation, microaspiration, and direct mucosal dispersion. Despite recurrent exposure to the lower respiratory tract, *S. aureus* is rarely an etiological agent of lung disease in immunocompetent hosts. As microorganisms progress through the bronchi and into the lungs they encounter pulmonary surfactant, a lipid rich complex primarily produced by type II pneumocytes that can act as the first line of defense against potential pathogens. While the antimicrobial effects of the fatty acids and collectins found in pulmonary surfactant have been well documented, only recently have several studies suggested that fatty acids may influence bacterial gene expression. Interestingly, our lab recently observed that *S. aureus* virulence production is delayed upon exposure to the healthy lung environment. Taken together, we hypothesize that the components of pulmonary surfactant suppress *S. aureus* virulence. Recent experiments revealed that the active fraction of pulmonary surfactant isolated from healthy mice suppressed *S. aureus* USA300 (LAC) virulence gene transcription. Moreover, supernatants from cultures grown in the presence of murine surfactant for 5 hours failed to lyse human PMNs compared to untreated controls. Using a plasmid based $_{hlgA}$-sGFP reporter assay, preliminary results indicated that palmitate (a major constituent of pulmonary surfactant) reduced the expression of the bi-component toxin *hlgA*. Treatment of USA300 with palmitate mirrored the phenotypes observed with treatment of murine surfactant. On-going studies are aimed at characterizing the changes that occur in pulmonary surfactant composition, production, and concentration during disease. Data suggests preceding influenza A infection leads to reduced pulmonary surfactant production through the destruction of type II pneumocytes, resulting in a loss of suppression of *S. aureus* virulence expression and the increased host morbidity observed during secondary bacterial pneumonia.

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Alveolar Macrophage Phenotype Following the Resolution of RSV Infection

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Background: Respiratory syncytial virus (RSV) infects virtually all children before the age of two. Children that are hospitalized for RSV have increased incidence of wheeze and asthma later in life, suggesting that RSV induces long-term changes in the immune landscape of the lung. Alveolar macrophages are long-lived cells that are sentinels of the lung, maintain tissue homeostasis against inappropriate inflammation, and have been shown to inhibit asthma

development. We therefore hypothesized that alveolar macrophage populations are phenotypically altered following the resolution of RSV infection, and as a result are less protective against asthma.

Methods: We challenged balb/c mice with RSV, allowed 25 days for mice to recover from infection, and collected alveolar macrophages from the lavage fluid to conduct *in vitro* analysis of cell phenotype. We depleted recipients of alveolar macrophages by administering intratracheal clodronate, and reconstituted the mice with alveolar macrophages harvested from RSV-challenged or saline-challenged animals. Animals were then subjected to a cockroach allergen (CRA) model.

Results: We found that 25 days after RSV infection, alveolar macrophages from RSV-infected mice had significantly higher MHCII expression. Alveolar macrophages harvested from RSV-infected mice expression levels of IL-1B and TNF when stimulated with CRA *in vitro* compared to those harvested from sham-treated mice; no differential cytokine expression was observed against lipopolysaccharide nor PAM3CSK4. We found mice that received clodronate prior to CRA sensitization had exacerbated allergic response against CRA, characterized by enhanced recruitment of inflammatory dendritic cells and monocytes during the sensitization phase and enhanced mucus production and eosinophilia after the final CRA challenge. Transfer of alveolar macrophages from both saline-treated and RSV-treated donors partially reversed the effect of clodronate depletion. These results suggest that alveolar macrophages can be phenotypically altered following RSV infection, and have a protective role against allergen sensitization.

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Neutrophil-Derived LTA4 Hydrolase Contributes to Bacterial-Induced Neutrophil Transepithelial Migration

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Rationale: Cystic fibrosis (CF) exhibits dysregulated and detrimental neutrophil-driven inflammatory responses that contribute to airway pathology and diminished lung function. Modeling neutrophil breach of the airway mucosa has revealed that epithelial cells apically release the bioactive lipid hepoxilin A3 (HxA3) in response to infection with *Pseudomonas aeruginosa*, which results in neutrophil transepithelial migration. Migrated neutrophils then release a second bioactive lipid, leukotriene B4 (LTB4), which is synthesized via neutrophil expressed cPLA2aand 5-lipoxygenase. LTB4 serves to substantially augment the magnitude of the neutrophil transmigratory response initiated by HxA3. Approaches that diminish LTB4 synthesis at the airway mucosa represent a possible therapeutic strategy for alleviating neutrophil-mediated damage in the airways of CF patients. LTA4 hydrolase (LTA4H) executes the final step in the synthesis of LTB4 through epoxide hydrolase activity on the substrate LTA4. Although LTA4H has the capacity to promote neutrophil recruitment by generating the neutrophil chemoattractant LTB4, LTA4H also exhibits aminopeptidase activity through a distinct active site

that functions to diminish neutrophil recruitment. LTA4H aminopeptidase activity degrades a different neutrophil chemoattractant, proline-glycine-proline, thereby reducing neutrophil migratory responses driven by this peptide. Given the bi-functional enzymatic activities displayed by LTA4H that feature opposing effects on inflammation and neutrophil recruitment, we sought to determine whether LTA4H impacts bacterial-induced neutrophil trans-epithelial migration.

Methods: Polarized airway epithelial cells were grown on the underside of permeable transwells. Neutrophils isolated from healthy volunteers were placed in the basolateral compartment and migrated towards apical signals released by *Pseudomonasaeruginosa*(PAO1)-infected epithelial cells or imposed chemotactic gradients (IL8). Neutrophils were pre-treated with LTA4H inhibitors or vehicle controls prior to migration. Total neutrophil migration was quantified using myeloperoxidase assays. LTB4 production was quantified by ELISA. Micro-Optical Coherence Tomography (µOCT) imaging allowed real-time visualization of PAO1-induced neutrophil transepithelial migration at 1µm resolution, without fixation or staining.

Results: Pre-treatment of neutrophils with LTA4H epoxide hydrolase inhibitor, ARM1, reduced PAO1-induced neutrophil transepithelial migration in a dose dependent manner. In contrast, pre-treatment of neutrophils with LTA4H aminopeptidase inhibitor, bestatin, resulted in no observable impact on PAO1-induced neutrophil trans-epithelial migration. µOCT imaging of PAO1-induced neutrophil transmigration following inhibition of LTA4H epoxide hydrolase in neutrophils reveals a pattern of diminished neutrophilic breach of the epithelium.

Conclusions: Our findings suggest that neutrophil-derived LTA4H plays a key role in bacterial-induced neutrophil transepithelial migration. The epoxide hydrolase activity of LTA4H, which converts LTA4 to LTB4, appears to drive this response. Further understanding the role of LTA4H as it pertains to airway inflammation is critical for optimizing LTA4H as a potential anti-inflammatory therapeutic target in the airways.

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Chronic Alcohol Exposure Decreases Expression of Tight Junction Proteins and Impairs Lung Barrier Function

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Objective: Alcohol use disorder (AUD) affects over 15 million people in the United States. Alcohol consumption is the third leading cause of preventable death and increases the mortality risk from acute respiratory distress syndrome (ARDS) to 65% in individuals with AUD, compared to 40% in non-alcoholics. Further, exposure to ethanol (EtOH) increases the risk of ARDS, lung injury, and susceptibility to respiratory infections. One major contributing factor to the increased risk of ARDS is an impaired alveolar epithelial barrier. Previous studies from our laboratory have demonstrated that chronic EtOH exposure decreases the antioxidant glutathione (GSH) and peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear hormone receptor with antioxidant effects, in bronchoalveolar lavage fluid. Therefore, we hypothesize that **EtOH**

exposure promotes alveolar barrier dysfunction, but strategies to attenuate oxidant stress via treatment with GSH or the PPARγ ligand, pioglitazone (PIO) will improve lung barrier integrity.

Methods: MLE-12 cells, a mouse alveolar epithelial cell line, were treated ± 0.08% EtOH for 3 d ± treatment with GSH (500 µM, last 1 d) or PIO (10 µM, last 1 d). mRNA and protein expression of claudin-1, ZO-1, and occludin were measured by qRT-PCR and western blot analysis, respectively.

Results: *In vitro* EtOH exposure: 1) decreased claudin-1, occludin, and ZO-1 expression; and 2) impaired transepithelial electrical resistance. Treatment with GSH or PIO reversed these EtOH-induced impairments in alveolar epithelial barrier function.

Conclusions: Chronic alcohol consumption reduces lung barrier function as measured by transepithelial resistance and expression of tight junction proteins (claudin-1, occludin and ZO-1). Our studies suggest that therapeutic interventions targeting oxidative stress will ameliorate EtOH-mediated barrier dysfunction. Future work will investigate the mechanisms by which alcohol consumption results in impaired barrier permeability. Current data demonstrate that GSH or PIO may provide novel therapeutic strategies to improve alveolar epithelial barrier function and could possibly reduce alcohol mediated complications in patients with ARDS.

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Impact of the Microbiome on Immune Responses and Cancer

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Type 3 Adaptive Cell and MDSC Expansion: Dynamics Mediated by IL-6 in a Mammary Carcinoma Model

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In the past decade immunotherapies harnessing anti-tumor immunity have been investigated as treatments for cancer. Initially, type I targeted immunotherapies were shown to be curative in some forms of leukemia but remain less effective in solid tumors such as breast cancers. As a consequence, the polarization away from type I immunity toward pro-tumor type II immunity as well as the induction of myeloid-derived suppressor cells (MDSCs) have been well characterized in breast cancer. However, little attention has been given to type III immunity in the tumoral context. The pro-tumor effects MDSCs have on $CD8^+$ and regulatory T cells and well-studied, but comparably less attention has been directed at the relationship between MDSCs and Type III immunity in breast cancer, despite their linkage in other inflammatory settings. The 4T1 mouse mammary carcinoma cell line is syngeneic to Balb/c mice and is thus an efficient immunocompetent tumor model to study the type of immune response elicited by triple-negative breast cancer. In this study we characterized MDSCs and type III T helper (Th) cells: Th17,

Th1/Th17, and Th22 responses to 4T1 tumors as well as the role of tumor-derived IL-6 in these inductions. IL-6 plays a key role as fate determining in Th maturation as well as induced MDSCs. Thus, the removal of IL-6 from 4T1 tumors, using the CRISPR-Cas9 system, allowed us to better characterize the source of these interactions. Populations of type III Th cells were defined as follows: Th17 ($CD3^+CD4^+RORγt^+IFN\text{-}γ^-IL\text{-}17A^+IL\text{-}22^{+/-}$), Th1/Th17 ($CD3^+CD4^+RORγt^+IFN\text{-}γ^+IL\text{-}17A^+IL\text{-}22^{+/-}$), and Th22 ($CD3^+CD4^+IFN\text{-}γ^-RORγt^-IL\text{-}17A^-IL\text{-}22^+$), and these cells were characterized in peripheral blood, spleen, tumor and bone marrow of mice bearing wild-type (4T1-WT) and IL-6 knockout (4T1-IL6-KO) tumors. We observed a reduction of Th17s in peripheral organs but expanded in 4T1-WT tumors. Knocking out tumoral IL-6 increased Th17 recruitment in peripheral organs but no change was seen in the tumor. Th1/17s were decreased in the blood and bone marrow of 4T1-WT but expanded in 4T1-IL6-KO. Th1/17s were present in both 4T1-WT and 4T1-IL6-KO tumors. Th22s were expanded in peripheral organs and in tumors of 4T1-WT mice compared to healthy controls. In 4T1-IL6-KO tumor-bearing mice, tumoral IL-6 reduced Th22 recruitment in peripheral organs but remained constant in the tumors themselves. MDSCs were defined as monocytic ($CD11b^+Ly\text{-}6G^-Ly\text{-}6C^{hi}$) or polymorphonuclear MDSCs ($CD11b^+Ly\text{-}6G^+Ly\text{-}6C^{low}$), $^+$) and were verified to be induced in peripheral organs and tumors of 4T1-WT bearing mice. Conversely, MDSCs were only reduced in peripheral organs of 4T1-IL6-KO mice as tumoral MDSC levels remained unchanged. In conclusion, our findings provide further insight into immune changes in the context of a triple-negative highly metastatic breast cancer. Specifically type III immunity, which may inform how to better polarize towards a type I immune response which would improve immunotherapy efficacy and outcomes in human breast cancer.

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Effect of IL-15 Deficiency on Thymocytes

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Leukemia is a diverse group of hematopoietic malignancies that cause significant morbidity and mortality in children and adults. T cell acute lymphoblastic leukemia (T-ALL) is an aggressive tumor that accounts for 10-15% of pediatric ALL. Even though intensive chemotherapy can cure 80% of pediatric cases, chemo-resistant and relapse cases have poor prognosis. Treatment of such cases requires the development of new approaches through better understanding of the molecular mechanisms of leukemogenesis.

We recently reported that NOD.Scid (NS) mice lacking either interleukin-15 NOD.*Scid*.*Il15*$^{-/-}$ or IL-15 receptor alpha chain NOD.*Scid*.*Il15ra*$^{-/-}$ *spontaneously* develop T cell leukemia with 100% penetrance by 30 weeks of age (***Leukemia*, *2016***). Leukemic cell lines established from NOD.*Scid*.*Il15*$^{-/-}$ mice express the T-ALL marker TdT, display constitutive NOTCH1 activation and are sensitive to NOTCH inhibition. Depletion of NK cells did not promote the development of leukemia in NOD.*Scid* mice, indicating NK-independent role for endogenous IL-15 in preventing the survival and outgrowth of leukemic cell precursors. Analysis of thymi from 4 wks-old

NOD.*Scid*.*Il15*$^{-/-}$ mice show discernible changes in the phenotype of DN thymocytes compared to those of control mice. Whereas NOD.*Scid* mice showed developmental arrest at the DN3 stage, substantial numbers of DN4, DP and SP cells were observed in NOD.*Scid*.*Il15*$^{-/-}$ thymi.

These findings strongly suggest that IL-15 signaling within the thymus controls the emergence of leukemogenic precursors from developing thymocytes.

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Alcohol Alters MAIT Cell Profile in Gut-Lung Axis Associated with Gut Dysbiosis

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Introduction: Mucosa-associated invariant T (MAIT) cells are programmed by the host microbiota and have a critical role in host defense against infection. Pneumonia is prevalent in people with alcohol use disorder and the alcohol-associated gut microbiota mediates alcohol-related impairments in host defense. However, the role of alcohol on MAIT cells is unknown. We hypothesized that MAIT cell profiles in the intestine and respiratory track are altered by alcohol and alcohol-induced pathobiosis likewise alters MAIT cells in the gut and lung.

Methods: C57BL/6 mice were treated with chronic-binge alcohol for 10 days and were then sacrificed at day 11. MAIT cells in lung and intestine were characterized by flow cytometry. To investigate the contribution of alcohol pathobiosis, alcohol microbiota from the chronic-binge alcohol experiment were co-cultured with PBMCs in vitro and was adoptive transferred into antibiotics pretreated mice. MAIT cells in lung and intestine were analyzed.

Results: In chronic-binge alcohol exposed mice, MAIT cell (MR1-tetramer+TCR+) numbers were reduced and activation (CD25+) was suppressed in the intestine ($p < 0.05$). Meanwhile, the frequency of cytolytic products (granzyme B), inflammatory cytokines (IL-17, IFN-, TNF-), and transcription factors (PLZF and T-bet) were lower in alcohol-fed mice than pair-fed mice ($p < 0.05$). Controversially, alcohol increased the activation of MAIT cells in the lung. Granzyem B and cytokine IL-17 were also increased notably with alcohol use ($p < 0.05$). After 4 h exposure to cecal microbiota, alcohol-associated pathobiosis increased the production of granzyme B within MAIT cells comparing with pair-fed microbiota ($p < 0.05$). Mice adoptively transferred alcohol-associated intestinal microbiota had higher frequency of MAIT cells and higher portion of MAIT cells with functionality in the lung, while lower MAIT cell frequency and lower expression of inflammatory cytokines in the intestine than the mice receiving pair-fed microbiota.

Conclusion: These data demonstrates that chronic-binge alcohol alters MAIT cell profiles, particularly in the intestinal tract. Simultaneously, alcohol treated microbiota contributes to alcohol's effects on MAIT cells.

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Treponema Denticola interrupts Activity of Phosphoinositol Processing in Neutrophils

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Periodontal disease is a worldwide health burden, including in the United States where almost 50% of the population suffers from some form of this disease. Dysbiosis of the oral biofilm and dysfunction of the immune system create a chronically inflamed state, causing significant tissue damage and alveolar bone destruction with eventual tooth loss. *Treponema denticola,* a spirochete found in the gingival pocket of patients with severe periodontal disease, perturbs neutrophils function by modulating phosphoinositide signaling leading to downstream restriction of Rac1 and Akt activation to inhibit chemotaxis. The major outer sheath protein, Msp, is a significant virulence factor for *T. denticola* that modulates neutrophil function and is present within outer membrane vesicles (OMVs) shed by the bacterium. Through a series of immunoblots and qPCR experiments, we show that Msp does not alter gene transcription or protein content of the key enzymes responsible for PIP3 signaling; PTEN, PI3K or SHIP1. Instead, using immunoblots and ELISA assays, we found that Msp activates PTEN through dephosphorylation specifically at site S380. Whole bacteria and OMVs also modulate PTEN phosphorylation and phosphatase activity. SHIP1 phosphatase activity was assessed using chemical inhibition and immunoprecipitation assays in combination with modified malachite green assay to detect free phosphate, to show that Msp also decreases the activity of SHIP1. Msp also prevents secondary activation of PTEN/PI3K response by fMLP stimulation. We speculate this result is due to the redirection of the PIP3 substrate away from SHIP1 to PTEN. Immunofluorescence microscopy revealed a redistribution of PTEN from the cytoplasm to the plasma membrane following exposure to Msp which may contribute to PTEN activation. Despite the significant presence of *T. denticola* found in association with other oral pathogens in the most severe forms of periodontal disease, the mechanisms of how *T. denticola* modulates and evades the host immune response or contributes to the dysbiotic biofilm are not well described. Repressing neutrophil signaling and chemotaxis is likely an important mechanism by which *T. denticola* contributes to the dysbiotic bacterial community and disease progression. Furthermore, an increasing number of reports indicate that the overall health and disease state of the oral cavity has significant impact on other chronic inflammatory diseases such as cardiovascular disease and diabetes. Therefore, understanding how oral bacteria evade the host immune response to perpetuate the cycle of inflammation and infection is critical to combating periodontal disease to improve overall health outcomes.

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Abnormal Eating Pattern Promotes Alcohol-induced Colon Carcinogenesis via Dysbiosis and Colonic Inflammation

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Background and aims: Alcohol intake increases the risk of colon cancer. Recent data uncovers a previously unrecognized role for circadian rhythm dyssynchrony (CD) in promoting alcohol-associated colon pathologies including cancer. Eating during or close to the physiologic rest time is a common habit in our modern society. We hypothesized that wrong time eating (WTE) causes CD which promotes alcohol-associated colon carcinogenesis.

METHOD: Circadian rhythms were measured in B6 PER2::LUC mice. TS4Cre APClox^{468} mice were used to model colon polyposis, and underwent study conditions: alcohol (vs. water) and feeding schedules and prebiotic treatment. B6 mice were used to assess the effect of time on alcohol associated mucosal immune profile. Histopathologic exam and tissue staining for CD3, CD4, RORgt (Th17), Foxp3 (Treg) and GPR109A and P-Stat3 were performed. Flow analysis was done. Fecal microbiota was assessed. Short chain fatty acid (SCFA) was measured. GPR109A was knocked down in cells. Annotated colon cancer samples from The Cancer Genome Atlas (TCGA) were interrogated.

Results: Wrong Time eating (WTE) caused CD by shifting the phase of the colon rhythm. In polyposis mice, WTE+Alcohol (WTE+A) caused worsening polyposis, with a pro-inflammatory profile characterized by hyperpermeability and increased mucosal Th17/Treg ratio, associated with an altered microbiota. Specifically, SCFA-producing bacteria and the butyrate ratio levels were decreased by WTE+A; this preceded the polyposis exacerbation and its recovery resulted in an improved mucosal inflammatory profile and polyposis. Decreased butyrate signaling resulted in epithelial Stat3 activation *in vitro*. Stat3 expression was increased in polyposis from WTE+A. Analysis of TCGA database confirmed the expression pattern of GPR109A with RORgt and FOXp3 in human CRCs.

Conclusion: Abnormal food timing interacts with alcohol to promote colon carcinogenesis, in part through promoting a pro-tumorigenic inflammatory milieu sustained by modulating microbiota and butyrate signaling.

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Reduced IL-27 Receptor Expression Following Ethanol Intoxication and Burn Injury: Role of HIF1a

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Background: Ethanol intoxication combined with burn injury leads to hypoxic conditions in the gut and contributes to gut barrier dysfunction, such as increased intestinal epithelial cell (IEC) apoptosis and decreased IEC proliferation. Recent studies from our lab have demonstrated reduced IL-27 production in the small intestine following ethanol intoxication and burn injury, as well as reduced IL-27 receptor expression on IECs. Since IL-27 signaling is critical to IEC proliferation and barrier maintenance, we assessed whether hypoxia has any role in reduced IL-27 production and its receptor expression. Our hypothesis is that alcohol in combination with burn injury leads to gut hypoxia and hypoxia inducible factor 1α (HIF1α) stabilization, resulting in decreased IL-27 receptor expression, which contributes to decreased IEC proliferation and gut barrier restitution.

Methods: C57BL/6 male mice (~25g) were gavaged with ~2.9g/kg ethanol 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 alone or with HIF1α inhibitor PX-478 (5mg/kg). One day following injury, intestinal epithelial cells were collected from the small intestine and analyzed by flow cytometry and real-time PCR (qPCR) for IL-27RA. To further confirm the role of HIF1a in modulating IL-27 receptor signaling, Mode-K mouse intestinal epithelial cell line was treated with cobalt chloride ($CoCl_2$), which stabilizes HIF1α, for 24 hours prior to analysis by qPCR.

Results: As compared to sham animals, mice subjected to burn injury in conjunction with ethanol intoxication showed a ~40% reduction in IL27RA by gene expression analysis of IECs, whereas burn injury alone did not show a significant decrease. Analysis of IL27RA protein expression on IECs by flow cytometry showed a decreased trend in ethanol and burn injured mice compared to sham injured mice. Mode-K cells treated with HIF1α stabilizer $CoCl_2$ for 24 hours showed a dose dependent reduction in the mRNA expression of IL27RA by qPCR, with a concentration of 200uM leading to a ~54% reduction in IL27RA mRNA expression. Ethanol intoxicated and burn injured mice receiving HIF1α inhibitor PX-478 in resuscitation fluid showed a ~6.7-fold increase of IL-27RA gene expression compared to untreated injured mice. To investigate the role of IL-27 on IEC growth, intestinal crypts were isolated from healthy mice and grown in a 3D Matrigel dome with or without 100ng/mL rIL-27 for 48hrs to measure organoid growth. Organoids treated with rIL-27 for 48 hours showed ~1.46-fold increase in area growth compared to untreated organoids.

Conclusions: Our findings suggest that gut hypoxia, as a result of ethanol intoxication combined with burn injury, leads to a reduction in the expression of the IL-27 receptor. This in turn can contribute to decreased IEC proliferation and barrier integrity.

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MicroRNA Expression Profile of Intestinal Epithelial Cells Following Alcohol and Burn Injury

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Previous findings from our laboratory have shown that ethanol intoxication at the time of burn injury promotes gut barrier disruption, however the underlying mechanisms are not fully understood. MicroRNAs are small noncoding RNA molecules that control gene expression by binding to mRNA and mediating repression of transcription or by promoting mRNA degradation. Previous work in our lab has demonstrated reduced intestinal epithelial cell proliferation and increased inflammation following the combined insult of ethanol and burn injury, both of which impact gut barrier function. In this study, we identified microRNAs associated with regulation of proliferation (miR-34c, miR-127, miR-192, miR-381 and miR-495) and inflammation (miR-146, miR-150, miR-194, miR-574 and miR-671) and then assessed their expression in small intestinal epithelial cells following ethanol and burn via RT-qPCR analysis. Male C57BL/6 mice were gavaged with ~2.9g/Kg ethanol and four hours later were given a ~12.5% total body surface area full thickness scald injury. One day following combined insult, mice were euthanized, and small intestine tissue was harvested. The small intestinal epithelial cells were isolated and total RNA was extracted and used for RT-qPCR analysis of microRNA expression. Among those tested, miR-34c, miR-127-3p and miR-451a were all confirmed to be significantly elevated ($p < 0.05$) in IECs isolated from mice receiving ethanol and burn injury compared to sham. Additionally, the expression of miR-192 was elevated but failed to reach significance ($p = 0.057$). Two microRNAs, miR-150 and miR-194, were found to be significantly downregulated ($p < 0.05$) in ethanol burn mice compared to sham vehicle. MiR-381, miR-495, miR-146, miR-574 and miR-671 did not show any significant alterations in expression between sham and ethanol burn mice. These findings indicate that multiple microRNAs exhibit altered expression in small intestinal epithelial cells from mice receiving a combined insult of alcohol and burn injury. In particular, microRNAs which are known to target proliferation pathways exhibit increased expression while those targeting inflammatory pathways exhibit decreased expression. Overall, these changes in microRNA expression could serve as a mechanism of gut barrier disruption following alcohol and burn injury.

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Innate Immune Response to Infection

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Augmenting Emergency Granulopoiesis with CpG-ODN Conditioned Mesenchymal Stromal Cells for the Treatment of Neutropenic Sepsis

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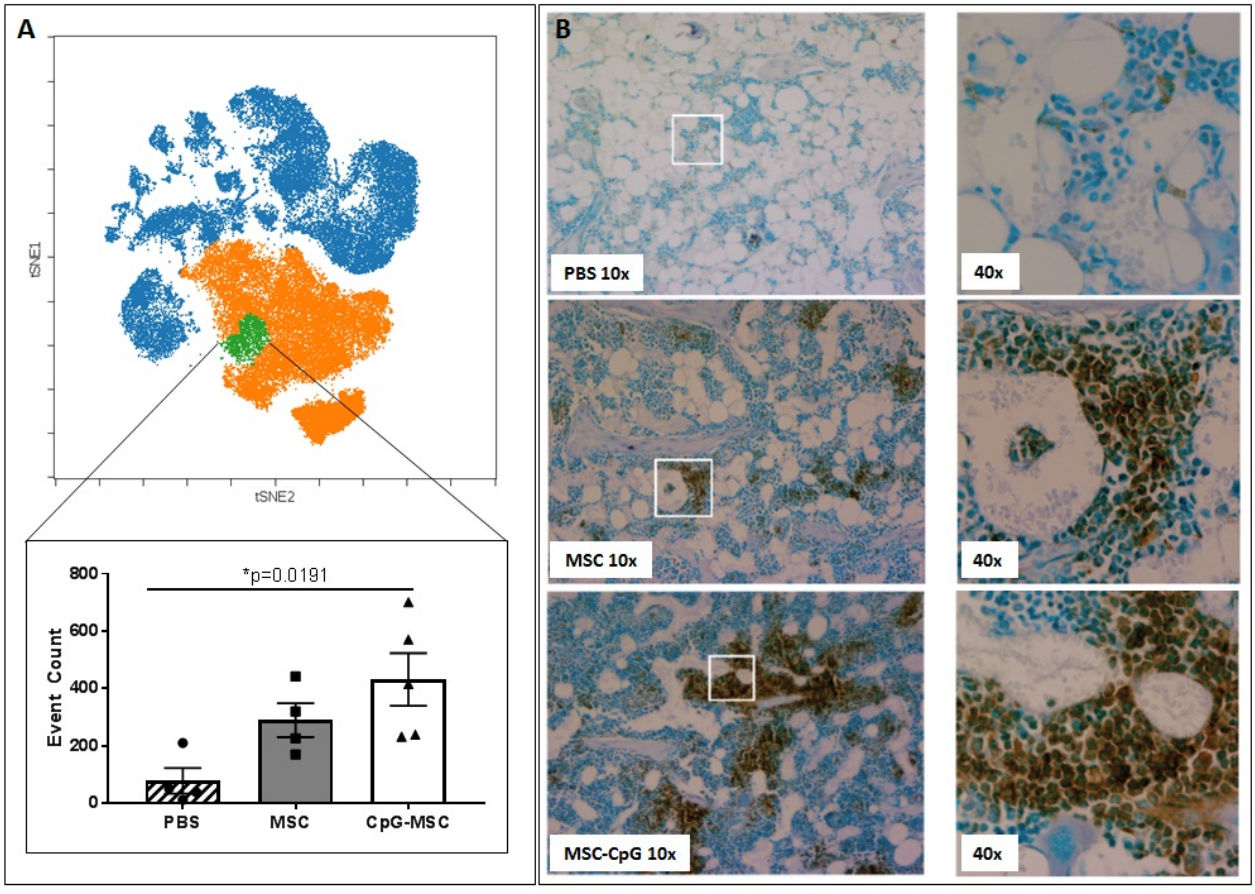
Introduction: Total body irradiation (TBI) is an important part of many preparative regimens for hematopoietic stem cell transplantation. The loss of hematopoietic cells and the damaging effects of radiation therapy put patients at risk for opportunistic infections, particularly during the peri-transplant period. Mesenchymal stromal cells (MSC)'s are fibroblast-like cells that contribute to the hematopoietic niche. They express toll-like receptors (TLR) and have antimicrobial and immunomodulatory functions in sepsis, which can be augmented by conditioning MSCs prior to administration. TLR9, which recognizes unmethylated CpG sequences prevalent in bacterial and mitochondrial DNA, can stimulate hematopoietic progenitor cells to differentiate into effector cells. Given this, we investigated the role of MSC's alone, and MSC's conditioned with the TLR9 agonist CpG-ODN, to rescue ineffective granulopoiesis in the setting of sepsis and radiation-induced bone marrow aplasia.

Methods: Male 8-10-week-old CD-1 mice were exposed to TBI (^{137}CsCl, 5Gy) to induce bone marrow aplasia. Three days after irradiation, mice were administered 100µl of 1) phosphate buffered saline (PBS), 2) 5 x 10^5 MSC's in PBS, or 3) 5 x 10^5 CpG conditioned MSC's (CpG-MSC) in PBS. MSC's were conditioned with 3µg/ml CpG-ODN 2336 in complete alpha MEM supplemented with 20% fetal bovine serum for 30 minutes prior to harvesting for injection. Seven days after irradiation, mice were infected intranasally with 1 – 2 x 10^6 CFU of Pseudomonas (P.) aeruginosa. 48 hours after infection, the mice were euthanized and single cell suspensions were prepared from the bone marrow. Cell populations were determined using flow cytometry, cytometry by time of flight (CyTOF) and immunohistochemical staining. C-kit$^+$ cells isolated from the bone marrow were cultured in mouse methylcellulose complete media to generate granulocyte-macrophage colony forming units (CFU). Blood leukocyte counts were analyzed using the Advia 120 Hematology System.

Results: Mice given CpG-conditioned MSC's prior to P. aeruginosa infection had improved survival compared with mice given PBS control ($p < 0.05$). This survival benefit correlated with decreased organ damage in the kidneys ($p=0.0002$) and lungs ($p=0.0143$) by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in mice that received CpG-MSC's

compared to PBS control. Hematoxylin and eosin staining of bones harvested 48 hours after infection show increased cellularity within the bone marrow of mice given CpG-MSC's. CyTOF analysis of the bone marrow at 48 hours demonstrate an increase in the abundance of a cluster of cells identified as $Ly6G^{low}$ and $Ki\text{-}67^{+}$ (Figure 1A), suggesting that proliferating neutrophils were driving the phenotype within the bone marrow. Flow cytometric analysis of bone marrow cell populations demonstrate an increase in a lineage restricted progenitor population ($Sca1^{+}C\text{-}Kit^{+}CD150^{-}CD48^{+}$) in mice treated with CpG-conditioned MSC's. Colony forming assays performed on bone marrow $C\text{-}kit^{+}$ cells harvested 24 hours after infection demonstrated increased myeloid colonies per plate in mice treated with CpG-conditioned MSC's compared to unconditioned MSC's and PBS control. This corresponded with higher peripheral total white blood cell count (p=0.0396) and neutrophils in the bone marrow (p=0.0293) by CyTOF analysis in mice that received CpG-MSC's. Immunohistochemical staining of Ly6G expression further validated an increased number of Ly6G expressing cells (neutrophils) in the mice treated with CpG-conditioned MSC's compared with mice receiving unconditioned MSC or PBS (Figure 1B)

Conclusions: Mesenchymal stromal cells conditioned with CpG-ODN augment emergency granulopoiesis in the setting of radiation associated neutropenic sepsis.



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Uric Acid Pathway Activation During Respiratory Syncytial Virus Infection Promotes Th2 Immune Responses via Innate Cytokine Production and Type 2 Innate Lymphoid Cells Activation

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Respiratory Syncytial Virus (RSV) infects a majority of infants and can cause severe disease leading to increased risk to develop asthma later in life. In the present studies, we detected high levels of uric acid pathway components during RSV infection and examined whether they altered the pathogenesis of RSV infection. Inhibition of Uric acid (UA) pathway activation during RSV infection in airway epithelial cells using xanthine oxidase inhibitors (XOI) decreased the expression of IL-33, thymic stromal lymphopoietin (TSLP), and CCL2. In addition, treatment of RSV infected bone marrow-derived macrophages with XOI decreased the production of IL-1b. Thus, UA activation of different cell populations contributes to different innate immune mediators that promote immunopathogenesis. When mice were treated with XOI or interleukin-1 receptor antagonist (IL1-ra) during RSV infection decreased pulmonary mucus was observed along with significantly reduced numbers of innate lymphocyte cells type 2 (ILC2) and macrophages, accompanied by decreased IL-33 in bronchoalveolar lavage of the treated mice. These findings provide mechanistic insight into the development of RSV immunopathology and indicate that xanthine metabolites and UA are key immunoregulatory molecules during RSV infection.

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Intermittent Rolling Is a Striking Novel Defect of the Extravasation Cascade Caused by Myosin1e-deficiency in Neutrophils

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Neutrophil extravasation is a migratory event in response to inflammation that depends on cytoskeletal dynamics regulated by myosins. Myosin-1e (Myo1e) is a long-tailed class-I myosin that has not yet been studied in the context of neutrophil-endothelial interactions and neutrophil extravasation. Intravital microscopy of TNFα-inflamed cremaster muscles in Myo1e-deficient mice revealed that Myo1e is required for efficient neutrophil extravasation. Specifically, Myo1e deficiency caused increased rolling velocity, decreased firm adhesion, aberrant crawling, and strongly reduced transmigration. Interestingly, we observed a novel discontinuous rolling behavior termed “intermittent rolling”, during which Myo1e-deficient neutrophils showed alternating rolling and jumping movements. Chimeric mice revealed that these effects were due to Myo1e deficiency in neutrophils. This was a surprising finding as neutrophils have been previously reported to not express Myo1e mRNA. Thus, we performed immunoprecipitation

assays and for the first time detected Myo1e protein in neutrophils. Vascular permeability was not significantly altered in Myo1e KO mice suggesting that endothelial Myo1e is not critically involved in endothelial barrier regulation or endothelial-specific support of neutrophil extravasation. Mechanistically, Myo1e deficiency caused aberrant CXCL-1-induced neutrophil arrest and spreading due to defective actin polymerization and integrin clustering. Moreover, chemotaxis of Myo1e-defcient neutrophils towards the chemokine CXCL1 was significantly reduced. In conclusion, Myo1e critically regulates adhesive interactions of neutrophils with the vascular endothelium and thus neutrophil extravasation. Myo1e may therefore be an interesting new target in chronic inflammatory diseases characterized by excessive neutrophil recruitment.

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Innate Trained Immunity Can Influence Immune Cells Recruitment and Phenotype at the Fetomaternal Interface and Pregnancy Outcomes in Mice

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Pregnant women are more susceptible to infection than non-pregnant women and more severely affected by these infections. It is due to the physiological changes happening during this specific time when their body tolerate and allow the growth of a semi-allogeneic fetus. Infection during pregnancy are known to cause preterm birth, fetal growth retardation and fetal developmental abnormalities. Innate immune cells, such as macrophages and Natural Killer (NK) cells are involved in fetal tolerance and placental/fetal development through their secretion of immunosuppressive and pro-angiogenic factors. Furthermore, these innate immune cells are susceptible to be modified following exposition to infectious agent, by an epigenetic reprograming and a switch in their metabolism. This phenomenon defines innate immune memory, also called trained immunity.

In our team, we showed that *in vivo* treatments with repeated low doses of LPS or with BCG vaccine can induce changes in tissue resident murine peritoneal macrophages, at the phenotypic level and in their capacity to secrete specific cytokines. This way, repeated low doses of LPS decrease the capacity of splenic and peritoneal macrophages to respond to an inflammatory challenge, making these cells immune-tolerant or immuno-paralyzed. On the contrary, BCG vaccine training was able to increase their pro-inflammatory potential. This indicated that trained immunity can modify tissue resident innate immune cells.

The impact of infection before pregnancy, as a potential modifier of innate immune memory, on the outcome of pregnancy is unknown. To investigate this concept, we used an abortion-prone mouse model that we exposed to low doses of LPS to induce immune tolerance and to BCG to induce a pro-inflammatory phenotype before mating. Gestations were then followed by ultrasound, and placental tissues were analyzed at 2 time points during the gestation. While we were not able to reduce the abortion rate with LPS training, we noticed than BCG training was associated with intra-uterine growth retardation. This phenotype was associated with changes in

immune recruitment early during gestation: the total frequency of immune CD45+ cells was unchanged but the proportions of NK and macrophages were reduced while the proportion of T cells was increased. The phenotype of the immune cells was also altered. These changes were not detectable at the end of gestation. This early immune imbalance at the fetomaternal interface may explain the fetal growth retardation. To better understand how the training impact the immune cells at the fetomaternal interface, we are currently investigating the uterine horn immune cells after *in vivo* training.

These results suggest than pre-conceptional infections could influence the immune cells in our reproductive system and have consequences during pregnancy. We are currently trying to assess this possibility using an epidemiological approach from the data available from the Cochin hospital in Paris.

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Anti-inflammatory Role of Intestinal Microbiota Helps Balance Immune Response After Pathogenic Infection

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After pathogenic infection, an unrestrained inflammatory response can lead to collateral damage of the tissue. As such, the immune system must carefully calibrate response strength against an invading pathogen. Within the intestine, we find the intestinal microbiota are key for intestinal antigen presenting cells (APCs) to promote tissue homeostasis. In the presence of the normal microbiota, intestinal APCs expressing the chemokine receptor CX_3CR1 reduced expansion of inflammatory T helper-1 (Th1) cells against pathogens and the microbiota itself. They also promoted regulatory T cells recognizing soluble proteins as well as the microbiota. We found disruption of the microbiota resulted in CX_3CR1^+ APC-dependent inflammatory Th1 cell responses with increased pathology after pathogen infection. Colonization with epithelial adherent microbes rescued the anti-inflammatory functions of CX_3CR1^+ APCs. This demonstrates that, while microbes that bind to the epithelium can be pathogenic, they can also activate homeostatic mechanisms. Our results identify a network by which the microbiota interacts with the host to limit intestinal inflammation and promotes tissue homeostasis.

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Serum- and Glucocorticoid- inducible kinase1 (SGK1) Restrains *P. Gingivalis*-mediated Inflammation and Protects Against Periodontal Bone Loss

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Objectives: Serum/glucocorticoid regulated kinase (SGK) 1 is a eukaryotic serine/threonine-protein kinase which acts downstream of PI3K and functions pleiomorphically in, e.g., the

regulation of ion channels and in cell proliferation and apoptosis. Whereas the immunomodulatory consequences of PI3K activation in innate and adaptive immunity are well established, the potential involvement of SGK1 in the inflammatory response has not been previously addressed. Periodontal diseases are chronic conditions characterized by failure to resolve inflammatory processes. The anaerobe *Porphyromonas gingivalis* is a major pathogen in periodontal disease, and in this study we addressed the role of SGK1 in the regulation of *P. gingivalis*-induced inflammation.

Methods: The hypothesis that SGK1 would inhibit inflammatory mediators induced by *P. gingivalis* was tested *in vitro* in primary human monocytes, and *in vivo* using the murine alveolar bone loss model. Cytokine production was measured by ELISA and phosphorylation of signaling molecules determined by western blotting. Inflammatory cell infiltrate was examined by HE staining and immunohistochemistry.

Results: Gene silencing, or pharmacological inhibition of SGK1 with EMD638683, significantly enhanced the production of TNF, IL-6 and IL-1bby *P. gingivalis*-stimulated monocytes (all $p< 0.05$). Furthermore, inhibition of SGK1 led to a robust increase in phosphorylation of IKKa/b, IkB, NF-kB-P65, MAPK-P38, and JNK in the same cells (all $p< 0.05$). Systemic administration of EMD638683 elevated infiltration of neutrophils and macrophages into the gingival tissues and aggravated the severity of alveolar bone resorption in *P. gingivalis*-infected mice ($p < 0.05$). In addition, a reduction of alternatively activated macrophage markers (CD206, CD163) was observed upon SGK1 inhibition, as compared with *P. gingivalis* infection only.

Conclusions:SGK1 plays an important protective, anti-inflammatory, and immunomodulatory role during *P. gingivalis* infection, and represents a potential novel therapeutic target for the control of periodontitis and other chronic inflammatory diseases.

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Modulation of Phagocytosis-induced Cell Death of Human Neutrophils by Neisseria Gonorrhoeae

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Infection with *Neisseria gonorrhoeae*, an exclusively human pathogen, is characterized by an exuberant inflammatory response secondary to recruitment of polymorphonuclear leukocytes (PMN). Phagocytosis-induced cell death (PICD), the accelerated apoptosis that follows phagocytosis, involves intrinsic and extrinsic pathways, through caspase 9 and caspase 8, respectively. Caspase 3, the executioner caspase of apoptosis, drives PICD. Prior studies have

shown that *N. gonorrhoeae* delay PICD of adherent PMN, however the underlying mechanism has not been elucidated.

Human PMN in suspension were fed *N. gonorrhoeae* FA1090 wild-type (GC) at 10:1 MOI for 30 minutes at 37°C, after which unbound bacteria were removed and bacteria-laden PMN in suspension were incubated. PICD was assessed in three ways: by measuring surface exposure of phosphatidylserine (PS), mitochondrial depolarization and DNA fragmentation. At both 2 and 6 hours, PMN fed GC externalized less PS, measured by binding of Annexin V, than did PMN fed pooled human serum-opsonized zymosan (OPZ) [GC at 2h=40 ± 3.2% vs. OPZ at 2h=56 ± 4.5%; GC at 6h=40 ± 4.5% vs. OPZ at 6h=63 ± 5.9%, $p< 0.05$, n=3]. Mitochondrial depolarization, measured by JC-1 staining, of PMN fed GC was decreased compared to that of PMN fed OPZ at 2 hours [GC=15 ± 2.8% vs. OPZ=46 ± 8.2%, $p< 0.05$, n=3]. DNA fragmentation of PMN fed GC, measured by TUNEL assay, was reduced compared to that of PMN fed OPZ at 6 hours [GC=21 ± 5.3% vs. OPZ=52 ± 6.6%, $p< 0.05$, n=3].

Caspase activities of PMN were assessed by Caspase-Glo, a luminescence assay. Caspase 3 activity was less in PMN fed GC compared to that of PMN fed OPZ [GC=37 ± 5.8% vs. OPZ=100%, $p< 0.05$, n=3]. Caspase 9 activity of PMN fed GC was decreased compared to that of PMN fed OPZ, whereas caspase 8 activities were similar [Caspase 9: GC=39 ± 3.1% vs. OPZ=100%, $p< 0.05$, n=3; Caspase 8: GC=105 ± 12.7% vs. OPZ=100%, n=3]. When PMN were fed with GC followed by OPZ, the caspase 3 activity stimulated by OPZ was reduced; furthermore, as the MOI of GC increased, the extent of inhibition increased in parallel [GC at 10:1MOI=80.5 ± 5.5%; GC at 25:1MOI=72 ± 7.5%; GC at 50:1MOI=56 ± 1.5 vs. OPZ only=100%, $p< 0.05$, n=2]. Similarly, caspase 9 activity of PMN simulated by OPZ was inhibited by GC after sequential phagocytosis of GC followed by OPZ [GC at 50:1MOI followed by OPZ=70 ± 6.5% vs OPZ only=100%, $p< 0.05$, n=3]. In contrast to results with caspase 3 and caspase 9, caspase 8 activity of PMN that ingested OPZ was not reduced by GC [GC at 50:1MOI followed by OPZ=151 ± 26% vs OPZ only=100%, n=3].

Taken together, these data demonstrate that GC delayed PICD of PMN in suspension by modulating the intrinsic apoptosis pathway through inhibition of caspase 9, thereby inhibiting caspase 3 of PMN. Delayed PICD of PMN by GC could contribute to the robust inflammatory response by PMN in GC infection.

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Neutrophil Maturation and Their Response to Infectious Pathogens Are Regulated by Microbiota

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It has long been considered that a neutrophil's response to various infectious challenges is innately predetermined. Here, we provide data that demonstrates that neutrophil proteomes are modulated by the microbiota. We found that the proteomic signatures of mature neutrophils derived from germ free (GF) and specific pathogen free (SPF) mice were significantly different at baseline and during infection, demonstrating significant plasticity. In the absence of microbiota, mature neutrophils lacked sufficient GM-CSF-driven priming.

GF-serum exposed neutrophil progenitors did not mature efficiently and had compromised bactericidal properties when compared to progenitors matured in SPF-derived serum. To identify molecular pathways, we set-up an *in vitro* system where neutrophil progenitors were transduced with lenti-guides to knock-down key microbiota-driven gene targets. To identify which of the microbiota-regulated proteins directly impacted bactericidal functions of neutrophils, we knocked out 19 candidates and tested their killing of *P. aeruginosa*. Excitingly, few of the targets demonstrated a significant decrease in the neutrophil bactericidal capacities. Hence, we identified novel neutrophil specific targets that are regulated by microbiota to control innate immunity to pathogens.

Cumulatively, our data support the concept that microbiota affects neutrophil maturation by defining not only the quantity, but also the quality of mature neutrophils. We predict that neutrophil responses can be specifically tailored to pathogens. In conclusion, neutrophil responses, although innately determined, are adapted and molded by the commensal presence.

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Neutrophil & Iron – the First Identification of a Primed Phenotype in an Iron Overload Disease

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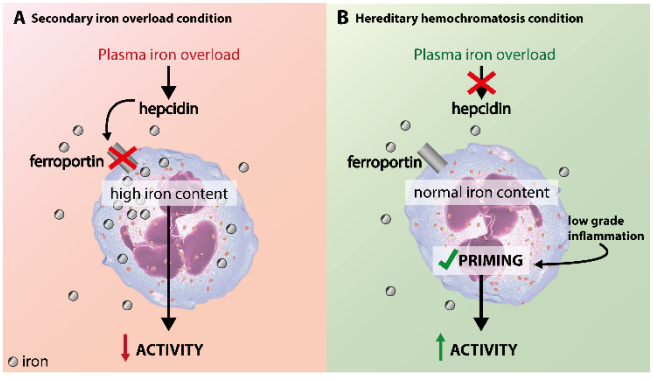
Iron is an essential element for humans and other vertebrates, as well as for their microbial invaders. Therefore a "fight for iron" takes place between the host and the intruders. Patients with secondary iron overload (owing to iron supplementation, erythrocyte transfusions...) are well known to be

associated with a predisposition to a variety of infections. In these patients, iron overload has been shown to have deleterious effects on neutrophil functions (phagocytosis and bactericidal activity). Paradoxically, despite high iron levels, increased susceptibility to infections has not been reported in hereditary hemochromatosis (HH) patients (except from isolated case reports of sepsis by *Y. enterocolitica*). HH is one of the most frequent genetic disorders in Occident, due to mutations in proteins regulating the key iron regulator hepcidin. Hepcidin is secreted by the liver in response to iron overload and circulates into the bloodstream to bind the only known iron exporter ferroportin (FPN) at the cell membrane to induce its degradation. At the systemic level, this interaction stops the intestine iron absorption and results in a decrease in plasma iron. At the cellular level, it increases the intracellular iron level. While secondary iron overload patients have high hepcidin levels, HH patients have abnormally low hepcidin levels. Neutrophil functions have never been investigated in HH patients.

We hypothesize that neutrophils from HH patients may be more active and more efficient in killing bacteria explaining the paradox of: "more iron/ not more infection". To test our hypothesis, we analyzed blood neutrophils from 40 HH patients vs 40 healthy donors (HD) and took advantage of an HH mouse model we generated in the laboratory.

Compared to HD, we found in the HH patient neutrophils (1) an increase in the oxidative burst response to different stimulating agents (fMLF, Opsonised Zymosan, PMA) (2) an increase in the phagocytosis capacity and (3) a decrease in L-selectin surface expression. Altogether, these results indicated an activated phenotype of neutrophils from HH patients in comparison with HD. As we found a positive correlation between the plasma iron parameters and the oxidative burst capacity in each patient, we next investigated the mechanisms by which iron could affect the neutrophil activity. Interestingly, while HH patients present with an iron overload in many cells and tissues the level of iron in the neutrophils was similar to the level detected in the neutrophils of HD. This result suggests that the absence of hepcidin secretion in HH patients resulted in the stabilization of FPN at the neutrophil cell membrane, limiting intracellular iron retention and subsequent impaired neutrophil functions. Moreover, iron accumulation in other cell types and tissues may induce a low grade inflammation responsible for the priming of the neutrophils. By evaluating 40 circulating factors linked to inflammation in our cohort, we observed an increase in some cytokines (TNFa, IFNg…) that could explain the increased neutrophil activity in HH patients. To confirm that the activated phenotype of HH neutrophils is sufficient to provide a better resistance to infection despite the iron overload, we evaluated the response to a *S. aureus* subcutaneous infection in mice 1) under a 2 week-iron rich diet (to mimic secondary overload) vs control diet and 2) in HH mice vs WT mice. While mice with secondary iron overload were more susceptible to infection than control mice, HH mice and WT mice presented with a similar response to infection.

This study highlights the central role of iron in the host/pathogens interaction and suggests that in HH, which is the most frequent genetic disorders in Occident, patients have acquired a selective immune advantage, by enhancing neutrophil functions, that enable them to cope with iron load.



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Mechanosensing Regulates Effector Functions of Human Neutrophils

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Neutrophils (PMNs) are the most abundant circulating white blood cell in the human body and the first responders to tissue injury and infection. Once out of the blood vessels, PMNs utilize chemotactic gradients to directionally migrate to the site of tissue injury or infection. To do this, PMNs must navigate through a three-dimensional tissue microenvironment that is densely packed with parenchymal cells and extracellular matrix. Within these tissues, PMNs sense the physical property of elasticity as a regulatory element as it pertains to motility. Historically, rigid tissue culture surfaces like glass and plastic have been used to study many key anti-microbial effector functions of PMNs such as generation of oxidative radicals, release of cytokines, phagocytosis, and release of extracellular traps; however, both fail to mimic the elasticity of peripheral tissues. Importantly, we show that elasticity plays a key regulatory role not just for PMN motility but also in anti-microbial effector functions, specifically those seen with fungal pathogens. Systemic fungal infections such as those caused by *Candida* sp. are particularly problematic in patients maintained in the surgical ICU for extended periods of time. Accordingly, we used *Candida* hyphae as our disease model, as PMNs are the primary line of host defense against tissue infection with *Candida*. Results showed clustering and NETosis regulatory for immune elimination of *Candida* not only in the previous tissue culture system, but also with the use of tunable elasticity gels. In addition, morphology and expression of NETosis foci were found to be highly mechanosensitive. For instance, NETotic foci were larger on stiff matrices as opposed to soft. Subsequently, NETotic foci were much more numerous on softer matrices.

While extensive work has been done elucidating the biochemical interactions of the PMN anti-fungal response, understanding important mechanosensitive mechanisms underlying chemotaxis, clustering, and NETosis may provide further therapeutic targets to benefit candidiasis patients in the future by reducing harmful PMN behaviors and/or promoting behaviors crucial to fungal clearance.

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Bacillus InhA Metalloproteases Contribute to Ocular Infection and Inflammation

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Bacterial endophthalmitis is a devastating infection that causes inflammation, and usually blindness, due to the introduction of organisms into the posterior segment of the eye. Unlike endophthalmitis with other bacterial pathogens, over half of *Bacillus* endophthalmitis cases result in significant vision loss, and the majority of these eyes are enucleated. *Bacillus* produces many virulence factors in the eye that may contribute to retinal damage and massive inflammation. This study analyzed the roles of the *Bacillus* Immune Inhibitor A (InhA) metalloproteases, which digests extracellular matrix and antimicrobial proteins. To test the hypothesis that InhA to intraocular virulence and inflammation, we analyzed the progress of experimental endophthalmitis in mouse eyes infected with 200 CFU of *B. thuringiensis* wild type (WT) (Bt407) or its InhA1-deficient mutant (ΔInhA1), or 200 CFU of InhA1, A2, and A3-deficient mutant (ΔInhA1-3). Infections were analyzed by quantifying intraocular bacilli and retinal function loss. Inflammation was analyzed by histology and quantifying the amount of intraocular myeloperoxidase from 0-12h post infection. ΔInhA1-infected eyes contained greater numbers of bacteria than WT *B. thuringiensis* during the infection ($p < 0.05$). However, eyes infected with ΔInhA1 experienced similar inflammation and retinal function loss that was not significantly different to eyes infected with the WT *B. thuringiensis* ($p \geq 0.05$, 6-10h). Eyes infected with ΔInhA1-3 had less bacterial burden, less retinal function loss, and less inflammation compared to the WT-infected eyes ($p < 0.05$). In vitro analysis of growth, proteolysis, and immune evasion from neutrophils were compared between the *B. thuringiensis* strains. Mutant ΔInhA1 were able to enter log phase growth faster than WT, but its proteolysis was significantly reduced ($p < 0.05$). There were also higher concentrations of extracellular and intracellular ΔInhA1 bacteria when incubated with neutrophils compared to WT ($p < 0.05$). Overall, these results indicate that the InhA metalloproteases may contribute to the severity of infection and inflammation in *Bacillus* endophthalmitis.

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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 hypersegmentation, 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 significantly decreased neutrophil chemotaxis to all tested stimuli, as indicated by measurements of cell directionality and instantaneous velocity. 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. We also determined this was not a general consequence of phagocytosis as infection with *F. tularensis* LVS and uptake of formalin-killed *H. pylori* did not elicit a chemotactic defect. 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 GTPases and actin and dynamics in are in progress. 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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Mechanisms of Human Neutrophil Apoptosis Delay and Metabolic Reprogramming by *Francisella Tularensis.*

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Francisella tularensis (Ft) is the etiological agent of tularemia, one of the most infectious pathogens known and one of few pathogens capable of infecting host neutrophils (polymorphonuclear leukocytes; PMNs). PMNs are vital innate immune cells that undergo constitutive apoptosis 24 hours after entering circulation, and disruption of this tightly-regulated process leads to an impaired immune response that is incapable of resolving infection. Our laboratory discovered that Ft delays human PMN apoptosis by inhibiting the intrinsic, extrinsic and phagocytosis-induced cell death pathways, but the mechanisms of Ft-mediated extension of PMN lifespan are not fully understood. Intriguingly, Ft-infected PMNs exhibit upregulation of genes encoding glucose transporters and 10 of 11 glycolytic enzyme genes. Congruently, Ft-infected PMNs produce and release significantly more lactate, and inhibition of glycolysis disrupts Ft capacity to delay apoptosis. These data highlight a potential role for glycolysis in Ft-mediated apoptosis inhibition, and we hypothesize that metabolic reprogramming of Ft-infected PMNs contributes to prolongation of cell lifespan.

p38 mitogen-associated protein kinase (MAPK) and class IA phosphoinositide-3 kinase (PI3K) are known to influence PMN lifespan in a stimulus-specific manner, and also have established roles as regulators of glycolysis. Our laboratory demonstrated that inhibition of p38 MAPK or PI3K signaling blocks Ft-mediated apoptosis delay, and we hypothesize that Ft-mediated glycolytic upregulation is mechanistically linked to extension of PMN lifespan by host p38 MAPK and PI3K signaling. In addition, Toll-like receptors (TLRs) are upstream of both p38 MAPK and PI3K, and our data demonstrate that Ft bacterial lipoproteins (BLPs) delay PMN apoptosis via a TLR2/1-dependent mechanism. Nevertheless, all of the factors that function to prolong PMN lifespan, and the mechanisms by which these factors are interfering with the major apoptosis pathway in PMNs remain undefined. Ongoing experiments are focused on further analysis of PMN metabolism and determining the specific roles of p38 MAPK, PI3K and TLR signaling in Ft-mediated apoptosis inhibition and glycolytic upregulation.

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Novel Synthetic Toll-like Receptor 4 Agonists Enhance Survival and Augment Resistance Against Gram-negative and Gram-positive Infection

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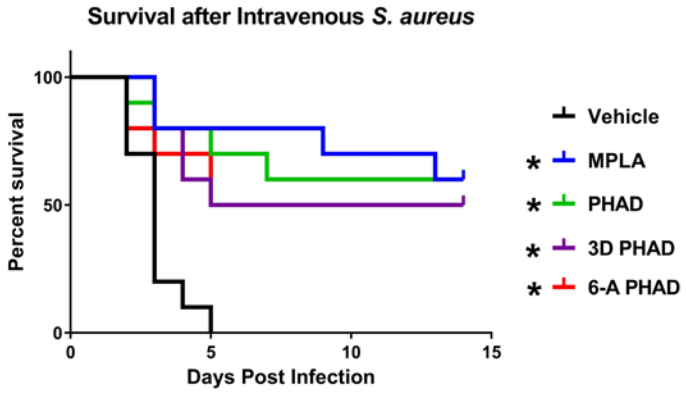
Introduction: The TLR4 agonist Monophosphoryl lipid A (MPLA) is FDA-approved as a vaccine adjuvant with potent immunomodulatory properties that augment innate immunity and infection resistance. However, MPLA is unavailable as a standalone immunotherapeutic agent for patients. Synthetic phosphorylated hexaacyl disaccharides (PHADs) are synthetic TLR4 agonists that are structurally similar to MPLA and are available for clinical development. We hypothesized that PHADs would augment innate immunity and enhance resistance to infection with common bacterial pathogens. To test our hypothesis, we compared the efficacy of PHAD, 3-deacyl (3D) PHAD and 3D-6 acyl PHAD to MPLA in models of *Pseudomonas aeruginosa* and *Staphylococcus aureus* infection.

Methods: BALB/c mice were treated with PHADs at 48 and 24 hours prior to infection. Monophosphoryl lipid A (MPLA) and Lactated Ringers solution (LR) served as positive and vehicle controls, respectively. In an intraperitoneal (IP) *P. aeruginosa* infection model, leukocyte, cytokine, and bacteria were measured in peritoneal lavage fluid 6 hours after infection. An additional cohort received intravenous (IV) *S. aureus* infection, with one group monitored for survival up to 14 days and the other examined for *S. aureus* burden 3 days after infection. A third cohort received IP 3D (6-Acyl) PHAD or vehicle 3, 10, and 14 days prior to IP *P. aeruginosa* infection to evaluate the duration of antimicrobial protection. The effect of PHAD treatment on leukocyte phagocytosis and respiratory burst functions was examined. Lastly, PHAD-induced protection against *P. aeruginosa* infection was assessed in TLR4 knockout mice.

Results: After IP *P. aeruginosa* infection, treatment with all PHADs or MPLA prevented infection–induced hypothermia, augmented leukocyte recruitment to the site of infection, attenuated local and systemic cytokine production and augmented bacterial clearance. During *S. aureus* infection, bacterial clearance from lungs, spleen, and kidneys was greatly enhanced in MPLA- and PHAD-pretreated mice compared to controls and was associated with significantly improved survival. A time course study demonstrated MPLA- and 3D (6-Acyl) PHAD-mediated antimicrobial resistance against *P. aeruginosa* lasts for up to 10 days. Leukocyte phagocytosis and respiratory burst functions were augmented after PHADs or MPLA treatment. Lastly, PHAD-induced antimicrobial protection was partially altered in TLR knockout mice treated with 3D (6-Acyl) PHAD.

Conclusions: PHADs protected against clinically relevant Gram-negative and -positive infection by augmenting bacterial clearance, phagocytosis and respiratory burst functions of innate leukocytes. Improved antimicrobial protection persisted for 10 days after treatment and was partially mediated via TLR4.



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Intestinal Epithelial Cells Drive Autoimmunity After Stimulation by Virally Derived Ligands

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Background: Host-environment dialogues are appreciated to precipitate and drive autoimmune disease, including that of human type 1 diabetes (T1D). The intestine, a dynamic and highly regulated immunologic organ, is the largest single site of environmental exposure in the human body. Though previous studies have implicated dysfunctional intestinal immunity in T1D, mechanisms of any mis-communications taking place as well as their potential impact on T1D disease initiation and progression remain largely unknown. Our aim is to understand the innate immune function of intestinal epithelial cells (IEC) and their potential contribution to the immune dysregulation observed in human T1D. Protein tyrosine phosphatase non-receptor type 22 (PTPN22) rose to prominence via genome-wide association studies, which correlated a single nucleotide polymorphism (SNP) in PTPN22 to enhanced risk for several human autoimmune diseases, including T1D. While previous work has characterized the function of PTPN22 in

lymphoid cells such as T cells and B cells, it is now evident that PTPN22 plays a major regulatory role in viral sensing by myeloid cells. In myeloid cells, PTPN22 promotes viral sensing and enhances the secretion of type 1 interferon (IFN-I) following stimulation by either viral ligands or viral infection. Our group has demonstrated that, similar to myeloid cells, IECs also express PTPN22, whereby T1D derived IECs express less IFN-I and IFN-responsive genes following stimulation with a viral ligand (poly I:C). *The goal of this study was to determine the role of the T1D PTPN22 risk SNP (1858T) in IEC innate immune function.*

Methods: A major hurdle to dissecting functional consequences of SNP and thus translation of preclinical findings, is a lack of tools that represent human processes, with enough power to detect minor effects of these SNPs that are normally undetectable due to a mixed genetic background. To overcome this barrier, we developed a human isogenic system for use of a disease-in-a-dish strategy that pairs human induced pluripotent stem cells (iPSCs) with CRISPR/Cas9 gene modification to produce clones hemizygous for the risk (1858T) and resistant (1858C) PTPN22 SNPs. iPSCs clones were then differentiated into primary human IECs (iIECs) and their poly I:C-induced innate immune function evaluated. IFN-I production and cyto/chemokine gene expression were evaluated 6 hours following stimulation. Antigen presentation capacity was evaluated using exogenously loaded MART1 and T cell avatars specific for the MART1 antigen. Finally, the metabolic activity induced by poly I:C activation was assessed using Seahorse technology.

Results: Poly I:C stimulation of iIECs with the T1D PTPN22 risk allele (1858T) produce significantly less IFN-I compared to that observed in iIECs with the T1D PTPN22 resistant allele (1858C), suggesting decreased ability to control viral replication. In addition, while poly I:C also induced a robust induction of *IL18*, *TSLP*, and *IL17C* mRNA in 1858C iIECs, significantly less induction of these mRNAs was observed in 1858T iIECs cultures, suggesting decreased capacity to regulate mucosal inflammation. Conversely, poly I:C induced a more significant upregulation of HLA Class I in iIECs with the T1D PTPN22 risk allele (1858T) compared to those iIECs with the T1D PTPN22 resistant allele (1858C) suggesting increased antigen presentation capacity. Indeed, 1858T iIECs induced a higher level of proliferation of MART1 T cell avatars than 1858C iIECs. More importantly, 1858T iIECs induced a higher frequency of Tc1 and Tc1/Tc17 within the proliferating population, with increased cytotoxic capacity. Finally, these poly-I:C induced phenotypic and functional effects were at least in part attributed to a decreased oxygen consumption rate (OCR) and increased extracellular acidification rate (ECAR) within the 1858T iIECs cultures as compared to 1858C iIECs.

Conclusions: Together these data suggest that within IEC, the T1D PTPN22 risk allele (1858T) promotes a response to viral infection which results in suboptimal viral control but enhanced inflammation and activation of adaptive immunity. Coupled, these phenomena implicate the intestinal epithelial cell and the PTPN22 risk SNP (1858T) in a host-environment (e.g. viral) dialogue with the potential to expand autoimmunity.

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Suppression of HTLV-1 Infection by Lactoferrin

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Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that transforms human T lymphocytes, and mother-infant infection through breast milk is the main infection route. Breast milk is rich in various protective factors against infection, and lactoferrin (LF) contained in it has an antiviral effect and an activity to activate immune cells such as T lymphocytes, monocytes, and dendritic cells. Therefore, is it possible to establish an HTLV-1 infection via breast milk because the infection prevention effect is reduced? Or lactoferrin is abundantly contained in colostrum but is not contained in continuous milk, so is the infection established? To clarify the possibility, we report HTLV-1 infection and tumor growth inhibitory effect by lactoferrin administration.

First, we examined the ability to form syncytia in intercellular infection in vitro. The formation ability was suppressed to 16.9% in the lactoferrin-administered group compared to the untreated group. Further, when changes in cell adhesion factor expression in infected cells were examined, ICAM-1 expression decreased. In addition, analysis of the lactoferrin inhibitory effect during virus adsorption and entry processes during particle infection showed that it was suppressed to 68.3% during the adsorption process.

Furthermore, we administered lactoferrin pre-administration to mice and transplanted EL4 lymphoma cells expressing HTLV-1 Tax (EL4 / Gax) to investigate whether the growth inhibitory effect of the resulting tumor cells was observed. As a result, it suppressed 45.8% significantly in the lactoferrin administration group compared with the untreated group. As a result of histopathology, hypertrophy of the marginal zone was observed in the spleen of the administration group, and activation of dendritic cells and helper T cells could be confirmed.

From the present results, it is expected not only to prevent the development of HTLV-1-related diseases for which there is currently no effective treatment but also to provide important findings in clarifying the infection dynamics in individuals.

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Impaired Airway Epithelial Host Defense as a Mechanism for Bronchiectasis in Patients with STAT3 Mutations

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Job Syndrome is an autosomal dominant primary immune deficiency characterized by recurrent infections and elevated IgE levels. While this disease can be caused by a number of mutations, patients with mutation in the STAT3 gene are particularly prone to developing pulmonary complications including bronchiectasis, pneumatoceles, bronchopleural fistulas, and airway infections with bacterial and fungal pathogens. Genetic studies in mice suggest STAT3 may participate in directing airway epithelial differentiation from airway basal stem cells to ciliated cells, instead of mucus-secreting goblet cells. Notably, mucus hypersecretion and impaired mucocilliary clearance are hallmark features of bronchiectasis. In this work, we examined (1) if STAT3 point mutation results in STAT3 functional deficiency and (2) if this STAT3 deficiency leads to abnormal epithelial differentiation and response to pathogens. To this end, we have derived primary airway basal stem cells from a Job syndrome patient undergoing a lobectomy for a refractory Aspergillus infection in a section of bronchiectatic lung. This patient harbors a p.Sdel560 STAT3 mutation in the linker domain. Our preliminary analysis suggests that the patient-derived airway basal cells have no obvious defect in STAT3 expression, phosphorylation and dimerization. In addition, they exhibit normal cellular morphology and can give rise to complete airway epithelium at air-liquid interface (ALI), similar to that of healthy control. These data indicated that the p.Sdel560 STAT3 mutation does not result in STAT3 functional insufficiency and airway differentiation. However, in response to *Pseudomonas aeruginosa* infection, p.Sdel560 STAT3-mutated airway epithelial cells fail to initiate neutrophil transepithelial migration. This impaired ability of airway epithelium to recruit neutrophils may contribute to chronic fungal and bacterial infection and bronchiectasis that is observed in Job syndrome patients. Currently, we are seeking to determine if this epithelial derived defective neutrophil chemotaxis is generally applicable for other STAT3 mutations. In addition, we are exploring whether cytokine and eicosanoid chemoattractant mechanisms could be exploited to understand this defective airway epithelial host defense.

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Neutrophil Level and Neutrophil Extracellular Traps (NETs) Formation in Early Postnatal Life

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Background: Neonates are more susceptible to infection than adults due to their immature immune system. Neutrophils make up the majority of leukocytes in blood, playing an important role in innate immunity in neonates. Interestingly, neonatal neutrophils were different from adult neutrophils not only in terms of quantity but also in their functions. Neutrophil extracellular traps

(NETs) are networks of extracellular DNA, chromatins, and antibacterial proteins that kill extracellular microbes. Reactive oxygen species (ROS) activates neutrophil elastase (NE), myeloperoxidase (MPO), and protein-arginine deiminase type4 (PAD4) to promote chromatin decondensation followed by releasing of NETs. NE is commonly used to determine the amount of NETs formation. From previous studies, both term and preterm neonates were found to have decreasing NETs formation compared to adults; however, the ontogeny of NETs formation and association with neutrophil count in early postnatal life are still not well established.

Objectives: To determine the ontogeny of neutrophil level and NETs formation in newborn mice after birth. Perform a quantitative comparison of the survival rate from *E.coli* sepsis between newborn mice and adult mice.

Methods: Neutrophils were isolated from the bone marrow (at femurs, iliac crests, tibias, and fibulas) of neonatal mice aged 1, 4, 7, 14, 21 postnatal days old; as well as from adult mice aged 28 postnatal days old. Using positive and negative selection via Histopaque followed by flow cytometry, the number of neutrophils present at different time point were assessed.

After stimulated to release NETs with phorbol myristate acetate (PMA), NETs associated elastase was measured by using NETosis Assay Kit (Cayman, Michigan, USA) to determine NETs formation.

A mortality study was performed by intraperitoneal injection of E.coli serotype K1 to neonatal mice aged 3-14 postnatal days old and adult mice.

For statistical analysis of neutrophil level and NETs formation, Student's t-test was used to compare differences between neonatal and adult mice. Kaplan-Meier log-rank test was used for survival analysis from *E.coli* infection

Results: Neutrophil level peaked at day 3 of life and decreased to adult level at 14 days of life. While neutrophil elastase was significantly deficient in neonatal mice but trended to increase by age until reaching adult level at 21 days of life.

Survival rate from E.coli sepsis was significantly different in neonatal mice and adult mice. After being challenged with intraperitoneal *E.coli*, neonatal mice had 50% survival rate at day 2 post infection and 0% survival rate at day3. In contrast, adult mice had 100% survival rate after day 6.

Conclusion: Even though, neutrophil level increased in the first 3 days of life, they still experienced impaired function determined by the low ability to form NETs. However, after 14 days of life, neutrophil level normalized to adult levels followed by significant improvement in NETs formation at 21 days of life. Correlated with the mortality study, neonatal mice were more susceptible to *E.coli* sepsis than adult mice.

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Nitric Oxide Inhibition Enhances Immunity of Neonatal Mice to *E. Coli*-induced Meningitis in an IL-1 Dependent Manner

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Escherichia coli-induced meningitis is the leading cause of bacterial meningitis in pre-mature infants. It is known that the neonatal immune system has several differences compared to adults, including impaired IL-1 secretion. However, it is unknown what role inflammasomes and IL-1 play in *E. coli* meningitis. We therefore wanted to determine whether Neonatal Meningitis Associated *E. coli* (NMEC) induced IL-1, and how it contributes to the outcome of NMEC infection. Interestingly, we found that induction of IL-1 in macrophages and microglial cells *in vitro* by NMEC was dependent on the NLRP3 inflammasome. Survival studies performed in adult wildtype and IL-1 receptor knockout (IL-1R$^{-/-}$) mice infected intracranially with NMEC indicated a protective role of IL-1. Additionally, we found adult IL-1R$^{-/-}$ mice had significantly increased bacterial loads in the brain. Surprisingly, when we investigated the role of IL-1 in a neonatal model of meningitis, we did not see a significant effect of IL-1R blockade on bacterial loads in either the blood or brain. Neonates are known to have increased nitric oxide (NO) levels, and NO can inhibit NLRP3 inflammasome activation and IL-1 production. Therefore, we wanted to test whether inhibition of IL-1 maturation by NO could contribute to the susceptibility of neonates to infection. Similar to reports of others, we found treatment of neonates with an inducible nitric oxide synthase (iNOS) inhibitor significantly decreased bacterial loads. Furthermore, this protective effect of iNOS inhibition was lost in anti-IL-1R treated pups. This suggests that the protective effect of nitric oxide inhibition during NMEC infection is due to improved IL-1 signaling, and may indicate a target for future therapeutics.

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Macrophage Functional Phenotyping by Metabolite Immunomodulation

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As of 2013, there were nearly 30 million Americans with diabetes or one in ten adults, but by 2050, estimates from the American Diabetes Association indicate that one in three adults (~350 million Americans) will be diabetic. At current rates of amputation (~25%) due to non-healing wounds, this 2050 population will have to absorb the social and economic burden of over 85 million diabetic amputees. While normal wound healing proceeds through a well-described iterative process, in non-healing wounds this process stalls at the transition from inflammation

to tissue repair. Our central hypothesis is that macrophage plasticity is key to this transition and directly facilitates shifting the wound environment from pro-inflammatory to promoting tissue repair.

While more established signaling molecules of immunity (such as cytokines and chemokines) remain essential to driving functional phenotype in the immune system, recent advances in metabolomics profiling have revealed a pivotal role for metabolites in immunomodulation. Presented herein, an *ex vivo* macrophage culture model, developed in our lab, produces six macrophage functional phenotypes and is a systems-level model for understanding the contribution of metabolism to macrophage functional plasticity. Specifically, CD14+ peripheral blood monocytes are isolated, differentiated into M0 MΦs, and polarized into the M1 (IFN-γ/LPS), M2a (IL-4/IL-13), M2b (IC/LPS), M2c (IL-10), and M2d (IL-6/LIF) phenotypes. Each phenotype is then characterized by a bioanalyte matrix of four cell surface markers, ~50 secreted immunomodulating proteins (cytokines, chemokines, and growth factors), ~800 myeloid genes, and ~450 identified metabolites (including lipids).

Signal protein profiles and pathway enrichment analysis of expressed genes generally groups the phenotypes into pro-inflammatory MΦs (M1 and M2b) and tissue repair/regeneration MΦs (M2a, M2c, and M2d); however, clear distinctions between each phenotype can be made. For example, M1 MΦs activate processes that facilitate acute inflammatory responses through control of nitric oxide (NO) and reactive oxygen species (ROS), whereas M2b MΦs activate processes that regulate chronic inflammation and chemotaxis of lymphocytes, endothelial cells, and epithelial cells. Furthermore, both M2c and M2d MΦs activate angiogenesis processes, but M2c MΦs promote extracellular matrix (ECM) assembly while M2d MΦs activate processes that drive ECM degradation. Finally, metabolomics profiles further validate recent findings that shifts between aerobic glycolysis, the pentose phosphate pathway, and oxidative phosphorylation distinguish pro-inflammatory MΦs and tissue repair/regeneration MΦs; however, our model provides further evidence to support the association between metabolism (such as decoupling of the TCA cycle, fatty acid synthesis, and b-oxidation) and functional phenotype.

Metabolomics is fundamentally changing our understanding of immunomodulation in diverse macrophage populations and that deeper understanding is informing our long-term goals of developing novel diagnostic and therapeutic approaches to wound healing. By integrating metabolomics into our systematic characterization of these phenotypes, we have developed quantifiable metrics that not only define metabolic and functional phenotype, but also provide insight into the cellular functions fundamental to macrophage plasticity.

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A Novel Therapeutic Target for Controlling Toll-like Receptor 4 Signaling

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Mitochondrial Dysfunction in Obesity with Sepsis: Role for SIRT2?

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Introduction: Immune response in sepsis transitions from early/hyper-inflammatory to a late/hypo-inflammatory and immunosuppressive phase with multiple organ failure. Mitochondrial dysfunction contributes to multiple organ failure to ultimately increase sepsis-mortality. Obesity increases morbidity in sepsis patients. We showed previously that obesity exaggerates hyper- and prolongs hypo-inflammatory phase in sepsis mice via sirtuin 2 (SIRT2) modulation. The effect of obesity on sepsis-related mitochondrial dysfunction is not well understood. In this project, using a cell model, we studied mitochondrial dysfunction and further elucidated the role of SIRT2 in prolonged hypo-inflammation of obesity and sepsis.

Methods: We used a RAW 264.7 (RAW) cell model with overnight stearic acid (FFA) or vehicle bovine serum albumin (BSA) feeding model of obesity with sepsis. We stimulated cells with E. coli lipopolysaccharide (LPS) and studied immuno-metabolic responses during hyper-inflammation (4 h post LPS challenge: sensitive cells) and hypo-inflammation (24h post-first LPS: tolerant cells) in response to LPS (4 h) re-stimulation. Specifically, using western blot analysis and immunocytochemistry, we studied total SIRT2 expression and subcellular localization of SIRT2. Seahorse XF24 analyzer was used to study oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in FFA- or BSA-fed sensitive and tolerant RAW cells with LPS re-stimulation.

Results: We observed that in both, the FFA and BSA-fed tolerant cells, total SIRT2 expression increased with or without LPS re-stimulation; in FFA-fed cells, this increase was significantly more pronounced. We also observed that during hypo-inflammation, the SIRT2 was localized in mitochondria, especially in FFA-fed tolerant cells with LPS re-stimulation. Extracellular acidification rate (ECAR) decreased in response to LPS re-stimulation in both, FFA and BSA-fed tolerant cells vs. sensitive cells, however this response was significantly more pronounced in FFA vs. BSA-fed tolerant cells. Moreover, while the maximum glycolysis was unchanged in BSA fed tolerant cells, FFA-fed tolerant cells showed a significant reduction in maximum glycolysis with LPS re-stimulation. Spare respiratory capacity (SRC)) significantly decreased in response to LPS in both FFA and BSA-fed sensitive cells vs. medium; SRC was profoundly diminished in tolerant cells especially in response to LPS re-stimulation.

Discussion: We have shown previously that obesity exaggerates hyper-inflammatory, prolongs hypo-inflammatory phase and increases mortality in sepsis mice. Mitochondrial dysfunction contributes to multiple organ failure in sepsis. We studied the effect of obesity on mitochondrial dysfunction in sepsis here. In a cell model of obesity and sepsis, we show that the hypo-inflammation (tolerant phenotype) is associated with significantly worsened mitochondrial

dysfunction suggested by diminished spare respiratory capacity and maximum glycolysis in FFA-fed vs. BSA-fed RAW cell macrophages. Interestingly, this change was associated with a significant increase in mitochondrial (and total) SIRT2 expression in FFA- vs. BSA-fed tolerant cells; SIRT2 is known for its cytoplasmic localization. We are currently investigating the role of mitochondrial SIRT2 in modulation of mitochondrial dysfunction in obesity with sepsis.

Conclusion: Obesity with sepsis is associated with worsening of mitochondrial dysfunction associated with increased mitochondrial localization of SIRT2.

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Fate of *Francisella Tularensis*-infected Human Neutrophils

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Francisella tularensis (Ft) is a Gram-negative facultative intracellular bacterium and the causative agent of the disease tularemia. This pathogen infects a variety of cell types including macrophages and neutrophils. Neutrophils are short-lived and are inherently programmed to undergo apoptosis ~ 24 hours after release into the circulation. Rapid clearance of dying neutrophils by macrophages, a process called efferocytosis, prevents release of toxic cell components and drives resolution of inflammation via effects on macrophage polarization state and cytokine production. Our published data demonstrate that Ft significantly delays neutrophil apoptosis but the fate of these cells is unknown. Exposure of phosphatidlyserine (PS) on the surface of apoptotic cells is an ‘eat me’ signal that favors efferocytosis. As PS is low on Ft-infected neutrophils, we predicted that interaction of these cells with human monocyte-derived macrophages (MDMs) would be impaired. In marked contrast, we found that MDMs interacted more avidly with infected neutrophils than the aged, uninfected controls. After uptake, Ft infected neutrophils evaded trafficking to lysosomes and instead acted as Trojan horses with live Ft escaping from PMN-laden efferosomes to replicate in MDM cytosol. Current studies are focused on analyses of MDM polarization and cytokine secretion and identification of receptors that mediate binding and uptake of aged and infected neutrophils. Candidates of interest are being evaluated using blocking antibodies in conjunction with microscopy and a multicolor flow cytometry assay and include complement receptors (CR1, CR3, CR4) and the vitronectin receptor (avb3) as well as calreticulin and scavenger receptors and the ‘don’t eat me’ molecule CD47. Results of these studies will advance understanding of how Ft evades the host immune system to persist and cause life-threatening infection.

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The TLR2 Tango: Contributions of Neutrophils and the Putative Oral Pathogen *Filifactor Alocis* to Periodontitis

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Periodontitis is an irreversible, chronic inflammatory disease where inflammophilic pathogenic microbial communities accumulate in the gingival crevice. Advances in culture-independent techniques have facilitated the identification of new bacterial species in periodontal lesions, such as the Gram-positive anaerobe, *Filifactor alocis*, whose pathogenic potential has only begun to be characterized. Neutrophils are a major component of the innate host response and the outcome of their interaction with *F. alocis* may be a key determinant of oral health status. While neutrophil microbicidal functions typically protect the host against periodontal disease, oral pathogens have adapted to evade or disarm neutrophil killing mechanisms while promoting mechanisms that drive inflammation, which provides a source of nutrients for growth. Thus, this study had two goals, to determine how *F. alocis* interferes with microbicidal mechanisms to survive in neutrophils and to examine how *F. alocis* contributes to the chronicity of periodontitis by promoting inflammation. Previous work from our laboratory showed that despite being efficiently phagocytized, *F. alocis* can persist within neutrophils by inducing minimal production of intracellular reactive oxygen species (ROS) and minimizing the fusion of antimicrobial granules with its phagosome. Our results showed that *F. alocis* is recognized by TLR2/6, so we examined the killing capacity of bone marrow neutrophils from TLR2$^{-/-}$ mice. We found that compared to WT, TLR2$^{-/-}$ neutrophils were more efficient at killing *F. alocis*, but the increase in killing capacity was not due to a difference in phagocytosis or production of ROS. Instead, it was due to an increase in fusion of myeloperoxidase granules with the *F. alocis* phagosome. Moreover, *F. alocis* continued to promote inflammation by inducing the release of pro-inflammatory cytokines through TLR2 signaling and extending the lifespan of neutrophils. *F. alocis*-stimulated cells also had higher functional capacity than untreated cells as tested by phagocytosis after 18 and 24 hours of challenge. Finally, the extended lifespan of *F. alocis*-challenged cells led to a delay in efferocytosis as tested by CD47 expression and uptake by macrophages. Collectively, our data suggest that *F. alocis* interferes with TLR2 signaling to promote its survival within neutrophils and promotes chronic inflammation by extending neutrophil lifespan and delaying the resolution of inflammation.

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Glucocorticoid-induced Leucine Zipper (GILZ) Promotes Annexin a1 Expression in Migrating Neutrophils and Downregulates Toll-like Receptor 2 in Peripheral Neutrophils Under Dexamethasone Treatment

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The Glucocorticoid-induced leucine zipper (GILZ) gene is a pivotal mediator of the anti-inflammatory effects of glucocorticoids (GCs) and regulates the functions of both adaptive and innate immunity cells. The aim of this work was to study the role of GILZ in the activity of neutrophils and in their migration during an inflammatory response under GC treatment, in GILZ-knock-out (GILZ-KO) and wild type (WT) mice.

Like in other cell types, GILZ was found to be up-regulated by dexamethasone (DEX), a synthetic GC, in bone marrow-derived, mature and activated neutrophils. Since an important function of neutrophils is their ability to migrate into inflamed tissues, we studied the migration in the thyoglicolate-induced peritonitis, which is an experimental model of acute inflammation, under DEX treatment. We found that DEX prevented migration of neutrophils only in WT mice, but not in GILZ-KO mice. This was because DEX was unable to up-regulate annexin A1 (Anxa1) expression, one of the most known GC-induced anti-inflammatory genes, in the absence of GILZ. Furthermore, we showed that GILZ mediates DEX-induced Anxa1 expression acting at the Anxa 1 promoter level *via* binding with the transcription factor PU.1, a negative regulator of Anxa1.

We next analyzed TLR2 expression on neutrophils, another critical receptor that is involved in neutrophil migration. TLR2 was not modulated by DEX treatment in peritoneal neutrophils, but was down-regulated in peripheral neutrophils, both in WT and GILZ-KO mice. Since TLR2 is involved in pathogen recognition, we explored the possibility of a modulation by DEX in neutrophils of healthy mice. We found that TLR2 is down-regulated by DEX in circulating neutrophils only in WT but not in GILZ-KO mice, and this down-regulation was correlated to a reduction of bacterial killing activity.

The present findings shed light on the role of GILZ in neutrophil functions and, for the first time, in the mechanism of induction of the anti-inflammatory Anxa1 by GCs. Furthermore, GCs reduce TLR2 expression in neutrophils, through GILZ, thus limiting their functions and being a mediator of GC effects. Therefore, GILZ may represent a druggable new target for the control of neutrophil activity in inflammatory diseases.

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Glucocorticoid-induced Leucine Zipper (GILZ) Removal Exacerbates the Neutrophil Functions

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GILZ (Glucocorticoid-induced leucine zipper) is an early glucocorticoid-inducible gene that mediates the anti-inflammatory effects of glucocorticoids. Glucocorticoids are endogenous hormones regulating a variety of physiologic responses, but represent also the most powerful pharmacological treatment for inflammatory diseases. The aim of this study was to investigate about the role of GILZ in microbicide activity of neutrophils during an inflammatory response. We found that peritoneal GILZ-knockout (KO) neutrophils showed an increased phagocytosis, killing activity and oxidative burst if compared to wild-type (WT) cells *in vitro*. In a mouse model of colitis, the highly activated GILZ-KO neutrophils caused a more severe disease than in WT mice. To study GILZ function in counteracting an infection, we used an *in vivo* model of *Candida albicans* intra-abdominal peritonitis, and observed that GILZ-KO neutrophils had a greater ability to eliminate the infectious pathogens than the WT neutrophils. To unravel the intracellular signaling pathway regulated by GILZ, we analyzed some players of the oxidative pathway in peritoneal neutrophils, in *C. albicans* induced peritonitis. NOX2 (NADPH oxidase 2) and p47 phox proteins, both involved in microbicide activity through production of reactive oxygen species (ROS), were found to be more activated in GILZ-KO neutrophils. Furthermore, ERK and p38 proteins of the MAPK pathway, both involved in the ROS production, were highly phosphorylated in GILZ-KO neutrophils. Overall, our data demonstrate that GILZ expression restrains the activation status of neutrophils to avoid a persistent response of these cells, possibly resulting in a dangerous chronic inflammation.

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Stimulating Pyruvate Dehydrogenase Complex Reduces Itaconate Levels and Enhances TCA Cycle Anabolic Bioenergetics in Acutely Inflamed Monocytes

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The pyruvate dehydrogenase complex (PDC)/pyruvate dehydrogenase kinase (PDK) axis direct the universal principles of immune resistance and tolerance in monocytes by controlling anabolic and catabolic energetics. Immune resistance shifts to immune tolerance during inflammatory shock syndromes when inactivation of PDC by increased PDK activity disrupts the tricarboxylic acid (TCA) cycle-supported anabolic pathways. The transition from immune resistance to

tolerance is also associated with a diversion from citrate-derived cis-aconitate to itaconate, which breaks the TCA cycle at isocitrate. Itaconate promotes immune tolerance via inhibition of succinate dehydrogenase and its support of ATP generation, and other mechanisms. We previously reported that inhibiting PDK in septic mice with dichloroacetate (DCA) increased TCA cycle activity, reversed septic shock, restored innate and adaptive immune and organ function, and increased survival. Here, using unbiased metabolomics in a monocyte culture model of severe inflammation, we showed that DCA-induced activation of PDC restored anabolic energetics in inflammatory monocytes by increasing TCA cycle intermediates, decreasing itaconate, and increasing amino acid anaplerotic catabolism of branched-chain amino acids (BCAAs). DCA also decreased itaconate and increased succinate levels in immune tolerant monocytes in response to a second inflammatory stimulation. Our study provides new mechanistic insight that the DCA-stimulated PDC homeostat reconfigures the TCA cycle and restores anabolic energetics by reducing itaconate levels and increasing BCAA catabolism, and supports that the concept that PDC is an energy-sensing and signaling homeostat that restores metabolic and energy fitness in monocytes.

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Calcineurin Inhibitors Reduce NFAT Dependent Gene Expression in Human Monocytes

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Calcineurin inhibitors are effective clinical immunosuppressants but leave patients vulnerable to infections. This study tested the hypothesis that calcineurin inhibition interferes with several innate immune mechanisms responsible for protection against infections. We showed that NFAT is expressed by human monocytes, and is activated by exposure to fungal ligands. We confirmed that NFAT translocation potently activated target gene transcription using a human monocytic reporter cell line. Inhibition of calcineurin-NFAT by cyclosporine A significantly reduced monocyte production of TNF-α, IL-10 and MCP-1 proteins in response to fungal ligands. Moreover, we revealed that human monocytes express the anti-fungal protein pentraxin-3 under the control of NFAT. We confirmed the likely molecular basis of this interaction by identifying an NFAT1 binding site in the murine and human ptx-3 gene. In conclusion, clinical calcineurin inhibitors have the potential to interfere with the novel NFAT-dependent Ptx-3 pathway as well as anti-fungal cytokine production in human monocytes, thereby impeding monocyte-mediated defenses against fungal infection in immune-suppressed patients.

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Conditions of Inflammasome Activation by *Streptococcus Gordonii*

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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, which release inflammatory cytokines, including inflammasome-dependent IL-1β. IL-1β and other inflammasome components increase in periodontal disease and are involved in disease progression. *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 in these conditions *S. gordonii* is able to damage phagolysosomes. Interestingly, *S. gordonii* infected macrophages release more IL-1β than macrophages infected with other oral microbes, both classical pathogens and commensals. Current work aims to determine the mechanism by which *S. gordonii* is activating macrophage inflammasomes resulting in the release of mature IL-1β and creating an environment that may perpetuate inflammation. We have found that macrophage cell death, inflammasome activation, and IL-1β release are increased in the presence of *S. gordonii* when macrophages are pre-activated to an inflammatory state. Overall, our results suggest *S. gordonii* is capable of evading immune destruction, increasing inflammatory mediators, and increasing inflammatory macrophage response; and that this ability is increased under conditions of existing inflammation.

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Mechanisms Responsible for the Inflammatory Polarization of Monocytes by Subclinical Low-dose Lipopolysaccharide (LPS)

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Chronic inflammation and related diseases such as atherosclerosis are some of the major health risks factors highlighted in recent studies. Prolonged inflammatory conditions along with lack of immunosuppressive responses, and disruption of cellular homeostasis play a critical role in initiating and exacerbating such diseases. Despite rigorous research, the underlying mechanisms of an imbalance in pro and anti-inflammatory phenotype, that further lead to prolonged non-resolving inflammation is not clearly understood. Patients with chronic inflammatory diseases have compromised mucosal barrier that allows continuous permeation of very low levels of bacterial endotoxin into their blood circulation. Here, we focus on the crucial role of subclinical-low dose bacterial endotoxin/ lipopolysaccharide (s-LPS), and further characterize the molecular mechanisms that may skew monocytes to a prolonged pro-inflammatory phenotype. We

observed that s-LPS induces the expression of immune-stimulating markers such as mcp-1 and CD40 while suppressing the immune-suppressive markers such as fpn and catalase. These phenotypic characteristics were further validated by differential levels of upstream signaling molecules such as activation of STAT1, STAT5, p38, CamKII and NFkB, and inhibition of Sirt3. Taken together, our study shows novel insights regarding the role of s-LPS in polarizing monocytes to a prolonged non-resolving inflammatory state.

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Microarray Analysis Reveals Down-regulation of Toll-like Receptor Genes in Activated HTLV-1-infected Cells from Patients with HTLV-1-associated Neurological Disease

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Introduction: Human T-cell leukemia virus type 1 (HTLV-1) is a human retrovirus and mainly infects CD4+ T cells *in vivo*. HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is an inflammatory disease of the central nervous system caused by HTLV-1 infection. Comprehensive analysis of host gene expression by HTLV-1 activation has been poorly studied in patients with HAM/TSP. Also, the mechanisms of the increased HTLV-1 viral load in HAM/TSP patients remain to be elucidated.

Objectives: We investigated the gene expression profile of activated HTLV-1-infected cells from patients with HAM/TSP compared with non-active infected cells to search molecules affecting HTLV-1 infection.

Methods: CADM1 is currently the most specific surface marker of HTLV-1-infected cells *ex vivo*. To enrich fresh HTLV-1-infected cells, we collected CD4+CADM1+ cells from peripheral blood mononuclear cells (PBMCs) of 4 HAM/TSP patients using a cell sorter. To obtain activated HTLV-1-infected cells, PBMCs from 2 HAM/TSP patients were cultured for 18 hours to spontaneously express the viral proteins, and CD4+Env+ cells were collected. Total RNA extracted from these cells was subjected to microarray analysis using a 60k-formatted slide from the Agilent Technologies company. The raw data were processed and analyzed with GeneSpring and Subio Platform software.

Results: Based on the criteria of p-value < 0.05 and fold change >2, over 2000 genes of activated HTLV-1-infected cells showed significant changes in gene expression levels. Gene ontology analysis showed that innate immune response was ranked at the top 10 with a p-value of 6.11E-6. Pathway analysis revealed that Toll-like receptor (TLR) signaling pathway and vitamin D receptor pathway were dysregulated. The expression levels of TLR1, 2, 4, 5, 6, and 8 genes, some of which induce viral destruction within cells as an innate immune system, were significantly decreased. The mRNA levels in some of the genes related to vitamin D receptor, which are known to play a role in the reduction of intracellular viruses, were also reduced.

Conclusions: These data suggest that HTLV-1 affects the expression of several molecules related to innate immunity and the reduced expression of TLR genes may influence the increased viral load in HAM/TSP patients.

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Role of the Calcium Regulatory Protein CarP in Impairment of Immune Cell Migration by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen that can cause severe biofilm- associated infections in immunocompromised individuals. Increased calcium levels enhance *P. aeruginosa* biofilm formation and increase the production of several secreted virulence factors. In *P. aeruginosa* PA01, calcium homeostasis is regulated by a two-component system called CarSR. One of the genes regulated by this system is *carP*, which encodes for a β-propeller protein. The serine/threonine protein kinase Akt is an important regulator of motility and other cell functions of many cells, including innate immune cells such as neutrophils and macrophages. Neutrophils and macrophages are phagocytes that respond to bacterial infections and also play a role in the inflammatory response through cytokine production. We hypothesized that the CarP protein would elicit increased rates of chemotaxis and Akt phosphorylation from phagocytes. Murine bone marrow neutrophils or the murine macrophage cell line RAW264.7 were treated with 100µL (OD = 0.1) or 10µL (OD = 0.1) of wild type (WT), a *carP* deletion (Δ*carP*), or a *carP* complemented strain of P. aeruginosa in a chemotaxis assay. Akt phosphorylation was measured with western blots and analyzed by densitometry. We found that macrophages and neutrophils migrate at a higher rate when treated with 100µL of WT *P. aeruginosa* when compared to treatment with 100µL of Δ*carP*. We also found that there was more migration with 100µL of WT when compared to the 10µL WT treatment. However, when treated with 10µL of Δ*carP* we observed increased migration of macrophages and neutrophils when compared to the 100µL Δ*carP* treatment. In both macrophages and neutrophils, treatment with 100µL Δ*carP* bacteria resulted in lower levels of Akt phosphorylation than treatment with the wild type or complemented strains. However, treatment of cells with 10µL Δ*carP* shows increased levels of Akt phosphorylation. We conclude that CarP plays a role in macrophage and neutrophil chemotaxis and affects the Akt pathway in phagocytes, suggesting a role for carP in interaction of P. aeruginosa with the host.

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Filifactor Alocis-derived Molecule(s) Modulate Neutrophil Granule Exocytosis

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Neutrophils play an important role both in microbial surveillance and immune regulation within the gingival tissue. However, pathogenic oral bacteria have evolved means to evade neutrophil killing and to propagate inflammation. Our laboratory has shown that the newly appreciated oral pathogen, *Filifactor alocis*, is able to manipulate neutrophil effector functions to subvert bacterial killing. In this study we aimed to define the ability of *F. alocis*-derived molecule(s) in filtered culture supernatant to modulate neutrophil granule exocytosis. Secretory vesicles and specific granule exocytosis was determined using flow cytometry to measure the expression of CD35 and CD66b, respectively. Pre-treatment of neutrophils with *F. alocis* culture supernatants significantly reduced *N*-formyl peptide (fMLF)-stimulated CD35 and CD66b expression. In addition, exposure of neutrophils to *F. alocis*-derived molecule(s) inhibited TLR-induced granule exocytosis but not the response elicited by phorbol 12-myristate 13-acetate (PMA). To determine if high or low molecular weight proteins were responsible for the inhibitory effect, a spin column system was used. Only the flow through from a 3 kDa spin column retained the inhibitory activity. However, treatment of the 3kDa flow through bacterial supernatant with DNAse, RNAse, Proteinase K or 2 h at 95°C, did not affect the inhibitory activity. Together, these data show that a low molecular weight compound(s) released by *F. alocis*, which is neither a protein nor a nucleic acid, exerts an inhibitory effect on neutrophil granule exocytosis. This inhibition may undermine neutrophils' antimicrobial response against other oral bacteria present in the gingival crevice.

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Oral Bacteria Promote Matrix Metalloproteinase Activity by Differentially Regulating Immune Cell Cytokine Responses

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Periodontitis is a serious condition leading to destruction of soft tissue and alveolar bone that causes tooth loss. The spirochete *Treponema denticola* is one of the bacteria typically found together with other pathogens such as *Porphyromonas gingivalis* in the dysbiotic dental biofilm associated with periodontitis. Neutrophils and macrophages are a critical part of the host response during both oral health and disease. These immune cells play a role in regulation and development of the inflammatory response through production and release of various cytokines. Oncostatin M (OSM) is a pleiotropic cytokine belonging to the IL-6 family with both pro- and anti-inflammatory effects. OSM is elevated in several inflammatory conditions including periodontitis, but the source and functional role of OSM during periodontitis is not well understood. OSM may exacerbate disease conditions by increasing the production of matrix metalloproteases (MMPs)

from tissue resident cells such as gingival fibroblasts (HGFs), the primary connective cell of the gingival tissue. Human neutrophils and macrophages were exposed in vitro to *T. denticola* and OSM protein (ELISA) and gene expression (qRT-PCR) levels were measured. We observed an elevation in OSM protein release and prolonged synthesis from human macrophages and neutrophils when co-incubated with *T. denticol*a. A murine air pouch model of inflammation was used to measure neutrophil recruitment and OSM production following exposure to *T. denticola* or *P. gingivalis* in vivo. *T. denticola* induced neutrophil recruitment to the air pouch, along with OSM release in the air pouch lavage fluid. Interestingly, when compared to *P. gingivalis*, *T. denticola* differentially recruited less neutrophils but a more robust OSM response in this model. OSM levels and the presence of different oral spirochete species were also examined in saliva samples and gingival tissue specimens from human subjects with (n=4) or without (n=3) periodontitis. OSM levels were elevated in the saliva from subjects with periodontitis compared to healthy, which correlated with the salivary presence of several oral *Treponema* species, including *T. denticola*. Immunohistochemistry analysis of gingival tissue sections from patients with periodontitis demonstrated strong OSM protein staining in the gingival epithelium and immune cell infiltrate, compared to healthy control gingival tissue. Following OSM exposure to HGFs in vitro, multiple members of MMPs were increased. A MMP screening array was used to characterize MMP protein release and MMP gene transcription was quantified using qRT-PCR from the saliva samples as well. Our results indicate that *T. denticola* induces OSM release and synthesis from neutrophil and macrophages in vitro and differentially induces OSM from neutrophils in vivo, compared to other oral pathogens. Furthermore, OSM has the capacity to promote MMP production from HGFs. Overall the interaction of *T. denticola* with innate immune cells to drive OSM-mediated MMP elevation may promote the soft tissue damage characteristic of periodontitis.

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Treatment with TLR Agonists Protect Against Severe Nosocomial Infections via Activation of MyD88 Signaling

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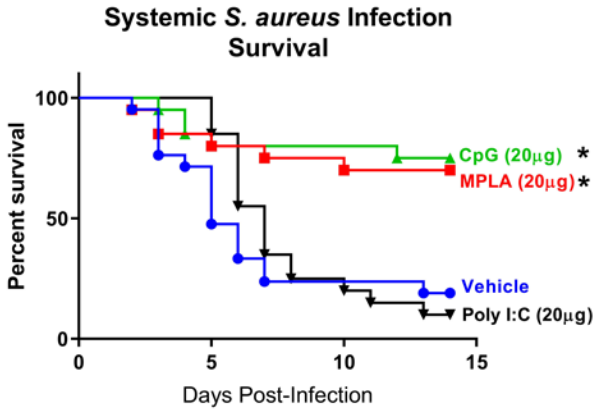
Infections by antibiotic-resistant organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* are among the leading causes of death in immunocompromised patients. Thus, strategies aimed at augmenting the host response to infection may decrease the incidence and severity of infections in these patients. We showed 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 clinically relevant models of 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 mice. To test this hypothesis, 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 both systemic *P. aeruginosa* infection and systemic *S. aureus* infections, 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. We found that both CpG and MPLA treatment led to a greater enhancement of leukocyte antimicrobial functions, phagocytosis and respiratory burst, as compared to Poly I:C treatment. 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. 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 imperative. Further defining the distinct roles of MyD88- and TRIF-dependent signaling in generating protective antimicrobial responses against a variety of infectious pathogens will likely reveal new insights that will advance current knowledge and can be harnessed to facilitate protection against infection in high risk patients.



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The Emerging Oral Pathogen, Filifactor Alocis, Manipulates MAPK Signaling Pathway in Human Neutrophils

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Periodontitis is a common chronic inflammatory infectious disease that affects ~50% of the adult population in the USA. Neutrophils are the primary leukocyte recruited to the gingival tissue to maintain periodontal health. However, emerging oral pathogens, such as *Filifactor alocis*, are able to evade neutrophil killing and contribute to disease progression. Preliminary results from our laboratory show that pre-treatment of human neutrophils with *F. alocis*-derived molecule(s) in

filtered culture supernatant inhibited both fMLF and TLR-induced granule exocytosis. In this study we aimed to define the ability of *F. alocis*-derived molecule(s) to manipulate the MAPK signaling pathway associated with neutrophil functional responses. Phosphorylation of p38 MAPK and ERK1/2 was determined by immunoblotting. Pre-treatment of neutrophils with *F. alocis* culture supernatants significantly reduced *N*-formyl peptide (fMLF)-induced ERK1/2 but not p38 MAPK phosphorylation. Similarly, pre-exposure of neutrophils to *F. alocis*-derived molecule(s) inhibited ERK1/2, but not p38 MAPK, activation induced by *F. alocis* challenge. The inhibitory effect on ERK phosphorylation, provoked by *F. alocis* culture supernatant, was reversible. Moreover, treatment of the bacterial supernatant with DNAse, RNAse, Proteinase K or 2 h at 95°C, had no impact on the inhibitory activity. Together, these data show that bacterial compound(s) released by *F. alocis*, which is neither a protein nor a nucleic acid, proactively inhibited ERK1/2 activation. This inhibition may compromise several key neutrophil functions, putting it at a disadvantage in the fight against the dysbiotic microbial community present in periodontitis.

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Cell Surface Interleukin-1α Is Tethered to the Membrane via IL-1R2 and GPI Anchors

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IL-1α is a powerful cytokine that drives inflammation and modulates adaptive immunity to help clear infections. IL-1α is translated as a pro-form that requires cleavage for full cytokine activity and is released after inflammasome activation or upon necrosis. An alternative cell surface form of IL-1α (csIL-1α) is reported on macrophages, but this is controversial as subsequent findings showed that the cell fixation used in assays causes leakage of intracellular IL-1α (icIL-1α), which is falsely detected as csIL-1α. Subsequently, csIL-1α has been reported on various cell types including bone marrow-derived dendritic cells (BMDCs), human monocytes and fibroblasts. csIL-1α is also the key driver of the senescence-associated secretory phenotype (SASP). However, we do not understand how IL-1α, which lacks a transmembrane domain and hydrophobic regions, is tethered to the membrane, or the signals that can control this.

Using flow cytometry with anti-IL-1α antibodies verified in *Il1α*$^{-/-}$ cells, we show that macrophages express bona fide csIL-1α in response to Toll-like receptor (TLR)-4 or TLR-2 ligation. Importantly, significant false-positive csIL-1α staining occurs without the use of a robust dead cell gate. Inhibitors of translation prevent csIL-1α expression, showing that csIL-1α comes from *de novo* synthesized IL-1α. We also find that csIL-1α is the full-length precursor form and that cleavage by thrombin releases active mature IL-1α from the macrophage surface. High concentrations of IL-1RA displace csIL-1α and csIL-1α levels are significantly lower on *Il1r2*$^{-/-}$ macrophages compared to wild-type, together indicating that csIL-1α is tethered in part to its cognate receptor IL-1R2. In addition, treatment with phosphoinositide phospholipase C reduces csIL-1α, indicating that further tethering occurs via GPI- anchors. Finally, we find that pre-treatment of macrophages with very low concentrations of interferon-γ significantly inhibits trafficking of IL-1α to the surface.

We believe the induction of csIL-1α may serve as a mechanism for low-level immune responses to try and resolve an infection with minimal tissue damage, before full activation of inflammasomes. Thus, exploring how csIL-1α is expressed and the factors that regulate this is important to understand the local or systemic context in which IL-1α can contribute to disease and/or physiological processes.

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Integrin VLA3 Mediates Endothelial Barrier Damage by Human Sepsis Patient Neutrophils in Vitro

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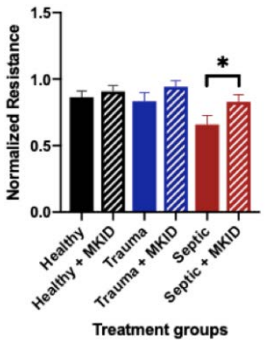
Background: Sepsis, a dysregulated host immune response to infection, can result in life-threatening organ failure. Neutrophils become hyperactive during sepsis and they mediate much of the morbidity and mortality associated with the disease. Integrin VLA3 has been shown to be significantly up-regulated in human neutrophils during sepsis. Our aim is to determine the role of VLA3 in an in vitro model of neutrophil-induced damage of endothelial barrier function.

Methods: Prospective study enrolling patients in surgical and trauma intensive care units at Level 1 Trauma Center. Septic patients were identified as those with two or more SIRS criteria with a source of infection confirmed by clinical evidence or microbiological data. Trauma patients were those with injuries severe enough to warrant ICU admission. Patients were enrolled within 24 hours of their diagnosis or admission. Blood samples were collected from patients and healthy volunteers the same day. Neutrophils were isolated by dextran sedimentation, pretreated with anti-VLA3 antibody or isotype control, and allowed to adhere to TNFα-activated human umbilical vein endothelial cell monolayers. Electrical cell-substrate impedance sensing was used to quantify real-time barrier disruption after neutrophil adhesion.

Results: Neutrophils from healthy donors and TICU patients did not induce significant differences in barrier function, measured as a decrease in normalized resistance (nR=0.86 +/- 0.16 and 0.83 +/- 0.15 at 120 minutes respectively). Neutrophils from sepsis patients, however, resulted in more loss of barrier function compared to neutrophils from healthy donors (nR= 0.66 +/- 0.19 at 120min). Neutrophils from septic patients that were pre-treated with the VLA3 blocking antibody showed significantly less barrier damage than septic cells alone (nR= 0.85+/-0.12 vs. 0.66+/-0.19 at 120min; $p < 0.05$).

Conclusions: Functional blocking of integrin VLA3 attenuated the loss of barrier function by septic patient neutrophils supporting the hypothesis that VLA3 mediates barrier dysfunction. Therefore, VLA3 may serve as a therapeutic target in the treatment of endothelial dysfunction in sepsis.



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Characterization of Macrophage Functions in Response to *Filifactor Alocis.*

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The etiology of periodontitis involves complex communities of polymicrobial pathogens that promote chronic inflammation of the periodontium, enhancing the rate of bone resorption and tooth loss. Macrophages are key members of the innate immune system and play a major role in initiation and maintenance of inflammatory processes, as well as in resolution of inflammation. *Filifactor alocis* was recently identified as a potential periodontal pathogen, and recent studies from our laboratory have shown that *F. alocis* can manipulate the antimicrobial functions of neutrophils to survive in the periodontal pocket. The aim of this study was to investigate the role and possible anti-microbial mechanisms associated with macrophages in response to *F. alocis.* Our results showed that *F. alocis* was phagocytized by human macrophages (~40%), a percent similar to what we demonstrated with human neutrophils. However, only 25% of *F. alocis*-containing phagosomes fused with lysosomes as shown by lysotracker and LAMP-1 recruitment. Further, the interaction of *F. alocis* with macrophages resulted in sustained induction of ERK1/2 and p38 MAPK after 30 min of infection. *F. alocis* induced significant changes in gene expression for cytokines and chemokines in human macrophages compared to unstimulated cells. The upregulation of IL-6 and IL-10 mRNA expression was also confirmed by a significant release of both cytokines measured in cell-free supernatant by ELISA. In summary, these results suggest that *F. alocis* withstands macrophage antimicrobial responses by preventing LAMP-1 recruitment to the bacterial phagosome, however, the organism can contribute to inflammation by upregulation of pro-inflammatory cytokine production by macrophages.

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CRM1-mediated Nuclear Export in *Helicobacter Pylori* Induced Neutrophil Hypersegmentation

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Neutrophils are the first line of defense against microbial invaders and are integral for disease caused by *Helicobacter pylori* infection. *H. pylori* is a human-specific Gram-negative bacterial pathogen that infects the gastric epithelium and can cause peptic ulcers, gastritis, and gastric cancer. *H. pylori* induces a massive recruitment of neutrophils to the infection site where they sustain a chronic inflammatory response. *H. pylori* also induces nuclear hypersegmentation, increasing the number of nuclear lobes from 3-4 to as many as 17 lobes/cell. The mechanisms by which it occurs are not well understood. We have previously shown that this hypersegmentation can be prevented if either host or pathogen translation is blocked. Treatment of infected cells with cycloheximide or chloramphenicol reduced hypersegmentation by 66.1 ± 24.1% (n=4, p < 0.01) and 81.3 ± 7.3% (n=3, p < 0.01) respectively. Further evidence supporting a role in nuclear export is our observation that CRM1 using Leptomycin B significantly decreases

hypersegmentation induced by *H. pylori* infection. In keeping with this, RNA-seq transcriptional profiling revealed significant differential expression of nuclear pore proteins and components of the Ran GTPase/CRM1 nuclear export pathway. Current studies are focused on confocal microscopy and Western blot analysis of CRM1 as well as its nuclear exporting partners. Completion of these studies will advance understanding of *H. pylori* infection as well as mechanisms that regulate neutrophil nuclear morphology.

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Formate Production by *Staphylococcus Aureus* biofilms Is Critical for Inhibiting Leukocyte Pro-inflammatory Activity and Promoting Biofilm Persistence *in Vivo*

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Biofilms are bacterial communities that are encased by a self-produced extracellular matrix, which adhere to a surface and cause chronic infections. *Staphylococcus aureus* (*S. aureus*) represents a significant healthcare burden through its ability to form biofilms with an intrinsic tolerance to antibiotics. *S. aureus* is a leading cause of medical device-associated biofilm infections, including prosthetic joints. The major cellular infiltrate during these infections are myeloid-derived suppressor cells (MDSCs), which release an array of anti-inflammatory molecules at the site of infection, including IL-10, which promote *S. aureus* biofilm persistence by inhibiting monocyte/macrophage pro-inflammatory activity. To further examine biofilm-leukocyte crosstalk, RNA-Sequencing was performed on biofilms co-cultured with both MDSCs and macrophages to identify *S. aureus* genes that were differentially regulated following leukocyte exposure that may promote their anti-inflammatory properties. Many fermentative genes were significantly upregulated, including formate acetyltransferase (*pflB*), which catalyzes the conversion of pyruvate and coenzyme-A into formate and acetyl-CoA. A *S. aureus pflB* mutant (Δ*pflB*) did not exhibit any growth defects; however, biofilm structure was dramatically affected as Δ*pflB* formed taller and more diffuse biofilms compared to the wild type (WT) strain as revealed by confocal laser scanning microscopy. *S. aureus* Δ*pflB* biofilms also induced an exaggerated pro-inflammatory response by both MDSCs and macrophages during co-culture compared to WT biofilms, including significant increases in IL-6 and IL-12, while TNF-α remained unchanged. In a mouse model of orthopedic implant biofilm infection, bacterial burdens were significantly reduced with Δ*pflB* during later stages of infection, which was accompanied by a corresponding decrease in infiltrating MDSCs and an increase in macrophages recruited to the inflamed area. Overall, *S. aureus*-derived formate is important for attenuating leukocyte pro-inflammatory cytokine production and bacterial clearance and represents a novel metabolic virulence factor.

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Mechanisms and Functional Consequences of *Helicobacter Pylori*-Induced Neutrophil Nuclear Hypersegmentation

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It is now clear that neutrophils are heterogeneous and can exhibit considerable phenotypic and functional plasticity. In keeping with this, we discovered that *Helicobacter pylori* infection induces N1-like subtype differentiation of human neutrophils that is notable for profound nuclear hypersegmentation. Herein, we utilized biochemical approaches and super-resolution microscopy to gain insight into the underlying molecular mechanisms and to analyze neutrophil nuclei in unprecedented detail. Sensitivity to inhibition by nocodazole and taxol indicated that hyper-segmentation requires microtubule (MT) dynamics; and STED imaging demonstrated that MTs were both longer and significantly more abundant following *H. pylori* infection. LINC complexes that connect the cytoskeleton to the nucleus in other cell types are absent in neutrophils. Our data implicate dynein in this process and suggest an additional role for dynein-driven retrograde transport of cargo along MTs in hypersegmentation. Remarkably, additional STED imaging of cells stained with antibodies to the nuclear membrane protein lamin B receptor showed that nuclear volume doubled prior to the onset of hypersegmentation whereas DNA content was unchanged. We therefore hypothesized that *H. pylori* infection may markedly alter chromatin structure, a notion that is in keeping with ability of transcription and translation inhibitors to ablate hypersegmentation. To test this hypothesis, we are currently mapping open chromatin by ATAC-Seq (assay for transposase-accessible chromatin using sequencing). Preliminary results suggest there are more open regions in the chromatin near transcriptional start sites after *H. pylori* infection, and RNA-Seq transcriptional profiling demonstrates significant differential expression of molecules associated with nuclear transport and the cytoskeleton. Based on these findings, our working model is that within the first hours of infection *H. pylori* alters chromatin structure in a manner that increases nuclear volume. Subsequent changes in gene expression, nuclear transport and MT abundance and dynamics act in concert to induce hypersegmentation of enlarged nuclei. 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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Effects of Pseurotin Alkaloids on Selected Immune Cell Functions

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Pseurotins A and D are secondary metabolites that are synthetized by filamentous fungi. Interestingly, pseurotins show significant effects on functions of different body cell types including immune system cells. However, effects of pseurotins on function of leukocytes is still unclear.

In this study we tested effects of pseurotins on function of two different leukocyte types, murine macrophages and human lymphocytes. Results obtained with murine macrophages RAW 264.7 reveals that pseurotins attenuate response of macrophages to bacterial lipopolysacharide. Pseurotins also slow down proliferation of this murine macrophage cell line. Analysis of selected signaling pathways revealed that pseurotins interfere with STAT signaling pathway in these cells. Next, the effect of pseurotins on human T- and B-lymphocytes isolated from blood of healthy donors was tested. T-lymphocytes were activated by anti-α CD3 and anti-α CD28. B-lymphocytes were activated by IL-4. Interestingly, significant inhibitory effects of pseurotins on expression of surface markers CD69 and CD25 was observed. Similarly, an inhibition of lymphocyte proliferation was observed. These effects were accompanied by inhibitory effects of pseurotins on JAK/STAT signaling pathway.

Overall, it can be concluded that natural pseurotins show inhibitory effects on leukocytes of both myeloid and lymphoid origin that is connected with inhibition of JAK/STAT signaling pathway inhibition.

The study was supported by the GACR of the Czech Republic (17-18858S).

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Importance of Adenylate Cyclases in Regulation of Differentiation of T Cell Subpopulations

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Cyclic AMP (cAMP) is an important intracellular second messenger, which is produced by adenylate cyclases (ACs). Ten mammalian AC isoforms identified up to date differ in their tissue distribution and biochemical regulation. In leukocytes, three dominant AC isoforms (AC3, AC7 and AC9) are suggested. The only known specific activator of membrane AC isoforms 1 to 8 is labdane diterpene forskolin. This compound was used experimentally to modulate immune

response, however, specific effects of forskolin and cAMP on Th cells differentiation and function is still unclear.

Our study is aimed to clarify importance of different AC isoforms in regulation of differentiation and function of T cell specific subpopulations and to elucidate possibilities how to specifically modulate production of cAMP in these cell types. Human T helper cells were sorted and stimulated by selected cytokines and activators to induce differentiation into Th1, Th2, Th17 and Threg subpopulations. The expression of membrane bound AC isoforms was screened and confirmed that AC7 is the most predominant in Th cells. Formation of cAMP was measured by homogenous time-resolved fluorescence resonance energy transfer together with cell proliferation and an immunophenotypic characterization of Th cell differentiation into particular subpopulation and cell activation state. Data clearly show differences in effects of forskolin on Th cell differentiation into subpopulations. Overall, AC isoform selective modulation may represent a new therapeutic approach for the treatment of T cells related pathological processes.

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Effects of β-hydroxybutyrate Receptor (HCA2/GPR109A) Agonists on Granule Release, Neutrophil Extracellular Trap Generation, and Proinflammatory Cytokine Expression in Bovine Neutrophils

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An elevated concentration of the ketone body β-hydroxybutyrate (BHB) in dairy cattle during lactation is associated with an increased incidence of inflammatory diseases, such as metritis and mastitis where neutrophils play a particular role. BHB was identified as an endogenous ligand of the Hydroxycarboxylic acid receptor 2 (HCA2 or GPR109A), a G protein-coupled receptor. Recent studies suggest that HCA2 activation modulates the inflammatory response in human macrophages, monocytes, and neutrophils, where this receptor is highly expressed. The effect of specific ligands of the HCA2 receptor on bovine immune cells have not been previously studied. We studied the effect of HCA2 activation on different neutrophil responses such as granule release, the generation of neutrophil extracellular traps (NETs), and proinflammatory cytokine expression. We found that treatment with MK-1903 and nicotinic acid, two HCA2 fully selective agonists, and BHB elicited the release of matrix metalloproteinase 9 (MMP-9). On the contrary, the structurally related compound Nicotinamide, which does not bind to the HCA2 receptor, had no effect on MMP-9 release. We also observed that nicotinic acid and MK-1903 increased NETs formation. Finally, we demonstrated that BHB increased IL-6 expression and nicotinic acid and MK-1903 increased IL-8 and IL-1β expression in bovine neutrophils. These results can contribute to our knowledge about novel modulatory HCA2 mechanisms in the innate immune system that could be involved in various cattle pathologies.

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A Unique Subset of Neutrophils Marked by Olfactomedin-4 Expression

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Neutrophil heterogeneity is well described, but the question remains if heterogeneity arises from differences in activation, maturation, or age of a single cell population or if there are truly unique subsets of neutrophils that are present at an early stage of differentiation. We provide data to support the latter, where olfactomedin-4 (OLFM4) expression marks a subset of neutrophils that are determined during neutrophil differentiation. We show that this subset is conserved from mouse to man and that mice are an excellent model for studying the function of this subset of neutrophils. Importantly, mice null for OLFM4 are protected from death when challenged with sepsis, suggesting that OLFM4 not only marks this subset of neutrophils but also has important mechanistic functions. While OLFM4 may be detrimental during acute inflammatory processes, we believe the function of this protein is important during recovery and repair. Expression of OLFM4 is intracellular, making sorting these neutrophils for side by side comparisons difficult and will require the development of novel reporter murine lines. We show longitudinal human data demonstrating that OLFM4 expressing neutrophils are independently regulated from other neutrophils. Finally, we present human data showing different disease states, including septic shock and trauma, illustrating differential regulation of OLFM4 expressing neutrophils during disease and recovery.

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The Accessory Gene *SaeP* of the SaeR/S Two-Component Gene Regulatory System Impacts *Staphylococcus Aureus* Virulence During Neutrophil Interaction

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Staphylococcus aureus (*S. aureus*) causes a range of diseases spanning from superficial skin and soft-tissue infections to invasive, life-threatening infections. *S. aureus* utilizes the SaePQRS sensory system to adapt to neutrophil challenge. Although the role of the SaeR response regulator and its cognate sensor, SaeS, have been demonstrated to be critical in surviving neutrophil interaction and causing infection, the roles for the accessory proteins SaeP and SaeQ remain incompletely defined. To characterize the functional role of *saeP* and *saeQ* during innate immune interaction we generated isogenic deletion mutant strains in USA300 (USA300Δ*saeP* and USA300Δ*saeQ*). Preliminary results demonstrate a gene regulatory function for *saeP* during

interaction with human neutrophils. *S. aureus* survival and cellular damage of neutrophils was increased following phagocytosis of USA300Δ*saeP* compared to USA300. Additionally, supernatants from USA300Δ*saeP* significantly increased neutrophil plasma membrane damage compared to USA300. Deletion of *saeQ* demonstrated a similar phenotype to the USA300Δ*saeP* strain but effects did not reach significance during neutrophil interaction. Increased neutrophil lysis correlated with increased gene expression of *lukF-PV*, and *lukGH*, in USA300Δ*saeP* compared to USA300. A USA300Δ*saeP*/Q *S. aureus* strain was comparable to USA300Δ*saeP* and showed significant increases in survival following neutrophil phagocytosis. However, only a USA300*saeP/Q* double mutant strain showed increased mortality compared to USA300 following murine bacteremia. These data provide evidence that *saeP* is important in regulating the SaeR/S response during interaction with human neutrophils and suggest that *saeQ* and *saeP* together impact pathogenesis *in vivo*.

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Leishmania Major Degrades Murine CXCL1: An Immune Evasion Strategy

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Leishmaniasis is a global health problem with an estimated report of 2 million new cases every year. Innate immune system plays a central role in controlling *L. major* infection by initiating a signaling cascade that results in production of pro-inflammatory cytokines and recruitment of both innate and adaptive immune cells. Upon infection with *L. major*, CXCL1 is produced locally and plays an important role in the recruitment of neutrophils and macrophages to the site of infection. Herein, we report that *L. major* specifically targets murine CXCL1 for degradation and this allows *L. major* to establish infection in the host. The degradation of CXCL1 is not dependent on host factors because *L. major* can directly degrade recombinant CXCL1 in a cell-free system. Using mass spectrometry, we have identified that the *L. major* protease cleaves at the C-terminal end of murine CXCL1. Finally, our data suggest that *L. major* metalloproteases are involved in the direct degradation of CXCL1. Currently, we are testing the hypothesis that the peptide spanning the cleavage site can be used to inhibit *L. major* protease activity and pathogenesis.

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Kindlin-3 Organizes a Ring of Clustered High-affinity β_2 Integrins During Human Neutrophil Spreading Under Flow

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Neutrophils are vital for inflammation and immune defense. Dependent on β_2 integrins, spherical neutrophils spread on vascular endothelium after arrest, which is critical for their recruitment from circulation to resist high shear flow and to initiate intravascular crawling. Here, we use high-resolution quantitative dynamic foot printing microscopy to monitor neutrophil spreading on a substrate of recombinant ICAM-1 and P-selectin under flow. A homogenous binding assay using the conformation-reporter antibodies mAb24 (reporting high-affinity β_2, H^+) and KIM127 (reporting extended β_2, E^+) showed three conformations of activated β_2 integrins. E^-H^+ β_2 integrins increased before E^+H^- and E^+H^+ conformations at the beginning of neutrophil spreading. Integrin extension depended on Syk-mediated integrin outside-in signaling. The ring of E^-H^+ and E^+H^+, but not E^+H^- β_2 integrins was fully formed during late neutrophil spreading just before migration. Using kindlin-3-GFP fusion proteins, a ring of kindlin-3 was observed before the ring of H^+ integrins appeared. These findings show spatially coordinated integrin activation during spreading. The previously unrecognized E^-H^+ conformation is the pioneer integrin for neutrophil spreading and appears to be organized by kindlin-3.

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Pro-inflammatory extracellular ASC Specks Released Through Pyroptosis Perpetuate Inflammasome Activation in Macrophages and Hepatocytes in Murine and Human Alcoholic Hepatitis

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Background: Alcoholic Liver disease is characterized by NLRP3 inflammasome activation and release of interleukin (IL)-1β. The NLRP3 adaptor protein Apoptosis-associated Speck-like protein Containing CARD (ASC) oligomerizes into specks for inflammasome activation. Inflammasome effector protein caspase-1 or caspase 11 activation can lead to pyroptosis. In disease condition, ASC specks are released from pyroptotic cells, remains bioactive extracellularly and activate

inflammasome in neighboring cells. Here, we hypothesized that ASC specks are released and propagate inflammation in alcoholic liver disease.

Aim: To investigate the role of extracellular ASC specks released through pyroptosis in alcoholic hepatitis.

Methods: We analyzed plasma of healthy volunteers and cirrhotic human patients with alcoholic hepatitis for ASC protein. ASC specks were evaluated in mouse models of AH, acute-on-chronic (10 days with binge) and chronic alcohol feeding (4 weeks plus 3 binges). NLRP3 inhibitor MCC950 was administered in vivo. Liver macrophages and hepatocytes were isolated from transgenic mice ectopically expressing fluorescent ASC (ASC-citrine mice) for visualization of speck formation.

Results: Patients with acute alcoholic hepatitis had increased ASC protein levels in the circulation compared to healthy controls, as measured by ELISA. Extensive ASC aggregates were highly expressed in livers from alcohol-induced cirrhosis patients compared to controls. In mice, chronic ethanol feeding induced ASC aggregates in the liver. A single EtOH binge plus lipopolysaccharide (LPS) increased ASC oligomers in mice plasma. To identify which cell type was responsible for ASC specks formation and release in the liver in alcoholic liver disease, we treated liver macrophages and hepatocytes from ASC-citrine mice with EtOH alone or EtOH plus LPS. On confocal microscopy evaluation, we found formation of intracellular ASC specks both in liver macrophages and hepatocytes after EtOH treatment alone. This effect was not enhanced in by LPS in EtOH treated cells. Furthermore, EtOH exposure resulted in release of ASC specks into the supernatants of macrophages and hepatocytes, measured by flow cytometry, raising the possibility of pyroptosis resulting in ASC release. Transfer of ASC specks, derived from activated macrophages triggered significant IL-1β release in hepatocytes in vitro. Furthermore, we found increased levels of GSDMD-N fragment in acute-on-chronic alcohol fed mice livers consistent with cleavage of Gasdermin D (GSDMD) downstream of inflammasome activation and ASC speck formation, indicating ongoing pyroptosis. Finally, in vivo inhibition of NLRP3 inflammasome activation with MCC950 reduced levels of Caspase-1 activation, macrophage pyroptosis and steatohepatitis in alcohol-fed mice.

Conclusions: Inflammasome-dependent pyroptosis in the liver results in ASC speck release in alcoholic hepatitis. Our novel findings suggest that increased circulating ASC protein and liver ASC aggregates in patients and mice with alcoholic hepatitis could be responsible for the progression of the disease and could also serve as biomarkers of alcoholic hepatitis.

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Elevated Levels of Specific Damage-Associated Molecular Patterns in Serum After Burn Injury Correspond with Altered Levels in the Skin

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Introduction: Cutaneous burns can generate a profound and pathological systemic inflammatory state implicated in long-term multiple organ failure. The signaling mechanisms underlying this process are poorly understood, but damage-associated molecular patterns (DAMPs) are believed to play a key role. DAMPs are biomolecules released into circulation following acute cellular injury. They interact with cells of the innate immune system bearing pattern recognition receptors, thereby promoting non-infectious pro-inflammatory and pro-fibrotic pathways. Elevated levels of specific DAMPs, including S100A8/A9 (calprotectin), cytochrome C, hyaluronan, and high mobility group box 1 (HMGB1), have been measured in serum following burn injury. To our knowledge, this study represents one of the first attempts to coincidentally examine levels of DAMPs directly in burned and burn-adjacent skin to determine whether it represents a significant source of DAMPs seen in systemic circulation.

Methods: Male C57BL/6 mice were anesthetized, shaved, and subjected to either a uniform 15% full-thickness dorsal scald burn injury or sham water immersion. Animals were euthanized 24 hours following burn injury and skin and blood samples were immediately harvested. Specifically, full-thickness 5 mm circular punch biopsies were taken at four locations for each injured animal: (1) central to the burn; (2) spanning the burn margin; (3) abutting the burn margin; and (4) at an uninvolved distal/ventral site. These locations were approximated for sham animals. Skin samples were mechanically homogenized for protein analysis by ELISA, targeting S100A8/A9, cytochrome C, hyaluronan, and HMGB1. All DAMP measurements were normalized to total protein and comparisons were made by one-way ANOVA.

Results: Relative to sham animals, significant elevations in levels of S100A8/A9 protein were observed in serum at 24 hours following burn injury (2.0-fold higher, $p < 0.05$). Corresponding increases in S100A8/A9 were seen in skin margin biopsies (1.9-fold higher, $p < 0.05$). With respect to cytochrome C, serum levels increased markedly (30-fold higher, $p < 0.05$), though only non-significant decreases were seen in skin biopsies at all four locations. A significant increase in serum hyaluronan was measured after burn injury (2.1-fold higher, $p < 0.05$), while significant decreases in hyaluronan levels were measured in skin specimens central to (2.6-fold lower, $p < 0.05$) and abutting (1.7-fold lower, $p < 0.05$) the full thickness burn. We were unable to measure HMGB1 in serum in sham or burn animals and changes in skin levels of HMGB1 were variable, decreasing in the specimen abutting the burn margin (1.3-fold lower, $p < 0.05$), and increasing in the distal specimen (1.4-fold higher, $p < 0.05$).

Conclusions: These data demonstrate significant changes in skin DAMP levels following full-thickness burn injury in a murine model. Notably, even distal and grossly normal skin, outside the

traditional zones of coagulation, stasis, and hyperemia, sometimes exhibits these changes. This is suggestive of an autocrine or systemic signaling response triggering DAMP upregulation and release following remote burn injury. Ongoing and future work will elucidate the kinetics of DAMP release and examine end-organ localization of DAMPs. Ultimately, specific DAMPs may be useful as quantitative burn injury biomarkers or therapeutic targets

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Innate Immunity in Cancer

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Growing Tumor Modifies Neutrophil Anti-bacterial Properties, Leading to the Increased Susceptibility of Tumor-bearing Hosts to Acute Lung Infections with *Pseudomonas Aeruginosa*

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Patients with solid tumors, such as head-and-neck cancer (HNC), are predisposed to infections with nosocomial bacteria, even in the absence of immunosuppressive therapies. One of such common nosocomial pathogens is *Pseudomonas aeruginosa.* At the same time, the treatment of cancer patients with granulocyte colony-stimulating factor (G-CSF), but not current neutropenia, predisposes them to bacterial infection. G-CSF is a growth factor that is known to modulate neutrophil survival and turnover. Importantly, one of the potent sources of G-CSF during tumor growth is the tumor tissue itself. Moreover, G-CSF levels in plasma and tumor correlate positively with tumor progression. Therefore, here we aimed to study mechanisms involved in G-CSF-mediated alteration of neutrophil anti-bacterial activity and its consequence for the course of acute lung infection with *P. aeruginosa*. Mouse HNC (tonsil epithelial carcinoma, MTEC) cell lines, control or transfected with G-CSF, were injected subcutaneously into WT mice and tumor growth was followed. At the day 14 *P. aeruginosa* was applied intratracheally ($2x10^6$CFU/mouse). 20 hours after infection mice were sacrificed, the clinical state, bacterial load in the lower respiratory tract and the lung tissue damage were assessed. The levels of cytokines and growth factors elevated in plasma, tumor and lung were estimated using ELISA. The investigation of anti-bacterial properties of isolated neutrophils included the analysis of gene and protein expression, using RT-qPCR, flow cytometry and western blot. Further, ROS production, phagocytosis and NET release in response to *P. aeruginosa* were assessed. In mice bearing G-CSF expressing tumors we could observe massive neutrophil infiltration into periphery, e.g. tumor, spleen and lungs. Nevertheless, such mice displayed an increased *P. aeruginosa*load in the lower respiratory tract, loss of aerated area and worse clinical outcome. Plasma levels of G-CSF, TNFα and MMP9 were significantly elevated in such mice. Isolated lung neutrophils showed diminished expression of anti-bacterial molecules (def1, NE), suppressed NET formation and lower phagocytic activity in mice bearing G-CSF-producing tumors, while the expression of molecules responsible for the tissue damage, such as MMP9 or ROS, were elevated. Taken together, here we could demonstrate that G-CSF that is released by the growing tumor affects not only tumorigenic

activity of neutrophils, but also their anti-bacterial properties. Moreover, such neutrophils release factors that are responsible for tissue damage. All this deteriorates the bacterial clearance in tumor-bearing hosts and results in their susceptibility to infections.

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Kinetic of Natural Killer Cells Showing an Unconventional Immunological Memory in Haploidentical Hematopoietic Stem Cell Transplanted Patients Undergone Human Cytomegalovirus Reactivation

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Haploidentical hematopoietic stem cell transplantation (h-HSCT) represents a promising therapeutic approach to cure patients affected by hematologic malignancies. h-HSCT induces a state of immunologic tolerance between donor and recipient cells and it allows to rapidly find a donor for almost all patients in need. Despite the positive results in terms of overall/disease free survival and reduced tumor relapse, the HLA mismatch between donor and recipient has not been yet fully exploited and the clinical outcome of h-HSCT patients is hampered by life-threatening side effects. Among them, the human Cytomegalovirus (HCMV) infection/reactivation represents one of the major causes of morbidity and mortality after h-HSCT.

Immune cell reconstitution (IR) is certainly key in determining a positive h-HSCT clinical outcome. In this context, we demonstrated that Natural Killer (NK) cells represent the first innate lymphocytes recovering after h-HSCT, thus highlighting their role in ensuring a prompt alloreactivity early after transplant as well as protection against opportunistic viral infections. Other and we previously reported that HCMV infection/reactivation greatly impact NK cell maturation and effector-functions, by providing a rapid expansion of mature, long-lived and hyper-functional NK cells showing memory-like (ml) properties.

To investigate the impact of HCMV infection/reactivation on NK cell IR after h-HSCT, we set up a complex multi-parametric flow cytometry panel and we characterized immune-reconstituting NK cells at different time points up to one year after the transplant. Data where then analyzed by an unsupervised PhenoGraph algorithm, that evaluates, at single cell level, differences/similarities of marker expression, building up clusters of phenotypically identical cells.

This system biology approach identified a subset of NK cells showing a peculiar CD158b1b2j+NKG2A-NKG2C+NKp30- phenotype that is expanded only in h-HSCT patients experiencing HCMV reactivation. Interestingly, this latter NK cell population showing memory-like functional features is maintained even after the resolution of the infection and its frequency positively correlates with HCMV viral load and with the numbers of reactivation events. These findings show in a human setting *in vivo* the expansion and the kinetic of those NK cells that "remember" the HCMV challenge in patients experiencing viral reactivation. Our data are important to better understand the ability of NK cells to control this life-threatening infection

after h-HSCT as well as to deep our knowledge in regard to human NK cell maturation in response to viral challenges.

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Pleiotropic Roles of Tumor-Associated Macrophages (TAMs) in Promoting Cancer Metastasis During Chemotherapy Treatment

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Neoadjuvant chemotherapy (NAC) is commonly used for treatment of locally-advanced breast cancer to downstage the disease and improve surgical outcome. Although patients who achieve complete pathologic response following NAC have excellent long-term prognosis, patients with residual disease do not. We hypothesize that chemotherapy may drive pro-metastatic changes in the residual tumor by inducing recruitment of bone marrow-derived myeloid progenitors (i.e. monocytes/macrophages) into the tumor microenvironment. Here, we evaluated our hypothesis using mouse mammary carcinoma models and patient-derived xenografts.

We demonstrate that treatment with paclitaxel and doxorubicin/cyclophosphamide promotes an influx of specialized tumor-associated macrophages (TAMs) into the tumor microenvironment. In particular, Tie2-expressing macrophages home at the perivascular niche where they assemble tripartite microanatomical doorways for cancer cell dissemination, known as “Tumor Microenvironment of Metastasis” (TMEM). Chemotherapy-induced increase in TMEM doorways correlates with increased incidence of circulating tumor cells (CTCs) and disseminated tumor cells (DTCs). Indeed, we confirmed by intravital imaging that these post-chemotherapy assembled TMEM doorways are active sites of transient vascular permeability and cancer cell intravasation. Moreover, we show that pharmacological inhibition of TMEM function, using a Tie2 inhibitor, counteracts this chemotherapy-induced pro-metastatic phenotype.

Furthermore, we demonstrate that a subpopulation of TAMs induces increased expression of an invasive isoform of the actin-regulatory protein Mammalian Enabled (Mena), called $Mena^{INV}$, in a subset of breast tumor cells. The $Mena^{INV\text{-}HIGH}$ tumor cells have phenotypic advantage for dissemination via TMEM doorways over $Mena^{INV\text{-}LOW}$ cells. Moreover, we found that these $Mena^{INV\text{-}HIGH}$ tumor cells also co-express high levels of stemness-inducing transcription factors Sox2/9.The partial ablation of TAMs by clodronate-liposomes eliminates the induction of both $Mena^{INV}$ and Sox2/9 in cancer cells and brings the CTCs and DTCs back to the basal levels, confirming the involvement of TAMs in inducing pro-metastatic changes in tumor microenvironment. Moreover, the targeted pharmacological inhibition of Notch1 receptor or its respective ligands (i.e. Jagged1), or shRNA-mediated knockdown of Notch receptors in tumor cells lead to significant decrease of $Mena^{INV}$ and Sox2/9 expression in tumor cells, indicating that

the above described TAM-tumor cell interactions are mediated through the Notch pathway. Importantly, the spatial analyses using multichannel immunofluorescence unraveled that cancer cells that co-express Mena^{INV} and Sox2/9 utilize TMEM doorways to disseminate to distant sites. Our preliminary observations additionally demonstrate that TAMs accompanying Mena^{INV} and Sox2/9 co-expressing tumor cells express high levels of programmed death ligand-1 (PD-L1), suggesting that PDL1^{+} TAMs provide an immunosubversive tumor microenvironment during cancer cell streaming towards TMEM.

In summary, we provide conclusive mechanistic underpinnings by which chemotherapy gives rise to metastasis-initiating cancer cell subpopulation that utilizes TMEM doorways for hematogenous dissemination. Unraveling further the cellular and molecular details of chemotherapy-induced pro-metastatic effects will be crucial for designing targeted approaches to improve current systemic management of locally advanced and metastatic breast cancer.

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ADAM17 Inhibition Enhances Human NK Cell Proliferation by IL-15 in a Mouse Xenograft Model

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Natural killer (NK) cells can kill tumor cells by direct and indirect means. Exploiting these cells for cancer therapies is a rapidly developing field, which includes autologous and allogeneic NK cell infusion strategies. L-selectin (CD62L) is a well described adhesion protein known to play a critical role in leukocyte adhesion, migration and signal transduction. The majority of CD56^{bright} and a subset of CD56^{dim} NK cells express CD62L, and these subsets appear to represent early and intermediate stages of NK cell maturation, respectively, and have a greater potential to

proliferate than CD56$^{dim\ CD62Lneg}$ NK cells. IL-15 treatment following the adoptive transfer of NK cells enhances their expansion *in vivo*, and is an important strategy being investigated in the clinic to improve NK cell persistence. Using an *in vivo* xenogeneic model, the infusion of human IL-15 causes the expansion of adoptively transferred human NK cells, and we found that this was completely abrogated upon administering an CD62L blocking mAb. IL-15 is known to induce the downregulation of CD62L expression, and we show this can be blocked by ADAM17 inhibition. Interestingly, the administration of function blocking ADAM17 mAbs, including one specific to human ADAM17, dramatically increased NK cell expansion *in vivo* in the presence of IL-15. These findings demonstrate that CD62L is important for NK cell expansion by IL-15 in a xenograft model, and that this process is impaired upon CD62L shedding by ADAM17 in the cytokine-stimulated NK cells. Our findings could have clinical relevance for NK cell immunotherapies involving cytokine stimulation. By blocking ADAM17 function it may be possible to increase NK cell expansion and persistence as well as certain cytolytic activities to enhance their anti-tumor effector activities.

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Removal of Innate Suppressors Enhances Anti-tumor Immune Responses

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Although there is an immense interest in identifying novel immune-based therapeutics for colorectal cancer, the role and regulation of innate leukocytes such as neutrophils during the modulation of tumor immune environment remain controversial and less defined. Our current study tested hypothesis that the removal of innate suppressor “check point” in neutrophils may facilitate tumor-immune surveillance *in vitro* and *in vivo*. To this regard, we examined two key innate signaling suppressors Tollip and IRAK-M and their roles in modulating neutrophil anti-tumor immune functions. We observed that selective deletion of Tollip enhanced tumor immune surveillance in the AOM-DSS chemically induced colon cancer model. Tollip deficiency neutrophils demonstrated enhanced capability to promote, instead of suppress, the proliferation and activation of effective T cells. Functionally, we observed that the transfusion of Tollip deficient neutrophils can potently render an enhanced anti-tumor immune response in the murine inflammation-induced colorectal cancer model. Likewise, the study of another innate suppressor IRAK-M revealed that IRAK-M expression was up-regulated in the human patients with colorectal cancer. We also demonstrated that IRAK-M deficient mice exhibited reduced tumor burden following AOM-DSS challenge. Together, our data reveal a novel anti-tumor immune-enhancement strategy through utilizing reprogrammed neutrophils with targeted removal of innate signaling suppressors.

New Technologies

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Infection Dissemination by Leukocytes

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For microbes to cause disseminated infection, they must first avoid destruction by innate immune cells. This can be achieved either by evading initial detection, or by manipulating the host cell to avoid activation of antimicrobial pathways following phagocytosis. Here, we hypothesized that commandeering the intra-leukocyte niche might provide bacteria both with protection against other elements of the immune system, while also acting as a motile vehicle that could actively promote dissemination to distal sites. We developed microfluidic assays to investigate key steps in dissemination: recruitment of leukocytes; phagocytosis of bacteria; suppression of antimicrobial responses; maintained migration of infected leukocytes; and microbial escape. Using these assays, we assessed the dissemination potential of several oral microbes, some of which are thought to cause disseminated infection in humans. We found that *Fusobacterium nucleatum* are readily engulfed by human neutrophils but appear to avoid induction of neutrophil ROS. In the absence of ROS induction, commandeered neutrophils remained motile and retained the ability to migrate in response to chemokine gradients. Following chemotaxis, we observed bacterial escape from infected neutrophils. Collectively, these observations suggest that neutrophils may act as “Trojan horses” during dissemination of *Fusobacterium nucleatum*.

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Regulation of Macrophage Low Density Lipoprotein Uptake by Microenvironmental Mechanics

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Macrophages are key mediators in the development of atherosclerosis: they scavenge lipid, transform into foam cells, and produce proinflammatory mediators. At the same time, the arterial wall undergoes profound changes in its mechanical properties. We recently showed that macrophage morphology and proinflammatory potential are regulated by the stiffness of the growth microenvironment. Here we asked whether changes in extracellular stiffness/microenvironmental mechanics also regulate uptake of lipid by macrophages. We cultured murine bone marrow-derived macrophages (BMMs) on polyacrylamide gels that model stiffness of healthy (1kPa) and diseased (10-150kPa) blood vessels. Our studies show that in unprimed BMMs, mechanics play an important role in the uptake of oxidized (oxLDL) and

acetylated (acLDL) low density lipoproteins, but not in the phagocytosis of bacteria or silica particles. Additionally, we found that macrophages adapted to stiff growth surfaces had increased mRNA and protein expression of two key lipid uptake receptors, CD36 and scavenger receptor b1. Regulation of a third uptake receptor, lectin-like receptor for ox-LDL, was more complex; mRNA expression decreased but surface protein expression increased with increased stiffness. Uptake of oxLDL and acLDL was independent of rho-associated coiled coil kinase, a key protein involved in actomyosin contractility. Focal adhesion kinase was required for maximal uptake of oxLDL, but not of acLDL. Through pharmacologic inhibition and genetic deletion of transient receptor potential vanilloid 4 (TRPV4), a mechanosensitive ion channel, we found an inhibitory role for TRPV4 in the uptake of acLDL, but not oxLDL. Together, these results implicate mechanical signaling in the uptake of acLDL and oxLDL, opening up the possibility of new pharmacologic targets to modulate lipid uptake by macrophages in vivo.

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Automated Microfluidics-Based System for Isolating Leukocytes from Human Peripheral Blood

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Despite its many limitations, density gradient centrifugation has remained the primary method of leukocyte isolation for many decades due to a lack of viable alternatives. To address this lack of innovation in upstream sample preparation, we are developing an automated microfluidic system based on inertial focusing that enables label-free white blood cell separation and concentration from large volumes of blood in short timescales with high consistency.

In this study we compared the performance of the Microfluidic System with density gradient method (DGM). The Microfluidic System consistently processed 40 mL of anticoagulated blood in approximately 20 minutes with minimal hands-on time as opposed to 60 – 90 minutes for DGM with considerable hands-on time. It is worth noting that the Microfluidic System isolates and concentrates the total white blood cell (WBC) population including granulocytes, whereas DGM collects only peripheral blood mononuclear cells (PBMCs). In terms of performance, the Microfluidic System achieved 87.6 ± 5.4% PBMC (88.6 ± 3.8% WBC) yield, 98.8 ± 1.3% WBC viability (via propidium iodide staining), 93.2 ± 7.5% WBC purity, 4.4 ± 0.5 log red blood cell depletion, and 4.0 ± 0.5 log platelet depletion (n = 91). In contrast, DGM achieved 62.7 ± 11.3% PBMC yield, 97.4 ± 8.5% PBMC viability (via propidium iodide staining), 5.0 ± 1.9% PBMC purity, 2.8 ± 0.3 log red blood cell depletion, and 1.1 ± 0.2 log platelet depletion (n = 42). The Microfluidic System resulted in over 30% and 70% improvement in viable yield of lymphocytes and monocytes relative to DGM (based on Annexin-V staining). The microfluidic cell separation process does not bias towards any specific cell subsets analyzed, including monocytes, CD4/CD8 T cells, and B cells.

The Microfluidic System offers a faster, more reliable method of obtaining high yield, high purity leukocytes than DGM. This technology has the potential to transform cell separation by automating an outdated, variable, and labor-intensive process.

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Neutrophil Swarming Restricts Fungal Growth

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The objective of this study was to leverage our microscale technologies to directly quantify the containment of microbes by the novel biological process of neutrophil swarming.

Methods: Neutrophil swarming is thought to be a key process for sealing off sites of infection and protecting healthy tissues and may be particularly relevant to pathogens like fungi that can be too big to be dealt with by a single immune cell. To directly examine the role of swarming in the containment of microbes, we have developed a novel tool that enables the study of thousands of swarming processes at once. Neutrophil swarming is triggered on large arrays of clusters of microbes, for which we control geometric features such as size and spacing. Single cell-resolution tracking of neutrophils provides us with information about the recruitment processes, while the real-time observation of the swarms allows the detailed monitoring of microbe-neutrophil interactions.

Results: We tested the swarming of human neutrophils against microbial targets. These targets included live fungi like *Candida albicans* and *Aspergillus fumigatus*. In control experiments, live microbes incubated alone grew well on the patterns. Swarms significantly delayed the growth of all microbes tested and also contained *C. albicans* and *A. fumigatus* hyphae for up to 16 hours. Disruption of swarming mediators compromised the ability of neutrophils to swarm and limited the ability to contain *C. albicans*. Neutrophil extracellular traps were formed during neutrophil swarming and disruption of NETs and ROS production also compromised swarming control of fungi. Swarming could be enhanced by the addition of cytokines like GM-CSF. This enhancement could partially restore swarming function during chemical inhibition of myeloperoxidase or ROS function.

Conclusions: Neutrophil swarming occurs against live microbes and restricts their growth. NETs are released inside the swarm, at the interface between the microbes and the neutrophils. Perturbations of swarming and NET formation enable *Candida* to escape the swarms. Swarming could also be enhanced by the addition of exogenous mediators. These results establish swarming as a potentially important mechanism of fungal control that warrants further investigation.

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Prostaglandin E2 Dependent Migration of Human Brain Endothelial Cells Are mediated Through Rho-Kinase-II

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Prostaglandin E_2 (PGE_2) plays a crucial role in angiogenesis as well as in ischemic and inflammatory disorders of the brain associated with breakdown of the blood-brain barrier. However, the effects of PGE_2 on brain endothelial cell migration, a key process in the angiogenic response and blood-brain barrier stability, are not well defined. Exposure of human brain endothelial cells (HBECs) to PGE_2 elicited a chemotactic response in a time-&-dose-dependent manner. The maximum migratory response was detected following 8 hrs exposure of HBECs to PGE_2(100 nM). Migration of HBECs in response to PGE_2 was accompanied by profound changes in the reorganization of actin filaments. Fluorescence microscopy examination of NBD-phallacidin-labeled endothelial cells showed increased formation of stress fibers, lamellipodia and podosomes after treatment with PGE_2(100 nM) compared to control. Based on these results, we hypothesized that Rho-kinase (ROCK), an enzyme involved in regulation of actin dynamics and cell migration, mediated the effects of PGE_2 on HBECs migration. Western blot analyses revealed that ROCK-II (type-α), but not ROCK-I (type-β), was expressed in HBECs. To examine ROCK-II activation, we performed immunocomplex kinase assays using myosin light chain (MLC) as a substrate. PGE_2 (100 nM) induced a 2-fold increase of ^{32}P-incorporation into MLC indicating activation of ROCK-II. Pretreatment of HBECs with the selective ROCK inhibitor, Y27632 (150 nM), blunted HBECs migration in response to PGE_2 but had no effect on migration induced by fetal bovine serum (10%). Knockdown of ROCK-II by siRNA also abrogated the migratory response of HBECs to PGE_2. In contrast, similar treatment had no effect of HBECs migration stimulated by hepatocyte growth factor. Taken together, these results are consistent with the hypothesis that stimulation of HBECs with PGE_2 leads to activation of ROCK-II, reorganization of the actin cytoskeleton and ultimately migration. A better characterization of the molecular events that regulate migration of HBECs are critical for the development of novel strategies to treat cerebrovascular diseases associated with deregulated angiogenesis.

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Resolution of Immune Responses

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Rewiring of the Placenta Immune Landscape with Pregravid Obesity

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The immune cell landscape of the placenta undergoes a compositional and phenotypic transformation over the course of gestation to establish fetal tolerance, facilitate vascular remodeling and fetal growth, and finally orchestrate the inflammatory events preceding labor. Evidence from epidemiological studies and experimental models strongly hint at the disruption of these cellular adaptations with pregravid obesity. Specifically, obesity has been shown to upregulate inflammatory macrophage markers in the decidua, and alter NK cell functionality. These observations are critical considering the high rate of pathologies such as placental dysfunction, preeclampsia, and preterm births associated with pregravid obesity. However, our understanding of the placental immune landscape at term and the disruptions induced by obesity remains incomplete. We, therefore, profiled the CD45+ fraction isolated from human term deciduas from lean and obese pregnant women following cesarean using droplet-based single-cell RNA sequencing. Clustering analyses of single cell transcriptomes revealed T cells, NK cells, and macrophages as major components of term placentas. As described for the early gestational decidua, we observe three NK cell subsets with distinct transcriptional signatures. We were also able to identify CD4+ and CD8+ T cells in equal proportions and a smaller subset of regulatory T cells. Interestingly, t-SNE analysis of the clusters identified three populations of macrophages with distinct gene expression profiles: a) a population of pro-inflammatory macrophages expressing high levels of S100 proteins, *IL1B* and *ITGAX*; b) a second population of hybrid macrophages expressing high HLA molecules and canonical M2 macrophage-like markers such as *MSR1* and *VCAN*; and finally c) a minor population of macrophages with a regulatory phenotype expressing high levels of *CD163* and *IGF1*. Pregravid obesity was associated with a dramatic drop in all subsets of T cells, and an expansion of all three sub-populations of macrophages. Moreover, macrophages from obese placentas expressed higher transcript levels of *NFKB1* and *REL*, suggestive of *in vivo* activation. These findings strongly support the concept of immune cell rewiring as a significant source of obesity associated inflammation in the decidua. We are currently analyzing the shifts in the spectrum of macrophage polarization and integrating the data with matched fetal placental immune cells to identify parallel changes on the villi. This high-resolution single cell atlas of the term maternal-fetal interface will reveal the immunological adaptations close to labor, the interactions between the maternal and fetal compartments that facilitate labor, and their aberrations with pregravid obesity.

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Phenotypical microRNA Screen Reveals a Noncanonical Role of CDK2 in Regulating Neutrophil Migration

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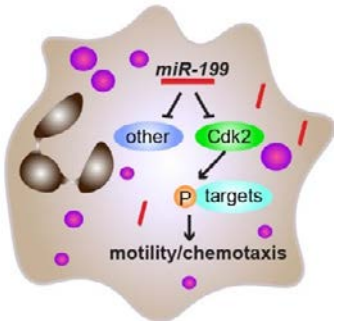
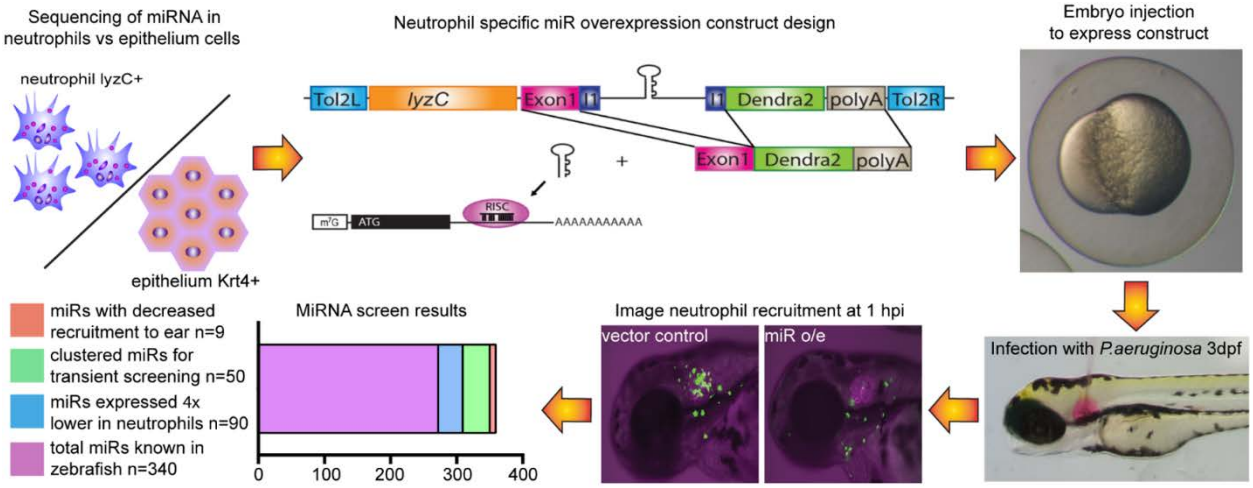
Neutrophil migration is essential for inflammatory responses to kill pathogens, however excessive neutrophilic inflammation also lead to tissue injury and adverse effects. To discover novel therapeutic targets that modulate neutrophil migration, we performed a neutrophil-specific microRNA overexpression screen in zebrafish, and identified eight microRNAs as potent suppressors of neutrophil migration. Among those, *miR-199* decreases neutrophil chemotaxis in zebrafish and human neutrophil-like cells. Intriguingly, in terminally differentiated neutrophils, *miR-199* alters the cell cycle-related pathways and directly suppresses cyclin-dependent kinase 2 (cdk2), whose known activity is restricted to cell cycle progression and cell differentiation. Inhibiting CDK2, but not DNA replication, disrupts cell polarity and chemotaxis of zebrafish neutrophils without inducing cell death. Human neutrophil-like cells deficient with CDK2 fail to polarize and display altered signaling downstream of the formyl peptide receptor. Chemotaxis of primary human neutrophils are also reduced by CDK2 inhibition. Furthermore, *miR-199* overexpression or CDK2 inhibition significantly improves the outcome of lethal systemic inflammation challenges in zebrafish. Our results therefore reveal previously unknown functions of *miR-199* and CDK2 in regulating neutrophil migration and provide new directions in alleviating systemic inflammation.





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RNA-sequencing Identifies Key Eosinophil Mediators Involved with Remodeling of Damaged Muscle Tissue in the Mdx Mouse Model of Duchenne Muscular Dystrophy

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Introduction: Duchenne Muscular Dystrophy (DMD) is a genetic disorder of progressive muscle degeneration caused by mutations in the gene encoding dystrophin. In healthy muscle tissue, dystrophin links the intracellular cytoskeleton of muscle fibers with the extracellular matrix, thereby providing mechanical support. Inactivating mutations in the dystrophin gene prevent expression of functional, full-length dystrophin, resulting in cycles of muscle damage and inflammation, followed by regeneration and repair. Eosinophils are among the leukocytes recruited to the dystrophic muscle tissue, where they were thought to exacerbate muscle damage by releasing cationic proteins stored within their cytoplasmic granules. In our earlier work, we have challenged this assumption by demonstrating that the level of eosinophil infiltration in the muscle tissue did not correlate with the degree of dystrophic muscle damage. Consistent with the recent reconsideration of eosinophils and their function in tissue microenvironments, we hypothesize that eosinophils may contribute to remodeling and repair of damaged muscle tissue.

Methods: In this study, we utilized mdx mice, which maintain a spontaneous nonsense mutation in exon 23 of the dystrophin gene and phenocopy DMD. In addition, we utilized interleukin-5 transgenic (*IL5*tg) mice, in which eosinophils are prominent in the muscle tissue in the absence of damage.

To identify eosinophil-derived mediators promoting regeneration, we sequenced RNA extracted from eosinophils isolated from the skeletal muscle tissues of mdx and *IL5*tg mice. For each genotype, we utilized three biological replicates, each consisting of pooled groups of 3-5 male mice at 4 weeks of age. We sorted eosinophils (LiveCD45^{+}CD11c^{-}Gr1^{-}MHC-II^{lo}SigF^{+} cells) from muscle single cell suspensions directly into TRIZOL-LS solution and prepared RNA according to protocol. All RNA samples submitted for sequencing had an RNA Integrity Number (RIN) greater than 8.2, as determined using an RNA PicoChip read on an Agilent Bioanalyzer. We prepared RNA libraries and sequenced pair-end reads using an Illumina HiSeq 4000 NextGeneration sequencer. Then, we trimmed and mapped sequencing reads to the mouse reference genome before quantifying transcript abundance using the RSEM program.

To identify differentially expressed transcripts in muscle eosinophils, we compared normalized gene counts from mdx muscle eosinophils against that of *IL5*tg muscle eosinophils. We identified genes that were significantly upregulated more than 5-fold in mdx vs. *IL5*tg muscle eosinophils using limma's empirical bayes t-test with multiple test correction and voom normalization.

Results & Conclusions: Eosinophils recruited to dystrophic muscle exhibit a distinct transcriptomic profile suggesting that they may contribute to the repair and remodeling processes. Of the more than 390 transcripts that were significantly upregulated in mdx muscle eosinophils, many encoded enzymes that remodel the extracellular matrix (e.g., matrix metalloproteinases 12, 14, 19; cathepsins B, K, S) as well as structural components of the muscle tissue (e.g., collagens 3-6, fibronectin). Furthermore, gene ontology (GO) enrichment analysis revealed that these transcripts detected primarily in mdx muscle eosinophils are associated with biological pathways including collagen binding, integrin binding, and structural components of the extracellular matrix. Interestingly, and consistent with our earlier findings, we detected no differential transcription of pro-inflammatory cytokines (IL-6, TNFa, IFNg) in mdx muscle eosinophils.

Taken together, our findings suggest that eosinophils may modulate the repair of dystrophic muscle damage, and thereby promote resolution of the accompanying tissue inflammation. Moving forward, we are evaluating the extent to which these findings have a direct impact on regeneration of the dystrophic muscle tissue. Accordingly, we are evaluating the impact of eosinophil-deficiency on the regenerative process in dystrophin-deficient muscle tissue, which occurs in mdx mice between 6 and 12 weeks of age. Ultimately, findings from this study will augment our understanding of the molecular mechanisms by which eosinophils promote tissue repair and may reveal novel therapeutic strategies for the treatment of DMD.

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Resolvin D4: Biosynthesis and Actions in Mice Vasculature

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Resolvins are specialized pro-resolving mediators (SPM) that actively stimulate resolution of inflammation. Six members of the D-series resolvins are biosynthesized from docosahexaenoic acid. Resolvin D4 (RvD4) is endogenously produced by human plasma, serum, breast milk, skin, and bone marrow, as well as in murine brain and spleen. Earlier, we established the complete stereochemistry of RvD4 (4*S*,5*R*,17*S*-trihydroxydocosa-6*E*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid) and its potent biological actions including the reduction of neutrophil infiltration in peritonitis and *S. aureus* infection. RvD4 also enhances human macrophages, monocytes and neutrophils phagocytosis. In this presentation, we demonstrate that administration of RvD4 during deep vein thrombosis (DVT) in mice, SPM naturally enriched at the onset of thrombus resolution, significantly reduced thrombus burden, with significantly less neutrophils and more pro-resolving monocytes in the thrombus, as well as an increased number of cells in an early apoptosis state (Cherpokova, D., Jouvene C.C., et al, in press, Blood). Moreover, RvD4 stimulated the biosynthesis of other pro-resolving and anti-inflammatory D-series resolvins. Neutrophils from RvD4-treated mice released less neutrophil extracellular traps (NETs), a meshwork of decondensed chromatin lined with histones and neutrophil proteins critical for DVT development, after induction by ionomycin. These results show that RvD4 attenuates the severity of thrombo-inflammatory disease *in vivo* and improves thrombus resolution.

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Injury Induces MHCII-Dependent Expansion of Specific CD4+ Regulatory T Cell Subpopulations

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Objectives: Traumatic injury causes suppressed adaptive immune function characterized by enhanced CD4+ regulatory T cell (Treg) activity and suppressed Th1-type immunity. Our research group showed that injury triggers acute Treg activation and expansion in injury-site draining lymph nodes, while conventional CD4+ or CD8+ T cells do not react to or expand in response to injury. Thus, we hypothesize that Tregs are uniquely reactive to injury and that danger associated molecular pattern (DAMP) factors or antigens trigger their activation. An objective of this study was to determine if there is a specific subpopulation of CD4+ Tregs that are injury-reactive and to test if Treg expansion by injury is MHC class II dependent.

Methods: Male C57BL/6J and Balb/c mice were anesthetized and subjected to 25% TBSA 3rd degree burn trauma by 9 seconds exposure to 90 C water. Sham mice underwent the same procedure, but were exposed to room temperature water. Injury-site draining lymph nodes were harvested 7 days later. Flow cytometry stains were performed to identify Treg subsets using a panel of non-overlapping fluorescently-tagged CD3, CD4, CD44, and FoxP3 antibodies. Specific T cell receptor variable (TCR-Vβ) chain expression on Tregs was accomplished using a panel of 14 different TCR-Vβ specific antibodies (BD Biosciences). Flow cytometry analysis was performed on a MACS-Quant Analyzer (Miltenyi Biotech). Data analysis was performed using the FlowJo software program (BD Biosciences). Cell events were calculated from equal downsampled CD4+ T cells. Fab fragment of anti MHC class II antibody (clone M5/114) was prepared to block MHCII function and produced using papain digestion (Pierce). Mice were treated with 20 µg/g of Fab anti-MHC class II at 2 hours before and 1 day after burn or sham injury.

Results: We compared relative levels of CD44-high and CD44-low Treg subset expansion in the lymph nodes at 7 days after burn injury. In both C57BL/6 and Balb/c mice, we detected significant expansion of CD44-high, but not CD44-low Tregs in burn as compared to sham mice. Next, we tested if these expanded CD44-high Tregs demonstrate monoclonal, oligoclonal, of polyclonal expansion by staining for TCR-Vβ chain expression on CD44-high or CD44-low Tregs. Among the 14 different TCR-Vβ chain staining antibodies that were tested, we observed that Tregs that express TCR-Vβ 4, 6, 8.1/8.2, 8.3 and 14 were expanded in the CD44-high Treg population in both C57BL/6 and Balb/c mice. No selective TCR-Vβ expansion was observed on CD44-low Tregs or conventional CD4+ T cells. This result provides clear evidence that injury-induces oligoclonal expansion of the CD44-high Tregs. This finding suggested that Treg expansion by injury may be dependent on TCR activation by antigens. To test this hypothesis, we used Fab fragment of MHCII antibody to block MHCII-dependent TCR activation. Burn-injured mice that were treated with Fab MHCII antibody did not show oligoclonal expansion of CD44-high Tregs, which indicates that injury-induced expansion of specific TCR-Vβ expressing Tregs is dependent on functional activation of Tregs by MHCII.

Conclusions: These findings demonstrate for the first time that the CD44-high Treg population is the primary injury-reactive Treg subset. The specific oligoclonal TCR expansion of CD44-high Tregs and the ability to block their expansion by Fab MHCII treatment provides strong evidence that certain Treg subsets react specifically to injury-associated antigens.

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Novel Activation of Immunoresolvent Circuits by Hypoxia Environment

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Targeting hypoxia-sensitive pathways in immune cells is of interest for the treatment of inflammatory diseases, yet little is known about the role of hypoxic local environment in modulating resolution of inflammation. Physiological hypoxia drivescell proliferation, maturation, development, and tolerance in immunologic niches for example bone marrow and lymphoid tissues. In contrast, pathological hypoxia in ischemic tissues and sites of chronic inflammation experience severe and disrupted oxygen gradients that can arise from loss of blood supply and increased oxygen demand from infiltrating neutrophils to sustain their oxidative burst required for microbial killing. Pro-inflammatory lipid mediators including leukotrienes and prostaglandins are produced in pathologic hypoxic tissues, yet the connection between physiologic hypoxic local environments and the resolution of inflammation via lipid mediators remained of interest. In this presentation, we demonstrate that physiological hypoxic environments, accelerate human M2 macrophage efferocytosis of apoptotic neutrophils and senescent erythrocytes via lipolysis-dependent biosynthesis of Specialized Pro-resolving mediators (SPMs), including resolvins, protectins, maresins and lipoxins (Libreros et al., Science Advances, in press). SPM biosynthesis was significantly enhanced in human M2 macrophages interacting with neutrophils and erythrocytes maintained in physiologic hypoxia which enabled the structural elucidation of a novel pro-resolving mediator. We coined this new molecule, as resolvin E4 (RvE4), derived from eicosapentaenoic acid, contains two conjugated dienes with alcohols at carbons 5 and 15, and stimulates efferocytosis of both senescent erythrocytes and apoptotic neutrophils. In a hypoxic environment, metabolic targeting via glycolysis inhibition enhanced neutrophil RvE4-SPM biosynthesis. Human macrophage-erythrocyte co-incubations in physiologic hypoxia produced RvE4-SPM from erythrocyte membrane sources of EPA and DHA. RvE4 demonstrated greater potency for stimulating efferocytosis than DHA-derived resolvins, namely RvD5 and RvD6, as well as enhanced resolution of hemorrhagic exudates *in vivo*, thus establishing a new EPA-derived lipid mediator biosynthetic circuit. These results indicate that hypoxic environments including bone marrow and spleen as well as sites of inflammation activate novel resolvin biosynthetic circuits that in turn stimulate resolution, tissue protection, and clearance of senescent erythrocytes and apoptotic neutrophils.

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Novel Prodrug Formulation of BCL6 BTB-specific Inhibitor Represses T Cell Activation, Tfh Cells, & T-cell Dependent Immune Responses *in Vivo*

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Background: Inhibition of the BCL6 BTB domain results in killing large B-cell lymphoma cells in an *in vivo* xenograft model and reducing the T-cell dependent germinal center (GC) reaction in mice, as well as reversing GC hyperplasia in nonhuman primates. However, the available BCL6 BTB-specific inhibitor, FX1, is a highly lipophilic compound lacking suitable water solubility for *in vivo* use. In this study, we synthesized a pro-drug (AP4-287) of the BCL6 BTB inhibitor, FX1, with the goal of improving water solubility, *in vivo* biodistribution, and increased activity. Our study is expected to provide a foundation for the clinical development of drug targeting the BTB domain of BCL6 for the treatment of diseases associated with abnormal elevated BCL6 expression, eg. B-cell lymphoma.

Methods: First, we first compared aqueous solubility, plasma stability *ex vivo*, and pharmacokinetics (PK) in mice and macaques for FX1 and AP4-287. We synthesized AP4-287 at the Molecular and Cellular Oncogenesis Program, The Wistar Institute. Male adult CD-1 mice (8 weeks old) were used in the PK study: (1) eighteen mice received one administration of FX1 at 25mg/kg in 100µL vehicle that comprised 30% propylene glycol, 65% PEG-400, 5% Dextrose (5%) intraperitoneally (i.p.) and underwent six blood collection with three mice at each time point (0.5, 2, 4, 6, 8 and 24hr later); (2) another eighteen mice received one administration of AP4-287 at 25mg/kg in 100µL vehicle i.p. and underwent similar six blood collections; (3) additional three mice received one injection of 100µL vehicle, underwent blood collection at 24hr later and used as negative control. Plasma samples were fractionated for analysis of AP4-287 and/or FX1 level by HPLC-MS/MS. Second, we compared in *vivo* biological activity using sheep red blood cells (SRBC)-vaccination T-cell dependent immune responses in mice. SRBC-vaccination was performed using male C57/BL6 mice (8-12 weeks old). We first performed one i.p. administration of 100uL 10% SRBC followed with a 3-day resting before drugs or vehicles treatment for an 8-day course. FX1 administration was proceeding once daily at 80mg/kg in 100uL vehicle; AP4-287 treatment was performed three times daily (TID, with 5hr apart) with doses at 25, 10 and 5mg/kg. Vehicle was administered as control with volume and treatment strategy matched either FX1 or AP4-287. After the 8-day course treatment, some mice were euthanized with CO_2 for collecting necropsy samples (blood, spleen and other tissues), and some were resting for 20 days before receiving 2^{nd}SRBC-vaccination and then euthanized at 11 days after 2^{nd}SRBC-vaccination. The blood was used to fractionate plasma for measuring pan and SRBC-specific antibody with ELISA and flow cytometry method; the spleen was split into two parts [half for mononuclear cell isolation following with flow cytometry analysis of activated GC B cells and T

follicular helper (Tfh) cells; half for histology studies including H&E staining and immunochemistry staining].

Results: AP4-287 had a significant higher aqueous solubility (~150 fold comparing to FX1) and readily converted to FX1 2-4 h after administration *in vivo* by the i.p.route. Although AP4-287 administration offered a higher peak FX1 concentration (C_{max}= 26uM vs. 7uM) at 0.5hr after injection, C_{max} declined rapidly due to first pass clearance showing a shorter half-life ($T_{1/2}$, 2.54hr vs. 9.51hr) *in vivo*. We observed that AP4-287 was effective and well tolerated in mice at 25mg/kg (three times daily) for an 8-days course treatment. Comparing to the vehicle, AP4-287 treatment led to (1) the reduction in the frequency of splenic $Ki67^+CD4^+$T cells, $CXCR5^+PD1^+BCL6^+CD4^+$Tfh cells, and activated GC B cells ($B220^+GL7^+CD95^+$); (2) lower GC formation following SRBC-vaccination; (3) decrease in the titers of SRBC-specific IgG and IgM but not pan antibodies. Importantly, AP4-287-mediated repression on GC formation and antibody responses is reversible by resting drug treatment for 20 days.

Conclusions: Our results indicate AP4-287 as a prodrug formulation can be successfully used to improve water solubility and *in vivo* biodistribution of hydrophobic FX1. Our identification of AP4-287 advances the clinical development of drug targeting BCL6 BTB domain, where a BCL6 BTB-specific inhibitor could help diseases associated with abnormal higher GC reaction, aberrant higher autoreactive antibody production, or retained Tfh cells (as a source of viral reservoirs) in ART-treated HIV-infected individuals.

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Identification of Novel A2Ar-dependent $TIGIT^+$ Tregs Associated with Resolution of Ocular Inflammation

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Experimental autoimmune uveitis (EAU) is used to gain a better understanding of human autoimmune uveitis. Resolution of EAU is in part due to emergence of ocular antigen specific regulatory T cells (Tregs) that are found in the spleen of EAU-resolved mice. Another function of the Tregs found in EAU-resolved mice (post-EAU Tregs) is to prevent relapse. The adenosine 2A receptor (A2Ar) is required for emergence of post-EAU $PD\text{-}1^+FoxP3^+CD25^+CD4^+$ Tregs in the spleen. However, $A2Ar^{(-/-)}$ mice still recover from EAU, suggesting there may be multiple subsets of Tregs that emerge during EAU. As such, we utilized $A2Ar^{(-/-)}$ mice as a negative control to identify different Treg cell subsets. We also utilized a $FoxP3^{GFP}$ mouse to monitor Treg cells in the eye and in secondary lymphoid tissues. An A2Ar deficiency delayed the timing of Tregs entering the eye during the course of EAU. Because post-EAU Treg cells express PD-1 we asked if another checkpoint inhibitor, T cell immunoreceptor with Ig and ITIM domains (TIGIT), is expressed on post-EAU Treg cells. We found that post-EAU $A2Ar^{(-/-)}$ mice have reduced $TIGIT^+$ $FoxP3^+$ Tregs compared to post-EAU A2Ar sufficient mice, and we identified three distinct subsets that are PD-

$1^{+}TIGIT^{+}$, PD-$1^{+}TIGIT^{-}$, and PD-$1^{-}TIGIT^{+}$. We next asked which of these subsets have the capacity to suppress EAU by transferring each isolated subset into recipient EAU mice. The mice that received the PD-$1^{+}TIGIT^{-}$ subset showed the most dramatic suppression of disease, and while the $TIGIT^{+}$ subsets did suppress disease compared to mice that did not receive an adoptive transfer, it was not as dramatic as the PD-$1^{+}TIGIT^{-}$ subset. These results suggest that multiple Treg subsets are involved in the suppression of an immune response. Importantly, we have identified a novel subset of post-EAU Treg cells that may have a different role in the suppression of ocular inflammation than the previously characterized PD-$1^{+}FoxP3^{+}CD25^{+}CD4^{+}$ A2Ar-dependent Treg. This work indicates that there may be different Treg subsets that may represent different mechanisms to suppress ocular inflammation.

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RvE1 Promotes Phenotypic Stability of Regulatory T Cells in Periodontitis

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Objectives: Resolvin E1 (RvE1) is an omega-3 eicosapentaenoic acid (EPA)-derived lipid mediator with pro-resolving inflammatory activity and participates in the regulation of chronic inflammation. Regulatory T cells (Tregs) are a subset of T CD4+ lymphocytes whose immuno-suppressive mechanisms and tissue repairing functions are necessary to sustain health. Exposure of Tregs to an inflammatory environment leads them to become pro-inflammatory and osteoclastogenic through losing the expression of their transcription factor master-switch Foxp3 and production of IL-17. The aim of this study was to test the hypothesis that RvE1 restores Treg phenotypic plasticity in periodontitis.

Methods: Experimental periodontitis was induced by placing silk ligatures around the maxillary molar teeth in mice in 10 days. RvE1 (10 nM) was delivered by oral gavage using the ligatures. After sacrifice, the frequency and the total number of IL-17-producing Tregs of cervical lymph nodes and spleen were measured by flow cytometry. The gingival mucosa was processed to analyze mRNA levels of IL-10, IL-6, and IL-17 by q-PCR. Tregs from cervical lymph nodes were purified by immunoseparation; their mRNA and DNA was purified for transcriptional and epigenetic analyses. Splenic Tregs were sorted from healthy animals and cultured in the presence of IL-6 with or without RvE1; the expression of Foxp3 was analyzed by flow cytometry.

Results: Experimental periodontitis resulted in increased Th17-related pro-inflammatory mediators in the gingival mucosa and infiltration of Tregs and Th17 cells in cervical lymph nodes (>2 and >5 fold-change, respectively). Tregs from mice with periodontitis exhibited reduced expression of Foxp3 (loss MFI) and expressed IL-17 (>15%). RvE1 treatment reduced the expression of IL-17 and IL-6 in the gingiva and decreased the frequency and number of IL-17

producing Tregs in cervical lymph nodes. Treatment with RvE1 increased the expression of Foxp3 (10%) in Tregs exposed to IL-6.

Conclusions: RvE1 reduces the expression of Th17 cytokines and the number of IL-17-producing Tregs and enhances the expression of Foxp3 in Tregs suggesting that RvE1 promotes phenotypic stability of Tregs in experimental periodontitis.

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Solute Carrier (SLC) 37A2 Controls Macrophage Inflammation by Regulating Glycolysis Zhan Wang, Yan Nie, Yi Yu, Qingxia Zhao, Manal Zabalawi, Biswapriya Misra, Jeff Chou, Matthew Quinn, Anthony Molina, Martha Alexander-Miller, Charles McCall, Xuewei Zhu
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Macrophages rewire cellular metabolism to support its immune function. Increased flux of glucose through glycolysis and the pentose phosphate pathway relative to mitochondrial oxidation is a prerequisite for pro-inflammatory macrophage activation and function. Solute carrier (SLC) 37A2 is endoplasmic reticulum-anchored phosphate-linked glucose-6-phosphate transporter and is highly expressed in macrophages and neutrophils, but its function is unclear. Here we demonstrated that SLC37A2 plays a pivotal role in macrophage inflammatory activation and cellular metabolic rewiring. We discovered that SLC37A2 is rapidly down-regulated upon innate immune challenge by lipopolysaccharide (LPS). Using unbiased metabolomics analysis, we further showed that deletion of SLC37A2 reprogramed resting macrophages to a pro-inflammatory state, as evidenced by 1) increased glycolytic intermediates 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate; 2) increased TCA cycle anabolic intermediates citrate and a-ketoglutarate, while decreasing TCA cycle-derived immune suppressive mediator itaconate; and 3) decreased kynurenine, while increased nicotinamide, nicotinamide mononucleotide, and increased NAD+ levels. LPS activation for 3 hr accelerated glycolysis, increased mitochondrial respiration, and enhanced proteolysis and lipolysis in SLC37A2 deficient vs. control macrophages. Transcriptomic analysis showed that SLC37A2 deficient macrophages were more pro-inflammatory in response to LPS, as shown by increased expression of M1-like cytokines including IL-6, IL-1b, and TNF-a, and that inhibiting glycolysis normalized the pro-inflammatory response. Conversely, overexpressing SLC37A2 decreased both glycolysis and pro-inflammatory cytokine gene expression. Taken together, the results support the new concept that SLC37A2 is a gatekeeper for glucose-dependent immunometabolic reprogramming in macrophages.

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Resolvin D1 Engages Myeloid Cell-Dependent Revascularization During Ischemia via Its Receptor, ALX/FPR2

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Ischemic injury causes tissue damage and leads to a rapid sterile inflammatory response. The transition from the initial stages of inflammation to resolution and tissue repair is governed in part by endogenous mediators, like the resolvins. Macrophages are cellular targets of resolvins and are important for tissue repair but how resolvins affect transcriptional programs in macrophages is not completely understood. Therefore, we assessed how resolvin D1 (RvD1) modulates global changes in gene expression in bone marrow-derived macrophages using RNA sequencing. Gene ontology enrichment analysis indicated that, consistent with its pro-resolving role, RvD1 increases expression of genes important in innate immunity and host defense. Surprisingly though, RvD1 stimulation also induces a distinct vascular transcriptomic signature in macrophages. The specific genes driving this vascular signature include several critical receptors, ligands and/or effectors of well-established signaling pathways for vascular growth and remodeling. To test whether RvD1 regulates tissue vascularization following injury, we used a murine surgical model of permanent limb ischemia. In this model, we found that RvD1 is temporally produced in the ischemic limb bone marrow, as well as, skeletal muscle. Pharmacologic delivery of RvD1 enhanced perfusion recovery as measured by laser speckle contrast imaging while mice lacking the RvD1 receptor, *Alx/Fpr2*, showed an endogenous defect indicating this pathway is necessary for limb revascularization. Enhancement of perfusion by exogenous RvD1 was completely lost in *Alx/Fpr2*-deficient mice. To determine the contribution of myeloid-derived cells to this defective perfusion recovery, we generated myeloid-specific *Alx/Fpr2*-deficient mice and found a similar impairment in revascularization. Further, several of the vascular genes that were induced by RvD1 in macrophages *in vitro* were downregulated in the ischemic gastrocnemius muscle of myeloid-specific *Alx/Fpr2*-deficient mice. Collectively, these results indicate that the RvD1-ALX/FPR2 axis may reprogram macrophages to promote revascularization and facilitate tissue repair after ischemia.

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Regulation of Adenosine Receptor 2A and Its Role in the Development of Neutrophil Extracellular Trap Formation

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Introduction: Neutrophils are a sub-type of leukocytes that play a key role in the innate immune system. During times of tissue injury and infection, neutrophils are recruited to the site of

inflammation where they are capable of responding to damaged tissue. Neutrophils release their chromatin, known as neutrophil extracellular traps (NETS), into the extracellular environment which can cause inflammation and trigger a host of pro-inflammatory pathways by a process known as NETosis. We discovered that co-culture of mesenchymal stem cells (MSCs) with human neutrophils decreased NETosis and hypothesized as to whether MSC-produced adenosine was involved. Recent literature suggests possible mechanisms that may contribute to NET formation. However, it remains unclear how NETs are regulated and what factors contribute to their production.

Methods: Quantification of cAMP production was used to assess adenosine signaling and determine whether cAMP is a regulator of NETosis in both resting and stimulated neutrophils. Other biochemical assays and techniques including liquid chromatography and mass spectrometry will be used to examine potential enzymes implemented in NET formation.

Results: We have found that adenosine receptor 2a ($A_{2a}R$) mediates the anti-NET effects of adenosine. In addition, treatment with an A2a receptor agonist is effective in increasing cAMP signaling in isolated human neutrophils.

Conclusion: These preliminary experiments suggest that A2a receptor activation is involved in suppression of NETs and that regulation of downstream signaling factors, such as cAMP, may influence their production. Furthermore, MSCs can reduce NETs *in vitro*. Reducing NETs *in vivo* may improve functional recovery after MI/R by reducing inflammation, preventing thrombosis, and other complications. Better understanding of NET properties and examination of downstream mediators may identify therapeutic targets for disease states.

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Stereochemical Assignments of Resolvin Conjugate in Tissue Regeneration 1-3 (RCTR1-3) in Human Tissues Stimulates Proresolving Phagocytes Functions

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Resolvin conjugates in tissue regeneration (RCTR) are a new group of chemical signals that coordinate host responses to accelerate resolution of inflammation and infection via enhancing efferocytosis, organ protection and tissue regeneration. In this presentation, we identified a new member of the resolvin conjugates denoted RCTR3 (8*R*-cysteinyl,7*S,*17*S*-dihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid). This third member, RCTR3 was identified using LC-MS/MS based profiling metabololipidomics in human brain, lymph node, bone marrow, and spleen. After addition of Staphylococcus aureus to human spleens, we obtained endogenous production of RCTR1, RCTR2, and RCTR3. Addition of deuterium labeled-DHA substrate (d_5-DHA) increased production of RCTRs and their precursor by human spleens. We matched each [RCTR1 (8*R*-glutathionyl 7*S*,17*S*-dihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid), RCTR2 (8*R*-

cysteinylglycinyl,7*S*,17*S*-dihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid, and RCTR3] unique stereochemical assignments and actions using materials prepared by total organic synthesis. Synthetic RCTRs confirmed their ability to stimulate human macrophage phagocytosis of Escherichia coli (E.coli), and apoptotic neutrophils giving a rank order potencies of RCTR3 >RCTR2 >RCTR1. Both RCTR2 and RCTR3 significantly reduced neutrophil infiltration into the lungs following hind limb ischemia reperfusion, and reduced eicosanoid amounts (LTB_4, TxB_2), where RCTR3 was found to be the most potent of the RCTRs (de la Rosa et al. *AJP* 2018). Using in vitrochemotaxis with human PMN, all three RCTRs limited migration of human neutrophils towards a potent chemoatractant gradient of LTB_4 where RCTR3 gave greatest action (rank order of potency RCTR3 >RCTR2 >RCTR1). Each RCTR dose-dependently (1-100nM) accelerated tissue regeneration in planaria shortening tissue regeneration index (TRI_{50})~0.5 days at the optimal concentration of 1nM. RCTRs were less potent than either MCTRs or PCTRs in promoting tissue regeneration. Taken together these results identify a new RCTR and establish the complete stereochemistry and rank order potencies for RCTR1, RCTR2 and RCTR3. In addition, RCTRs are produced in human organs, exert potent anti-inflammatory and pro-resolving actions with human leukocytes.

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Transcriptional and Epigenetic mechanisms Underlying Heightened Pro-inflammatory Responses of Monocytes with Chronic Heavy Alcohol Consumption

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It is well established that chronic heavy drinking (CHD) increases the incidence of a multitude of health problems including cardiovascular disease, certain types of cancers, and alcoholic liver disease. CHD is also associated with increased susceptibility to infection as well as decreased wound healing and tissue repair capacity. Evidence suggests that these defects are mediated by a dysregulated inflammatory response originating from myeloid cells, notably monocytes and

macrophages. However, most of the data is derived from *in vitro* studies where cells are treated with alcohol leaving the physiological mechanisms by which CHD disrupts monocyte/macrophage function poorly understood. We aim to uncover the mechanisms leading to altered monocyte function using a rhesus macaque model of voluntary ethanol self-administration. We have shown previously that 12 months of CHD results in large gene expression changes in peripheral blood mononuclear cells (PBMC) from female macaques that enrich to innate immune processes. Indeed, stimulating these PBMC with LPS resulted in a hyper-inflammatory response based on the pattern of immune mediator production as well as the transcriptional response. Next, we profiled PBMC miRNA from the same female macaque cohort to assess possible post-transcriptional modifications contributing to the gene expression changes. Our studies revealed several differentially expressed miRNA with validated gene targets amongst our published PBMC differentially expressed genes. We next examined the response of purified monocytes to delineate their role in this hyper-inflammatory response. This analysis confirmed that CHD leads to an over-production of inflammatory mediators including increased production of TNFα. We then for the first time showed large transcriptional differences in the response of purified monocytes from CHD animals to LPS compared to control samples. These transcriptional changes suggest that CHD may alter the activation/regulation of the adaptive immune system, which could play a role in poor responses to infection. Finally, we profiled changes to chromatin accessibility within purified monocytes by ATACseq and identified a greater number of open promoters and intergenic regions with CHD. In the future we plan to further elucidate the epigenetic mechanisms leading to the altered inflammatory responses by CHD monocytes.

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Neutrophil Extracellular Traps (NETs) Are Induced by Alcohol and Neutrophil Depletion Attenuates Liver Injury in Alcoholic Liver Disease in Mice

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Background: Continued excessive alcohol consumption causes alcoholic liver diseases (ALD) and its most severe clinical manifestation of alcoholic hepatitis (AH). Neutrophil infiltration in the liver is considered as a key pathologic finding in the diagnosis of AH. However, little is known about neutrophil functions in the pathogenesis of AH. Neutrophils release decondensed chromatin mixed with granular proteins including neutrophil elastase, called neutrophils extracellular traps (NETs) in response to various pathogen- and damage-associated molecules. NETs release is an effective anti-microbial strategy; however, uncontrolled NETs production prolongs inflammation, which can exacerbate tissue damage. A recent study reported that alcohol itself induces spontaneous NETs formation, suggesting that neutrophils may contribute to the pathogenesis of alcoholic hepatitis by releasing NETs.

The aim of this study was to examine NETs production in the liver in patients and in a mouse model of ALD and to evaluate the effect of neutrophil depletion on liver damage, inflammation and macrophage differentiation in a mouse model of ALD.

Method: To induce alcoholic liver disease, 8-10 week old C57Bl/6 female mice received chronic alcohol treatment, with Lieber-DeCarlie diet containing 5% ethanol (EtOH) or calorie matched liquid diet (pair-fed, PF) for 4 weeks. *In vivo*NETs formation was accessed by double immunofluorescence (IF) staining (Neutrophil elastase and histone H3) with human and mouse liver specimens. 200ug of either anti-Ly6G (1A8) or isotype antibody (average 20g body weight) was injected into mice twice (48 hours of time interval) prior to the end of chronic alcohol feeding for neutrophil depletion *in vivo*. We measured serum ALT and cytokines by ELISA. The liver tissues were perfused with Collagenase and used to quantify neutrophils as well as infiltrating macrophages and monocytes by flow cytometry. Unpaired student t-test was used for statistical analysis.

Results: Chronic alcohol feeding resulted in features of ALD, indicated by increase of serum alanine aminotransferase (ALT) and inflammatory cytokines including monocyte chemoattractant protein 1 (MCP-1) and interleukin-6 (IL-6) as well as steatosis in the liver. We found significant neutrophil infiltration by using flow cytometry and increased NETs formation as determined by double IF staining in the livers of ALD patients and EtOH-fed mice compared to the healthy controls and PF mice, respectively. *In vivo* neutrophil depletion, confirmed by flow cytometry, prevented EOH-induced NETs formation in the liver from EtOH-fed mice. Alcohol-induced ALT increase was reduced in the mice treated with anti-Ly6G antibody compared to the mice group received isotype control antibody. Serum levels of MCP-1 and IL-6 were also decreased upon administration of the anti-Ly6G antibody compared to treatment with isotype control antibody, showing alcohol-induced liver damage and inflammation were attenuated. In addition, we observed that CD80 (a classically activated macrophage marker) and CD206 (an alternative activated macrophage marker) were both upregulated upon neutrophil depletion in alcohol-fed mice. Moreover, F4/80 high CD11b high Ly6C high macrophage and Ly6C high inflammatory monocyte populations were increased in alcohol-fed mice and the cell counts decreased with neutrophil depletion, while the number of Ly6C low anti-inflammatory monocyte population increased in the liver from the mice received anti-Ly6G antibody.

Conclusion: Increased NETs formation in the mouse model of ALD contributes to liver damage, and depletion of “pathogenic” neutrophils can alleviate liver damage through prevention of NETs release and reducing inflammatory macrophage and monocyte populations in ALD.

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The Role of Checkpoint Regulators in Trauma Induced Immunosuppression: T-Lymphocyte Co-expression of HVEM and BTLA and Immunoparalysis

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Introduction: Trauma induces significant immune-deficiency associated with secondary infection, long term organ dysfunction and increased risk of death, and, patients who develop secondary infections fair worse than their counterparts when matched for injury severity and age. Co-regulatory molecules are central mediators of the immune dysfunction of critical illness, associated with lymphocyte loss and dysfunction. HVEM, a TNF family transmembrane receptor expressed diffusely on solid organs and immune cells, is one such coregulatory molecule. HVEM serves a bidirectional switch, poised to both stimulate and inhibit immune function based on cues from its surrounding environment. HVEMs interacts with a variety of ligands and its ultimate signal is dependent on which it binds. In addition to the HVEM can be co-expressed with ligand BTLA, forming an inert complex which prevents immune cells from responding to environmental stimuli. Altered expression of HVEM and its ligand BTLA have been associated with poor outcomes and increased nosocomial infections in critically ill septic patients, and altered HVEM expression has been noted after trauma. As infections drive poor long-term trauma outcomes, we hypothesized critically ill trauma patients will display altered T lymphocyte BTLA expression with an increase in HVEM/BTLA co-expression.

Methods: Following IRB approval, critically ill trauma patients, aged 18 years and older, were prospectively enrolled from the Trauma ICU, and compared with age matched healthy controls. Whole blood was obtained, leukocytes were isolated using gradient separation and stained using monoclonal antibodies for CD3 (lymphocytes), BTLA, and HVEM and analyzed using flow cytometry. Post-analysis was completed using SigmaPlot software. Charts were reviewed for injuries sustained, APACHE II score, and hospital course including development of secondary infections.

Results: Trauma patients (N=9) compared with healthy controls (N=4) were slightly older (51.78 +/-6.98 vs 29.5+/- 4.11 years; p=0.069) but matched for male sex (70% vs 54%; p=0.52). Trauma patients had elevated presenting White Cell Counts (17.96 +/- 3.22 x106/ml), and APACHE II scores (17.89 +/- 3.04), with an overall mortality of 11%. When compared to healthy controls, trauma patients demonstrated lower percentage of CD3+ lymphocytes (16.3% +/-6.1 vs 54.24% +/-5.7; p=0.025), significantly greater expression of HVEM on CD3+ lymphocytes (59.2% +/-4.5 vs 9.1% +/-4.7; p< 0.001), but similar BTLA expression on CD3+ lymphocytes (36.45% +/- 4.8 vs 25.48% +/- 2.2%; 0.172). Trauma patients demonstrated a significant increase in co-expression of HVEM and BTLA on CD3+ lymphocytes (36.0% +/- 6.5 vs. 2.73% +/- 0.56, p=0.01).

Discussion: Trauma induced immune suppression represents a well-documented, but poorly understood, phenomenon. Lacking direct targeted treatment, it accounts for substantial

morbidity, and mortality in trauma patients. HVEM signaling has been shown to mediate host immunity at mucosal barriers in mice, and HVEM deletion increases septic mortality. In humans, HVEM expression increases on T-lymphocytes after trauma, with a strong correlation to secondary infections in trauma patients. In keeping with these prior observations on critically ill patients, we demonstrate increased HVEM expression on CD3+ cells after traumatic stress. BTLA expression on CD3+ cells was found to be similar in trauma patients and healthy controls, however there was significantly higher co-expression of HVEM and BTLA on CD3+ lymphocytes after trauma, with an average of 36% of remaining T lymphocytes demonstrating co-expression. Given co-expression results in inert complexes where HVEM is unable to provide its usual signal instructing the T-cell on the most appropriate response to its environment, this represents a plausible mechanistic explanation of trauma induced immune suppression/paralysis, and potential therapeutic target, warranting further exploration.

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Clearance of Extracellular Proteins by Immune Cells During Resolution of Inflammation in the Liver Visualized by Intravital Microscopy

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Intravital (*in vivo*) microscopy is a revolutionary imaging technique that allows to follow dynamic processes in living animals in real time. With appropriate tissue preparation and use of target-specific monoclonal antibodies it is possible to track the cells of interest and observe their interactions with other cells and structures in their natural environment. We aimed to study such processes during resolution of inflammation when leukocyte activity is quenched and debris clearance occurs. In the study we followed resolution of lipopolysaccharide-induced sepsis in C57BL/6J mice, focusing on kinetics of clearance of extracellular proteins deposited in vasculature during the immune response. Furthermore, we aimed to verify which cells are involved in the clearance. Applying the spinning-disk confocal microscopy we focused on the engulfment of neutrophil elastase by the immune cells in the sinusoids of the inflamed liver. Using advanced image analysis software (MeasuremntPro, IMARIS), we reconstructed a 3D structure of the liver and cells present therein from a serial optical-scans (*z-stacks*) of the imaged organ. First, we verified specificity of our approach using appropriate isotype controls and Fc block antibodies. Next, we were able to identify the engulfing cells (phagocytes) and measure the amount of intracellular elastase (MeasuremntPro). Having determined that, now we can focus on identification of specific receptors involved in this process. Altogether, this novel approach allowed us to create the basis for further research on the clearance of various molecules and structures from blood and/or endothelium.

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Lipid Dysregulation of Immune Mediated Intestinal Epithelial Healing

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It is clear that long term exposure to high fat diets (HFD) results in obesity and systemic inflammation and can exacerbate many disorders including inflammatory bowel disease (IBD). However, the direct effect of lipids on tissue homeostasis and repair remain undefined. We show here that short term exposure to HFD results in reduced barrier repair after intestinal epithelial damage with increased accumulation of apoptotic neutrophils. After barrier breach, neutrophils are recruited to clear invading bacteria after which they undergo apoptosis followed by uptake by tissue macrophages in a process termed efferocytosis. Efferocytosis initiates a pro-repair program including upregulation of the anti-inflammatory cytokine IL-10. We find dietary lipids directly interfere with macrophage recognition and uptake of apoptotic cells, and subsequent IL-10 production, after intestinal damage by blocking interactions between apoptotic cells and the efferocytosis receptor CD36, which also binds dietary lipids. Overexpression of IL-10 rescues repair defects after HFD, but not if epithelial cells lack the IL-10 receptor, highlighting the key role of IL-10 in barrier repair. These findings demonstrate a previously unidentified mechanism by which dietary lipids, a risk factor for intestinal disease, can directly interfere with homeostatic processes required to maintain tissue integrity.

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Uveitic Retinal Pigment Epithelial Cells Do Not Suppress the Phagocytic Antigen Processing Pathways in Antigen Presenting Cells.

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Purpose: We have reported that the use of the neuropeptide α-MSH is effective in suppressing experimental autoimmune uveitis (EAU), a mouse model of human ocular autoimmune disease. Through melanocortin 5 receptor (MC5r), α-MSH-therapy induces systemic regulatory immunity to retinal antigens. When MC5r is knocked-out, α-MSH suppresses the inflammation of ocular autoimmune disease, but does not induce the regulatory immunity. In addition, their post-EAU retinas are greatly damaged, and susceptible to a subsequent induction of EAU. This has suggested that MC5r may be important in α-MSH restoring ocular immune privilege. We examined for the recovery of retinal pigment epithelial cells (RPE) following α-MSH-treatment of EAU mice to suppress the phagocytic pathway associated with antigen processing and presentation.

Methods: EAU was induced in C57BL/6 mice by immunizing the mice against Interphotoreceptor binding protein in complete-CFA with two injections of pertussis toxin. At the chronic phase of EAU, based on clinical scoring, the eyes were collected from euthanized mice, or the mice were injected twice, one day apart, with α-MSH (50 µg/mouse), MC5r-agonist PG901 (50 µg/mouse),

or PBS carrier. When the uveitis was resolved in the eyes treated with α-MSH, the eyes were collected. The anterior segment and the retina were removed to make the RPE eyecup. The RPE eyecups were incubated in serum-free medium for 24 hours, and the conditioned medium (CM) was collected. CM from healthy RPE eyecup cultures served as expected immunosuppressive controls. To assay the effects of RPE CM on APC to antigen-stimulate T cell activation, peritoneal macrophages were fed opsonized-OVA and treated with CM from healthy or EAU mice for24 hours. The cells were washed, and $CD4^+$OVA-specific T cells were added to the cultures. After 72 hours, T cell proliferation was measured. To assay for RPE regulation of phagolysosome activation, macrophages were fed 0.02 mg of opsonized pHrodoRed-Staphylococcus aureus bioparticles, and incubated for 24 hours. The cells were imaged for red fluorescence intensity. Ten images were captured per well, and total cell fluorescence minus the background was calculated.

Results: The macrophages treated with healthy CM were significantly suppressed by 55 ± 6% in stimulating OVA-specific T cell proliferation. In contrast, the EAU RPE-CM had no significant affect, 90 ± 4% proliferation of OVA-specific T cells. RPE CM from EAU mice treated with α-MSH, but not with MC5r-agonist suppressed phagolysosome activation in macrophages. While α-MSH-treatment of $MC5r^{(-/-)}$ mice suppressed EAU, it did not recover RPE CM suppression of phagosome activation in macrophages.

Conclusion: There is a beneficial therapeutic effect of α-MSH-treatment on suppressing uveitis. While there is a dependency on MC5r-expression for α-MSH to promote RPE recovery of immunosuppressive activity, it is not the only melanocortin receptor required for α-MSH mediated suppression of inflammation, and potential recovery of ocular immune privilege.

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IFN-γ and IL-17A Selectively Induce and Regulate Intestinal Crypt Production of CXCL10 in the Healthy and Inflamed Colon

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Inflammation in the Gastrointestinal (GI) tract drives recruitment of inflammatory leukocytes including T_H1 and T_H17 T lymphocytes and subsequent production of effector molecules, including potent cytokines and chemokines. Whether colonic crypts remain responsive to these cytokines during active damage and repair, and how T_H1/T_H17-associated cytokines directly influence the damaged and regenerating colonic epithelium remains unclear. Here, using laser-capture microdissection and primary colon spheroid culture we show that IFN-γ induces the T_H1-recruiting, pro-inflammatory chemokine CXCL10/IP10 in the primary murine intestinal crypt epithelium, and *ex-vivo* in colonic spheroids derived from mice with active, experimentally-induced colitis, suggesting that the crypt can actively secrete CXCL10 in select cytokine microenvironments. Colon expression of *cxcl10* further increased during infectious and non-

infectious colitis in *Il17a*$^{-/-}$ mice, demonstrating that IL-17A exerts a negative effect on CXCL10 *in-vivo*. Further, IL-17A directly antagonized CXCL10 production in spheroids derived from healthy murine colons. Interestingly, direct antagonism of CXCL10 was not observed in spheroids derived from colitic mouse colons bearing active lesions. These data, highlighting the complex interplay between the cytokine milieu and crypt epithelia, demonstrate pro-inflammatory chemokines can be induced within the colonic crypt and suggests the crypt remains responsive to cytokine modulation during inflammation. Ultimately, cytokine modulation of the crypt may influence whether pro-inflammatory leukocytes continue to be recruited to the actively inflamed intestines. These results highlight complex interactions between leukocytes and regenerating intestinal crypt epithelium that critically promote tissue regeneration and restore barrier integrity. Moreover, our observations suggest damaged/regenerating colon crypts differ in responses to cytokines during inflammation of the GI mucosa.

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Short Chain Fatty Acid Analysis in Traumatic Brain Injury Patients

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Introduction: Traumatic brain injury (TBI) is a leading cause of morbidity and mortality in the United States. It is well known that TBI can lead to both organ and immune dysfunction and there is evidence to suggest that the gastrointestinal tract may play a central role in driving this dysfunction in the post-TBI period. Stress and inflammation in the post-TBI period may lead to dysbiosis and gut barrier disruption, which allow extra-intestinal translocation of bacteria and bacterial products that can activate the immune response, resulting in secondary insult. Short chain fatty acids (SCFAs), i.e. acetate, propionate, and butyrate, are the main metabolites of our gut bacteria. They are reduced in states of dysbiosis and may contribute to upregulation of the pro-inflammatory pathway. SCFAs are also thought to be involved in gut-brain signaling via vagal, endocrine, and immune pathways. We hypothesize that patients who have sustained a TBI will have changes in their SCFA profile different from those of non-head injury trauma patients, and that the changes will be time dependent.

Methods: Male and female patients ≥ 18 years or older were recruited to our study upon admission to our ED after sustaining a traumatic injury. Patients were identified as either TBI, diagnosed by evidence of head injury on CT scan, or trauma control, which is trauma without evidence of TBI on CT. Blood was collected at three time points: within 24 hours of admission, 3-5 days after admission, and 7-10 days after admission. Samples were spun at 2,000 rpm for 10 minutes at 4° C. Plasma and cells were separated, and plasma was sent to the University of Illinois at Chicago Core for HPLC-MS to isolate and quantify levels of acetate, propionate, and butyrate. Plasma levels were reported as µM. SCFA data for TBI and trauma control samples were stratified

on various parameters, including sex, race/ethnicity, alcohol use at time of injury, antibiotic use during sample collection, and injury severity (GCS).

Results: Of our enrollees, 71% were in the TBI group and 69% were male. Caucasians made up 76%, African Americans 12%, Asians 7.3%, and Hispanics 5%. A positive blood alcohol level (BAC) on admission was found in 26%, antibiotics were administered during the hospital stay in 57%, and GCS level was ≤ 8 in 14%. We found no significant difference in any SCFAs between TBI and trauma controls at any time point. We observed a downward trend as time progressed for all three SCFAs in both TBI and trauma control groups from 24h to 3-5d. However, only acetate had a further decrease from 3-5d to 7-10d and all decreases were statistically insignificant. Due to inadequate sample size for each time period, we grouped data from different time points together for further analysis. We observed a significant increase in propionate in females compared to males in both the TBI and trauma control groups. We found significantly increased levels of all three SCFAs in the Caucasian population compared to non-Caucasians in the TBI group. There was also a significant increase in propionate in the non-Caucasian group in trauma control compared to TBI. Positive BAC on admission was associated with a significant decrease in acetate and butyrate in the TBI group and significant decrease in acetate and propionate in the trauma control group. Antibiotic use was associated with a significant decrease in butyrate for the TBI group only and GCS had no apparent impact on the level of SCFAs.

Conclusion: Together these data suggest no difference in SCFA changes in response to TBI and trauma control. However, being female and Caucasian appear to be associated with increases in plasma SCFAs levels after TBI. While we did not see a significant change in time, we did observe a stepwise downward trend. Further sample collection and analysis are in process to substantiate our findings.

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The Effects of CpG-ODN Therapy for Trauma on Immune Cell Cytokine Production Profiles

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Objectives: Traumatic injuries remain the leading cause of death in people under the age of 45, especially in patients that develop nosocomial infections. It is critical to advance our understanding of how traumatic injuries alter immune system phenotype and function. Many studies have demonstrated that CpG oligodeoxynucleotides (ODNs), acting as TLR9 agonists, have immunostimulatory effects on cellular and cytokine responses to infections, injury, and cancer. Our research group is developing CpG-ODNs as a treatment to restore immune system function and balance after traumatic injury in a pre-clinical mouse burn trauma model. In this study, we tested the effects of CpG-ODN therapy on modulating cytokine expression in different

immune cell subsets over time in burn-injured mice using CyTOF mass cytometry as a single-cell phenotyping approach.

Materials and Methods: Male wild type C57BL/6 were subjected to 25% total body surface area (TBSA) 3rd degree burn injury. Groups of mice receiving A-class CpG-ODN 2336 (CpG) treatment were subcutaneous injected with 0.2 mg/kg CpG in saline at 2 h after burn-injury. Lymph nodes were harvested from individual mice at 1 day or 7 days after burn. To induce cytokines, cells were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate and 1ug/ml Ionomycin at 37℃ and under 5% CO_2 for 4h with Protein Transport Inhibitor Cocktail to allow for intracellular accumulation of cytokines. CyTOF mass cytometry was used to profile immune cell populations and cytokines expression simultaneously by staining with a 33-marker antibody panel. CyTOF data analysis was conducted using viSNE, Phenograph, SPADE, and clustering in Cytobank and R.

Results:

Influence of burn and CpG treatment on the composition of immune cells (Figure 1a). At day 1, burn injury increased CD4+ T cells and decreased B cell numbers in the lymph nodes. CpG treatment reversed these changes. At day 7 after burn innate immune cell types increased as compared to sham mice. Although CD4+ T cell numbers did not increase at day 7, CD4+ regulatory T cells (Tregs) increased significantly after burn. Interestingly, CpG treatment further enhanced this increase in innate cell types and Tregs.

Influence of burn trauma and CpG treatment on cytokine production in different immune cell subpopulations at day 1 (Figure 1b). In CD4+ T cells, the percentage of TNFα+ cells was not affected by burn. CpG treatment increased the percentage of TNFα+ CD4+ T cells. In CD8+ T cells and γδT cells, burn injury reduced TNFα production. CpG treatment restored TNFα production by γδT cells. Moreover, CpG treatment caused higher percentages of γδT cells expressing IL-2 and IFNγ. We also found that γδT cells produced high IL-6 levels after burn, but CpG treatment lowered IL-6 to normal levels. In Tregs, IL-10 production was low after burn and CpG treatment had not effect, but CpG treatment caused high TNFα and IL-2 by Tregs in burned mice. IFNγ production by NK cells was suppressed by burn injury and CpG treatment reversed this suppression.

Influence of burn and CPG treatment on the production of cytokines in different immune cell subpopulations at day 7 (Figure 1c). At day 7, more CD4+ T cells produced TNFα, while less produced IL-6 after burn and CpG treatment did not change this trend. The IL-2+ CD4+ T cells increased after burn, and CpG treatment increased it further. In CD8+ T cells, burn injury increased TNFα, but decreased IL-6. CpG treatment induced more CD8+ T cells to produce IFNγ and IL-2. After burn injury, γδT cells produced more TNFα, IFNγ and IL-2 than sham mice, but the IL-17A decreased. CpG treatment restored IL-17A production to normal levels, and increased TNFα and IFNγ expression in γδT cells. In Tregs, burn increased TNFα and decreased IL-10 production and CpG treatment restored Treg IL-10 production. In NK cells, CpG treatment increased the IL-17A and GM-CSF production.

Conclusion: We demonstrate by a multidimensional CyTOF staining approach that traumatic injury alters cytokine production by γδ T cells and Tregs and that these changes in cytokine production can be reversed by CpG-ODN treatment. Taken together, these findings are the first to identify the cellular targets and influence of CpG-ODN immunotherapy on cytokine production by different immune cell subsets.

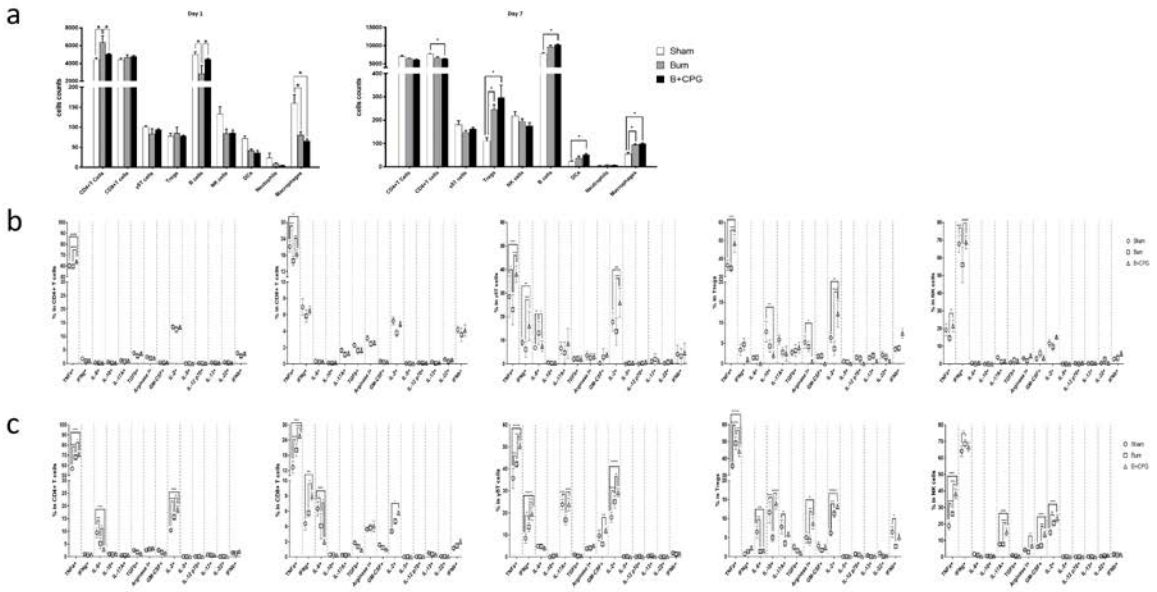

Figure 1 a: Composition of immune cells at day 1 and day 7; b: Production of cytokines in different subpopulations at day 1; c: Production of cytokines in different subpopulations at day 7

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Resolvin D-series Mediate Phagocyte Functions During Inflammation and Resolution by Regulating Phospholipase D

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A successful acute inflammatory response results in the elimination of infectious agents by neutrophils and monocytes, followed by resolution and repair by tissue-resident and recruited macrophages. D-series resolvins are pro-resolving mediators involved in resolution and tissue repair, whose signaling mechanism remains of interest. In this study, we found that resolvins activate phospholipase D (PLD), a ubiquitously expressed membrane lipase that hydrolyzes phosphatidylcholine (PC) into free choline and phosphatidic acid (PA), and has a pivotal role in various phagocyte functions. Resolvin-D4 (RvD4) and, to a larger extent, Resolvin-D5 (RvD5) regulated macrophage PLD gene expression (Pld1-Pld6), in naïve (M0), pro-inflammatory (M1) and anti-inflammatory (M2) macrophages, as well as increased PLD protein expression, enzyme activity, phagocytosis and efferocytosis. PLD2 itself induces macrophage polarization from

CD80+-M1 to CD206+-M2 phenotypes, resulting in increased efferocytosis by M2 macrophages, enhanced by RvD5. The mechanism for PLD-mediated actions of RvD5 in polarizing macrophages (M1-M2) is two-pronged: (a) RvD5 inhibits post-transcriptional mechanisms such as miRs and 3'exonulceases that process PLD2 mRNA transcripts, thus increasing PLD2 expression and activity; (b) RvD5 enhances PLD2-S6K signaling and Actin expression required for membrane expansion and efferocytosis. Further investigating RvD5's pro-resolving actions on second organ reflow injury, we found that RvD5 reduced lung neutrophil myeloperoxidase (MPO) levels in WT and PLD1-/- mice, but not in PLD2-/- mice, pointing to a novel role of PLD2 as the relevant isoform in RvD5-mediated resolution. This study provides compelling evidence that RvD5-PLD2 may be an attractive target for therapeutic intervention in inflammatory conditions like I/R injury, cardiovascular diseases.

Respiratory Allergic Diseases

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The Enigmatic Role of Sphingolipid Signaling in Asthma

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The global burden of respiratory diseases is staggering. A recent report in JAMA highlighted the significant impact respiratory diseases have in the US and that regions of Appalachia lead the nation in both incidence and mortality from chronic lung disease. Previous work by our group has shown that sphingolipids play an important role in lung immunopathology. Our laboratory has shown that ceramide is upregulated in pulmonary inflammation in mouse models of pneumonitis and is elevated in the exhaled breath condensate of mechanically ventilated patients with severe asthma. Using murine models including *Alternaria alternata* and house dust mite, we observe a significant increase in sphingolipids post allergen challenge in both the BALF and lung tissue of mice. We also see corresponding increases in inflammatory cell infiltration, mucus hypersecretion, and airway hyperresponsiveness (AHR). We also demonstrate that administration of FTY720/fingolimod, fumonisin B1, or myriocin had differing effects based on timing of administration. We found a surprising role for sphingosine 1 phosphate (S1P) in airway inflammation, especially in early, innate events. Exciting preliminary data suggest that S1P may specifically be important in the development of allergic airway disease. We also found that ceramide levels strongly correlate with AHR. Additionally, we find a novel role for ceramide in obesity exacerbated asthma, which is a clinically relevant and hard to treat endotype. These data highlight an important and emerging multi-faceted role of sphingolipids in pulmonary biology and that pharmacologic targeting of this pathway may be beneficial for the control of airway inflammation and AHR. Blocking these lipid mediators may open the door for novel, steroid sparing therapeutics, especially for the control of asthma.

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Defining Cellular Heterogeneity in Asthma with Multiparameter Single Cell Profiling of Airway Inflammatory Cells

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Asthmatic patients have wide variation in clinical severity and severely affected patients are not controlled by current medication regimens. Recent studies of airway hyper-reactivity suggest that asthma reflects distinct disease endotypes with distinct inflammatory mechanisms. Elucidating cellular phenotypes may distinguish novel immunophenotypes of airway immune cells that are associated with uncontrollable disease and identify novel therapeutic targets. Detailed investigation of airway cells has historically been challenging but recent technical advances of mass cytometry or CyTOF (Cytometry by Time-Of-Flight) allows the in-depth characterization of cells. We have taken advantage of multiparameter detection by CyTOF for detection of >40 markers in airway cells to facilitate unprecedented multidimensional cellular analyses.

We collected primary airway cell samples by sputum induction with hypertonic saline from healthy controls and well-characterized adult asthmatic subjects. Sputum samples were processed immediately on the day of isolation using sputolysin to generate cell suspensions. Cells were washed, and processed for labeling with a 40-marker antibody panel comprised of cell surface lineage markers and intracellular markers. Prior to sample acquisition on CyTOF, metal-labeled calibration beads were added to each sample for instrument normalization. CyTOF files were bead normalized and assessed for standard gating based on key cellular markers and excluding debris, dead cells, doublets, and epithelial/squamous cell populations. Statistical analysis of live single cells was performed using Prism 6 software and further automated clustering, computational analysis, and visualization using Cytobank, FlowSom, and tSNE.

Airway immune cells in both controls and asthmatic subjects are CD45+ and predominantly granulocytes and monocyte/macrophages with low frequency of T- and B-lymphocytes and NK cells. We quantified cell frequency and functional status of sputum cells and detected distinct differences between healthy controls and asthmatic subjects. In initial studies, asthmatic subjects showed a higher frequency of both eosinophils and neutrophils with elevated levels of IL-6, CD69, and IL-5, critical markers of cell activation. In contrast, sputum granulocytes from healthy subjects were enriched for baseline activation status. Differences in of cellular activation were also noted between macrophage populations of healthy and asthmatic subjects, with healthy subjects having a higher proportion of $CD11c^{+}$ $HLA\text{-}DR^{+}$ activated myeloid subsets, reduced proportions of key markers including IL-3 receptor, IL-7 receptor, and distinct profiles of multipotent $IL\text{-}8^{+}$, TNFa+, $CD14^{+}$ macrophages and expression of MIP-1b.

We have developed a method for sensitive and reproducible deep immunophenotyping of airway immune cell subsets in asthmatic patients. Our method is being utilized in an ongoing multicenter clinical trial protocol that will provide a rich dataset on the effects of a therapeutic intervention on airway inflammation. Integrating such findings with transcriptional profiling in induced sputum of cells from asthmatic subjects can lead to predictive methods to identify distinct patient subtypes. This single cell resolution dramatically improves our understanding of immune responses in the airway and will support detailed classification of patients and distinct inflammatory mechanisms. This approach is especially relevant to our understanding of the effectiveness of therapies given the growing use of biologics to treat severe asthma.

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Aspergillus Fumigatus **cell Wall Promotes Airway Epithelial Recruitment of Human Neutrophils**

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*Aspergillus fumigatus*is a ubiquitous fungal pathogen capable of causing multiple pulmonary diseases including invasive aspergillosis, chronic necrotizing aspergillosis, fungal colonization, and allergic bronchopulmonary aspergillosis. Intact mucocilliary barrier function and early airway neutrophil responses are critical for clearing fungal conidia from the host airways prior to establishing disease. Following inhalation, *Aspergillus* conidia deposit in the distal airways where they are likely to make their initial host encounter with epithelial cells. Challenges in airway infection models have limited the ability to explore early steps in the interactions between *A. fumigatus* and primary human airway epithelium. Here, we use inverted air-liquid interface cultures to demonstrate that human airway epithelium can respond to apical stimulation to *A. fumigatus* to promote transepithelial migration of neutrophils from the basolateral membrane surface to the apical “airway” surface. Promoting epithelial transmigration with live *Aspergillus* required prolonged exposure with live resting conidia. Forming swollen conidia did not expedite epithelial transmigration of neutrophils. Using *A. fumigatus* strains containing genetic deletions of cell wall components, we identify that the lack of a hydrophobic rodlet layer or DHN-melanin in the conidial cell wall accelerate the epithelial transmigration of neutrophils. This activity was confirmed in primary human airway epithelium as well. Ultimately, we show that an as-yet unidentified non-secreted cell wall component is required to promote early epithelial transmigration of human neutrophils into the airspace in response to *A. fumigatus*.

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The Role of Neutrophil Extracellular Traps in Rhinitis of Infectious and Allergic Origin

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Introduction: Neutrophil extracellular traps (NETs) are structures released by neutrophils in response to infections. The biocidal role of NETs has been demonstrated against bacteria, fungi, viruses and parasites. Depending on the situation, these structures protect the host from pathogen invasion or contribute to the development of many autoimmune diseases such as cystic fibrosis and rheumatoid arthritis, often onerous and life-threatening. In our work, we focused on the NET occurrence in the secretions of the upper respiratory tract in infectious and allergic diseases.

Methods: The nasal mucus was collected from donors qualified as infectious rhinitis (in influenza season) or allergic (in the spring season). The material was weighed and divided for further analyzes. Each sample was microscopically analyzed to determine the content of extracellular DNA using SytoxGreen staining. The samples were also digested with micrococcal nuclease (MNase), and the total protein pool was determined by the micro BCA method. The proteins contained in NETs, i.e., elastase, myeloperoxidase, cathepsin G, proteinase 3, were identified by Western blotting, and the enzymatic activity of elastase and myeloperoxidase in these structures was analyzed.

Results: We found that the nasal mucus collected from patients with infectious rhinitis contained an extracellular DNA that is responsible for increased density and viscosity of secretions. Enzymatic degradation of DNA significantly reduced the viscosity of the tested samples. The microscopic imaging of extracellular DNA showed that it is the main component of mucus, with the structure similar to that in NETs. The DNA component was not identified in the samples of allergic patients. The total concentration of proteins in the samples collected from donors with the infection was much higher than in samples collected from allergic patients. Moreover, the presence of the NET proteins and the activity of elastase and myeloperoxidase was confirmed in infection-induced secretions.

Conclusions: Inflammation of the upper respiratory tract leads to the release of large amounts of mucus into the nose and paranasal sinuses. Our current findings suggest that the composition of the nasal mucus is variable and depends on the cause of mucosal irritation. We demonstrated that NETs are a part of the nasal secretions from donors with infections of the upper respiratory tract. NETs were not identified in allergic patients, indicating a different response of the neutrophils recruited to the irritated mucosa. The presence of DNA and NET proteins have severe consequences for the therapeutic process. The low viscosity of nasal mucus in allergic patients facilitates washing the mucosa and removal of allergens. However, the presence of DNA increases the viscosity of the mucus, making it difficult to remove, similarly to the observations in cystic fibrosis cases. The neutrophilic proteolytic enzymes associated with the secretions can lead to further mucosal irritation and exacerbation or prolongation of the treatment process. On the other hand, a sticky mucus full of biocidal agents is an excellent barrier against invading pathogens.

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Advanced Age and Ethanol Exposure Alter Alveolar Macrophage Gene Expression and the Pulmonary Innate Immune Response to *Streptococcus Pneumoniae.*

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Background: The proportion of the world's population that is ≥ 65 years old (termed "elderly") is expected to double by 2050. Nearly half of this age group is drinking, on average, 1-2 alcoholic beverages/day 3 days a week. Even though they typically drink less than younger individuals, the negative health effects of alcohol may be more potent in elderly drinkers due to "inflamm-aging," slower alcohol metabolism in the liver, and interactions with medications taken for co-morbidities. Together, the effects of inflamm-aging and alcohol may attenuate and/or delay the innate immune response to *Streptococcus pneumoniae* and contribute to the increased morbidity and mortality seen in elderly alcohol consumers with pneumonia. We hypothesize that moderate alcohol use, especially in aged subjects, leads to dysregulated gene expression in tissue-resident alveolar macrophages and contributes to impaired pulmonary immunity following *S. pneumoniae* infection.

Methods: To test our hypothesis, young and aged mice were given ethanol by oral gavage for 3 consecutive days (dose of 0.75-1.25 g/kg ethanol; designed to raise blood ethanol levels to ~80 mg/dL at 30 min). One hour after the final gavage, alveolar macrophages (AMs) were collected via bronchoalveolar lavage (>98% purity by microscopy) and RNA was sequenced (Novogene). For *in vivo* infection experiments, intranasal inoculation with 10^5 cfu of *S. pneumoniae* occurred one hour after the final gavage and mice were euthanized 24h later.

Results: RNAseq data indicate that AMs from aged animals have significant upregulation of 582 genes and downregulation of 616 genes compared to cells from young animals. Further, ethanol treatment in young mice led to 426 upregulated genes and 623 downregulated genes in AMs. Notably, AMs from aged animals given ethanol showed less transcriptional variation, with only 54 genes significantly upregulated and 47 genes downregulated compared to aged animals given vehicle (adjusted $p < 0.05$ for all above). Finally, several important immune pathways are significantly and differentially affected due to ethanol and/or advanced age, including chemokine signaling, Toll- and NOD-like receptor signaling, and antigen processing and presentation.

Following *S. pneumoniae* infection, lungs from ethanol-treated aged animals showed the least amount of leukocyte extravasation into large airways and decreased leukocyte clustering at sites of infection in the lower airways. Quantitative PCR of infected lungs showed that aged mice had significantly lower *Cxcl1* and *Il6* gene expression compared to young mice (33% ($p< 0.01$) and 29% ($p< 0.0001$) reduction, respectively), and this was further lowered when aged mice were given ethanol before infection (66% ($p< 0.0001$) and 39% ($p< 0.0001$) reduction compared to young vehicle, respectively); similar trends were seen with *Ccl2*. Age alone had no effect on expression of *Cxcl2* at this time point, however ethanol treatment reduced *Cxcl2* expression in young mice

by 17% (p< 0.0001) and it was further decreased in aged ethanol-treated mice (36% reduction compared to young vehicle; p< 0.0001). Aged mice had a significant increase in lung *Il10* expression following infection (2.5-fold higher compared to young vehicle, p< 0.001), which was lost in aged animals given ethanol. Despite these differences in cytokine and chemokine expression, we found no difference in lung bacterial burden between treatment groups at 24h post-infection.

Conclusion: Multi-day ethanol exposure and advanced age contribute to significant gene expression differences in AMs, the resident antigen-presenting cells in the lung. Dysregulated transcription may lead to functional and phenotypic changes in AMs and contribute to the diminished pulmonary immune response after infection. Future experiments aim to define the mechanisms altered due to alcohol and advanced age following infection.

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Inhibition of Stem Cell Factor Decreases Food Allergic Reactions in Sensitized Mice

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Food allergy is a growing public health problem, with approximately 15 million people affected in the United States. In IgE-mediated disease, allergen binds to IgE on mast cells in the intestines, leading to the activation and degranulation of these mast cells, resulting in an anaphylactic reaction. Therefore, preventing the accumulation and activation of mast cells may be a valuable target. Mast cells express the receptor c-kit, which is targeted by the cytokine stem cell factor (SCF). Additionally, c-kit is expressed on other cell types that contribute to allergic disease, including eosinophils and innate lymphoid cells. We therefore targeted SCF in a model of food allergy to determine whether blocking SCF could decrease the severity of the anaphylactic reaction. In this study, we sensitized mice systemically with ovalbumin, then challenged mice by oral gavage three times a week for two weeks. Mice were monitored for symptoms of anaphylaxis such as respiratory distress, diarrhea, and a drop in body temperature. During the second week of challenges, mice were injected with an antibody to block SCF, or were given IgG control. We found that mice injected with anti-SCF had a decreased incidence of diarrhea and were protected from a significant change in body temperature. We then restimulated cells from the mesenteric lymph nodes and measured cytokine production, and found that cells from anti-SCF treated mice had decreased OVA-specific Th2 cytokine production compared to IgG control. The mesenteric lymph node cells were also analyzed by flow cytometry, where we measured a decrease in the number of type 2 innate lymphoid cells in mice injected with anti-SCF. Together, these data suggest that blocking SCF in previously sensitized animals can reduce the severity of food allergy.

Late Breaking Research

LB01

Phospholipid Phosphatase 6 Regulates Phagocyte Function and Immune Responses

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Phospholipid phosphatase 6 (PLPP6) regulates polyisoprenoid diphosphate signaling in cell activation. In response to pro-inflammatory stimuli, PLPP6 converts presqualene diphosphate (PSDP) to PSMP. Because polyisoprenoids serve fundamental roles in cell immunology, we generated mice deficient in Plpp6 (Plpp6 -/-) to investigate its role in isoprenoid remodeling and cellular responses *in vivo*. Naïve Plpp6-/- had lower total and cellular cholesterol levels. In house dust mite (HDM)-induced lung allergic inflammation, Plpp6-/- mice had reduced conversion of PSDP into PSMP. Plpp6-/- mice had lower numbers of lung eosinophils, neutrophils and dendritic cells (DCs) relative to WT. Plpp6-/- mice also had lower expression of type 2 cytokines and serum IgE levels. Uptake of labeled HDM by DCs *in vivo* was decreased in Plpp6-/- mice and Plpp6-/- DCs uptake of labeled dextran by macropinocytosis was decreased *in vitro* with lower phosphoinositide 3-kinase (PI3K) expression and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) formation compared to WT. Together, these results indicate that PLPP6 deficiency is associated with lower cellular cholesterol biosynthesis, reduced polyisoprenoid diphosphate remodeling, decreased DC macropinocytosis and reduced allergen-driven tissue inflammation, and suggest a pivotal role for PLPP6 in mediating allergic responses to environmental stimuli.

LB02

Maresin Conjugates in Tissue Regeneration (MCTRs) Improve Post-influenza Pneumococcal Pneumonia by Modulation of Macrophage Phenotype

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Post-influenza (IAV) pneumococcal pneumonia is a major cause of morbidity and mortality during IAV epidemics and pandemics. Inflammation triggered by infection is crucial for pathogen clearance but an unregulated response can lead to increased lung damage and mortality during pneumonia. Alveolar macrophages (AM) are one of the first responders to a respiratory infection and coordinate the early stages of lung inflammation. Maresin conjugates in tissue regeneration (MCTRs) are a novel class of specialized proresolving mediators that are produced by and can act

on macrophages. The role of MCTRs during respiratory viral infections is not known. Here, we determined that murine IAV infection (H1N1 WSN/33, 500 PFU i.n) leads to transient AM depletion and increased susceptibility to infection with *Streptococcus pneumoniae* (serotype 3, ATCC 6330, 1000 CFU, i.n). Of interest, lung macrophages displayed long lasting phenotypic (flow cytometry) and transcriptional (Nanostring analysis) alterations after macrophage tissue repopulation. After IAV infection, lung macrophages displayed altered expression for pathogen receptors, and pro-inflammatory and host susceptibility genes for pneumococcal infection. Mice exposed to MCTRs (100 ng, i.n., protocol days 17-21) after IAV had significantly decreased bronchoalveolar lavage (BAL) leukocytes, especially neutrophils, and reduced BAL fluid total protein levels 48 hours after *Sp. pneumoniae* infection. Importantly, in addition to decreased lung inflammation, MCTRs significantly decreased lung bacteria counts and substantially decreased bacteremia. Potential mechanisms evident in post-IAV MCTR exposed lung macrophages include increased expression of CD36, decreased expression of platelet-activating factor receptor and IFN-pro-inflammatory genes. Together, these findings indicate that IAV alters macrophage responses to increase susceptibility for bacterial infection and suggest that macrophage-directed specialized pro-resolving mediators, such as MCTRs, can counter the IAV effects to promote resolution of lung inflammation and enhance host defense.

LB03

Highly Metastatic Breast Cancer Cells Recruit Neutrophils by Secreting Chemotactic Factors

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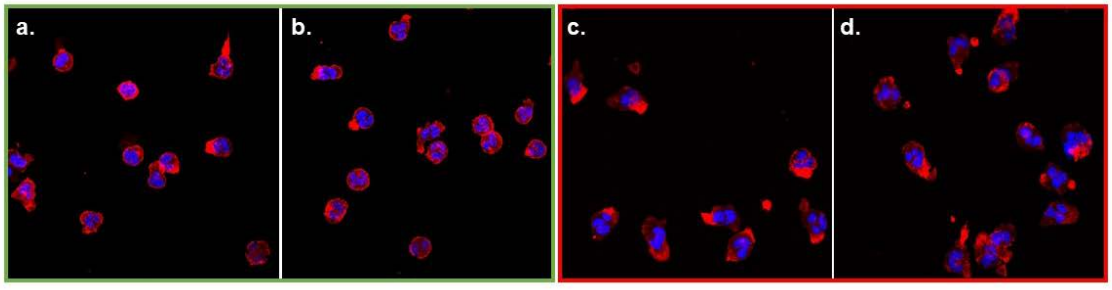
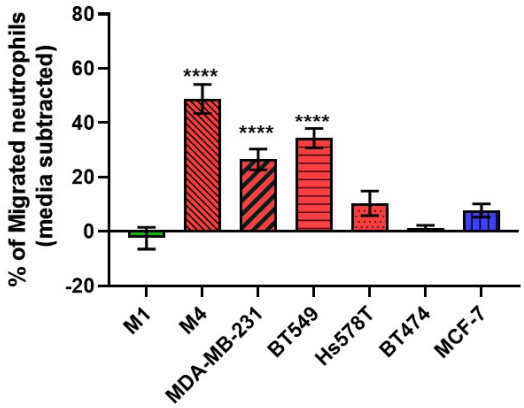
Neutrophils have been implicated in promoting breast cancer progression and metastasis. Interestingly, neutrophils are more frequently detected in aggressively metastatic, triple-negative breast tumors (TNBCs) compared to relatively less aggressive, hormone receptor-positive (HR+) subtypes. However, significant gaps remain in our understanding of the mechanisms that regulate neutrophil trafficking to the tumor microenvironment (TME). We hypothesize that neutrophil infiltration in the TME correlates with the metastatic potential of the breast cancer cells and is mediated by specific tumor-derived factors.

To establish the role of the tumor cells in driving neutrophil recruitment, we harvested tumor-conditioned media (TCM) from breast cancer cell lines of different metastatic potentials, cultured as two-dimensional (2D) monolayers. We then compared the abilities of the TCM to induce cell polarity in neutrophils, which is critical for migration. Neutrophils undergoing conversion from a resting circular to elongated polarized morphology, along with actin polymerization at cell protrusions were detected by phalloidin-TRITC staining and confocal microscopy imaging. We found that TCM derived from metastatic M4 cells of the MCF-10A breast cancer progression model as well as aggressively metastatic TNBC cell lines like BT549 were highly potent at inducing neutrophil polarization, compared to benign M1 cell-derived conditioned media (CM) or TCM from the relatively less aggressive HR+ subtype cell line BT474. Next, we compared the ability of TCM to induce neutrophil chemotaxis using a 2D (transwell) migration assay. Quantification of neutrophils that migrated to the bottom chamber of transwells was used to evaluate to what

extent different TCM induced neutrophil migration. As seen with cell polarity, we found that TCM from metastatic M4, BT549 and MDA-MB-231 cells induced robust neutrophil migration, while benign M1 derived CM or TCM from HR+ MCF-7 and BT474 cells had minimal activity. Notably, 2D and a more physiologically relevant 3D spheroid-derived TCM from MDA-MB-231 cells were similarly potent at inducing neutrophil migration. By keeping TCM in both upper and lower chambers of the transwells, we further confirmed the directional nature of neutrophil migration. In addition, using a 3D (type-I collagen matrix) migration chamber and environment controlled fluorescence microscope, we followed neutrophils in real-time migrating directionally towards M4 TCM. Remarkably, we found that the velocity (17-19 µm/min) of neutrophils migrating towards M4 TCM was similar to what is observed in response to IL-8, a potent neutrophil chemokine. Importantly, neutrophil migration towards TCM was significantly reduced with pharmacological inhibition of Gαi signaling, indicating the involvement of chemokine G-protein-coupled receptors. Using heat-inactivation, we further determined the chemical nature of the active factors. Neutrophil migration induced by TCM from MDA-MB-231 cells was significantly impaired with heat inactivation, suggesting a proteinaceous nature of the tumor-derived active factors. Surprisingly, the activity of heat-inactivated TCM from M4 cells remained largely unaffected, indicating the probable involvement of lipid mediators in recruiting neutrophils. Mechanistically, we identified a role for epithelial to mesenchymal transition (EMT) induced by a combination of TGF-β and TNF-α treatment in enabling breast epithelial cells to secrete neutrophil recruiting factors.

Together, our findings suggest that breast tumor cells secrete a unique profile of neutrophil seeking chemotactic factors that is dictated by the metastatic potential of the tumor cells. On-going studies are aimed at identifying the factors. Targeting the corresponding receptor system will offer insight into therapeutic interventions for metastasis.





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LB04

Phagolysosome Resolution Occurs via Phagosomal Shedding and Is Required to Regenerate Lysosomes and Mediate Phagocytic Flux

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Phagocytosis is the selective internalization of large particles and is critical for immunity, development and tissue homeostasis. The conversion of phagosomes into microbicidal compartments is a highly regulated process, which has been studied in great detail. Conversely, the fate of phagolysosomes, after their cargo is digested, is poorly understood. By utilizing fluorescent bacteria and labelling phagosomal markers, we followed mature phagolysosomes to their resolution. Despite previous assumptions, phagolysosomes are not exocytosed, but instead undergo a continuous dismembering process resulting in the shedding of myriads of vesicles containing degraded cargo. These phagosome-derived vesicles resemble lysosomes, based on the acquisition of lysosomal markers, acidification and fusogenicity with newly formed phagosomes. Combining pharmacological and genetic strategies, we found that phagosomal fragmentation is dependent on cargo degradation, clathrin and the integrity of the actin and microtubule cytoskeleton. In addition, we provide evidence that phagosome-derived vesicles are required to recycle lysosomal components allowing for the formation of new phagosomes and consequently maintaining the capacity of macrophages to ingest targets. Thus, we propose that phagolysosome resolution recycles cellular resources for the continuous generation of new phagosomes and the prevention of this phase will critically impair the immune and housekeeping functions of the phagocytes.

LB05

An Evaluation of Young vs. Old Neutrophils: A System of ER-Hoxb8-derived Neutrophils from the Bone Marrow Progenitors of Young and Aged Mice

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Elderly persons are at increased risk for serious pulmonary infection by *Streptococcus pneumoniae*, but the basis for this susceptibility is poorly understood. Neutrophils isolated from aged hosts are impaired for *S. pneumoniae* killing *ex vivo* [1, 2]. In addition, upon *S. pneumoniae* lung infection of aged (22 to 24-month old) mice, neutrophil influx is dysregulated and bacterial clearance is diminished [3]. Strikingly, adoptive transfer of neutrophils from young mice to aged mice provides protection from lethal *S. pneumoniae* lung challenge [4]. To determine whether

these functional deficiencies of neutrophils of aged mice are due to age-related changes in hematopoietic stem cells, we conditionally immortalized bone marrow-derived stem cells from four young (two-month old) and four aged (25-month old) C57Bl/6 male mice using a retrovirally expressed ER-HoxB8 fusion protein [4]. Upon differentiation of these stem cell factor dependent progenitors *in vitro*, the HoxB8 neutrophils derived from both young and aged mice developed characteristics of primary neutrophils, such as multi-lobed nuclei and CD11b and Gr-1 surface expression, with equivalent kinetics. Current work is ongoing to determine whether HoxB8 neutrophils derived from aged or young mice display differences in PMN functions, such as reactive oxygen species production, migration, degranulation, or opsonophagocytosis.

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LB06

Isoprenoid Depletion Reduces Expression of MicroRNAs That Regulate Inflammation

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Autoinflammatory diseases are caused by the improper activation of the innate immune system due to mutations in genes that regulate inflammation. Patients exhibit inflammatory symptoms such as fever, joint pain, and rashes and have increased plasma levels of acute phase proteins and cytokines during attacks. Mevalonate kinase deficiency (MKD) is an autoinflammatory disease caused by mutations in the mevalonate kinase (*MVK*) gene, which is an enzyme that acts early in the cholesterol biosynthetic pathway. This pathway is responsible for the production of both sterol and non-sterol isoprenoid compounds. Evidence indicates that the depletion of the non-sterol isoprenoid compound geranylgeranylpyrophosphate (GGPP) is responsible for MKD phenotypes. One function of GGPP is to modify the Ras superfamily of small GTP-binding proteins, which participate in inflammatory signal transduction. Activation of inflammatory signaling pathways cause increased expression of cytokines and molecules that regulate the inflammatory response such as miRNAs. miRNAs are approximately 22 nucleotide RNA molecules that regulate gene expression by blocking translation or causing degradation of target mRNA

molecules. It has been established that MKD causes changes to cytokine mRNA levels through alterations in inflammatory signal transduction, but miRNA expression has not been examined in MKD. In order to test the hypothesis that the expression of inflammatory miRNAs is altered in MKD, we isolated peripheral blood mononuclear cells (PBMCs) from the blood of healthy donors and treated them with lovastatin to block the cholesterol pathway and deplete isoprenoids. The cells were then stimulated with lipopolysaccharide (LPS) to induce an inflammatory response. A panel of LPS-induced miRNAs was chosen based on previous studies and miRNA expression was measured by real-time polymerase chain reaction (RT-PCR). The expression levels of miR-155, miR-9, miR-147b, and miR-204 were significantly lower in lovastatin as compared to carrier treated cells 24 hours following LPS stimulation and co-incubation of lovastatin-treated cells with mevalonate prevented these changes indicating that they are the result of isoprenoid depletion. These miRNAs all target molecules that are part of inflammatory signaling cascades and therefore our results indicate that reduced expression miRNAs that are negative regulators of inflammatory signaling may be a mechanism of increased inflammation in MKD.

LB07

Lysis of Human Neutrophils Following Phagocytosis of *Staphylococcus Aureus*

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Staphylococcus aureus is an important opportunistic pathogen that can cause a variety of pathologies such as skin and soft tissue infections, bacteremia, endocarditis and pneumonia. Healthy individuals are often colonized asymptomatically with *S. aureus*. The relative high burden of *S. aureus* colonization (~30-60% of the population) is compounded by antibiotic resistance. Notably, methicillin resistant *S. aureus* (MRSA) is a major concern in healthcare systems and the community worldwide. A MRSA strain known as USA300 is the predominant cause of community and healthcare infections in the US. The success of USA300 as a human pathogen is in part due to its ability to circumvent killing by the host innate immune system. Neutrophils are an important component of innate host defense against bacterial infections, including those caused by *S. aureus*. Although neutrophils phagocytose *S. aureus* readily *in vitro*, and the vast majority are killed within the phagocytic vacuole, some ingested bacteria can survive and eventually cause neutrophil lysis. We hypothesize that lysis of neutrophils after phagocytosis of *S. aureus* contributes to the previously reported enhanced-virulence phenotype of USA300. The mechanism for lysis of neutrophils after phagocytosis of *S. aureus* remains incompletely determined. To address this deficiency in knowledge, we used biochemical assays and fluorescence microscopy to investigate lysis of human neutrophils by USA300 and isogenic mutant strains that are deficient in selected cytolytic toxins. Our data demonstrate that neutrophil lysis occurs at bacteria-to-neutrophil ratios as low as two bacteria per neutrophil and increases with more bacteria and additional assay time. For example, lysis of human neutrophils was typically 60% after six hours using a ratio of ten bacteria per neutrophil. Live USA300 caused significantly more lysis than UV-killed bacteria, a finding consistent with the notion that

molecules produced by ingested *S. aureus* promote cytolysis, whether directly or indirectly. In addition, pre-opsonization of bacteria with human serum increased neutrophil lysis three-fold, presumable because phagocytosis was increased significantly. A detailed view of the mechanism that underlies neutrophil lysis after *S. aureus* phagocytosis is an important step toward a complete understanding of the enhanced virulence potential of USA300.

LB08

Protectin D1 and Protectin Conjugate for Tissue Regeneration 1 Regulate Lung Inflammation and Viral Load During Respiratory Syncytial Virus Infection

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Rationale: Respiratory Syncytial Virus (RSV) infection is a major cause of morbidity and mortality, driven by dysregulated lung inflammation that can cause respiratory failure (Openshaw et al, *Annual Review of Immunology* 2017). Therapies are urgently needed to resolve lung inflammation while enhancing viral clearance during RSV infection. Protectin D1 (PD1) and its peptide conjugate Protectin Conjugate for Tissue Regeneration 1 (PCTR1) are endogenous lipid mediators, enzymatically derived from docosahexaenoic acid, that have shown anti-microbial and pro-resolving properties in other experimental models of infection and inflammation (Bannenberg et al, *Journal of Immunology* 2005; Morita et al, *Cell* 2013; Dalli et al, *Immunity* 2017). The roles of PD1 and PCTR1 in host responses to RSV infection in the lung are not yet known.

Approach and Results: In an experimental model of murine RSV infection, endogenous PD1 and PCTR1 were detected at picogram levels in murine lung and correlated inversely with viral titers in the lung by qPCR. Intranasal treatment with PD1, 100ng daily for 3 days during the early phase of RSV infection, decreased RSV-induced lung eosinophils by 64%, concurrent with a 57% decrease in IL-13 mRNA transcripts. Similar intranasal treatment with PCTR1 not only decreased RSV-induced lung eosinophils by 67% but also decreased lung neutrophils by 74% and NK cells by 59%. Despite these reductions in cellular inflammation, PD1 and PCTR1 enhanced viral clearance by 80% and 60%, respectively. While PD1 treatment partially protected mice from RSV-induced decreases in antimicrobial interferon lambda expression, PCTR1 treatment decreased lymphocyte expression of interferon gamma.

Conclusions: Together, these results identify PD1 and PCTR1 as endogenous pro-resolving autacoids for lung RSV infection and its associated inflammation. These data suggest differential mechanisms of PD1 and PCTR1 in the resolution of inflammation and viral burden after RSV infection.

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LB09

S100A14 Promotes MX1 Expression and a Reduction of HIV-1 Infection and Replication in Macrophages

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Individuals that remain seronegative despite exposure to HIV-1 through high-risk behavior suggest the presence of host-mediated resistance in these individuals. Mechanisms of viral resistance can include host-related factors such as intrinsic anti-viral mechanisms that may impact infection. In our previous studies, we observed that S100A14 protein is significantly increased in NK cells and plasma from high-risk HIV-exposed seronegative people who inject drugs. We also established that S100A14 can interact with TLR-4 and induce secretion ofIL-12p40 and the expression of Mx1 in PBMCs. Here, we investigated further the potential of S100A14 to mediate an antiviral effect on *de novo* HIV infection in macrophages. To investigate the potential of S100A14 to promote an antiviral state pre-infection, we collected cell lysates from PMA-differentiated THP-1 cells or monocyte-derived macrophages(MDM) after 24 hours of treatment with S100A14, then we measured expression of Mx1. We further determined the role of PI3K in mechanism of action of S100A14 by using LY294002 in combination with S100A14 treatment. To measure antiviral activity, 18 hour-pretreated MDM from healthy donors with recombinant S100A14 were exposed to $HIV_{89.6}$ for 2 hours during spin-infection. HIV-1 replication was assessed by p24 ELISA on the supernatant of MDMs after 7 days post infection. Results show that 24 hour-exposure to S100A14 increases Mx1 expression in MDMs or PMA-THP-1 cells. Combination of LY294002 with S100A14 reduced the expression of Mx1. MDM pre-treated with S100A14 (or LPS) reduced HIV-$1_{89.6}$ replication after 7 days post infection. Altogether, these data show that S100A14 promotes limited HIV infection and replication of MDM by increasing expression of type I IFN-mediated antiviral host restriction factors. As a host factor, S100A14 may contribute to a mechanism of resistance in HIV-1-exposed seronegative individuals.

LB10

Mapping Flu-specific Memory B Cells and Clonal Networks in the Human Immune System

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Using a unique human tissue resource to map the human immune system in space and time, we are testing the overarching hypothesis that influenza-specific memory B lymphocytes are

preferentially maintained in the lung and lymphoid tissues compared to the blood. We are studying lung, lung lymph nodes, spleen, bone marrow and peripheral blood from human organ donors ranging in age from 19 - 65 years of age. The B-cell analyses use genetically engineered HA trimer probes (H1, H3) to allow estimates of the relative contributions of different flu viruses to the influenza repertoire within each donor. We will determine if H1 or H3-binding B cells are more frequent in a given donor and whether their frequency correlates with donor age. In addition, we will define the clonal interconnections of flu HA-binding B cells to reveal whether flu-reactive B-cell clones reside in individual tissues or form networks across multiple tissues, or if they circulate. Here we report initial results for the frequency and B-cell subset composition of HA-binding B cells in the tissues of several donors. We also show initial analyses of HA-binding B-cell repertoires and overlap of Tbet-positive and Tbet-negative B-cell clones from the spleens of four donors. Class switched Tbet-positive splenic B cells that bind HA harbor somatic hypermutations, consistent with these cells being antigen-experienced. Further analysis of clonal lineages of HA-binding B cells that span subsets and tissues may provide novel insights into the origins and manner of distribution of different flu-binding B-cell subsets in humans.

LB11

TotalSeq™ Antibodies: Standardized Antibody-Oligo Conjugates for CITE-Seq™, or Single-cell Proteogenomics

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High-throughput single cell RNA-seq techniques have transformed our understanding of complex cell populations and processes, however, these don't allow additional phenotypic analysis of the same cells. Stoeckius *et al*. (Nature 2017) described the use of antibodies coupled with oligonucleotides to simultaneously study protein and RNA expression at the single cell level. This method, termed Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq), combines highly multiplexed protein marker detection with unbiased transcriptome profiling and is compatible with scRNA-seq platforms based on a poly-A capture system. A collaboration between CITE-seq developers and BioLegend generated antibody-oligonucleotide conjugates under the brand name TotalSeq™.

As new technologies are developed, it is imperative to demonstrate that reagents deliver reproducible results, and that manufacturing complies with the highest standards possible. Here we demonstrate that our conjugates comply with BioLegend's quality standards, and the results are comparable with traditional flow cytometry. We also demonstrate the utility of CITE-seq using TotalSeq™ conjugates to improve cluster resolution as compared to samples analyzed via RNA-seq alone. We also show that the technology can produce equivalent data when compared to CyTOF. Finally we show how TotalSeq™ "Hashtags" are used to efficiently multiplex single cell samples, analyze multiple TotalSeq antibody dilutions simultaneously, or optimize the CITE-seq protocol and reagents.

In summary, we establish the utility of analyzing proteins and transcriptomic in the same cell in a highly multiplexed fashion, and show a completely new way to approach biomedical research.

LB12

NADPH Oxidase Modulates Inflammatory Responses in the Oral Mucosa

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Generation of reactive oxygen species (ROS) is largely conceptualized as a deleterious event often associated with oxidative stress, and inferred to have adverse consequences in periodontal disease. However, this perspective is largely derived from the use of non-specific ROS inhibitors, and is increasingly challenged by studies on nicotinamide adenine dinucleotide phosphate (NADPH) oxidases or NOX family of enzymes. Clinically, inherited genetic defects in the leukocyte NADPH oxidase (NOX2) subunits counterintuitively pre-dispose to hyperinflammation, and enhance risk for several chronic inflammatory and autoimmune disorders; supporting the premise that the NADPH oxidase derived ROS play an important modulatory role in limiting host inflammation. Using a murine model of ligature-induced periodontitis we measured alveolar bone loss by micro-CT in *Cybb*$^{-/-}$mice that specifically lack NADPH oxidase activity due to deletion of Nox2/gp91^{phox}, and wildtype (WT) mice. ROS deficiency significantly augmented alveolar bone loss and inflammatory burden in *Cybb*$^{-/-}$mice compared to WT mice. Gingival tissues from *Cybb*$^{-/-}$mice had significantly higher recruitment of immune cells, augmented expression of pro-inflammatory genes coupled with significant downregulation of Nrf2 regulated anti-oxidant genes. Neutrophils from *Cybb*$^{-/-}$mice were not deficient in killing of periodontal pathogens but were hyperactivated resulting in enhanced degranulation responses and *de novo*cytokine generation. Thus, our studies demonstrate that NADPH oxidase-derived ROS are essential for limiting gingival inflammation, in, part by redox modulating of Nrf2 mediated pathways and neutrophil responses. Contrary to the established paradigm, our studies demonstrate that low-level generation of oxidants is essential to limit inflammatory responses within the oral mucosa.

LB13

Investigating Iron Homeostasis in Erythroblastic Island Macrophages Using High Resolution Microscopy

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Dysfunctional iron homeostasis underlies a variety of diseases, including hematopoietic, cardiac, and neurodegenerative diseases. Macrophages exist in all tissues where they regulate tissue homeostasis, and they are critical for the systemic regulation of iron via their role in consuming and recycling iron-rich red blood cells. Bone marrow erythroblastic island (EBI) macrophages have been characterized as “nurse cells” because they nurture developing erythrocytes and

ingest the extruded nuclei of developing red cells. EBI macrophages are essential for promotion of efficient erythropoiesis and are functionally impaired during inflammation processes. EBI macrophages regulate erythropoiesis by i) adhering to erythroid progenitors (EPs), ii) producing growth factors, iii) ingesting nuclei extruded from reticulocytes, and iv) likely providing iron to EPs for incorporation into heme. However, the basic biology of iron homeostasis in EBI macrophages is poorly understood. Our long-term goal is to investigate the cellular mechanisms of iron import, utilization, storage, and export in EBI macrophages, which is an essential step for defining the functional role of the EBI macrophages in erythropoiesis and hematopoiesis. Although first recognized as the central cell of erythroblastic islands in the 1950s, study of EBI macrophages cells in health and disease has been hindered by little consensus regarding their markers, and phenotypic heterogeneity. Bone marrow macrophages separated from erythroid progenitors (EP) revealed that the EP-associated macrophages exhibited lower levels of CD11b and significantly higher transferrin receptor (TfR) expression, relative to macrophages that were not in association with EPs. The putative $CD11b^{lo}$ EBI macrophages also exhibited an increased labile iron pool and expressed ferroportin, suggesting the capacity to recycle iron. Our hypothesis is that $CD11b^{lo}$ macrophages are functionally distinct EBI macrophages displaying unique iron transport and metabolism, when compared to other bone marrow macrophages. Here, we will test this hypothesis by performing immunofluorescence staining of directly isolated bone marrow cell clusters. These isolated bone marrow cell clusters containing EPs and EBI macrophages will be subjected to immunofluorescence (IF) staining using multiple markers, including F4/80, VCAM-1, EPO receptor, CD11b, Ter119, and CD169 to determine the immune cell composition of bone marrow cell clusters. IF will be performed in permeabilized and non-permeabilized bone marrow cell clusters to discriminate cell surface from intracellular localization. Fast Airyscan confocal microscopy will be employed to collect z-stacks to visualize 3D morphology of bone marrow cell clusters. Bone marrow cell clusters will also be subjected to IF using antibodies against several proteins involved in iron metabolism such as TfR, ferroportin and ferritin to characterize their iron metabolism. This work will form the critical foundation for investigation of functional and dysfunctional erythropoiesis, which will ultimately provide insight into bone marrow function in myelodysplastic disease, myelofibrosis, and aging, where macrophages and iron have been associated with disease.

LB14

High CD4+ T Cell/B Cell Ratios in the Paranasal Sinus Mucosae of Patients with Eosinophilic Chronic Rhinosinusitis

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Objective: Chronic rhinosinusitis (CRS) is a frequently encountered disease in daily otorhinolaryngological practice. CRS is commonly classified based on the presence or absence of nasal polyps (NPs). In most cases of CRS with NP (CRSwNP) in the Europe and the United States, eosinophil infiltration is observed at the inflammatory regions of the nose and paranasal sinuses.

In Japan and some East Asian countries, the term eosinophilic CRS (ECRS) has been introduced to characterize CRSwNP patients with eosinophil infiltration in the NPs. It is demonstrated that the prognosis of CRS could be predicted by classifying CRS into ECRS and non-ECRS (NECRS) through comprehensive assessment of computed tomography findings and eosinophil ratio in the peripheral blood as well as the presence or absence of NPs by a large-scale study. However, immune status, especially the involvement of the acquired immunity on the paranasal sinus mucosae in ECRS has not been well investigated. As the first step to clarify this issue, we analyzed immune cells in the paranasal sinus mucosa of ECRS or NECRS patients.

Methods: The present study included 18 patients with CRSwNP who were operated at Toho University School of Medicine Omori medical center. Of the 18 patients, 6 had NECRS and 12 had ECRS. The sinus mucosa specimens from each patient had enzymatic treatment and mononuclear cells were collected. They were stained with anti-human antibodies against CD4, CD8, and CD20 and were analyzed by FACS analysis.

Result: The $CD4^+$ T cell/B cell ratio in the paranasal sinus mucosa was significantly higher in ECRS patients than in NECRS patients. No significant differences were observed in $CD8^+$ T cell/B cell or CD4+ T cell/CD8+ T cell ratios.

Conclusion: The relative increase in the counts of CD4+ T cells infiltrating the paranasal sinus mucosa in ECRS patients reflected the diagnostic criteria for ECRS, that suggests that the infiltrating CD4+ T cells might play an important role in the pathogenesis of ECRS.

LB15

Dissociation Between Rab7 Recruitment and Activation in Phagosomal Membranes

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Phagocytosis is triggered when membrane receptors in phagocytes recognize target particles. The activation of these receptors induces the extension of phagocytic cups that lead to the uptake of targets within phagosomes. Resembling the endocytic pathway, nascent phagosomes readily mature by sequentially fusing with and acquiring properties of endosomes and lysosomes.

Hence, phagosomes transiently acquire molecular characteristics of early endosomes that successively gives way to late endosomal properties and then to lysosomal markers. The end product of maturation is the transformation of a nascent, inert phagosome into a phagolysosome, where sequestered particles are digested.

Rabs are small GTPases that control the maturation of phagosomes. Rab5, a marker of early stages of phagosomal maturation, regulates the fusion of early endosomes with nascent phagosomes. Later stages in phagosomal maturation are instead marked by Rab7, which controls the movement of phagosomes along microtubules, as well as their fusion with late endosomes

and lysosomes. The replacement of Rab5 by Rab7 is a tightly controlled process known as Rab conversion.

We explored phagosomal Rab conversion utilizing long, filamentous bacteria as targets of phagocytosis. Phagocytosis of filamentous bacteria occurs through tubular phagocytic cups that last for several minutes, and mature by fusing with endosomes and lysosomes, before forming a phagosome. These features offer a greater spatial and temporal resolution to study phagocytosis, compared with the phagocytosis of spheroidal particles targets, like red blood cells or latex beads, for which phagosome maturation occurs at a much faster pace.

Our results indicate that the tubular phagocytic cups, formed for the uptake of filamentous bacteria, undergo Rab conversion. However, we detected a clear dissociation between Rab7 recruitment and its activation in tubular phagocytic cups. Intriguingly, Rab7 activation occurred with a delay of several minutes after its recruitment and depended on the transition of phosphoinositides in the phagosomal membrane.

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