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ABSTRACT BOOK

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Pioglitazone Attenuates Alcohol-Induced Alveolar Macrophage Oxidative Stress by Down-Regulating NADPH Oxidases

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Alcohol abuse increases risk of respiratory infections through impaired lung immunity mediated by enhanced alveolar macrophage (AM) oxidative stress, which results in phagocytic dysfunction. Peroxisome proliferator-activated receptor (PPAR)γ ligands reduce lung oxidative stress through down-regulation of NADPH oxidases (Nox) 1, 2 and 4. We hypothesized that treatment with pioglitazone (PIO), a PPARγ ligand, would attenuate alcohol-induced AM dysfunction by down-regulating Noxes and subsequently decreasing AM oxidative stress. AMs were obtained from the bronchoalveolar lavage (BAL) fluid of C57BL/6J mice fed ± ethanol (20% w/v) in the drinking water for 12 wks and treated ± 10 mg/kg/day PIO during week 12. In parallel, MH-S cells, a mouse AM cell line, were treated ± 0.08% ethanol for 3 d ± 10 μM PIO for 1 d. mRNA and protein levels of Nox1, Nox2, and Nox4 were assessed by qRT-PCR and western blot, respectively. Oxidative stress was measured with DCFH-DA and Amplex Red assays, and mitochondrial bioenergetics was determined using Seahorse Bioanalyzer mitochondrial stress testing. AM function was evaluated by phagocytosis assay (*S. aureus* internalization). *In vivo* and *in vitro*, ethanol: 1) increased Nox1, Nox2, and Nox4 expression, 2) enhanced oxidative stress, 3) decreased mitochondrial oxygen consumption rate, and 4) impaired phagocytic capacity. PIO treatment reversed these ethanol-induced AM derangements. Our studies suggest PIO as a clinically relevant intervention that will ameliorate alcohol-induced AM dysfunction by down-regulating Nox expression despite continued alcohol ingestion. (Supported by NIAAA 5T32AA013528-08, NIAAA NRSA 1F32AA020724-01, AHA SDG 13SDG13930003, NIAAA 1K99AA021803 (SMY), Emory Alcohol & Lung Biology Center 1P50AA135757 (LAB & CMH), and Merit Review Funding from the Atlanta VA Medical Center (CMH)).

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Chronic Alcohol Enhances Pulmonary Damage in a Mouse Model of Endotoxemia-Induced Acute Lung Injury: Novel Role of Plasminogen Activator Inhibitor-1

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Background. Alcohol consumption is a common custom worldwide, and the detrimental health effects of alcohol abuse are well established in many organ systems. The lung is recognized as a target of chronic alcohol abuse, and alcohol-related lung injury is estimated to account for tens of thousands of deaths in the United States each year. Alcohol is a significant risk factor in the morbidity/mortality of lung damage from other causes. For example, acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), occurs 3.7 times more often in people meeting the diagnostic criteria for alcohol use disorders. The mechanisms by which alcohol sensitizes the lung to development of serious injury are poorly understood. Plasminogen activator inhibitor-1 (PAI-1) is known to be involved in many models of ALI, and this group has previously demonstrated that PAI-1 plays a critical role in alcohol-induced liver injury. The goal of this project was therefore to determine the role of PAI-1 in a mouse model of alcohol-enhanced ALI.
Methods. Male mice (WT and PAI-$1^{-/-}$) were exposed to ethanol-containing Lieber-DeCarli diet or pair-fed control diet for 6 weeks. Some animals were administered intraperitoneal lipopolysaccharide (LPS) prior to sacrifice to cause endotoxemia-induced ALI. Lung injury was assessed using histological indices of pulmonary damage. Inflammation was assessed by measuring myeloperoxidase (MPO) activity and expression of inflammatory mediators including cytokines, chemokines, and adhesion molecules.
Results. Chronic alcohol feeding caused enhanced pulmonary damage after systemic LPS administration. Additionally, chronic alcohol

feeding enhanced induction of the chemokines MIP-2 and KC (murine IL-8 homologues) after LPS injection in wild type animals. This enhanced chemokine expression did not correlate with enhanced pulmonary neutrophil infiltration; however, animals exposed to chronic ethanol showed enhanced 4-HNE staining, indicative of oxidative stress and inflammatory damage. This enhanced pulmonary damage and chemokine expression was completely attenuated with PAI-1 knockout animals, despite the fact that PAI-1 expression after LPS was not significantly enhanced by ethanol.

Conclusions. This work characterizes an animal model which will be useful in future studies to determine the mechanisms by which alcohol sensitizes the lung to injury. Additionally, this work has identified PAI-1 as a key player in alcohol-enhanced pulmonary damage and chemokine expression in endotoxemia-induced ALI.

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Alcohol Abuse is Associated with Enhanced Pulmonary Xanthine Oxidoreductase Activity

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Rationale: The acute respiratory distress syndrome (ARDS) is a devastating disorder affecting ~190,000 people in the US yearly with an in-hospital mortality of 27 to 45%. People with alcohol use disorders (AUDs) are at increased risk for ARDS development and poor outcomes. Although specific mechanisms underlying these associations remain unclear, translational studies suggest that alcohol metabolism contributes to oxidative stress via the enzyme xanthine oxidoreductase (XOR). XOR activity is essential to innate immunity and inflammation, producing both reactive species and uric acid (UA). XOR is widely distributed in cells including alveolar macrophages (AMs). AM dysfunction, characterized by high reactive species production, has been implicated in ARDS susceptibility in AUDs. Our pre-clinical work demonstrates that XOR promotes inflammation in pulmonary mononuclear phagocytes, regulates leukocyte adhesion *in vivo*, and contributes to cytokine-induced acute lung injury. The effect of AUDs on human pulmonary XOR activity has not been reported. We hypothesized that AUDs would be associated with enhanced XOR activity in bronchoalveolar lavage (BAL) referable to AMs. We further postulated that BAL from ARDS patients would be characterized by augmented XOR activity and procollagen peptide III (PCPIII), a molecule implicated in fibroproliferation.

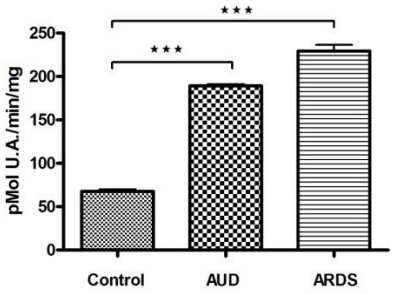
Methods: BAL was performed in (1)healthy subjects with alcohol use disorders (AUDs), (2)healthy controls, and (3)ARDS patients previously enrolled in a multicenter randomized trial. The Alcohol Use Disorders Identification Test (AUDIT) was used to characterize AUDs. BAL was centrifuged (900xg, 20 min) to separate cells from fluid. BAL fluid UA was quantified using the *o*-phenylenediamine dihydrochloride/uricase enzymatic method, measuring fluorescent oxidation products (Ex/Em: 410/550). Immunohistochemical staining of fixed BAL cells was performed for XOR. Total XOR enzymatic activity in fresh BAL cells was assessed via measuring oxypurinol-inhibitable uric acid synthesis over 72 minutes @293nm, corrected for total protein.

Results: BAL was obtained from subjects with AUDs (n=20) and controls (n=20) who did not differ in terms of age or gender; 50% of each group was current smokers. BAL was also obtained from 33 ARDS patients at day 3±1 after diagnosis (**table 1**). BAL fluid UA was higher in AUD subjects compared with controls (p=0.0003). AUD subjects displayed 3-fold higher UA values compared with controls (p=0.0006) in analyses excluding smokers. BAL fluid UA values were four-fold higher in ARDS patients compared to AUD and controls collectively (pfigure 1). BAL fluid UA correlated positively with PCPIII in ARDS patient samples (rho=0.59, n=13).

Conclusions: AUDs were associated with increased pulmonary XOR activity referable to AMs. ARDS patients demonstrated further enhancement in XOR activity compared to non-ARDS subjects. Our observations suggest that soluble UA in epithelial lining fluid and intracellular AM XOR have the potential to excessively stimulate innate immunity, and may be one driver of the predisposition for ARDS with poor outcomes in AUDs.

Table 1.

	Non-ARDS Subjects	ARDS Patients	P
Age (mean±SD)	42.3±6.7	48.5±12.6	0.008
Sex (% men)	65%	58%	0.63
% AUDs	50% (20/40)	33% (11/33)	0.16
BAL fluid UA, uM Group median (IQR) *AUD* *Non-AUD*	5.0 (3.3, 8.0) *7.1 (5.0, 8.2)* *3.3 (2.0, 4.9)*	21.7 (15.3, 38.1)	<0.001



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Combined Alcohol and Cigarette Smoke Exposure Decreases Repair/Restorative Cytokines in Mouse Lung

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Persons with alcohol use disorders are known to also be cigarette smokers. Cigarette smoke-induced lung injury has been studied extensively and the effects of alcohol on the lungs are now established. However, few model systems exist to examine the comorbidity of combined cigarette smoking and alcohol consumption on lung injury or restorative repair after injury. Previously, we have demonstrated in a mouse model that the combination of alcohol and cigarette smoke slows mucociliary clearance and enhances lung inflammation through the elevation of the reactive lung aldehydes, malondialdehyde and acetaldehyde. We hypothesize that combination exposure of lungs to cigarette smoke and alcohol will result in altered cytokine production as compared to individual exposures to either agent. To test this, we used the Meadows-Cook ad libitum alcohol feeding model and the Teague whole-body smoke exposure system to compare bronchoalveolar lavage fluid cytokines as measured by multiplex array from control mice, alcohol-fed mice, cigarette smoke exposed mice, and the combination smoke+alcohol mice. Certain inflammatory cytokines and chemokines stimulated by either alcohol or smoke were significantly decreased by combined smoke+alcohol (CXCL5, CXCL16, IL-12, IL-1a). However, several cytokines and chemokines involved in repair/homeostasis stimulated by either alcohol or smoke were significantly reduced by combined smoke+alcohol (CXCL4, CCL17, CCL19, CCL24, CCL25, CCL27, IL-4, IL-10). In addition, some growth factors and hormones were reduced by co-exposure (IGFBP-3, IGFBP-5, IGFBP-6, leptin, SCF). Collectively, the wound repair phenotype driven by Th2 responses appears to be blunted by the combination smoke+alcohol exposure. These data provide evidence that the combination of smoking cigarettes and drinking alcohol can decrease normative cytokine-mediated lung repair processes that would otherwise occur under conditions of just alcohol or cigarette smoke.

	Increases	Decreases	Alcohol Increases; blocked by Smoke	Smoke Increases; blocked by Alcohol
Alcohol	M-CSF		CXCL16, IGFBP-3, IGFBP-6, IL-1a, CCL27, IL-4, Leptin, CXCL5, CCL19, CXCL4, CCL17, CCL25	
Cigarette Smoke	MCP-1, MIP-2, MIP-3a	Lymphotactin, VEGF		IGFBP-5, IL-10, Leptin R, SCF
Alcohol + Smoke	sTNF-RI, sTNF-RII	CCL24, TPO IL-12		

Supported by NIAAA (AA017993, AA008769) and VA (BX000728).

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Is Liver Injury a Mechanism of Accelerated Immune Dysfunction (Immunosenescence) Among ART-Naive HIV-Infected Russians with a Recent History of Heavy Drinking?

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Background: Immunosenescence, the normal aging of the immune system, may be accelerated in HIV and leads to poor outcomes. The liver regulates innate immunity and adaptive immune tolerance. HIV-infected people have high prevalence of hepatotoxic comorbidities including unhealthy alcohol use and viral hepatitis. We hypothesize that HIV may be associated with pro-senescent alterations in T-cell subsets via a mechanism mediated by liver injury. This analysis investigates whether liver injury, as measured by FIB4, is associated with pro-senescent alterations in T-cell subsets.

Methods: By April 2015, 250 ART-naïve HIV-infected Russians with a recent history of heavy drinking were recruited into a clinical trial of zinc supplementation. In a subset of participants at baseline (N=114), flow cytometry was used to quantify T-cells. The two primary dependent variables were CD8+ and CD4+ T-cells expressing CD28-CD57+ (potentially senescent cell phenotype). Secondary dependent variables were CD8+ and CD4+ T-cells expressing CD45RO+CD45RA- (memory phenotype), CD45RO-CD45RA+ (naïve phenotype), or naïve:memory T-cell ratio (lower ratios associated with immunosenescence). FIB4, the independent variable, was calculated at baseline using liver enzymes and platelet count and dichotomized at 1.45. Analyses were conducted using multiple linear regression adjusted for potential confounders. Skewed outcomes were log-transformed.

Results: Patient characteristics were as follows: mean age (SD) 33.6 (6.3) years; 100% white; 74% male; and 86% hepatitis C antibody positive. Those with elevated FIB4 (N=46) had higher HIV-1 RNA and prevalent HCV versus those with FIB4 less than 1.45 (N=68). FIB4 greater than or equal to 1.45 was not significantly associated with CD8+ or CD4+ CD28-CD57+ T-cells in adjusted analyses (Table 1). Increases in CD8+ and decreases in CD4+ naïve:memory T-cell ratio were observed among those with elevated FIB4, however the results were not statistically significant (Table 1).

Conclusions: Elevated FIB4 was not significantly associated with the pro-senescent T-cell phenotypes assessed in this sample of ART-naïve HIV-infected Russians with a recent history of heavy drinking. Future studies should assess whether changes over time in liver injury are associated with changes in these and other potentially senescent T-cell phenotypes.

Table 1: Association between FIB4 and T-cell subsets

Dependent variables		Adjusted mean difference* in T-cell subsets for FIB4≥1.45 vs. <1.45	P-value
Denominator	T-cell subsets		
CD8+ T-cells	CD28- CD57+	0.01 (-0.06, 0.07)	0.83
	Memory T-cells	-0.01 (-0.07, 0.05)	0.67
	Naïve T-cells	0.04 (-0.02, 0.10)	0.21
	Naïve:Memory CD8 ratio*	1.27 (0.87, 1.86)	0.21
CD4+ T-cells	CD28- CD57+*	1.26 (0.54, 2.93)	0.59
	Memory T-cells	0.05 (-0.02, 0.12)	0.15
	Naïve T-cells	-0.04 (-0.10, 0.03)	0.24
	Naïve:Memory CD4 ratio*	0.81 (0.54, 1.22)	0.32

Models adjusted for age, sex, $\log_{10}$ HIV-1 RNA, heavy drinking (> 4 standard drinks in a day (or > 14 standard drinks/week) for men and > 3/ day (or > 7/week) for women) and hepatitis C antibody status (qualitative lab test)
FIB4 calculated as [Age*AST]/[Platelet count * (ALT)$^{0.5}$]
*Represents the ratio of means after back transformation from natural log scale.

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Generation of Murine Stem Cell Derived Hepatoblast as a Transplantable Cell Source to Treat Chronic Alcoholic Liver Disease

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Background: Although, liver transplantation is a major treatment modality for end stage liver diseases, the scarcity of available donor livers remains a major concern. Cell replacement therapy (CRT) using isolated hepatocytes has been proposed as short-term liver support until a suitable liver becomes available, however, procuring sufficient hepatocytes for this form of treatment is also limited by the availability of donor organs. To this end, stem cell derived hepatoblasts (SC-DH) offers an alternative to adult hepatocytes because they can be propagated in vitro, and preliminary studies in immunodeficient animals have shown that SC-DH can support liver function. To date, no studies have

been conducted to test the therapeutic utility of transplanted SC-DH in immunocompetent hosts. The aim of this project is to develop a SC-DH differentiation and transplantation system that can be used to study the therapeutic potential of SC-DH in treating chronic alcoholic liver diseases, as well as, the impact of chronic alcohol on alloimmune responses. **Method**: To generate a cellular graft that can be monitored non-invasively by bioluminescence, murine embryonic stem cells (mESC) were generated from FVB-Tg(CAG-luc,-GFP)L2G85Chco/J blastocysts that harbor the CAG-luc-eGFP L2G85 transgene. These reporter genes allow tracking of cells non-invasively by luciferase and post-mortem evaluation by GFP expression. The pluripotency of the generated mESC clones were characterized by karyotype analysis, teratoma formation assay and FACS analysis for pluripotency markers Nanog, Oct4 and SSEA-1. To generate SC-DH, a modified Gouon-Evans two-step differentiation protocol was employed that involved definitive endoderm induction following by hepatoblast maturation. To study the survival kinetics of transplanted SC-DH, $2x10^6$ SC-DHs were injected under the renal capsule of syngeneic FVB mice. **Results**: We have established an *in vitro* method to reproducibly generate SC-DH from murine embryonic and induced pluripotent CAG-luc-eGFP L2G85 transgene+ stem cells. These SC-DH display morphologic and functional features of the hepatocyte lineage: albumin and glycogen production, and express Afp, Hnf4a, Ck19, Cps1, Tat, Cyp7a and Cyp3a following thirteen days of differentiation. Furthermore, transplanted hepatoblast survive and can be monitored by bioluminescent imaging. **Conclusion:** We have developed a novel system for generating and transplanting SC-DH that behave and express hepatocyte lineage genes, and express a dual-reporter system that allows for *in vivo* fate tracking and post-mortem evaluation. Finally, this system can be used to study the utility of this cell type to treat chronic alcoholic liver disease in immunocompetent host, as well as study the effect of chronic alcohol feeding on the alloimmune response.

Ethanol Induces GILZ Gene Expression to Modulate Cell Immune Response Through a Non-Canonical GR Trans-Activation

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Alcohol abuse has long been associated with immune suppression, which leads to increased susceptibility to infection. However, the molecular mechanism underlying this alcohol negative impact on immune functions has not been fully defined. Our previous publication demonstrated that ethanol upregulates Glucocorticoid-Induced Leucine Zipper (GILZ), a prominent glucocorticoid (GC)-responsive gene and an important anti-inflammatory and immunosuppressive regulator (Gomez et al. (2012) J. Immunol. 184, 5715-5722). Here we report that alcohol induces GILZ expression to modulate cell immune response through a non-canonical activation of the glucocorticoid receptor (GR) signaling. The proximal GILZ promoter was cloned and confirmed to have five glucocorticoid-responsive elements (GREs). Deletion and/or mutation of the GREs abrogated the promoter responsiveness to alcohol, indicating that GR-GRE interaction conveys the alcohol action on GILZ gene. Gel mobility shift assay showed that the non-GC-bound GR was able to bind to GREs, and such a physical interaction was not influenced by ethanol exposure. To determine the potential role of GILZ in alcohol immune suppression, we depleted GILZ expression in Mono-Mac-6 cells via small RNA interference. LPS-stimulation of pro-inflammatory cytokine TNF-α production was assessed. All the cells, regardless of GILZ expression levels, had a robust TNF-α production after LPS stimulation. Strikingly, ethanol significantly suppressed TNF-α production by the parental cells and the SiRNA-control cells that had an unaltered GILZ expression. However, GILZ depletion in the SiGilz cells abolished the alcohol-induced TNF-α-suppressive effect. Therefore, alcohol modulates GILZ expression and cell immune response via a GC-independent, non-canonical GR activation, which

contributes to alcohol-associated immunosuppression. This work was supported by Grant 1R21AA021824 from NIH/NIAAA and a pilot grant from LSUHSC Comprehensive Alcohol Research Center.

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Alcohol Consumption Modulates Host Defense by Altering Gene Expression in Circulating Leukocytes

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Several lines of evidence indicate that chronic alcohol use disorder leads to increased susceptibility to several viral and bacterial infections whereas moderate alcohol consumption decreases incidence of colds and improves immune responses to some pathogens. In line with these observations, we recently showed that heavy ethanol intake (average blood ethanol concentrations (BECs) >80 mg/dl) suppressed, whereas moderate alcohol consumption (BEC <50 mg/dl) enhanced T and B-cell responses to modified vaccinia ankara (MVA) vaccination in a nonhuman primate model of voluntary ethanol consumption. No differences in frequency of key immune cells (T and B cell or monocytes) were detected between all three groups, which suggests that ethanol induces functional changes in immune cells. To uncover the molecular basis for impaired immunity with heavy alcohol consumption and enhanced immune response with moderate alcohol consumption, we performed a transcriptome analysis using peripheral blood mononuclear cells (PBMCs) isolated on day 7 post-MVA vaccination, the earliest time point at which we detected differences in T-cell and antibody responses. Overall, chronic heavy alcohol consumption reduced expression of immune genes involved in host defense and wound healing, and increased expression of genes associated with the development of lung inflammatory diseases and cancer. In contrast, chronic moderate alcohol consumption upregulated expression of genes involved in immune response to infection and reduced expression of genes involved in cancer.

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Elevated HIF-1α in the Gut Following Alcohol and Burn Injury

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Alcohol exposure is a major contributor to post burn pathogenesis. As the gut is the largest bacterial reservoir; breakdown of the gut barrier has been associated with the observed adverse effects following alcohol and burn injury. Gut barrier dysfunction allows for bacterial dissemination from the intestine to extra-intestinal sites which may lead to systemic inflammatory response syndrome and multiple organ failure. In this study we examined whether alcohol and burn injury results in hypoxic insult to the gut. We used hypoxia inducible factor (HIF)-1α as a marker of hypoxic insult and examined its expression following alcohol and burn injury. Male mice (~25g) were gavaged with alcohol (~3 g/kg) or water. Four hours following the gavage, mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and received a ~12.5% total body surface full thickness burn. One and three days post injury, mice were euthanized and the small and large intestines were harvested. The harvested tissues were used for intestinal epithelial cell (IEC) isolation. We found that HIF-1α expression is elevated in the small intestine (~2 fold) day 1 following alcohol and burn injury compared to sham injured animals. HIF-1α expression normalized to sham values day 3 following alcohol and burn injury. Similarly, HIF-1α expression was increased (~3 fold) in the large intestine following the combined insult compared to sham injured animals. To demonstrate that elevated HIF-1α also increased its activity we examined expression of heme-oxygenase-1(HO-1) which is a downstream target of HIF-1. HO-1 expression was increased by (~4 fold) in the small intestine day 1 following alcohol and burn injury compared to sham animals. In contrast, HO-1 expression was significantly reduced day 3 following burn injury alone (~2 fold) and alcohol and burn (~2 fold) injury compared to sham injured animals. The large

intestine exhibited (~5 fold) increased expression following the combined insult. Expression of HO-1 in large intestinal epithelial cells normalized to sham values day 3 following alcohol and burn injury. Together these data indicate that alcohol and burn injury increases HIF-1α mRNA expression. Future studies will address whether elevated HIF-1α has a role in intestinal barrier disruption following alcohol and burn injury. Supported by R01AA015731, T32AA013527.

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Alcohol-Induced S-Nitrosylation Drives Protein Phosphatase 1-Dependent Ciliary Dysfunction

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Background: There is an increase in lung infections and pulmonary diseases among individuals with alcohol use disorders. We previously identified that alcohol stimulates nitric oxide (NO) production in the airway and prolonged alcohol exposure impairs airway mucociliary clearance, known as alcohol-induced ciliary dysfunction (AICD). Increased levels of NO can lead to protein S-nitrosylation (SNO) serving as an oxidation-sensitive switch to regulate enzyme function. Modifications of cilia-localized regulatory enzymes, known as the ciliary metabolon, are key to the pathogenesis of AICD. Specifically in AICD, protein phosphatase 1 (PP1) is activated, altering phosphorylation of cilia motility regulator proteins and blunting of ciliary beat frequency (CBF) responsiveness to β-adrenergic stimuli. PP1 contains a putative oxidoreductase site that may regulate its phosphatase activity.

Hypothesis: We hypothesized that alcohol-driven PP1 activation is mediated by protein S-nitrosylation, driving changes in cilia motility responsiveness to β-agonists.

Methods: To test this hypothesis we compared the effects of prolonged alcohol exposure and the direct S-nitrosylating agent, S-nitrosoglutathione (GSNO) on airway epithelial and PP1 SNO, PP1 activity and CBF when stimulated by a β-agonist in isolated axonemes, primary tracheal epithelial cells and tracheal rings. To assess SNOwe used a combination of liquid chromatography – mass spectrometry (LC-MS) and the biotin switch technique (BST) for detection of SNO. The BST employs SNO specific reduction/oxidation chemistry to replace labile sulfur-NO bonds with covalent biotinylation that can be detected by techniques such as Western blotting and immunofluorescent microscopy. PP1 activity was determined from tracheal ring lysates by colorimetric detection of phosphate release by malachite green detection. CBF was measured by phase contrast high-speed video microscopy.

Results: Axonemes extracted from bovine trachea treated with ±alcohol (100mM X 24hr) demonstrated an approximate two-fold increase in SNO as detected by BST. LC-MS analysis for S-nitrosylation of similarly treated axonemes revealed increased SNO of 121 proteins. Of these proteins, 10 unique SNO sites, corresponding to 6 unique proteins, demonstrated >20 fold increase in SNO by alcohol exposure. Four of these unique sites corresponded to different residues of PP1. Super-resolution microscopy of tracheal rings from 12-14 wk old female C57Bl/6 mice treated ex vivo with ±alcohol (100mM X 10d) and ±GSNO (1mM X 4hr) revealed SNO that localized to the apical and ciliated areas of the airway epithelium. Alcohol and GSNO treatment activated of PP1 (116.3 ± 8.7 and 265.4±19.04, respectively, vs. 13.24±6.8 pmolXmin^{-1} Xμg^{-1}for media control; p

Conclusion: Alcohol exposure stimulates activation and S-nitrosylation of PP1 in airway epithelial cells. Direct S-nitrosylation drives ciliary dysfunction, recapitulating alcohol-induced cilia dysfunction in a PP1-dependent manner. These data reinforce our previous findings that PP1 activation at the level of the ciliary metabolon results in ciliary dysfunction and identify S-nitrosylation as a novel target in preventing or reversing AICD and airway disease.

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Ethanol Intoxication Amplifies Lung Histone Deacetylase Activity and Increases Levels of Histone Deacetylase 1

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Clinical data suggest that alcohol intoxication prior to injury or infection dampens the immune response, delaying infection clearance, extending hospital stays, and increasing morbidity and mortality. Epigenetic memory is likely a contributing factor in the "two-hit hypothesis" where the first hit, alcohol consumption, exaggerates physiological responses to the second hit, infection or injury. Using a mouse model, our lab previously demonstrated that compared to either ethanol exposure or injury alone, the combined insult increased lung pro-inflammatory cytokine production, leukocyte infiltration and congestion. Since histone deacetylases (HDACs) are important epigenetic regulators of inflammatory responses and since others have demonstrated ethanol exposure alters histone acetylation in other tissues, we hypothesized that HDAC dysregulation may drive exaggerated inflammation in our model by contributing to the "first hit." To test our hypothesis, male C57BL/6 mice were treated with saline vehicle or ethanol (1.12 g/kg), a dose corresponding with a blood alcohol concentration of approximately 150 mg/dL, 30 minutes after exposure, and were euthanized 24 hours later. Compared to controls, HDAC specific activity in lung nuclear extracts was 14% higher in ethanol-treated mice ($p<0.05$). No differences in transcript levels of *HDAC1-11* were observed between groups using qRT-PCR. However, Western blot of lung tissue demonstrated protein levels of HDAC1 were increased by 1.4-fold ($p<0.05$), while HDAC2, HDAC3, HDAC4, and HDAC10 levels were similar between ethanol and vehicle-treated mice. Minor increases in HDAC1 were observed in bronchioles and alveoli by immunofluorescence in ethanol-treated mice, but levels of activated (phosphorylated)-HDAC1 were similar between groups. There were no differences in levels of histone H3 acetylation, including global H3 acetylation (H3-Ac), as well as acetylation of specific lysine residues, including H3 Lysine (K)-9, H3K18, H3K27, and H3K56, but a 37% decrease in histone H4K12 acetylation was observed ($p<0.05$). Hence, our data reveal that ethanol exposure increases pulmonary HDAC specific activity and HDAC1 levels, corresponding with specific decreases in acetyl histone marks, which are apparent 24 hours after a single dose of ethanol. These findings suggest acute ethanol intoxication prior to injury or infection may prime "second hit" exuberant inflammatory responses by modifying HDACs. Supported by: NIH R01 AA012034 (EJK), R01 GM117257 (EJK) T32 AA 013527 (EJK), F32AA021636 (BJC), F31 AA022566 (JAS) and the Dr. Ralph and Marian C. Falk Medical Research Trust.

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Ethanol Modulation of Expression Profiling of TRP Channel Proteins

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Ethanol (EtOH), the main ingredient in alcoholic beverages, is well known for its behavioral and psychological effects. Binge drinking, or consumption of a large amount of alcoholic beverages in a very short time, is a serious societal problem. The detrimental effects of binge drinking of hard liquor that has 40% or higher alcohol by volume have been increasingly demonstrated. EtOH exerts its effects through a variety of protein targets, including transient receptor potential (TRP) channels, consisting of seven subfamilies, including ankyrin (TRPA), canonical (TRPC), melastatin (TRPM), mucolipin (TRPML), NOMPC (TRPN), polycystin (TRPP), and vanilloid (TRPV). TRP channels have their specific agonists and are expressed in various tissues and cells. EtOH has been reported to significantly affect various TRP channels, including activation of TRPA1 and TRPV1 and desensitization of TRPM8. Animal behavioral assessment also suggests roles of TRP channels in EtOH-induced intoxication and the avoidance of EtOH. Taken together, we have hypothesized that alcohol use modulates expression of TRP channels that are involved in EtOH-induced biological effects. Using a cell culture model, we have shown that TRPM7 is the most abundantly

expressed TRP in the brain microvascular endothelial cells (BMVECs). EtOH decreased TRPM7 expression in BMVECs and TRPM7 mediated EtOH-increased blood-brain barrier permeability. In this study, we have examined how binge exposure to EtOH affects expression of TRPs in various tissues including brain and liver using a rat model. F344 rats were administered water or 52% EtOH by intra gavage (i.g.) for 3 d (4.8 g/kg per d). Twenty four hours following the last EtOH treatment, both prefrontal cortex (PFC) and striatum were collected from each animal. Gene expression of 29 TRP channels were determined using custom-made real-time qPCR array. Our results indicated that, compared to water control, EtOH significantly decreased expression of TRPV2, TRPV3 and TRPV4 genes (2.1-2.7 fold) in the striatum and TRPM6 (2.0-fold) in the PFC. EtOH modulation of expression profiling of TRP channels appears to be tissue dependent. Currently, we are studying the TRP expression in liver of the F344 rats given binge exposure to EtOH (partially supported by NIH AA019415).

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Chronic Heavy Alcohol Consumption Increases Risk of Cardiovascular Diseases and Anemia in Female Rhesus Macaques

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It is well established that heavy alcohol consumption affects immune system and inflammatory processes, ultimately increasing the risk for infectious and chronic diseases. However, these processes have not been systematically studied in a dose and gender dependent manner. In this study, we investigated the impact of chronic heavy ethanol consumption on peripheral blood mononuclear cells (PBMC) isolated from female rhesus macaques using whole genome expression profiling. We used schedule-induced polydipsia to establish reliable self-administration of 4% ethanol in 9 female rhesus macaques 5 years of age. Following an induction period, 6 of these females were given open access to both water and 4% ethanol 22hr/day. PBMC were collected after 12 months of ethanol self-administration. Illumina HiSeq was used to sequence the transcriptome of PBMC from heavy drinkers (n=5) with an average consumption of 4.3 g/kg/day (considered very heavy drinking) and controls (n=2). In total, 1706 genes were differentially expressed (FDR <= 0.05) with at least a two-fold change in expression in heavy drinkers compared to the controls (755 up- and 951 down-regulated). Pathway analysis using GeneGo Metacore software of the 1382 genes that had human homologs showed that the most affected GO processes in the up-regulated genes included response to wounding, blood coagulation and immune system process. We also saw a strong up-regulation of genes involved in anemia and cardiovascular disorders. Regulation of signaling was the most enriched GO term among down-regulated genes. These changes in gene expression were seen despite the lack of differences in the frequency of any major immune cell subtype. These findings suggest that heavy alcohol consumption increases the risk of cardiovascular disorders and impairs regulation of cellular signaling.

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Alveolar Macrophage Phagocytic Activity and Apoptosis after Intoxication and Injury

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Clinical evidence reveals that half of all burn patients who present to the emergency department are intoxicated at the time of injury. This combined insult results in amplified neutrophil infiltration to the lung and pulmonary edema, with an overall increased risk of lung failure and mortality, relative to either insult alone. We and others believe that this excessive pulmonary inflammation, that also parallels decreased lung function, is mediated in part by alveolar macrophages (AMs). Restoration of tissue homeostasis is dependent on the eradication of neutrophil infiltration and removal of apoptotic cells, both major functions of AMs. With lung function impairment causing a likely decrease in gas exchange, we chose to examine the fate of these multi-functional cells. Thirty minutes after binge

ethanol intoxication, mice were anesthetized and given a 15% total body surface area dorsal scald injury. At 24 h, there was a 7-fold increase in $TUNEL^+$ apoptotic cells in the lungs of intoxicated and injured mice, relative to controls ($p<0.05$). To determine if AMs were undergoing apoptosis, bronchoalveolar lavage (BAL) cells were analyzed by flow cytometry for annexin IV and propidium iodide. We found a 50% decrease in the number of AMs ($p<0.05$) after intoxication and injury, compared to controls. This observation paralleled a 3-fold increase in the percent of apoptotic AMs and a 2-fold increase in the percent of dead AMs ($p<0.05$), above that of controls. In contrast to the reduction in cell numbers, AMs from intoxicated and injured mice had a 5-fold increase in phagocytosis relative to cells from controls ($p<0.05$). Immunofluorescent staining confirmed AMs were phagocytosing active-caspase 3^+ cells. Cultured AMs from intoxicated and injured mice also spontaneously produced 5-fold more tumor necrosis factor α than controls ($p<0.05$). In summary, following intoxication and injury, there is an increase in the number of apoptotic cells in lung tissue. Our data reveal that AMs are one population of these cells. Even though there are less AMs, the AMs that are present are functional and actively phagocytizing apoptotic cells. Overall, these data suggest that the loss of these important inflammatory regulators may delay resolution of inflammation, leading to aberrant lung function and elevated mortality. [Supported by NIH R01 GM115257 (EJK), R01 AA012034 (EJK), T32 AA013527 (EJK), F31 AA022566 (JAS), and the Falk Foundation (EJK)].

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Toll-Like Receptor 9 Stability and Signaling are Regulated by Phosphorylation and Cell Stress

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Innate sensing of pathogens elicits protective immune responses through pattern recognition receptors including toll like receptors (TLRs). However, some TLRs recognize ligands that are shared with the host, for example RNA and DNA recognized by TLR3/TLR7/TLR8 and TLR9, respectively. To avoid autoimmunity, there are multiple levels of regulation that control ligand recognition by the relevant receptors. We, and others, have previously shown that TLR9 is excluded from the cell surface, is primarily retained in the endoplasmic reticulum, and that critical tyrosine residues in the cytoplasmic tail contribute to regulation of TLR9 trafficking and signaling. We have also shown that TLR9 is proteolytically cleaved into mature and negative regulatory forms upon reaching the endosomal compartment. Very little is known about what extracellular signaling may regulate TLR9 localization, stability and signaling. In the present study, we investigated the role of tyrosine phosphorylation of the cytoplasmic tail of TLR9, as well as the role of various cell stress pathways in regulating TLR9 stability, cleavage and signaling. Using spleen tyrosine kinase (Syk) deficient cells, we identified a novel function for Syk activity in TLR9 protein stability and in TLR9 signaling. TLR9 protein stability was also sensitive to autophagy, the cellular stress response pathway, and infection by a DNA virus. Thus, our data unravel novel information about TLR9 biology related to stability and signaling and suggest that immune evasion mechanisms may involve targeted loss of innate sensing receptors.

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Lyn Delivers Bacterial Substance to Lysosomes for Eradication Through TLR2-Initiated and Autophagy-Associated Phagocytosis

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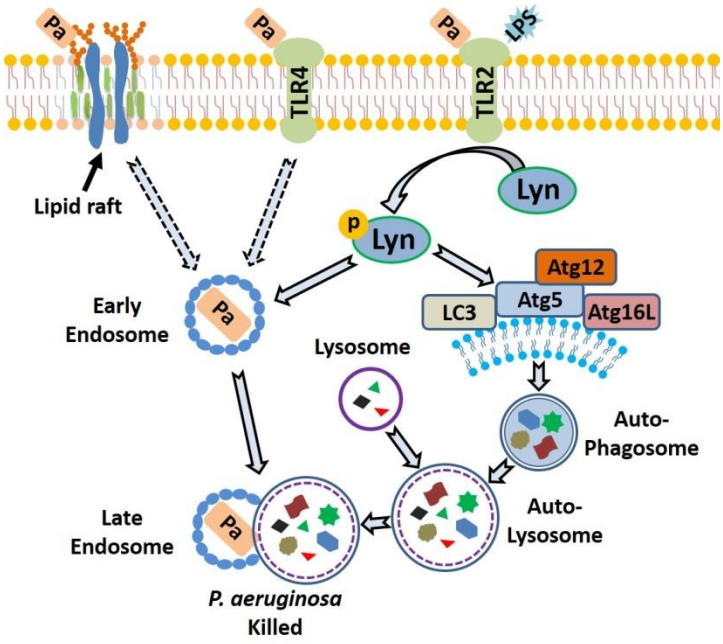
Extracellular bacteria, such as *Pseudomonas aeruginosa*, have been reported to induce autophagy; however, the role and machinery of infection-induced autophagy remain elusive. Here we show that the pleiotropic Src kinase Lyn mediates phagocytosis and enhances autophagosome maturation in alveolar macrophages (AM), facilitating eventual bacterial eradication. Mechanistically, Lyn is required for LC3 related autolysosome fusion. Further, blocking autophagy with 3-methyladenine (3-MA) or depleting Lyn decreased phagocytosis and subsequent bacterial clearance by AM. Morphological and biological evidence clearly shows that Lyn delivered bacterial components to lysosomes through autophagy-associated phagocytosis. TLR2 initiated the

phagocytic process and activated Lyn following infection. Cytoskeletal trafficking proteins, such as Rab5 and Rab7, critically facilitated phagocytosis-mediated bacterial degradation following LC-3-mediated phagolysosomal maturation. These findings reveal that Lyn, TLR2 and Rab modulate autophagy-associated phagocytosis and augment bactericidal activity, which may offer insight into novel therapeutic strategies to eliminate infection from the lung.



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A Novel Mechanism for the Human Host Defense Peptide LL-37-Induced Mast Cell Degranulation

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Background: A human neutrophil-derived cathelicidin peptide LL-37 was originally characterized as an antimicrobial, and now recognized to modulate inflammatory and immune responses in leucocytes, endothelial cells and epithelial cells. In addition, LL-37 induces chemotaxis, degranulation and chemokine production by mast cells; however, the mechanism for LL-37-induced mast cell activation remains unclarified.
Recently, a G protein-coupled receptor Mas-related genes X2 (MrgX2) was identified as a receptor for LL-37 for mast cell degranulation. This finding suggests that LL-37 binds with cell surface MrgX2 and activates G protein signaling cascade. In contrast, pruritogenic basic peptides (such as substance P) are reported to induce mast cell degranulation by translocating into the cells. Thus, we hypothesized that LL-37 induces mast cell degranulation by the translocation into the cells, and examined whether LL-37 could translocate into mast cells and induce degranulation, using LAD2 human mast cells.
Methods: LAD2 cells were incubated with LL-37 (0.1~10 μM), and the degranulation was measured by β-hexosaminidase release. Moreover, to examine the translocation of LL-37, LL-37-treated LAD2 cells were cytospun, fixed, permeabilized and stained with anti-LL-37 antibody followed by fluorescence-labeled anti-IgG antibody. The translocation of LL-37 was expressed as a percentage of LL-37-positive (internalized) cells.
Results and Discussion: LL-37 dose-dependently induced degranulation by LAD2 cells, and the degranulation was suppressed by pertussis toxin, confirming the involvement of a G-protein in the degranulation. Interestingly, LL-37 dose-dependently translocated into the cells, and the extents of translocation and degranulation are positively correlated.
To further clarify the cell-surface molecules involved in the LL-37-induced degranulation and LL-37 translocation, LAD2 cells were treated with neuraminidase (sialidase). By neuraminidase-treatment, both the LL-37-induced degranulation and LL-37 translocation were suppressed. Together these observations suggest that human host defense peptide LL-37 interacts with cell surface sialic acids, translocates into the cells and activates (induces) degranulation via a G-protein. However, it remains to be clarified how the translocated LL-37 activates a G-protein for mast cell degranulation.
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Different Dosages of LPS Differentially Modulate Monocytes Responses

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Depending upon the strength of external stimuli, innate monocytes and macrophages can be differentially programmed into distinct states. High dose lipopolysaccharide (LPS) can cause robust expression of pro-inflammatory mediators, while on the other hand, low grade endotoxemia is manifested in humans with adverse health conditions and may skew host immune environment into a mild non-resolving pro-inflammatory state, which is a risk factor for inflammatory diseases such as atherosclerosis. We hypothesize that varying dosages of LPS may differentially modulate monocyte responses including chemokine receptors and cytokines expression, therefore influence their behaviors in disease pathogenesis. The migration ability of monocytes is a good target to study the priming and tolerance phenomena induced by different dosages of LPS. To test this hypothesis, we used an *in vitro* mouse bone marrow derived macrophage (BMDM) culture system. Bone marrow cells cultured with murine M-CSF were stimulated with different dosages of LPS in the same time for five days. Chemotaxis assay was used to study monocytes migration.

We demonstrated that five days treatment of super low dose LPS significantly increased monocytes migration toward MIP-1α and RANTES, while decreased migration abilities were observed in monocytes treated with high dose LPS. The expression level of CCR5, receptor of MIP-1α and RANTES was also found increased in monocytes treated with super low dose LPS. We also discovered that super low dose LPS may enhance monocytes migration via upregulating phosphorylation level of JNK and suppressing phosphorylation level of ERK. On the other hand, migration of monocytes induced by super low dose LPS was suppressed by Tauroursodeoxycholic acid (TUDCA), an ambiphilic bile acid that known as the inhibitor of ER stress.

Our findings showed that different dosages of LPS differentially modulate monocytes chemotaxis through regulating MAPK pathways. The potential mechanisms behind LPS priming and tolerance revealed here will give us better understanding of monocytes development and activation under different circumstance.

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The Role of Sirtuin1 during RSV Infection: Autophagy, ER Stress, and Innate Immunity

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Our lab has established that the process of autophagy contributes significantly to anti-RSV immune responses. In addition, we have recently identified an important role for the NAD-dependent deacetylase Sirtuin 1 in driving autophagy during RSV infection *in vitro* and in a murine model of RSV infection. Dendritic cells from Sirtuin $1_{fl/fl}$-CD11c-Cre mice show significant inhibition of autophagosome formation via confocal and transmission electron microscopy. Sirtuin 1 is thought to be involved in many distinct cellular pathways, including endoplasmic reticulum stress (ER stress). The relationship between autophagy and ER stress has not been completely elucidated, but evidence suggests a potential role for Sirtuin 1. Our data show that RSV infection of bone marrow derived dendritic cells (BMDCs) from mice lacking functional Sirtuin 1 ($Sirt1^-$) have a significant increase in expression of PERK (gene EIF2AK3), a kinase that phosphorylates EIF2a and thereby contributes to the ER stress response. Cells expressing functional Sirtuin 1 (WT) did not upregulate PERK expression following RSV infection, however ER-stress protein CHOP (gene Ddit3) was upregulated in WT cells 12 hours after RSV infection compared to $Sirt1^-$ cells. BMDCs from both Sirt1 sufficient and deficient mice upregulated the expression of ER-stress gene IRE-1. Together, these *in vitro* data suggest that the role of Sirt1 in regulating ER stress may be pathway dependent. Our lab has previously shown that Sirt1 is also involved in anti-RSV immune responses and DC activation during RSV infection. Using lung cDNA from 48 hour RSV-infected WT and Sirt1- mice, we observed changes in expression of PERK, IRE-1, CHOP, IL-23, and IL-27. A full *in vivo* RSV infection time course will be performed and should illuminate numerous changes in the Sirt1 deficient

animals. When infected BMDCs were examined for innate cytokine production, a significant increase was observed with IL-1, IL-6, IL-12, IL-23, and IL-27 in Sirt1$^-$ cells compared to WT. Ongoing studies will test whether a link can be drawn between Sirtuin 1 and ER stress protein responses with control of innate cytokines, which could alter the severity of the virus-induced pathology. Our RSV/Sirtuin 1 knockout model provides a clinically relevant system to explore these questions and resolve the molecular mechanisms connecting Sirtuin 1 activity, autophagy, and ER stress to identify novel therapeutic targets for treatment of RSV infection.

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Induction of Autophagy Increases Enterococcus Faecalis Clearance During Infection

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Medical care costs associated with treating infections in humans due to antibiotic-resistant microorganisms are estimated to be over $ 21 billion annually. A leading cause of such infections are *Enterococcus faecalis and Enterococcus faecium*, Gram-positive bacteria which rank high among the most commonly encountered pathogens infecting the bloodstream, surgical sites and urinary tract and those that cause infective endocarditis. Despite being a commensal organism colonizing the gastrointestinal tract of all humans, enterococci are endowed with traits that make them an opportunistic pathogen. One of the mechanisms by which these commensals cause life-threatening infections is thought to be through bacterial translocation from the intestinal lumen to extraintestinal sites. After translocation into the peritoneal cavity or introduction into the bloodstream, *E. faecalis* survives in the host by evading host-defenses, mainly by resisting phagocytic killing by macrophages. While substantial progress has been made in recent years in understanding the genetic makeup of these organisms, much remains to be learned regarding specific virulence determinants and mechanisms by which they evade host defenses. Our studies and that of others have previously demonstrated that certain strains of *E. faecalis* are capable of surviving within macrophages for extended periods of time, however the exact mechanisms involved are unknown. The resulting failure of host cells to rapidly kill intracellular *E. faecalis* could ultimately lead to systemic spread of the infection. Here we analyzed the intracellular trafficking of enterococci after phagocytosis by macrophages and found that enterococci-containing vacuoles could resist fusion with lysosomes. Characterization of the enterococcus-containing compartment by Transmission Electron Microscopy revealed a single membrane vacuole, which precluded the role of autophagy in elimination of enterococci during macrophage infection. Examination of the conversion of LC3-I to LC3-II by Western blot showed that *E. faecalis* could trigger inhibition of the production of LC3-II during infection, indicating that enterococci could decrease the autophagy level in the infected macrophages. By employing RAW264.7 macrophages transfected with RFP-LC3 plasmid and infected with GFP-labeled *E. faecalis*, we further observed a decrease of RFP-LC3 positive punctae in macrophages infected with *E. faecalis* compared with uninfected cells and no colocalization of RFP-LC3 with *E. faecalis* during enterococcal infection. Physiological or pharmacological stimulation of autophagy in macrophages increased the colocalization of *E. faecalis* containing vacuole with LysoTracker, and suppressed the intracellular survival of *E. faecalis* in *vitro*. In *vivo*, rapamycin treatment also effectively decreased the burden of *E. faecalis* in the liver and spleen of infected mice. These results suggest that pharmacological induction of autophagy may offer a new approach to therapeutic intervention for the treatment of serious enterococcal infections.

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Evaluating the Toxicity of Flavored E-Cigarette Liquids on Human Peripheral Blood Neutrophils and Alveolar Macrophages

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Background: New and emerging tobacco products, such as flavored electronic cigarettes (e-

cigarettes), are advertised as safer alternatives to cigarettes and are frequently allowed in public venues that ban the use of traditional tobacco products. Although "vaping" is gaining popularity, little is known regarding the health effects of short-term and long-term use. Moreover, the increasing use of flavored e-cigarettes is greatly out-pacing toxicological studies evaluating the safety of these products.

Methods: To better understand the biological and physiological effects of exposure to flavored e-liquids, we challenged human peripheral blood neutrophils, human primary alveolar macrophages, and THP-1 monocytes with seven different flavored e-liquids diluted to 1.0% in cell culture media. Following a twenty-four hour e-liquid challenge, cytotoxicity (live/dead cell counts) and inflammation (IL-8 release) were assessed in alveolar macrophages and THP-1 monocytes. Formation of neutrophil extracellular traps (NETs) was used as a marker of neutrophil function. NET formation was determined by quantifying chromatin release every hour during a four-hour e-liquid challenge.

Results: Our preliminary data indicate that of the seven different e-liquids tested, three flavors had varying capacities to elicit adverse responses in each cell type studied. In particular, the cinnamon-flavored e-liquids ("HOT Cinnamon Candies" and "Sin-a-cide") induced significant cytotoxicity in both alveolar macrophages and THP-1 monocytes. Additionally, challenge of THP-1 monocytes with "Banana Pudding (Southern Style)" e-liquid resulted in increased IL-8 secretion as determined by ELISA. Furthermore, the "Sin-a-cide" e-liquid induced significant nucleic acid release from peripheral blood neutrophils over a four-hour exposure while other flavors had no effect on, or suppressed, neutrophil extracellular trap formation in the presence of phorbol 12-myristate 13-acetate (PMA), a potent NET agonist.

Conclusions: Together, these results suggest that the variable composition of individual flavored e-cigarette liquids contributes to measurable inflammatory and cytotoxic responses observed in our study. As such, we plan to further investigate specific chemical components shared among the flavored e-liquids causing these adverse effects and correlate them to specific physiological outcomes. We anticipate that the findings from this study will elucidate the potential negative health effects of e-cigarette usage, and may be useful in developing federal regulations for specific chemicals associated with e-liquid toxicity.

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Macrophage Production of Innate Immune Protein C1q is Modulated During Clearance of Pathogens and Altered-Self Targets

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C1q is an important recognition molecule of the innate immune response. It plays a dual role in vivo, triggering inflammation via activation of the classical complement pathway, and production of opsonin C3b, to clear pathogens. However, it can also directly opsonize targets including apoptotic cells and damaged-self molecules, leading to increased macrophage phagocytosis and M2-like anti-inflammatory cytokine production. C1q is primarily synthesized by macrophages. Therefore we investigated C1q production by macrophages during clearance of inflammatory or damaged-self targets. Murine bone marrow derived-macrophages were incubated with bacteria, immune complexes, apoptotic cells, or atherogenic lipoproteins. C1q gene expression levels were measured by quantitative RT-PCR from isolated mRNA. C1q protein expression and secretion was measured in cell media by hemolytic titer. The results showed a dose dependent decrease in C1q production at the gene level during clearance of inflammatory targets such as E.coli bioparticles, or immune complexes. Levels of C1q activity also decreased dose dependently in cell supernatants, after an initial release of active C1q above basal levels. Conversely, C1q activity increased dose dependently during clearance of altered-self targets such as oxidized LDL, acetylated LDL and apoptotic cells. These data suggest that macrophage responses are fine-tuned to their environment. Macrophages are able to reduce C1q production in the presence of high levels of pathogens, which may assist in resolving excessive complement activation, and increase C1q production upon encountering apoptotic cells or damaged-self to facilitate clearance. This would support a non-complement associated role for C1q in resolving inflammation and prevention of autoimmune disease.

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Transcriptomic Analysis of the Modulation of Programmed Responses in Human Macrophages by Complement Protein C1q During Clearance of Atherogenic Lipoproteins

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Atherosclerosis is a chronic inflammatory disease characterized by the deposition of modified forms of lipoproteins in the arterial intima. Macrophages form the focal point of cholesterol trafficking and are critical in maintaining cholesterol homeostasis. When the balance between cholesterol efflux and influx is disrupted, macrophages accumulate free cholesterol and transform into foam cells. In addition, defective clearance of apoptotic foam cells further drives the progression of atherosclerosis. A detrimental role for complement pathway activation in atherosclerosis is well characterized, however, complement protein C1q plays a dual role in this disease, with studies in C1q knockout mice highlighting a beneficial role for C1q in controlling early atherosclerosis. Beyond its role in the activation of complement cascade, and in the absence of co-activators C1r and C1s, C1q protein directly modulates phagocyte cell activation when bound to targets such as damaged-self molecules like modified lipoproteins. We have previously reported that C1q improves phagocytosis and promotes anti-inflammatory macrophage polarization during the clearance of oxidized low-density lipoprotein (oxLDL). However, detailed pathways and mechanisms of C1q's protective role in atherosclerosis remain unexplored.

To further characterize the role of C1q in programming human monocyte-derived macrophage (HMDM) responses, we used RNA-Sequencing as an unbiased screen to elucidate the non-complement functions of C1q protein and to characterize novel effector functions and pathways that C1q modulates in macrophages during the clearance of oxidized and acetylated LDL. Following library construction, we used bioinformatics tools to analyze and determine relationships between differentially expressed genes in the presence or absence of C1q. We observed 1679 genes and 2239 genes modulated by C1q in HMDMs treated with oxidized LDL and acetylated LDL respectively. Gene ontology analysis and gene network visualization using Cytoscape revealed dampening of genes involved in inflammasome activation and apoptosis, and upregulation of genes involved in promoting cholesterol efflux and lipid metabolism.

These results suggest C1q promotes macrophage survival by modulating apoptosis, the inflammatory response, cholesterol efflux, and macrophage activation. Ultimately, we hope to identify potential therapeutic targets and strategies to slow down the progression of atherosclerosis. These may include strategies to restore or augment defective apoptotic foam cell clearance, enhance or mimic anti-inflammatory macrophage polarization, and inhibit detrimental terminal complement pathway activation.

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Ceramide Plays a Critical Role in Allergic Airway Disease

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Asthma, defined as a chronic inflammatory condition characterized by episodic shortness of breath with expiratory wheezing and cough, is a serious health concern. The World Health Organization estimates that asthma affects more than 230 million people worldwide. Asthma is a serious public health concern here in the Commonwealth as well. The Asthma and Allergy

Foundation of America has deemed Richmond the 'Asthma Capital of the US' for the last two years, thus highlighting asthma as significant public health interest locally. There is a strong genetic component to asthma and numerous genome-wide association studies (GWAS) have identified ORM (yeast)-like protein isoform 3 (ORMDL3) as a gene associated with susceptibility to both childhood and adult-onset asthma in a number of ethnically diverse populations. Surprisingly however, the mechanism by which ORMDL3 contributes to asthma pathogenesis is not well understood and is a matter of great debate. The yeast ortholog of ORMDL3 is a negative regulator of serine palmitoyltransferase (SPT), the rate limiting step in *de novo* ceramide biosynthesis, yet elevations of ceramide rather than its reduction have been linked to inflammatory responses *in vitro*. Additionally, elevated levels of ceramide have been reported in lung disease, thus presenting a biological paradox. Therefore, we examined the role of ORMDL3 in asthma immunopathology. Consistent with its role in yeast, we have shown in preliminary studies that decreasing expression of ORMDL3 in lung epithelial cells and macrophages increases ceramide and conversely, modest increases in ORMDL3 decrease ceramide levels by decreasing *de novo* biosynthesis. Remarkably, however, higher levels of ORMDL3 overexpression had the opposite effect and significantly elevated ceramide. In a house dust mite (HDM) mouse model of allergic airway disease, allergen challenge induced expression of ORMDL3 and resulted in a concomitant increase in lung ceramide. Intriguingly, the use of specific inhibitors which block the increase in ceramide production completely prevented HDM-induced airway hyperreactivity (AHR) and suppressed airway inflammation. We and others have found that ORMDL3 is an allergen- and Th2 cytokine–inducible gene. Nasal administration of the orally available FDA approved prodrug FTY720/fingolimod reduced both ORMDL3 expression and ceramide production while mitigating airway inflammation, hyperreactivity, and mucus hypersecretion in HDM challenged mice. Intriguingly, we also see an elevation of ceramide in the exhaled breath condensate (EBC) of patients with asthma as compared to healthy controls. Thus the ORMDL3 -ceramide pathway may be a novel therapeutic target for the control of allergic asthma.

A Novel Role for Oxysterols in Resolution of Airspace Neutrophilia

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Recent literature has identified several lipid and non-lipid molecules that promote resolution of inflammation, yet many questions remain about the nature and function of pro-resolving mediators in vivo. We recently reported that treatment of mice with a synthetic agonist for the oxysterol-sensing nuclear receptor Liver X Receptor (LXR) attenuates lipopolysaccharide (LPS)-induced lung inflammation. Given this, here, we questioned whether native LXR agonists (oxysterols) might be generated in the inflamed lung and mediate resolution. Among several oxysterols surveyed, we found the LXR agonist 25-OH-cholesterol (25HC) to be induced in the airspace fluid and serum of mice following LPS inhalation. This was associated with robust MyD88- and TRIF-dependent upregulation of the 25HC-synthetic enzyme, cholesterol-25-hydroxylase (Ch25h), in airspace macrophages. 25HC induction by LPS was abolished in Ch25h-/- mice, indicating an absolute requirement for this enzyme. In profiling the effect of Ch25h deletion upon LPS-induced inflammation in the murine lung, we found that the induction phase of cellular inflammation was intact in Ch25h-/- mice, but that late-phase resolution of alveolar neutrophilia was attenuated. Chimeric mice generated through bone marrow transfer revealed that deletion of Ch25h in hematopoietic cells was sufficient to confer a deficit in resolution of neutrophilia. Similar deficits in resolution of end-organ neutrophilia in Ch25h-/- mice were noted after infection with K. pneumoniae in the lung and after injection of thioglycolate in the peritoneum. Suggesting a requirement for endogenous 25HC in native LXR activation in the inflamed lung, LXR target genes were induced in the Ch25h+/+ lung after LPS, whereas this LXR signature was abolished in Ch25h-/- mice. Further, consistent with a requirement for endogenous LXR activity in resolution, LXR-null mice, like Ch25h-

null mice, displayed sustained alveolar neutrophilia after inhaled LPS. Given the established role of efferocytosis (phagocytic clearance of apoptotic cells) in resolution and reports that the efferocytic receptor Mertk is a direct LXR target, we tested for a deficient Ch25h-25HC-Mertk-efferocytosis axis in Ch25h-/- mice. We found that 25HC induced Mertk and other LXR targets (i.e., Abca1) in cultured macrophages, whereas Mertk and Abca1 expression were deficient in both peritoneal exudate macrophages and LPS-exposed lung of Ch25h-/- mice. Apoptotic cells upregulated Ch25h in co-cultured wild type macrophages and were found to induce Mertk and Abca1 less robustly in Ch25h-/- macrophages than in wild type counterparts. Finally, in vivo efferocytic clearance of intratracheally instilled apoptotic thymocytes by alveolar macrophages was confirmed to be deficient in Ch25h-/- mice, as was induction in the airspace of TGFβ, an anti-inflammatory mediator induced during efferocytosis. Taken together, we identify a key requirement for Ch25h-generated 25HC in an LXR-mediated pathway to efferocytosis during resolution of inflammation.

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Pseudomonas Aeruginosa Promotes Persistent Inflammation in the Cystic Fibrosis Airway by Preventing the Generation of the Pro-Resolving Mediator 15-Epi Lipoxin A4

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Lipoxins are arachidonic acid-derived lipid mediators that promote the resolution of inflammation and the return to tissue homeostasis. The rapid generation of lipoxins by cell-to-cell interactions promotes healing of inflamed tissues by several mechanisms, including reducing neutrophil extravasation and enhancing efferocytosis of apoptotic leukocytes. Reduced pulmonary lipoxin levels have been correlated with hyperinflammatory diseases such as cystic fibrosis (CF), potentially contributing to the inability to resolve the inflammatory environment in the lung (Karp, et al. *Nature Immunology*, 2004). However, mechanisms by which lipoxin levels are decreased remain to be determined. Neutrophils can produce 15-epi lipoxin A_4 (15-epi LXA_4) when activated in the presence of 14,15-epoxyeicosatrienoic acid (14,15-EET), a cytochrome P450-derived eicosanoid produced by airway epithelial cells (AECS) (Ono, et al. *Am J Respir Crit Care Med*, 2014). Following antibiotic treatment, 15-epi LXA_4 levels increase in CF patient bronchial lavage fluid suggesting infection status may contribute to low levels of lipoxins in CF airways (Chiron, et al. *J Cystic Fibrosis*, 2008). CF patients are often chronically colonized in the airways with the bacterial pathogen *Pseudomonas aeruginosa*, resulting in a significant morbidity and mortality. *P. aeruginosa* infections are characterized by robust neutrophilic inflammation, resulting in extensive damage to host tissue and eventually respiratory failure. *P. aeruginosa* is able to thrive in the hostile hyper-inflammatory CF lung, persisting despite heightened immune responses that are unable to clear the infection. We hypothesized that the epoxide hydrolase activity of the secreted *P. aeruginosa* virulence factor Cif (Bahl, et al. *J Bacteriology*, 2010) catalyzes the hydrolysis of 14,15-EET, thus reducing the generation of 15-epi LXA_4 by neutrophils and impairing resolution of inflammation in the CF lung. Utilizing a polarized AEC model, our studies show that Cif hydrolyzes the epoxide moiety on 14,15-EET, secreted apically by AECs. Employing a catalytically-inactive mutant of Cif (D129S-Cif), we confirmed that Cif's epoxide hydrolase activity is required for this effect. Co-culture experiments with CF AECs and isolated human neutrophils demonstrate that through a reduction in 14,15-EET, Cif decreases the production of 15-epi-LXA_4 by neutrophils. To test whether Cif-mediated reduction of 15-epi LXA_4 had functional consequences, we examined neutrophil transepithelial migration though CF AECs grown in a modified Boyden chamber. We confirmed that 15-epi LXA_4 prevents neutrophil transepithelial migration through CF AECs and further, that Cif reverses this inhibition. In conclusion, our data demonstrates that *P. aeruginosa* can reduce neutrophil production of 15-epi LXA_4 by decreasing the bioavailability 14,15-EET, thus promoting an inflammatory environment in the CF lung. These findings suggest a novel function for a bacterial virulence factor and provide a potential mechanism to explain in part the clinical observation of

prolonged and destructive neutrophil derived inflammation caused by *P. aeruginosa* infections in chronic lung diseases.

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The Endocannabinoid N-Arachidonoyl Dopamine Modulates TLR-Dependent Endothelial and Systemic Inflammation

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Background/Rationale: Endocannabinoids are endogenous, arachidonic acid (AA)-derived lipid mediators with immunomodulatory activities. Endothelial cells (ECs) are centrally involved in the pathogenesis of organ injury during inflammatory critical illnesses, such as sepsis, since they express inflammatory mediators and regulate leukocyte trafficking, coagulation, and vascular barrier function. We recently discovered that the endocannabinoid *N*-arachidonoyl dopamine (NADA) reduces EC activation induced by microbial and endogenous inflammatory agonists, including LPS, bacterial lipopeptide, and TNFa. We tested the hypothesis that NADA reduces inflammatory activation of ECs via other AA-derived lipids, including the prostanoids, and assessed the effects of NADA treatment on inflammation and coagulation pathways *in vivo* in endotoxemic mice.

Methods: *In vitro:* Human lung microvascular ECs (HMVEC) were treated with LPS in the presence or absence of NADA. We performed a qPCR array using cDNA to determine NADA's effects on AA metabolism. Genes showing a >3-fold change in expression were verified by immunoblot, and levels of prostanoids were quantified in EC culture supernatants and lysates. To determine if prostanoids contribute to the immunomodulatory activity of NADA, we treated HMVEC with NADA and LPS in the presence of the COX-1/2 inhibitor indomethacin. *In vivo:* C57BL/6J mice (n = 4-6/group) were treated with NADA (IV, 1-10 mg/kg) just prior to challenge with LPS (IV, 5 mg/kg). We quantified plasma levels of cytokines, chemokines, and plasminogen activator inhibitor-1 (PAI-1). Data were analyzed using Mann-Whitney tests. P values < 0.05 were considered significant.

Results: *In vitro*: NADA significantly abrogates LPS-induced prostacyclin, thromboxane A_2, and prostaglandin E_2 production by HMVEC, but dramatically upregulates prostaglandin D_2 production. NADA also robustly upregulates COX-2 expression, and COX inhibition reverses the NADA-mediated decrease in cytokine secretion. *In vivo*: Treatment with NADA dose-dependently reduces levels of IL-6, TNFa, and MIP-1a, and the coagulation pathway intermediary PAI-1, but increases levels of the anti-inflammatory cytokine IL-10 in LPS-treated mice ($p<0.05$).

Conclusion: Our results suggest that the NADA modulates endothelial inflammatory activation via the regulation of COX-2-derived lipid mediators. By elucidating the mechanism by which endocannabinoids modulate prostanoid metabolism in ECs, we hope to better understand the therapeutic potential of manipulating the endocannabinoid system in acute inflammatory disorders characterized by endothelial activation. Maladaptive responses in sepsis and tissue injury are believed to result in part from the failure to shift to the pro-resolving phase of inflammation. Pro-resolving lipid mediators, such as resolvins, have been implicated as mediators of and potential therapies for inflammatory critical illness. We hypothesize that NADA represents a novel endogenous lipid mediator that promotes the resolution of endothelial inflammation and, as a result, may contribute to therapeutic strategies to combat organ failure during inflammatory critical illness.

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Docosahexaenoic Acid Ethyl Ester Enhances Antibody Levels Upon Influenza Infection in Murine Obesity Accompanied by a Reduction in Circulating Resolvin D1

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Obesity is associated with impaired humoral immunity, particularly in response to viral infections. Recent studies show that endogenous specialized pro-resolving lipid mediators, synthesized from the omega-3 fatty acid docosahexaenoic (DHA) acid, boost antibody secretion by increasing the production of antibody secreting cells. Therefore, the objective of this study was to determine if DHA as the parent molecule, upon dietary supplementation to an obesogenic diet, would boost murine B cell mediated responses upon influenza A/PR/8/34 (PR8) infection. DHA ethyl esters, modeling human intake at 2 grams per day, enhanced circulating hemagglutination inhibition antibody titers accompanied by a significant increase in the frequency of all major splenic B cell subsets including $CD19^{+}IgM^{+}CD138^{+}$ cells. DHA had no influence on hemagglutination inhibition antibody titers in bronchoalveolar lavage fluid although DHA did lower transcript levels of PR8 and inflammatory cytokines in the lungs. Strikingly, DHA decreased resolvin D1 levels in addition to PGE2 in circulation, suggesting potential flux of DHA through other metabolic pathways involved in the synthesis of pro-resolving lipid mediators. Altogether, our results indicate that a physiologically relevant dose of DHA can boost antibody production to a T-dependent antigen in obese mice. Moreover, these data suggest that dietary DHA may have therapeutic potential for targeting immune outcomes in the obese population upon respiratory viral infection.

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Aspirin-Triggered Resolvin D1 is Produced During Self-Resolving Escherichia Coli Pneumonia and Regulates Infiltrating Macrophages for the Resolution of Lung Inflammation

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Bacterial pneumonia is a leading cause of morbidity and mortality worldwide. Host responses to contain the infection and mitigate pathogen-mediated lung inflammation are critical for pneumonia resolution. Aspirin-triggered resolvin D1 (AT-RvD1; 7*S*,8*R*,17*R*-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) is a lipid mediator that displays organ-protective actions in sterile lung inflammation. Here, with a self-resolving murine model of *Escherichia coli* (*E. coli*) pneumonia, principal components analysis of the results of lipid mediator metabololipidomics on lung tissue obtained at baseline and 24 and 72 hours after infection pointed to a prominent role for the endogenous production of AT-RvD1. Early treatment with exogenous AT-RvD1 (1 hr post-infection) enhanced bacterial clearance *in vivo*, and lung macrophage phagocytosis of fluorescent *E. coli* particles *ex vivo*. Characterization of macrophage subsets during pneumonia identified efferocytosis of alveolar apoptotic neutrophils by infiltrating macrophages ($CD11b^{Hi}$ $CD11c^{Low}$) and exudative macrophages ($CD11b^{Hi}$ $CD11c^{Hi}$). AT-RvD1 increased efferocytosis by these cells *in vitro*, and accelerated neutrophil clearance in pneumonia *in vivo*. These anti-bacterial and pro-resolving actions of AT-RvD1 were additive to antibiotic therapy. Taken together, these findings suggest that the pro-resolving actions of AT-RvD1 for pathogen-mediated lung inflammation represent a novel therapeutic strategy to complement the current antibiotic-centered approach to combatting infections.

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Expression of the Free Fatty Acid Receptor 4 (FFAR4/GPR120) in Bovine Neutrophils

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Free fatty acids are increased in plasma around partum in cows, period which there is a high incidence of infectious disease, such as mastitis and metritis. Long chain fatty acids bind to G-protein coupled receptors, such as free fatty acid receptor 1 (FFAR1) and FFAR4, and a role of these receptors on inflammatory response has been suggested. FFAR1 has been related to proinflammatory response, whereas FFAR4 to antiinflammatory response. Recently, we cloned and demonstrated the presence of the functional FFAR1 in bovine neutrophils, however there is not data about FFAR4 in these cells. The aim of this study was to determine the presence of FFAR4 in bovine neutrophils.

Neutrophils were isolated from healthy bovine and total RNA and proteins were obtained. By RT-PCR and using specific primers to bovine FFAR4 we obtained a product of amplification, 134 bp, of the expected size for FFAR4. Using antibody against FFAR4 we detected by immunoblot a band of approximately 42 kDa, the predicted size for FFAR4. Also, it was possible to observe the presence of FFAR4 in neutrophils by immunofluorescence. Additionally, we observed that the natural agonist of FFAR4 docosahexaenoic acid (DHA) induced intracellular calcium mobilization in Fura-2AM-loaded neutrophils by spectrofluorometric assay. The EC_{50} value of DHA was 59 μM. When DHA was incubated in presence of GW1100, an antagonist of FFAR1, no inhibition of intracellular calcium mobilization was observed.

In conclusion, our results showed the presence and a functional role of FFAR4 in bovine neutrophils, thus suggesting that bovine neutrophil function could be modulated in the presence of fatty acids.

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Attenuation of Viral-induced Pulmonary Disease by Lactobacillus Johnsonii Supplementation: Plasma Metabolites and Dendritic Cell Modulation.

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The emerging field of microbiome research has demonstrated the key role of microbial communities in the modulation of host immune responses. Previous studies in our group observed changes in the murine gut microbiome that were associated with altered airway immune responses to allergen challenge in mice gavaged with pet-associated house dust. This alteration was replicated in RSV infected mice using a gut-derived bacterium from dust-supplemented animals, *Lactobacillus johnsonii*. The present studies demonstrate changes in immune responses at early time points of the RSV infection response due to changes in innate immune responses. Bone marrow-derived dendritic cells (BMDC) grown from L. johnsonii supplemented animals demonstrated reduced innate cytokines, TNF and IL-6, upon RSV infection, suggesting systemic changes in cell reactivity. Plasma metabolomic profiles of mice given *L. johnsonii* daily for 1 week showed a significant increase in docosahexaenoic acid (DHA), a key n-3 polyunsaturated fatty acid, as well as a butyrate metabolite. More striking were changes in a broad spectrum of immunomodulatory serum metabolites in *L. johnsonii*-supplemented mice during RSV infection at day 2 of infection. The use of plasma from supplemented mice in RSV-infected BMDC cultures reduced co-stimulatory molecule expression. When plasma-incubated BMDCs were used in RSV stimulated CD4 T cells isolated from RSV-infected mice a reduction in IL-4 and increase in IFNg was observed. Thus, supplementation of mice with *L. johnsonii* alters the gut microbiome and enhances circulating concentrations of the immune modulatory metabolites providing a potential mechanism for the modified immune response to RSV.

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Elevated MicroRNA-33 in Carbon Nanotube-Mediated Chronic Granulomatous Disease and Human Sarcoidosis Patients is Associated with Alveolar Macrophage Lipid Transporter Dysfunction.

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We established a murine model of multiwall carbon nanotube (MWCNT)-induced chronic granulomatous disease which bears many similarities to human sarcoidosis, a debilitating inflammatory disease of unknown cause. At 60 days after oropharyngeal MWCNT instillation, bronchoalveolar lavage (BAL) cells from wild-type mice express an M-1 phenotype with elevated pro-inflammatory cytokines and reduced peroxidase proliferator-activated receptor gamma (PPARγ) - characteristics also present in sarcoidosis. Because of this PPARγ repression, we hypothesized that MWCNT might also mediate repression of PPARγ-related pathway genes such as the lipid transporters ABCA1 and ABCG1, both of which display anti-inflammatory properties and are essential to pulmonary homeostasis. Results at 60 days after MWCNT instillation indicated significant repression of BAL cell ABCA1 (-1.7 fold) and ABCG1 (-2.0 fold) expression compared to sham controls. Exploration of potential regulatory factors revealed that microRNA (miR)-33, a lipid transporter regulator in atherosclerosis models, was strikingly elevated (13.9 fold, p=in vitro studies to determine whether lentivirus-miR-33 overexpression would affect alveolar macrophage lipid transporters. Results confirmed that miR-33 overexpression repressed both ABCA1 and ABCG1 (but not PPARγ) in cultured primary murine alveolar macrophages. Subsequent evaluation of BAL cells from sarcoidosis patients compared to healthy controls also revealed elevated miR-33 (5 fold) together with reduced ABCA1 (4.3 fold) and ABCG1 (-3.4 fold). Moreover, miR-33 was elevated in sarcoidosis granulomatous tissue. *In vitro* studies indicated that the pro-inflammatory cytokine, TNFα, which is overexpressed in the MWCNT model and in sarcoidosis lung, dose-dependently upregulated miR-33 in healthy human alveolar macrophages. Findings suggest that alveolar macrophage miR-33 is upregulated by pro-inflammatory cytokines and may perpetuate chronic inflammatory granulomatous disease by repressing anti-inflammatory functions of ABCA1 and ABCG1 lipid transporters. Data further suggest that alveolar macrophage lipid dysfunction may play a role in the dyslipidemia previously reported in sarcoidosis patients.

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Retinoic Acid and Helicobacter Pylori Differentially Regulate CD103 (αE integrin) Expression by Human Dendritic Cells

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Human gastric mucosal dendritic cells (DCs) have been shown to express CD103 (αE integrin) at lower levels than intestinal mucosal DCs. CD103 is thought to be a mediator of immune cell interactions with the epithelium. In this study, we assessed possible regulatory mechanisms of CD103 using monocyte derived dendritic cells (MoDCs) under normal and serum free culture conditions. Specifically, we determined whether MoDC expression of CD103 is regulated by physiologically relevant concentrations of retinoic acid (RA, 100 nM) and by Helicobacter pylori (H. pylori) infection. In serum-containing medium, the presence of RA had little impact on MoDC CD103 expression, possibly due to the retinoids present in the fetal bovine serum. In contrast, qPCR revealed a strong, 34-fold upregulation of CD103 when MoDCs were cultured with RA under serum free conditions. Correspondingly, CD103 surface expression analyzed by FACS significantly increased from 6.8±2.8% to 40.1±8.9% of total MoDCs cultured in the presence of RA. Intracellular CD103 showed a similar increased pattern of expression with 75.7±9.0% compared to 50.8±17.5% of CD103 positive MoDCs in RA-treated versus control cultures. Interestingly, RA also caused an increase in the ratio of extracellular to intracellular CD103. In contrast to RA, stimulation with H. pylori had down-regulatory

effects on MoDC CD103 expression. Consequently, MoDCs expressed less surface CD103 after H. pylori exposure versus the control culture, 2.2±1.0% and 6.8±2.8% respectively. Intracellular CD103 expression also decreased. Importantly, H. pylori stimulation completely abrogated the strong upregulation of CD103 seen with RA culture. Thus, MoDCs cultured in RA and concurrently stimulated with H. pylori showed decreased expression of extracellular and intracellular CD103, 3.2±1.1% and 15.1±8.1% respectively. Notably, these patterns were confirmed using reverse transcriptase qPCR. MoDCs stimulated with H. pylori showed a 4.3-fold decrease in CD103 gene expression compared to the control culture. Similarly, when MoDCs cultured with RA were stimulated with H. pylori, there was a 4.7-fold decrease in CD103 gene expression compared to the RA-treated, non-stimulated culture. Further investigation into the regulation of CD103 by H. pylori is needed to determine if the response is specific. The observed downregulation of CD103 could be a possible mechanism for dendritic cells to detach from the epithelium for migration to thelymph nodes upon bacterial infection. Insight into the regulation of CD103 may lead to a better understanding of epithelial and dendritic cell interactions within the gastric mucosa.

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Sensing Pseudomonas aeruginosa Lipid A Variants by Neutrophils and Monocytes, the Key Players of Lung Inflammation in Cystic Fibrosis

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In cystic fibrosis (CF) disease, the host immune system fails to clear *Pseudomonas aeruginosa* (PA) infection. PA alters the structure of the lipid A component of their lipopolysaccharide (LPS) to adapt to the CF airway. Robert K. Ernst's laboratory discovered unique penta- and hexa-acylated PA LPS in clinical isolates from patients with CF. A hepta-acylated PA LPS was also found in one third of the CF patients with severe disease as determined by FEV_1 measurements; these patients lack the penta and the hexa-acylated variants. The biological activity of the CF specific penta- and hexa-acylated LPS variants has been partially characterized in the human monocytic cell line (THP-1). Our study is the first to determine if these structural variants can modulate functional responses of human neutrophils and monocytes, the predominant innate immune cell types infiltrating CF lungs. Lipid A, the bioactive component of LPS, has been used for each of the PA LPS structural variants. First we tested each lipid A variant for human TLR4 stimulating activity in a HEK Blue 4 reporter cell line. We found that both the hexa- and hepta-acylated lipid A variants were equivalent in TLR4 activation, whereas, the penta-acylated variant was much weaker. Next, human neutrophils were stimulated with each lipid A and two immediate functions of neutrophils were investigated. Priming of isolated human neutrophils was measured as an increase in fMLF-stimulated superoxide release following incubation with each lipid A variants. Our data showed strong priming activity by the hexa and hepta- acylated variants whereas very weak activity by the penta-acylated structure. Similar to the priming response, hexa and hepta-acyl lipid A variants induced exocytosis of secretory vesicles and specific granules measured as increases in membrane expression of CD35 and CD66b, respectively, by FLOW cytometry. Interleukin (IL)-8 is a crucial chemoattractant for neutrophils in CF lungs, hence, IL-8 released after overnight stimulation of neutrophils with the different lipid A variants was quantified by ELISA. Strong IL-8 induction was observed by the hexa and hepta-acylated variants, while the penta-acylated form was a weak inducer of the cytokine. Both hexa- and hepta-acylated variants showed strong IL-8 induction in primary monocytes; however, unlike neutrophils, monocytes showed a robust IL-8 response to the penta-acylated variant. In addition, at very high dose, the penta-acylated structure induced similar level of IL-8 as hexa- or hepta-acylated lipid A treatment. To determine if the differential effect of the penta-acylated variant in monocytes was similar for other cytokines, we next compared their abilities to induce TNF-α, a major pro-inflammatory cytokine present in elevated level in CF lungs. Our data showed that the penta-acylated variant, even at very high dose, could not induce similar level of TNF-α as the hexa- or hepta-acylated structure. In addition, the penta-acylated lipid A elicited an antagonistic effect on hexa- and hepta-induced TNF-α response from monocytes.

Taken together, our data suggest that the presence of antagonistic penta-acylated lipid A variant which has very little inflammatory activity of its own, may suppress the inflammatory outcomes triggered by the hexa-acylated structure in the early stage of the disease. Absence of this penta-acylated structure and appearance of another inflammatory variant with seven fatty acid chains may contribute to more severe disease in the late stages of CF.

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Loss of Polyisoprenyl Diphosphate Phosphatase 1 (PDP1) Enhances Bacterial Clearance While Attenuating Neutrophil Functions

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Bacterial pneumonia continues to be one of the leading causes of morbidity and mortality. Neutrophils play critical roles in the defense against invading pathogens; however, their activation must be tightly regulated in order to avoid unwanted tissue injury. Polyisoprenyl diphosphate phosphatase 1 (PDP 1) is a lipid phosphatase that converts presqualene diphosphate to presqualene monophosphate in order to facilitate neutrophil activation. We have previously shown that 15-epi-lipoxin A_4, a specialized pro-resolving lipid mediator, blocks PDP1 activation and neutrophil superoxide production. In order to further elucidate the role of PDP1 during infection and neutrophil functions, we have generated mice deficient in PDP1. Bone marrow (BM) neutrophils deficient for PDP1 displayed reduced superoxide production compared to the littermate-control (WT) BM neutrophils in response to PMA as measured by the cytochrome c reduction assays. In addition, PDP1 deficient BM neutrophils show reduced release of neutrophil extracellular traps (NETs) in the presence of *E. coli* as measured by fluorescence plate reader assay and fluorescence microscopy. Moreover, human neutrophils transfected with anti-PDP antibody also show reduced NET release in response to PMA or *E. coli*. Despite reduced neutrophil functions, PDP1 deficient mice showed enhanced bacterial clearance *in vivo* in a mouse model of bacterial pneumonia. The analysis of bronchoalveolar lavage fluid 2 h post infection showed that there is increased number of neutrophils in PDP1 deficient mice. Together, these results suggest that PDP1 deficient mice enhance neutrophil recruitment to control bacterial infection while attenuating injurious functions from individual neutrophils. The results provide evidence that attenuating PDP1 enzymatic activities may be host protective during bacterial pneumonia.

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Antibodies Are Necessary for rVSV/ZEBOV-GP Mediated Protection Against Lethal Ebola Virus Challenge in Nonhuman Primates

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Ebola viruses (EBOV) cause hemorrhagic disease in humans and nonhuman primates with high fatality rates. These viruses pose a significant health concern worldwide due to the lack of approved therapeutics and vaccines as well as their potential misuse as bioterrorism agents. Although not licensed for human use, recombinant vesicular stomatitis virus (rVSV) expressing the filovirus glycoprotein (GP) has been shown to protect macaques from EBOV and Marburg virus (MARV) infections, both prophylactically and post-exposure in a homologous challenge setting. However, the immune mechanisms of protection conferred by this vaccine platform remain poorly understood. In this study, we set out to investigate the role of humoral versus cellular immunity in rVSV vaccine-mediated protection against lethal *Zaire ebolavirus* (ZEBOV) challenge. Groups of cynomolgus macaques were depleted of CD4+ T, CD8+ T or CD20+ B cells prior to and during rVSV vaccination. Unfortunately, CD20-depleted animals generated a robust IgG response. Therefore, an additional group of vaccinated animals were depleted of CD4+ T cells during challenge. All animals were subsequently challenged with a lethal dose of ZEBOV. Animals depleted of CD8+ T cells survived, suggesting a minimal role for CD8+ T cells in vaccine-mediated protection. Depletion of CD4+ T cells during vaccination caused a complete loss of GP-specific antibodies and abrogated vaccine protection. In contrast depletion of CD4+ T cell during challenge resulted in survival of the animals, indicating a minimal role for CD4+ T cell

immunity in rVSV-mediated protection. Our results suggest that antibodies play a critical role in rVSV-mediated protection against ZEBOV.

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Uncovering Mechanisms of Protection Against Ebola Virus Infection Conferred by Recombinant VSV-GP Vaccine

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Ebola virus (EBOV) continues to pose a significant threat to human health as evidenced by the recent outbreak in West Africa that claimed over 11,200 lives and lasted for over a year. Although there are no currently approved vaccines or postexposure treatments, a recombinant vesicular stomatitis virus expressing the EBOV glycoprotein (rVSV-GP) has entered clinical trials. We have shown that protection conferred by rVSV-GP is mediated via antibodies; however, the mechanisms by which this live attenuated vaccine elicits robust antibody responses against the GP antigen are poorly understood. To uncover these mechanisms, we carried out a longitudinal gene expression analysis of blood samples collected from four groups of animals post-vaccination and post Ebola challenge: 1) Negative controls vaccinated with rVSV expressing Marburg virus GP (rVSV-MARV, non-survivors); 2) Positive controls vaccinated with rVSV/EBOV-GP (survivors); 3) animals vaccinated with rVSV/EBOV-GP and depleted of $CD4^+$ T cells (non-survivors); 4) animals vaccinated with rVSV/EBOV-GP and depleted of $CD8^+$ T cells (survivors). Following vaccination with rVSV/EBOV-GP, changes in gene expression were transient and only detected on day 7 post-vaccination. Differentially expressed genes (DEG) were involved in host defense, cell cycle and protein metabolic processes. Following EBOV challenge, both groups of non-survivors had significantly larger transcriptional changes compared to survivors. At day 4 post-infection, rVSV-MARV animals had over 1900 DEG that enriched to pathways related to inflammation, defense to virus infection, lymphocyte activation and regulation of body fluids. In contrast, the $CD4^+$ T cell depleted group had limited transcriptional changes, indicating they had partial protection compared to the negative controls as evidenced by delayed viremia. Similarly, both groups of survivors had small transcriptional changes consistent with lack of viremia on day 4 post-infection. On day 7, both non-survivor groups showed over 2,000 DEG involved with immune response, cell death and coagulation, consistent with development of hemorrhagic viral fever and fatality. Interestingly, and despite the lack of viremia, $CD8^+$ T cell depleted survivors had a higher number of transcriptional changes (484) than non-depleted positive controls (197) on day 7 post infection indicative of higher subclinical viral burden. These data suggest that although antibodies are the primary mode of protection conferred by rVSV/EBOV-GP, $CD8^+$ T cells play a role in protection. Both groups of survivors upregulated genes involved in innate immune response, defense response to virus and type I interferon signaling. Finally, our analysis revealed a set of genes that were differentially expressed in survivors and non-survivors. Overall, our data gives insight into the mechanisms of protection conferred by this vaccine as well as pathogenesis of EBOV.

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Differential Effects of HIV Latency Reversing Agents on T Cell Phenotype and Function: Implications for HIV Cure

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HIV antiretroviral therapy (ART) is highly successful at lowering plasm viremia, restoring CD4 T cell levels and prolonging life. However, ART does not clear HIV-1 infected cells in which the virus is integrated into the host genome but does not express viral proteins. These HIV latently infected cells are rare but following treatment interruption consistently reactivate, these are likely stochastic events, resulting in virus rebound within weeks. The lack of HIV protein expression in latently infected cells also limits the detection of

these cells by humoral and cellular immune responses. Unsurprisingly, T cell immunotherapy studies have not limited virus rebound following treatment interruption.A major advance in the HIV cure field, has been the identification compounds, from different drug classes, that induce HIV reactivation from latently-infected cells in vitro. More recently, reactivation of HIV, as measured by HIV RNA, has also been demonstrated in vivo. Significant effort is now being made to use these latency-reversing agents (LRAs) in combination with vaccines capable of eliciting CD8+ T cells to detect and delete HIV-1 infected, reactivated cells.While this strategy wholes great promise, more studies are needed determine the effect of these drugs on T cells. We developed an in vitro system focusing on the impact of clinically-relevant exposures to leading HIV LRAs on T cell activation and function – three histone deacetylase inhibitors (HDACi), Vorinostat (VOR), Panabinostat (PAN) and Romidepsin (ROMI) and protein kinase C modulators (PKCm), ingenol dibenzoate (ING), Prostratin (PRO) and Bryostatin (BRYO) were tested in PBMC from both HIV-1-infected, durably treated and seronegative individuals.No effect of VOR was observed on either CD4 or CD8 T cell activation, whereas PAN and ROMI consistently increased expression of CD69 on CD4 and CD8 T cells HDACis did not impact PD-1 expression levels. In contrast, the PKCm consistently increased and sustained cellular T activation. BRYO was highly toxic to cells.When antigen specific T cell function was measured, VOR and ROMI did not impact ex vivo antigen-specific CD8 T cell responses as measured by degranulation and cytokine production (IFN-γ, TNFα and MIP-1β) after stimulation with influenza, EBV and CMV T cell epitopes (FEC). Ex vivo studies of VOR treated individuals suggested VOR impacted T cell distribution but not the circulating frequencies of HIV reactive T cells. In contrast, in vitro studies of PAN, produced a small but significant decrease in the frequency of both HIV and FEC specific CD8+ T cell responses. In contrast, PKCm induced significantly non-specific cytokine production; this effect was more marked in cells from durably suppressed, HIV seropositive individuals. No significant effect and antigen specific function was observed. These data suggest that even within the same class, LRAs vary in their effect on the phenotypic profile and functional capacity of T cells. These differences should be considered when optimizing HIV immunotherapy studies to achieve HIV Cure.

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Modulation of Th2-Associated Allergic Immune Responses With Mucosal Nanoemulsion-Based Vaccines

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Food allergy is an emerging epidemic that affects upwards of 15 million people in the United States, including 1 in 13 children. The pathology associated with food allergy is most often the result of a Th2-skewed immune response and increased IgE production, triggering the release of histamine from mast cells, which can lead to anaphylaxis. Immunotherapies to shift immune responses from Th2 to Th1 have generally required prolonged immunization protocols and have not induced long-lasting Th1 responses. We have demonstrated that nanoscale emulsion (NE), a novel mucosal adjuvant, induces robust IgA and IgG antibody responses and Th1/Th17-polarized cellular immunity, resulting in protection against a variety of respiratory and mucosal infections. We hypothesized that this strong induction of Th1/Th17 by nanoemulsion has the potential to modulate the Th2 immune responses associated with allergy, by re-educating the immune system to have a more balanced response when encountering the allergen.

A Th2-biased allergic phenotype was established by intraperitoneal sensitization with ovalbumin (ova) and alum. Subsequent IN immunizations with NE-ova resulted in increased Th1 associated immune responses (IFN-gamma, TNF-alpha, IgG2a and IgG2b) and IL-17, while decreasing Th2 cytokines (IL-4, IL-5 and IL-13) and IgG1. Additionally, the NE immunization also significantly increased IL-10 production, suggesting the upregulation of regulatory cells. Mice that received the therapeutic NE immunizations also had reduced IgE and increased IgA. Importantly, following inhalation challenge with ova, NE-treated mice had significant reduction in lung inflammation and mucus production, suggesting a strong reduction in allergic hypersensitivity. These data demonstrate that NE-

based vaccines can be used to modulate Th2 allergic responses to promote Th1/Th17 immunity and suggest the therapeutic use of NE vaccines for diseases associated with Th2 immunity.

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EMMPRIN Inhibition Using a Novel Epitope-Specific Antibody Reduces Tumor Progression

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Extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) mediates tumor cell-macrophage interactions, and has been shown to induce both MMPs and VEGF. However, the epitope responsible for MMP induction is controversial, and the epitope responsible for VEGF induction is yet unknown. We generated a novel anti-EMMPRIN polyclonal antibody directed against a specific epitope that successfully inhibited the production of both MMP-9 and VEGF in tumor cell-macrophage *in vitro* co-culture systems, exhibiting a U-shaped dose response ($p<0.05$). Furthermore, this antibody efficiently inhibited *in vivo* tumor progression in both the RENCA renal cell carcinoma and CT26 colon carcinoma tumor models by more than 97% ($p<0.05$). This was achieved by inhibiting angiogenesis as assessed by immunohistochemical staining for the endothelial marker CD31 (by 3-folds, $p<0.0001$), by inhibiting tumor cell proliferation as assessed by the staining for Ki-67 (by 3-folds, $p<0.005$), and by enhancing tumor cell apoptosis as assessed in the TUNEL assay (by at least 8-folds, $p<0.02$). Moreover, administration of the antibody recruited about 2-folds ($p<0.05$) more macrophages into the tumor, and altered the tumor microenvironment by decreasing the local concentrations of TGFβ (by about 11-folds, $p<0.05$). The antibody improved the ability of stimulated macrophages to perform antibody-dependent cell cytotoxicity (ADCC) and kill tumor cells ($p<0.01$). Thus, our new antibody maps a novel epitope which is capable of inducing both MMPs and VEGF, and its ability to immuno-modulate the tumor microenvironment as well as to affect tumor angiogenesis, proliferation and apoptosis, places EMMPRIN as an attractive target for cancer therapy.

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Echinacea Purpurea Root Polysaccharide Enhances TLR7 Binding Adjuvant Imiquimod Mediated Activation Of Innate Immunity

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In order to understand the mechanism(s) by which Echinacea may mediate its effect in the treatment of upper respiratory tract infections (URTI), Echinacea derived phytochemicals and extracts have been widely studied in terms of their immunomodulatory properties. We describe in this work the ability of a polysaccharide-containing water extract of *Echinacea purpurea* roots (EPRW) to enhance stimulation of human monocytic THP-1 cells by the TLR7 activating adjuvant imiquimod. In the presence of imiquimod, EPRW augmented production of TNFa by THP-1 cells. This pro-inflammatory activity was mediated by JNK as SP600125 abolished production of TNFa. EPRW and imiquimod also induced phosphorylation of JNK. Furthermore, imiquimod enhanced activation of NFkB in an NFkB-reporter gene assay and this was augmented by EPRW. Finally EPRW induced production of IFNa. These results may explain in part some of the therapeutic antiviral effects of Echinacea that have been observed *in vivo* in the treatment of URTI such as cold and flu and also suggest that polysaccharides derived from Echinacea may enhance responsiveness to imiquimod adjuvanted vaccines.

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TLR2, a Chitin Binding Protein, in Phagocytosis and M1 Activation by Chitin Microparticles

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Chitin, an N-acetyl-D-glucosamine polymer, is the second most abundant polysaccharide in nature, occurring as a structural component of crustaceans, fungi, helminthes and insects, but not mammals or

bacteria. We found -- and others confirmed -- that chitin microparticles (CMPs, 1 – 10 µm diameter) are neither antigenic nor allergenic, but they produce a strong Th1 adjuvant activity. Furthermore, administration of CMPs to animals induces immunoregulatory effects that are beneficial in asthma, infection, cancer and colitis. The mechanism includes, at least in part, M1 activation of macrophages; this M1 activation requires both a phagocytosable particle size and chitin chemical composition; therefore, neither large chitin beads (40 - 100 µm), nor soluble chitin, nor de-acetylated CMPs (chitosan microparticles [CsMPs], 1 - 10 µm) induce M1 activation. CMP-induced M1 activation is mediated by TLR2, MyD88 and MAPK activation. Consequently, not all tissue macrophages are activated by CMPs, even though they phagocytose CMPs. In order to further understand the mechanisms of phagocytosis-mediated M1 activation, we determined whether TLR2, as a chitin binding protein (CBP), is involved in internalization of the particles by macrophages. We isolated CBP-CMP complexes from macrophages during phagocytosis of CMPs. CsMPs were used as a control. Intraperitoneally activated M1 and M2 peritoneal macrophages as well as normal peritoneal macrophages and RAW264.7 cells were used for the study. TLR2 was identified cytometrically and by western blot as a CBP. We found that TLR2 bound to CMPs but not CsMPs during phagocytosis, and the amount bound per CMP became saturated by 30 minutes after phagocytosis, prior to MAPK activation. More TLR2 was detected in M1 than M2 macrophages. Based on our preliminary results, we hypothesize that CMP-induced M1 activation requires the exposed acetyl group, a rate-limiting for TLR2 binding in M1 activation. The magnitude of CMP-induced M1 activation will be diverse among alveolar, peritoneal/splenic, and intestinal macrophages that are activated by intranasal, intraperitoneal and oral administrations of CMPs.

Vaccine-Mediated Immunity to Experimental Mycobacterium Tuberculosis is Intact in the Absence of Toll-Like Receptor 9

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Given that the efficacy of BCG (Bacillus Calmette-Guérin) vaccine against the onset of pulmonary tuberculosis in adults is variable, several other live attenuated *Mycobacterium tuberculosis* (Mtb) strains have been developed as potential vaccine candidates. Some of these are already in pre-clinical assessments. Even so, the nature of the innate immune signals that influence the induction of memory immunity by these live attenuated vaccines has not been fully defined. Accumulating evidence indicates that innate inflammatory signals required for maximizing effector T cell generation have opposing effects on the development of memory T cell precursors. During Mtb infection, Toll-Like receptor (TLR)2, and TLR9 significantly contribute to the inflammatory milieu. *In vitro* studies from our laboratory have demonstrated that the expression of Interleukin-12 (required to polarize naïve T cells to the Th1 effector phenotype) is dependent on TLR2 and TLR9 signaling on dendritic cells and macrophages. Thus, we began to explore the development of memory immunity in the absence of signaling through TLR2 and/or TLR9. Previously, we have published that TLR2KO mice are able to mount an effective secondary immune response to Mtb. In this study, we therefore examined whether single gene deficiency in TLR9 or the combined gene deletion of TLR2 and TLR9 would affect vaccine-mediated immunity to Mtb. We found that TLR9KO and TLR2/9DKO mice vaccinated with a live Mtb auxotroph exhibited early control of Mtb growth in the lungs, akin to vaccinated WT mice. The granulomatous response, IFNg production and cellular recruitment to the lungs were also similar between all the vaccinated groups of mice. These findings indicate that the contribution from TLR2 and TLR9 in generating memory immunity to Mtb with live vaccines is minimal. We are now exploring the memory immune response mounted in the absence of other innate immune signals that are known to influence effector T cell

generation. These studies are directed at defining the innate milieu that can drive maximal memory T cell generation with a tuberculosis vaccine.

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Impact of Androgen Supplementation on Immune Senescence in Aged Macaques

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Aging leads to a progressive decline in immune function commonly referred to as immune senescence, which results in increased incidence and severity of infection. As males grow older, they experience a significant disruption in their levels of circulating androgens, testosterone and dehydroepiandrosterone (DHEA), which has been linked to sarcopenia, osteoporosis, cardiovascular disease and diabetes. Since sex steroid levels modulate immune function, it is possible that the age-related decline in androgen levels can also affect immune senescence. Therefore, in this study we evaluated the pleiotropic effects of physiological androgen supplementation in aged male macaques on immune cell subset frequency and response to vaccination. Compared to age-matched controls, androgen supplemented aged macaques had increased naïve CD4 and CD8 subsets, while the opposite was true for the memory T cell subsets. In addition, we also measured the immune response to modified vaccinia ankara and the seasonal influenza vaccine. Post MVA and flu vaccinations, androgen supplemented and age-matched controls had similar levels of T and B cell proliferation. However, these levels were lower than those observed in the young animals. Interestingly, despite comparable levels of proliferation, the androgen-supplemented males had a higher IgG antibody response post the MVA and flu vaccinations than the age-matched controls. However, immune response of the supplemented aged males did not reach the levels of the response generated by young macaques, indicating that physiological androgen supplementation can improve, but not restore, vaccine responses in older males.

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Characterizing the Nasal Resident Natural Killer Cell: Defining a Role in the Respiratory Innate Immune Response

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Natural killer (NK) cells are cytotoxic lymphocytes with critical roles in innate host defense against viruses and malignancies and have recently been implicated in resolution of allergic inflammation through clearance of eosinophils. Our group has demonstrated these cells comprise approximately half of the resident immune cells in the nasal cavity. We hypothesize that these nasal resident NK cells are phenotypically distinct from their circulating peripheral blood counterparts. NK cells were isolated from nasal lavage fluid of healthy human volunteers, along with matched NK cells from peripheral blood, and subjected to gene expression analysis via NanoString. These data suggest the nasal NK cell to have less expression of markers of cytotoxicity such as perforin and granzyme B, and increased expression of cytokines and cell signaling molecules such as TRAIL, IFNγR2, and IL-8. Further, our lab has previously demonstrated suppression of NK cell cytokine and chemokine production in individuals exposed to cigarette smoke and diesel exhaust. Taken together, these data propose a role for NK cells as orchestrators of the innate immune response in the nasal cavity, distinct from their role as a non-antigen-restricted cytotoxic lymphocyte in other parts of the body. It is not yet clear whether this difference is due to selective recruiting of a cytokine-producing phenotype of NK cells or conditioning by the mucosal microenvironment. Further, suppression of NK cell function, such as seen with exposure to cigarette smoke and diesel, may be a mechanism behind the increased susceptibility to respiratory viral infections and allergic exacerbations in individuals exposed to inhaled toxicants.

Changes in the Lung Microbiome Induced by Exposure to Tobacco Smoke in Healthy Mice and Mice with Chronically Inflamed Airways

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Introduction: The healthy lung contains a microbiome that is altered in many lung diseases, including tobacco smoke-induced lung injury and chronic obstructive lung disease (COPD). However, the effect of tobacco smoke itself, in the absence of lung disease, on the microbiome is not clear. Changes in the lungs of mice exposed to cigarette smoke partially mimic changes induced in human lungs exposed to cigarette smoke. Transgenic mice overexpressing the Scnn1b gene, which codes for the beta component of the epithelial sodium channel (BENaC), develop dehydrated airway mucus and chronic bronchitis. These studies tested the hypothesis that chronically exposing wild type (WT) and BENaC mice to either cigarette smoke or room air (sham) will uncover the changes in lung microbiome that are specifically due to cigarette smoke exposure in healthy airways and in chronic bronchitis.

Methods: Male WT or BENaC mice were exposed to six months of smoke or sham conditions. The microbiome was analyzed for 12 BENaC sham, 13 BENaC smoke, 12 WT sham, and 15 WT smoke-exposed mice. For each mouse, the lungs were lavaged with PBS, and total DNA was extracted from the lavage fluid. The phylogenetically discriminating variable region 4 (V4) of the bacterial 16S ribosomal RNA gene was amplified from each sample, barcoded according to mouse, pooled, and sequenced via Illumina Miseq. OTUs were clustered using UCLUST and the open reference of Greengene's most current version. OTUs that did not cluster with the reference were clustered de novo and retained. All subsequent analyses including diversity estimates and phylogenetic assignments were generated using QIIME version 1.8.0, and other statistics were performed using the Data Analysis ToolPak for Microsoft Excel 2015.

Results: The raw OTU sequence count was filtered to remove sequences found in the PBS control. There was no significant difference in the OTU count between treatment groups. Alpha diversity, scored as a Chao1 average, was significantly different after smoke exposure in both the WT and BENaC genotypes and was significantly different between WT and BENaC mice exposed to sham conditions, when tested using a paired T-test. Alpha diversity between WT smoke and sham mice and between BENaC smoke and sham mice approached significance when tested using an ANOVA analysis. Beta diversity calculated using abundance-based Jaccard similarity index was significantly different between BENaC smoke and sham conditions and between WT sham and BENaC sham conditions. Representation of taxa, measured as a percentage of total sequences, was also compared between groups. Significant differences in taxa among groups were identified. For example, Staphylococcus, Lactobacillus, Bradyrhizobiaceae and Chromatiaceae were found in significantly lower proportions, whereas Micrococcaceae, Rhizobiales and Aggregatibacter were present in significantly greater proportions in smoked WT mice compared to sham-exposed WT mice.

Conclusions: Smoke alone induced a decrease in the alpha diversity, a measure of species richness, similar to the observed changes in the human microbiome between healthy subjects and those with tobacco smoke-induced COPD. Smoke also altered the taxa present in murine lungs. Chronic bronchitis alone resulted in differences in the lung microbiome, and other changes were present after smoke exposure. Thus, both smoke exposure and chronic bronchitis appear to impact the lung microbiome. These changes may serve as a biomarker of smoke toxicity.

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Inflammation Associated CHI3L1 Alters the Pulmonary Microenvironment Supporting Breast Cancer Metastasis

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Metastasis is the primary cause of mortality in women with breast cancer. Metastasis to the lungs is greater in patients with pulmonary inflammatory illnesses. A glycoprotein commonly observed to be upregulated in inflammatory illnesses and cancer is chitinase-3-like-1 protein (CHI3L1). Although inflammation is one of the hallmarks of cancer, it is not well known how inflammation at a secondary site affects metastasis. To understand how pre-existing inflammation and CHI3L1 expression at a target organ such as the lung affects metastatic growth rate, we combined a mouse model of pulmonary inflammation with breast cancer. We hypothesized that allergic pulmonary inflammation establishes a pre-metastatic niche for infiltrating tumor cells by effecting tissue architecture, altering cellular composition and inflammatory mediators. Using the highly metastatic mouse breast tumor models (orthotopic 4T1 mammary tumor; the spontaneous MMTV-PyMT mammary tumor) induced for pulmonary inflammation with ragweed allergen, we found that pre-existing pulmonary inflammation increases the growth of primary tumor and metastasis. The lungs of allergic tumor bearing mice had higher levels of: myeloid populations, i.e., inflammatory monocytes ($CD11b^{+}Ly6G^{-}Ly6C^{mid}F4/80^{-}CD68^{-}CD11c^{-}$ $CX3CR1^{+}$) and an expansion of pulmonary macrophages ($CD11b^{+}Ly6G^{-}Ly6C^{-}$ $F4/80^{+}CD11c^{+}CD68^{+}$). Monocytes/macrophages were the key cells producing and/or expressing CHI3L1, pro-inflammatory chemokines CXCL2/IL-8, CCL2/MCP-1, chemokine receptors CXCR2, CCR2 and CCR4, and proinflammatory proteins S100A8 and S100A9. Additionally, the pulmonary architecture was disrupted in these mice as evidenced by increased smooth muscle actin (SMA); collagen I deposition and increased lipoxygenase 2 (LOX2). We next determined if all of these adverse effects were due to the establishment of the pre-metastatic niche by the tumor or if the pre-existing inflammation alters the pulmonary microenvironment before the entry of tumor cells. A similar expansion of cellular subpopulations and inflammatory molecules were found in allergic tumor free mice compared to tumor bearers. To understand if myeloid cells are the main contributor to the increased expression of inflammatory mediators and disrupted architecture in allergic tumor bearers, depletion studies by chlodronate liposomes and anti-F4/80, anti-Ly6C were performed prior to tumor implantation and continued post-tumor implantation. Depletion with either chlodronate or the monocyte/macrophage specific antibodies reduced the inflammatory responses resulting in decreased tumor growth and metastasis. Since our previous studies showed that CHI3L1 modulates inflammation, we determined its role in inflammation associated tumor progression. Allergic CHI3L1 KO tumor bearers displayed decreased myeloid derived cells, decreased pro-inflammatory mediators, and significant reduction in tumor volume and metastasis compared to wild type (allergic tumor bearer) controls. Further, the pulmonary architecture was not significantly affected by inflammation in the knockout mice. Pre-existing inflammation and CHI3L1 may be driving the establishment of a pre-metastatic milieu in the lungs and aiding in the establishment of metastasis. Understanding the role of allergen induced CHI3L1 and inflammation in tumor bearers and its effects on the pulmonary microenvironment could result in targeted therapies for breast cancer.

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Targeted Protein Kinase C-Delta Inhibition is Organ-Protective and Improves Pathogen Clearance in Sepsis

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Sepsis is one of the leading causes of death in the ICU and is characterized by a systemic inflammatory response leading to excessive neutrophil infiltration in multiple organs such as

lungs, kidneys and liver, producing tissue damage. This multiple organ dysfunction and eventually failure can lead to permanent disability and death. We identified Protein Kinase C-delta (PKCδ) as a critical regulator of the acute inflammatory response in sepsis and demonstrated that intra-tracheal (IT) administration of a PKCδ inhibitor was lung protective in a rat model of sepsis, suggesting targeting PKCδ as a potential strategy for preserving pulmonary function in sepsis. Since a critical issue in anti-inflammatory therapeutics is the risk of immune-suppression and inability to effectively clear pathogens, we aimed to investigate whether PKCδ inhibition could provide lung protection without compromising pathogen clearance and attenuate sepsis-induced damage to other organs.

Rats underwent sham surgery or cecal ligation and puncture (CLP) to induce sepsis. Following surgeries, vehicle (PBS) or a PKCδ-TAT peptide inhibitor (200µg/kg) were administered IT. Blood, peritoneal cavity fluid (PCF) and tissue samples were collected 24 hours post-surgery. We examined the effects of PKCδ inhibition on the spread of infection in blood (anaerobic) and the PCF (aerobic and anaerobic). We also evaluated leukocyte activation in cells of the PCF, by analyzing CD11b expression and in blood samples by studying platelets/leukocyte aggregate formation using flow cytometry. Finally we examined liver, lung and kidney damage by measuring Myeloperoxidase (MPO) activity and plasma markers.

At 24 hours after CLP surgery, the bacteria burden was significantly increased in both the PCF and blood samples. However, when rats were treated with the PKCδ inhibitor, the levels of bacteria were significantly decreased in both the PCF and blood, compared with vehicle-treated animals. Neutrophil counts were elevated in the PCF in response to sepsis but there was no difference between vehicle- and PKCδ inhibitor-treated septic rats. However, PKCδ inhibitor treatment reduced the sepsis-induced expression of CD11b in the cells of the PCF. Moreover, platelet/leukocyte aggregate formation was increased in the blood of septic animals, but PKCδ treatment reduced circulating aggregates compared to vehicle-treated animals. At 24 hours post-CLP surgery, there was evidence of lung, liver and kidney damage. IT delivery of the PKCδ inhibitor reduced blood urea nitrogen (BUN) and alkaline phosphatase (ALK) levels indicating remote organ protection. MPO activity was elevated in the lungs and kidneys but not in the liver at 24 hours post CLP. PKCδ inhibitor treatment reduced MPO levels in both the lung and kidneys indicating decreased inflammatory cell influx.

In conclusions, our data demonstrate that inhibition of PKCδ was not immunosuppressive and did not prevent pathogen clearance. Sepsis-induced neutrophil activation was modulated by treatment with the PKCδ inhibitor suggesting that PKCδ inhibition may protect against excessive cell activation in the tissue without altering cell efficiency. Moreover, PKCδ inhibition offered protection against sepsis-induced organ injury.

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Expression of the Melanocortin-Adenosinergic Pathway in Human Uveitis Patients

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Uveitis is a leading cause of blindness in developed countries. The majority of uveitis cases are acute, whereas, a smaller proportion of cases become chronic and lead to blindness. The mechanisms that provide resistance to relapse in the acute population and if these mechanisms can be used to treat the chronic population are unknown. Mice that have recovered from experimental autoimmune uveoretinitis (EAU) may represent acute human uveitis. These EAU-recovered mice have regulatory immunity in their spleen that provides resistance to recurrence of EAU. This regulatory immunity requires expression of melanocortin 5 receptor (MC5r) on the APC, and adenosine 2A receptor (A2Ar) on the T cells. Therefore, this conserved pathway is a potential therapeutic approach for uveitis. PBMC from healthy volunteers and uveitis patients were collected. Patients were grouped as suppressed (US) and active (UA). US patients had no uveitis within a year at the time of collection. PBMC were analyzed by FACS for CD14, CD16, CD4, MC5r, and A2Ar expression. Analysis of PBMC from US (n = 11) and UA (n = 21) patients

and healthy volunteers (n = 9) revealed a similar percentage of classical and non-classical monocytes. Further analysis of MC5r expression on each of the subsets showed no significant difference. The expression of A2Ar on $CD4^+$T cells was also similar. There was no difference in MC5r or A2Ar expression with age, type of therapy, or location of uveitis. In order to show MC5r and A2Ar were functional, the CD4 T cells were sorted, and the monocytes were sorted into classical ($CD14^+CD16^{lo}$) and non-classical ($CD14^-CD16^{hi}$) subsets. The monocytes were pulsed with tetanus toxin and treated with α-melanocyte stimulating hormone (α-MSH) to stimulate the melanocortin pathway. The T cells were added to the cultures, and the supernatants were assayed for TGF-β. Both monocyte subsets produced slightly more TGF-β from US (n = 5) and UA (n = 6) patients compared to healthy volunteers (n = 3), and treatment with α-MSH had no effect. When T cells were added to the monocyte cultures a similar pattern of TGF-β production was observed. While our initial functional analysis saw no effect on TGF-β production, the potential to activate the melanocortin-adenosinergic pathway to suppress effector T cells should be possible in uveitis patients.

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Beyond The Inflammasome: Regulatory NLRs in Inflammation and Tumorigenesis

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Aberrant inflammation is associated with the majority of human diseases and functions as an enabling characteristic of cancer. Thus, signaling cascades that regulate the activation and resolution of inflammatory signaling cascades are of specific relevance to disease pathogenesis. This is of particular concern to patients suffering from chronic inflammatory diseases, where prolonged or overzealous activation of the immune system results in excess collateral tissue damage and provides a rich microenvironment for tumor development. Pattern recognition receptors (PRRs) are essential mediators of inflammation and have emerged as critical elements affecting multiple facets of tumor pathobiology. The NLR proteins are intracellular PRRs that sense microbial and non-microbial products associated with pathogen exposure, damage, and cellular stress. Members of the NLR family can be divided into functional subgroups based on their ability to either positively or negatively regulate the host immune response. Recent studies have identified a novel subgroup of regulatory NLRs that do not function in inflammasome formation. These non-inflammasome forming NLRs function to negatively regulate diverse biological pathways that are highly relevant in maintaining immune system homeostasis. NLRP12 is one of the NLRs in this subgroup and is highly expressed in myeloid derived cells. In the context of IBD and cancer, NLRP12 attenuates inflammation in the gut through the negative regulation of either the canonical or non-canonical NF-κB signaling cascade. While the role of the canonical NF-κB pathway is well established during IBD and cancer, the role of the non-canonical signaling cascade is relatively uncharacterized. Our findings have identified a group of signaling molecules and chemokines associated with the non-canonical NF-κB signaling pathway that are significantly up-regulated in the absence of NLRP12 and associated with IBD pathobiology in mice. Subsequent data from our group has also revealed that these signaling pathways are up-regulated in human Crohn's disease and ulcerative colitis patients. We have subsequently discovered cell type and temporal specific regulatory events that implicate a role for NLRP12 in the regulation of inflammation through hematopoietic derived cells during the early, inflammatory, stages of disease progression. Here, we will discuss recent findings associated with NLRP12 modulation of canonical and non-canonical NF-kB signaling during IBD and cancer. Likewise, we will also review recent developments associated with the other NLRs in this functional sub-group and their roles in attenuating overzealous inflammation. Understanding the mechanisms underlying the function of these unique NLRs will assist in the rationale design of future therapeutic strategies targeting a wide spectrum of inflammatory diseases and cancer.

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Single-Cell Analysis of Natural Killer Cell Responses to Infection with West Nile Virus

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Key words: Natural Killer cells, West Nile virus, mass cytometry, aging

West Nile virus (WNV) is a mosquito-borne virus that may lead to severe neuroinvasive disease in humans, especially in elderly people. Subjects with a history of symptomatic WNV infection showed a striking increase in the frequency of $CD3^{-}CD56^{+}$ natural killer (NK) cells compared to those with asymptomatic infection. NK cells express an array of activating and inhibitory receptors leading to vast phenotypic and functional diversity. As the role of NK cells during human WNV infection remains incompletely defined, the goal of this study was to define NK cell subsets and functional responses associated with symptomatic vs. asymptomatic infection. NK cell phenotype and function were assessed using mass cytometry, also known as cytometry by time-of-flight (CyTOF), to simultaneously detect 23 NK surface receptors and 10 functional markers in each cell. Infection of peripheral blood mononuclear cells (PBMCs) with WNV at a multiplicity of infection (MOI) of 1 for 24 hours led to robust NK cell activation, with a 2-10 fold elevation of surface expression of CD107a (a marker of cytolysis) and production of MIP-1β and IFN-g. At baseline, in the absence of stimulation, the frequency of IFN-g-producing NK cells was markedly lower in symptomatic (2.7 ± 2.0%, n=12) than in asymptomatic (0.8 ± 0.6%, n=10) subjects. However, following stimulation with WNV infection in PBMCs *in vitro*, the frequency of NK cells producing IFN-g was not significantly different between asymptomatic and symptomatic groups. Furthermore, there were no differences in NK cell activation as measured by changes in the frequency of NK cells expressing CD69, CD107a, perforin, MIP-1β, TNF-α, or GM-CSF. Using the automated clustering algorithm Citrus to detect differences between the asymptomatic and symptomatic subjects, symptomatic subjects had a significantly lower abundance of an immature NK subset ($CD56^{bright}CD16^{-}CD94^{+}NKG2A^{+}$, 5.3 ± 4.4% vs. 2.0 ± 1.0%). As increasing age is a significant risk factor for severe WNV disease, to determine whether this difference was merely associated with age, NK cell responses to WNV were assessed in WNV-naïve, healthy young and old subjects (n = 14/group). Healthy young and old, WNV naïve subjects had comparable induction of CD69, CD107a, perforin, IFN-g, TNF-α, and GM-CSF by WNV infection, though older subjects had an increased abundance of a mature NK subset ($CD56^{dim}CD16^{+}CD57^{+}PD\text{-}1^{+}NKp30^{+}$, 60.3 ± 12.9% vs. 44.9 ± 12.4%) and elevated baseline IFN-g production (1.6 ± 0.9% vs. 0.5 ± 0.3%) in the absence of stimulation. This study demonstrates that human NK cells exhibit cytolytic activity and cytokine production in response to WNV infection, and suggest that the maturation status of NK cells may contribute to host susceptibility to infection with WNV.

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Population Distribution of Interleukin 1 Beta and its Receptor Antagonist in Standardbred Race Horses Via Novel Validated Enzyme-Linked Immunosorbent Assays

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Plasma concentrations of interleukin 1 beta (IL-1β) and IL-1 receptor antagonist (IL-1RA) were measured in Standardbred horses prior to racing (n=312) by novel validated enzyme-linked immunosorbent assays (ELISAs) and correlation between two cytokines was determined. The sandwich ELISAs were developed and validated using analyte-specific polyclonal antibody (PAb) to capture and detect IL-1β and IL-1RA. The recombinant protein or reference equine plasma was employed as calibrator to generate a standard curve for quantification. The results indicated that the methods did not exhibit significant cross-reactivity with other cytokines tested and when plasma samples were serially diluted, the reactivity

proportionally decreased. When biotinylated Ab was partially replaced with capture Ab or when the samples were pre-treated with capture Ab, assay signals decreased by 39 – 61 %. Concentrations of cytokines increased when whole blood was treated with lipopolysaccharide ($p<0.001$). The inter-assay precision was 11.8 % (IL-1β) and 12.1% (IL-1RA), the intra-assay precision was 9.8 % (IL-1β) and 9.4% (IL-1RA); the inter-assay accuracy was -6.2% (IL-1β) and 5.9% (IL-1RA) while the intra-assay accuracy was -4.6% (IL-1β) and 4.1% (IL-1RA). Finally, plasma IL-1β and IL-1RA concentrations showed great variation in Standardbred horses and ranged from 0~47987 pg/ml and 0~111739 pg/ml, respectively. Two cytokines showed positive concord correlation ($r=0.917$, $p<0.000001$). High concentrations detected in small population of horses may have clinical relevance and deserve further investigation.

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Absence of IL-6 Impairs Thrombus Resolution Through Reduced MMPs And uPA Expression in Murine Deep Vein Thrombosis Model

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Deep vein thrombosis (DVT) is frequently complicated with severe morbidity. In order to minimize the mortality of DVT-related pulmonary thromboembolism, accurate diagnosis and prompt therapy for DVT are necessary. In the process of formation and resolution of venous thrombi, the intrathrombotic accumulation of neutrophils and macrophages and the remodeling of extracellular matrices are essential.

IL-6 is a pleiotropic cytokine that plays an important role in host defense by regulating immune and inflammatory responses. In this study, we examined the pathophysiological roles of IL-6 in the resolution of DVT by the use of BALB/c and *Il6* KO mice.

Under deep anesthesia, a laparotomy was performed in mice, and the inferior vena cava (IVC) was exposed and ligated with silk suture. Mice were euthanized at 1, 3, 5, 7, 10, 14 and 21 days after ligation, and thrombi or thrombosed IVCs were harvested. Upon the IVC ligation of WT mice, venous thrombi formed and grew progressively until 5 days, and, thereafter, the thrombus weight decreased. Concomitantly, *Il6* expression was detected in the thrombi. When *Il6* KO mice were treated in the same manner, thrombus size was much larger in *Il6* KO mice than WT ones (Figure 1). IL-6 was expressed by intrathrombotic macrophages using double-color immunofluorescence staining. And, the number of intrathrombotic IL-6-positive cells changed in proportion to the number of macrophages. Moreover, the IVC blood flow was more recovered in WT than in *Il6* KO mice. And intrathrombotic matrix metalloproteinase (*Mmp*)*2, Mmp9*, and urokinase-type plasminogen activator (*Plau*) expression was significantly reduced in *Il6* KO mice than WT mice.

Then, to clarify the function of IL-6 for thrombus formation and resolution, we performed western blotting and real-time RT-PCR using mice-derived intraperitoneal macrophages. Stattic, a specific Stat3 inhibitor, significant suppressed IL-6-induced Stat3 phosphorylation and these gene expressions. As a result, these observations would imply that the lack of IL-6/Stat3 signal pathway can inhibit the gene transcription of MMPs and uPA, eventually resulting in the delay of thrombus resolution.

Based on these results, we performed in vivo experiments. After the IVC ligation, KO mice were received i.p. injection of recombinant murine IL-6 and 10 days after ligation, the thrombi were harvested. In KO mice treated with exogenous IL-6, the thrombus mass was smaller and blood flow recovery was faster than only PBS received mice (Figure 2, left). Furthermore, WT mice were received i.p. injection of anti-IL-6 and 10 days after IVCligation, the thrombus mass was larger and blood flow recovery was slower, compared with PBS-treated WT mice (Figure 2, right).

We demonstrated that the absence of IL-6 retarded thrombus resolution, together with suppressed the expression of MMP-2, MMP-9, and PLAU, compared with WT mice. This may reflect the observation that IL-6 could enhance the expression of *Plau, Mmp2,* and *Mmp9* in macrophages in an IL-6-dependent manner. These observations implied that the IL-6 may be a key molecule for the development of therapeutic strategies in DVT patients. Collectively, IL-6 can have a beneficial role in the thrombus resolution by the upreglation of

MMP-2, MMP-9 and uPA expression, and be a good molecular target for the DVT treatment.

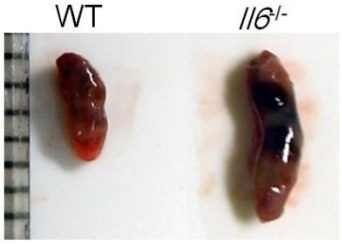

Figure 1 Macroscopic observation of thrombi (10 days after IVC ligation).

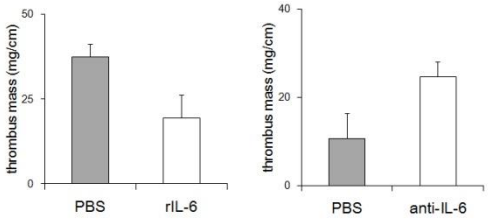

Figure 2 Recombinant murine IL-6 accelerated thrombus resolution on *Il-6-/-* mice (left). In contrast, anti-IL-6 antibody inhibited thrombus resolution on WT mice (right).

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Pellino-1 Positively Regulates Toll-like Receptor (TLR) 2 and TLR4 Signaling and is Suppressed upon Induction of Endotoxin Tolerance

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Endotoxin tolerance reprograms Toll-like receptor (TLR) 4-mediated macrophage responses by attenuating induction of proinflammatory cytokines while retaining expression anti-inflammatory and antimicrobial mediators. We previously demonstrated deficient TLR4-induced activation of IL-1 receptor associated kinase (IRAK) 4, IRAK1, and TANK binding kinase (TBK) 1 as critical hallmarks of endotoxin tolerance, but mechanisms remain unclear. In this study, we examined the role of the E3 ubiquitin ligase Pellino-1 in endotoxin tolerance and TLR signaling. LPS stimulation increased Pellino-1 mRNA and protein expression in macrophages from mice injected with saline and in medium-pretreated human monocytes, THP-1 and MonoMac-6 cells, while endotoxin tolerization abrogated LPS inducibilty of Pellino- 1. Overexpression of Pellino-1 in 293/TLR2 and 293/TLR4/MD2 cells enhanced TLR2- and TLR4- induced nuclear factor κB (NF-κB) and expression of IL-8 mRNA, while Pellino-1 knockdown reduced these responses. Pellino-1 ablation in THP-1 cells impaired induction of myeloid differentiation primary response protein (MyD88) - and Toll-IL-1R domain-containing adapter inducing IFN-β (TRIF)-dependent cytokine genes in response to TLR4 and TLR2 agonists and heat-killed *Escherichia coli* and *Staphylococcus aureus*, while only weakly affecting phagocytosis of heat-killed bacteria. Co-expressed Pellino-1 potentiated NF-κB activation driven by transfected MyD88, TRIF, IRAK1, TBK1, TGF- β-activated kinase (TAK) 1 and TNFR-associated factor 6, while not affecting p65-induced responses. Mechanistically, Pellino-1 increased LPS-driven K63-linked polyubiquitination of IRAK1, TBK1, TAK1, and phosphorylation of TBK1 and IFN regulatory factor 3. These results reveal a novel mechanism by which endotoxin tolerance re-programs TLR4 signaling via suppression of Pellino-1, a positive regulator of MyD88- and TRIF-dependent signaling that promotes K63-linked polyubiquitination of IRAK1, TBK1, and TAK1.

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The Role of Guanylate Binding Proteins as Regulators of Inflammasome Activation

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Interferon (IFN)-inducible Guanylate Binding Proteins (GBPs) mediate cell-autonomous host resistance to bacterial pathogens and promote inflammasome activation. The prevailing model postulates that these two GBP-controlled activities are directly linked through GBP-dependent vacuolar lysis. It was proposed that rupture of pathogen-containing vacuoles (PVs) by GBPs destroyed the microbial refuge and simultaneously contaminated the host cell cytosol with microbial activators of inflammasomes. Here, we demonstrate that GBP-mediated host resistance and GBP-mediated inflammatory responses can be uncoupled. We show that PVs formed by the rodent pathogen *Chlamydia muridarum*, so-called inclusions, remain free of GBPs and that *C. muridarum* is impervious to GBP-mediated restrictions on bacterial growth. Although GBPs neither bind to *C. muridarum* inclusions nor restrict *C. muridarum* growth, we find that GBPs promote inflammasome activation in *C. muridarum*-infected macrophages. We

demonstrate that *C. muridarum* infections induce GBP-dependent pyroptosis through both caspase-11-dependent noncanonical and caspase-1-dependent canonical inflammasomes. Demonstrating the importance of GBPs as regulators of inflammasome activation *in vivo,* we find that IL-1b and IL-18 serum levels are diminished in *GBP*-deficient mice undergoing LPS-induced sepsis. Together, our results reveal that GBPs can control inflammasome activation independently of their role in PV lysis.

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A Mechanism of indirect-Acute Lung Injury: Pulmonary Epithelial Cell HVEM Expression Promotes Inflammation/ Organ Injury.

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Background: Acute lung injury (ALI) has a high lethality of 40%, accounting for around 75,000 deaths per year in USA. But the underlying pathophysiology of ALI is not completely understood. The Herpes Virus Entry Mediator (HVEM), a member of the tumor necrosis factor receptor family, has been reported to be either pro-inflammatory or anti-inflammatory depending on the situation/environment and/or nature of ligand it engages. In this respect, while we have previously documented the expression of HVEM on leukocytes of septic mice/critically ill patients, its functional role in ALI has not been studied yet.
Objective: To determine the role of HVEM in the development of shock/sepsis- induced ALI.
Methods: A murine model of indirect ALI (iALI) was induced by hemorrhagic shock followed 24 hours after with cecal ligation & puncture-septic challenge (CLP). HVEM-siRNA or PBS was administrated by intra-tracheal instillation 2-4 hrs after hemorrhage. Samples were collected 24 hrs after CLP. Indices of lung injury were measured.
Results: 1. HVEM expression in both lung tissue (P=0.01 by Western Blot and P=0.03 by qrt-PCR) and lung epithelial cells (P=0.008 by flow cytometry) increased significantly in iALI mice compared with sham surgery mice. In HVEM-siRNA treated mice compared with PBS treated mice, HVEM expression was somewhat decreased in whole lung tissue (by Western Blot) and significantly decreased on isolated lung epithelial cells (P=0.001 by flow cytometry) 2. Total cell count (P=0.04) and protein concentration (P=0.02) in bronchoalveolar lavage, MPO activity in lung (P=0.002), chemokines [MCP-1 (P=0.006), KC (P=0.02) and MIP-2 (P=0.002)] and IL-6 levels (P=0.04) in lung decreased significantly in HVEM-siRNA treated mice compared with PBS treated mice. But there was no change in TNF-a and IL-10 levels in lung tissue. STAT-3 phosphorylation in whole lung tissue (by Western Blot) increased in iALI mice compared with sham mice (P=0.05), and decreased in HVEM-siRNA treated compared to PBS treated iALI group (P=0.02). 3. HVEM-siRNA administration also reduced lung inflammatory infiltrates and improved alveolar architecture (by H/E stain). The survival rate was also elevated significantly during the first 60 hrs after CLP (83.3 vs 47.6%; P=0.04), but this difference was lost by 10 days' survival (58.3 vs 41.7%; P=0.25).
Conclusion: While HVEM can ligate either immune suppressive receptors like BTLA or immune activating receptors such as LIGHT, its' own local pulmonary expression largely and HVEM ligation promotes inflammatory activity in lung epithelial cells of iALI mice. Thus, mitigating signaling through HVEM may have some novel lung protective effects relative to organ injury not seen by targeting its ligands.

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Endotoxin Tolerance Inhibits Phosphorylation of Lyn and c-Src and Their Recruitment to TLR4 and Increases Total Phosphatase Activity and Expression of PTP1b, PP2A and PTPN22

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Sepsis patients that survive initial "cytokine storm" develop immunosuppression, become immunocompromised and unable to clear secondary

infections, and their monocytes exhibit the endotoxin-tolerant phenotype. Endotoxin tolerance develops upon host exposure to endotoxin and re-programs TLR signaling by reducing expression of pro-inflammatory cytokines, while not inhibiting anti-inflammatory and anti-microbial mediators. The exact molecular mechanism of endotoxin tolerance is yet to be elucidated. This study shows that the induction of endotoxin tolerance in human monocytes, THP-1 and MonoMac-6 cells inhibited LPS-mediated phosphorylation of c-Src and Lyn, their recruitment to TLR4, but increased total phosphatase activity and expression of phosphatases PTP1b, PP2A and PTPN22. Chemical inhibitors of phosphatases, ocadaic acid, dephostatin and cantharidic acid, significantly decreased or abolished the induction of LPS tolerance. Overexpression of PTPN22 in 293/TLR4/MD2 cells decreased LPS mediated NFκB activation, phosphorylation of p38 and CCL8 gene expression while knockdown of PTPN22 in THP-1 cells up-regulated LPS-induced NF-κB activation and TNFα gene expression. Our results indicate that endotoxin tolerance decreases phosphorylation of c-Src and Lyn and their recruitment to TLR4, while enhancing total phosphatase activity and expression of PTP1b, PP2A and PTPN22, and suggest PTPN22 as an important mediator.

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Type I interferons as Suppressors of TLR Dependent Innate Responses to Salmonella Typhimurium

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At mucosal surfaces, innate immune sensing systems and their induced inflammatory responses are subject to complex layers of negative regulation. Such regulation is critical to balance pathogen recognition and clearance with host tissue integrity and an eventual return to homeostasis. In particular, mechanisms restricting innate responses are crucial in the intestinal mucosa, as the trillions of microbes composing the commensal microflora must be tolerated to prevent colitis-associated pathologies. However, the strict governance of innate responses at intestinal sites may provide an opportunity for pathogens seeking to gain an advantage over the host. The type I interferons, in particular, while essential for amplifying antiviral responses, have been shown to play a homeostatic role in models of gut auto-immunity. We have shown that mice lacking a functional gene for interferon beta (IFN-$\beta^{-/-}$) display significantly enhanced resistance to oral infection with pathogenic *Salmonella enterica subsp. enterica* serovar Typhimurium (*S*T). *In vivo* the resistance to *S*T in IFN-$\beta^{-/-}$ mice is associated with markedly increased neutrophil activity in the small intestine. *In vitro* infection of murine macrophages with *Salmonella* reveals that autocrine or paracrine action of IFN-β selectively restricts the transcriptional inflammatory responses mediated by both the Toll Like Receptors (TLRs) as well as the NOD-Like Receptors (NLRs). This "sculpting" of the innate response involves the suppression of IL-1 family cytokines as well as key neutrophil chemo-attractants. Further transcriptomic analyses have revealed the larger scope of type I interferons in governing Toll responses to this pathogen. This work provides mechanistic insight into homeostatic roles for type I interferons in the gut, and demonstrates how these effects may actually promote the pathogenesis of enteric pathogens. Supported by AI18797 (SV).

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NADPH Oxidase 2 has an Essential Anti-Inflammatory Role in the Lung

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An essential component of the host response to infectious and/or inflammatory stimuli is the production of reactive oxygen species (ROS) by NADPH oxidases, with NOX2 NADPH oxidase (Nox2) being the multi-subunit enzyme complex in leukocytes. Patients with chronic granulomatous disease (CGD), who lack functional NOX2, suffer from frequent serious bacterial infections. These patients also suffer from chronic inflammation, suggesting that NOX2 is critical for maintaining immune homeostasis. Previous studies in our

laboratory established the importance of NOX2 in limiting inflammation using a murine model of sterile systemic inflammation. Mice lacking NOX2 ($gp91^{phox-/y}$) had increased early mortality and unresolved chronic inflammation compared to wild type (WT) mice. In addition, $gp91^{phox-/y}$ mice had evidence of acute lung injury six hours following the induction of sterile inflammation that was absent in the WT mice. Given the rapid development of inflammation in the lung in $gp91^{phox-/y}$ mice, we reasoned that a resident cell, specifically the alveolar macrophage, plays an important role in regulating inflammation in the lung. We hypothesize that NOX2 has an essential anti-inflammatory role in the lung. Furthermore, we postulate that this anti-inflammatory role is mediated via macrophage polarization; specifically, that NOX2-derived ROS are essential for macrophage polarization from an inflammatory (M1) to an anti-inflammatory (M2) phenotype. We utilized a murine model of sterile inflammation, the zymosan induced generalized inflammation model (ZIGI). Mice received an intra-peritoneal injection of zymosan and were sacrificed at one and six hours. We examined bronchoalveolar lavage fluid (BALf) and whole lung digests from WT and $gp91^{phox-/y}$ mice. Samples were analyzed for cytokine expression using a Bio-Plex Multi-Plex assay, cell surface markers by flow cytometry, and gene expression by RT-PCR. Analysis of cytokines in BALf indicated that several cytokines, including IL-6, G-CSF, MCP-1 and MIP-1ß were substantially elevated in $gp91^{phox-/y}$ mice compared to WT mice 6 hours following injection. There were a greater number of neutrophils sequestered in the lung by analysis of lung digest of WT and $gp91^{phox-/y}$ mice one hour post-injection. Gene expression of IL-6 was higher in $gp91^{phox-/y}$ mice compared to WT mice one hour following injection. Additionally, expression of IL-6 and NOS2, markers of M1 macrophages, was significantly enhanced 6 hours following zymosan injection in whole lung digests in $gp91^{phox-/y}$ mice compared to WT mice. Our results suggest a requirement for NOX2 in resolving or preventing inflammation in the lung. We speculate that macrophage NOX2 has a critical anti-inflammatory signaling role in the lung.

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Oviduct Pathology During Chlamydia Muridarum Infection-The Exclusive Contribution of IL-1α Signaling

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Background: *Chlamydia trachomatis* infection of the upper female genital tract can lead to Pelvic Inflammatory Disease, causing infertility. Using a mouse model of genital *Chlamydia* infection, we have previously shown that IL-R signaling plays a dual role by controlling infection but contributing significantly to oviduct disease. Inflammasome adaptor ASC was essential for reducing infection levels in this model, but ASC deficient mice were not protected from pathology. This study delineated the individual protective/pathogenic contribution of canonical and non-canonical inflammasome pathways and their downstream cytokine mediators IL-1a and IL-1b, in this model.

Methods: Infection course during genital infection, oviduct pathology, antigen-specific T cell response, and inflammatory cell recruitment was determined in wild type (WT), caspase-11-, and caspase-1-11-, IL-1a-, IL-1b- and IL-1ab-gene knock out (KO) mice.

Results: Caspase-1 activation and inflammasome activation were essential for secretion of IL-1b during infection in vitro. However, during in vivo infection both caspase-1-11 and caspase-11 KO mice had minimal changes in their infection course compared to WT mice. Caspase-11 KO mice displayed a slightly reduced incidence of oviduct hydrosalpinx, a visual indicator of oviduct pathology. Since the phenotype of these mice did not resemble IL-1R KO, which showed increased infection but significantly reduced pathology, we investigated the upstream mediators IL-1a and IL-1 b. IL-1a KO mice displayed a similar infection course as WT mice and a normal T cells response, but were completely protected from genital tract pathology. These data suggest that activation of cell death pathways and release of IL-1a is sufficient to cause long-term damaging effect on the oviduct and demonstrate a hierarchy of events, with IL-1a

exclusively directed toward eliciting damaging local epithelial responses. [Supported by PHS funding NIAID AI067678 (UN)]

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Mesalamine Treatment Reduces Intestinal Inflammation and Restores Gut Barrier Integrity After Burn Injury

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Previous studies from our laboratory have shown that burn injury results in intestinal inflammation, decreased intestinal transit, and increased intestinal permeability. This disruption of the intestinal barrier has been implicated in the development of sepsis and multiple organ dysfunction syndrome (MODS) following burn injury. In this study, we examined whether therapeutic intervention with mesalamine (5-ASA), a common anti-inflammatory treatment for patients with inflammatory bowel disease, reduces intestinal inflammation and maintains normal barrier integrity after burn injury. Male C57BL/6 (8-10 week old) mice were anesthetized and administered an ~20% total body surface area dorsal scald burn using 85^0C water for ~7-9 seconds and resuscitated with either 1mL normal saline or 100mg/kg of 5-ASA dissolved in saline. One day following burn injury, small intestines were harvested and processed for the isolation of intestinal epithelial cells (IECs). For the measurement of intestinal permeability and transit, a group of sham and burn mice were gavaged with 0.4 ml of 22 mg/ml FITC-dextran 3 hours prior to sacrifice. Blood and the luminal contents of stomach, intestine and colon were collected, and FITC levels were determined. IEC protein levels of the pro-inflammatory cytokines IL-6 and IL-18 were examined via ELISA. We found that burn injury significantly increases $p<0.001$ levels of IL-6 (by ~1.5 fold) and (IL-18 by ~2.5) fold in small intestine IECs one day after injury. Similar to previous findings, we observed significant increases in intestinal permeability $p<0.0001$ one day after burn compared to sham. Furthermore, a significant decrease in intestinal transit was noted one day after burn injury. However, the treatment of mice with 5-ASA after burn normalized IL-6 and IL-18 to sham levels and prevented the increase in intestinal permeability following burn injury. Additionally, 5-ASA treatment partially restored normal intestinal transit in burn injured animals. Together these findings suggest that 5-ASA can potentially be used as treatment to decrease intestinal inflammation and normalize intestinal function after burn injury. Supported by DOD W81XWH-09-1-0619, RO1 AA01573, T32AA013527

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PI3P-Dependent Generation of ROS Regulates Inflammatory Response

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The leukocyte NADPH oxidase is a membrane-bound multi-subunit enzyme that generates superoxide that is rapidly converted into H_2O_2 and other reactive oxygen species (ROS) essential for microbial killing. NADPH oxidase-derived ROS are also increasingly recognized to regulate other aspects of the inflammatory response. While null mutations in most subunits of the oxidase completely abrogate plasma membrane and phagosomal ROS production, null mutations in the phosphatidylinositol 3-phosphate (PI3P)-binding $p40^{phox}$ subunit lead to a selective defect in NADPH oxidase activity on PI3P-enriched membranes such as phagosomes. Lack of a functional NADPH oxidase is associated with inflammatory complications but the underlying mechanisms for hyper-inflammation are incompletely characterized. We show that macrophages and neutrophils from $p40^{phoxR58A/R58A}$ mice, which express a non-functional form of $p40^{phox}$ due to a mutation in the (PI(3)P) binding domain, have reduced phagosomal ROS in response to *in vitro* challenge with IgG coated latex beads and serum opsonized *S. aureus*. These mice also exhibit exaggerated inflammation in a peritonitis model associated with significantly higher numbers of newly recruited neutrophils and

monocytes. While wild-type mice efficiently clear accumulated inflammatory cells, , $p40^{phoxR58A/R58A}$ mice are unable to resolve inflammation. This delay is caused by multiple events including prolonged recruitment of inflammatory leukocytes, delayed apoptosis of recruited inflammatory cells and subsequent delays in clearance of recruited cells. Our studies implicate an essential role for PI3P-regulated NADPH oxidase in resolution of inflammation by regulating inflammatory cell recruitment and subsequent clearance.

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Pulmonary Microvascular Endothelial Cells: A Significant Source of Angiopoietin-2 in the Pathogenesis of ARDS

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Loss of endothelial cell (EC) barrier function is characteristic pathology in patients with acute respiratory distress syndrome (ARDS). EC growth factors, Angiopoietin (Ang)-1 and Ang-2, are important mediators of EC function. When bound to their shared receptor, Tie2 (expressed on ECs), they have diametrically opposing effects on EC activation. Ang-1/Tie2 promotes a quiescent phenotype while Ang-2/Tie2 is associated with an activated phenotype; increased vascular permeability and neutrophil recruitment. Ang-2, stored pre-formed in EC storage vesicles, Weibel Palade bodies (WPbs), is rapidly released from activated ECs. We have reported increased Ang-2 in the blood of trauma patients with ARDS. We have also shown Ang-2 to be significantly elevated in plasma and lung tissue in our hemorrhage/sepsis model for the development of indirect acute lung injury /experimental ARDS in mice, and that blockade of Ang-2 protein following hemorrhage increases their survival. These findings point to Ang-2 as a potential therapeutic target in the treatment of ARDS. The pulmonary vascular endothelium has been show to have distinct/characteristic phenotypes depending upon the region of the vascular bed. To better elucidate the kinetics and mechanism(s) of release and the contribution of Ang-2 to the pathogenesis of ARDS, we used commercially available (PromoCell) primary human pulmonary arterial ECs (hPAECs) and microvascular ECs (hPMECs) ($5x10^6$ cells/well). Cells were grown to confluence on LabTek chambered slides and stimulated with 20ug/ml TNF-a for either 4, 10 or 24 hours. Ang-2 protein release in culture supernatant from hPMECs at all-time points was significantly (2-3 times) greater than from hPAECs. In addition, von Willebrand Factor (vWf), a protein stored and released from EC WPbs along with Ang-2, was also significantly elevated in hPMEC culture supernatant. TNF-a stimulated hPAECs, however, had significantly higher Ang-2 mRNA than hPMECs. These findings are significant because, while ECs in larger vessels, like arteries, contain WPbs, they are not observed in microvascular ECs (<10um diam), suggesting an alternative mechanism for release of Ang-2 and vWf from hPMECs. Further, this data identifies a phenotype distinctive to the microvascular region of the pulmonary vascular bed that may play a role in the pathogenesis of ARDS and as such presents a potential therapeutic target. (NIH GM103652)

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Expression Profile of Innate Immune Receptors, NLRs and AIM2, In Human Colorectal Cancer: Correlation with Cancer Stages and Inflammasome Components

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NLRs (nucleotide-binding domain leucine-rich repeat proteins or NOD-like receptors) are regulators of inflammation and immunity. A subgroup of NLRs and the innate immune receptor, AIM2 (absent-in-melanoma 2), can induce the assembly of a large caspase-1 activating complex called the inflammasome. Other NLRs regulate key signaling pathways such as NF-kB and MAPK. Since inflammation is a central component of colorectal cancer (CRC), this work was undertaken to analyze NLR and AIM2 expression in human CRC by combining bioinformatics analysis and

experimental verification using clinical human tissue samples. Inflammation plays a profound role in human CRCs, and these genes have been already implicated in mouse models of colitis and CRC. Additional experiments analyzed the association of (i) gene expression and cancer staging, and (ii) gene expression among inflammasome components. Ten public CRC datasets from the Oncomine® Platform were analyzed. The study focused on the expression of innate immune receptors and sensors called NLRs and AIM2, which regulate inflammatory responses. We additionally examined expression levels of these candidate genes using clinical CRC samples. Genes analyzed include NLRP1, NLRP3, NLRP6, NLRP12, NLRC3, NLRC4, NLRC5, NOD1, NOD2 and AIM2. Additionally, forty case-matched cancer samples and adjacent healthy control tissues isolated from a cohort of Chinese CRC patients were profiled. Three patterns of gene expression in CRC are shown. The expression of NLRC3, a checkpoint of inflammation, and the inflammasome components NLRP1, NLRP3, NLRC4 and AIM2 were reduced in CRC. Reduced expression of these genes showed significant correlation with major clinical characteristics of CRC, and a subgroup of NLRs revealed tumor stage-specific reduced expression. NOD1 and NOD2 expression was increased in CRC, while NLRC5, NLRP6 and NLRP12 showed little difference compared to controls. Reduced expression of NLRC3 in CRC was verified in all available databases analyzed and confirmed with our patient cohort. Furthermore, the extent of NLRC3 and AIM2 gene reduction was correlated with cancer progression. This report reveals the potential value of NLR and AIM2 genes as biomarkers of CRC and cancer progression. Thus, NLRs potentially have diagnostic values as biomarkers and may represent promising targets for cancer therapy and prevention.

Development of Aggressive Pancreatic Ductal Adenocarcinomas (PDAC) Depends on G-CSF Secretion from Carcinoma Cells

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Pancreatic ductal adenocarcinoma (PDAC), one of the most lethal cancers, is characterized by an expanded stroma with marked fibrosis. We previously generated pancreas epithelium–specific TGF-β receptor type II (Tgfbr2) knockout mice in the context of Kras activation (p48/Kras/TGFβRII-KO mice) and found that they develop aggressive PDAC that recapitulated the histological features of the human disease. The mouse PDAC tissue showed strong expression of CXCL1, 2, 5, and 16 chemokines known to affect functions of immune cells. In this study we investigated the immune response during PDAC development. We found by IHC increased number of $Gr1^+$ cells in tumor tissue of p48/Kras/TGFβRII mice vs. p48/Kras mice. Detailed flow cytometry analysis showed an increased number of granulocytic cells – $CD11b^+Ly6G^+Ly6C^{low}$. In established pancreas carcinoma cell lines from p48/Kras/TGFβRII-KO mice we found more than 4 fold increase in G-CSF secretion compared with pancreatic carcinoma cell lines expressing activated Kras and with intact TGFβ signaling. In agreement with this, we found that tumors, isolated from p48/Kras/TGFβRII-KO mice, had elevated level of G-CSF compare to tumors from p48/Kras mice and to normal pancreas. G-CSF regulates differentiation and functions of myeloid cells. Our *in vitro* studies showed that conditional medium from p48/Kras/TGFβRII-KO pancreas carcinoma cell lines potentiate differentiation of $Ly6G^+$ cells from bone marrow progenitors and stimulate pro-tumorogenic IL-10 secretion from dendritic cells and these changes were abrogated in presence of anti G-CSF Ab in culture. Different subpopulation of myeloid cells ($CD11b^+Ly6C^{high}Ly6G^{low}$, $CD11b^+F4/80^+Ly6C^-Ly6G^-$, $CD11b^+Ly6C^{low}Ly6G^{high}$) isolated from pancreas tumor tissue by FACS sorting had increased level of

mRNA for Arg, iNOS, VEGF, IL6, IL1b, suggesting their immunosupressive phenotype. Indeed, we found decreased CD3/CD28 stimulated and allogeneic T cell proliferation, confirming their immunosuppressive activity. Finally, deletion of G-SCF by shRNA in pancreas carcinoma cells or treatment mice with anti G-CSF Ab significantly decreased tumor growth. Moreover, anti G-SCF treatment in combination with Gemcitabine reduced tumor growth more effectively than Gemcitabine alone and was associated with increased number of T cells ($CD3^+$) and decreased number of $Ly6G^+$ cells in tumor tissue. We propose that TGF-β signaling on pancreas epithelium is a key regulator of G-CSF secretion and the elevated levels of G-CSF contributes to the immunosuppressive and tumor-promoting effects in development of PDAC. Inhibition of G-SCF by blocking Ab have a positive effect on tumor reduction especially in combination with Gemcitabine.

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Stress Induced Heat Shock Transcription Factor 1 (HSF1) Orchestrates Inflammatory Cytokines and Fibrogenic Genes to Regulate Liver Fibrosis

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Hepatic fibrosis is a hallmark of chronic liver injury and characterized by excessive deposition of extracellular matrix components, especially type I collagen, disrupting normal liver architecture. Previous studies show that hsp47, a collagen-specific ER molecular chaperone, is required for stabilization and appropriate folding of pro-collagen mediated by pro-fibrogenic cytokine, TGF-β via activation of stress induced heat shock transcription factor1 (HSF1). We hypothesize that HSF1 plays an important role in hepatic fibrosis via regulation of hsp47 and collagen synthesis. HSF1 deficient (HSF1KO) and WT littermates were treated with carbon tetrachloride (CCl4) twice weekly for 6 weeks. Liver fibrosis was assessed by Sirius Red staining and α-SMA expression. Hepatic stellate cell activation markers and pro-fibrogenic transcripts as well as pro-inflammatory cytokines and HSF1 DNA binding activity and target genes, hsp47, hsp70 and hsp90 were assessed by real-time PCR. Our results show induction of liver fibrosis after chronic CCl4 treatment was significantly higher in HSF1KO mice compared to WT controls, reflected in elevated serum alanine aminotransferase and accompanied by elevation of pro-fibrogenic markers, α1-(I)-collagen ($p<0.05$) and activated hepatic stellate cell marker, α-smooth muscle actin (α-SMA) ($p<0.02$). Interestingly, HSF1 target genes were differentially regulated in CCl4 treated HSF1KO mice. Collagen chaperone, hsp47 expression was significantly increased ($p<0.04$) whereas hsp70 was reduced ($p<0.001$) without any changes in hsp90 in CCl4 treated HSF1KO mice. Expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) was elevated, whereas extracellular matrix proteins MMP-8 and MMP-13 were significantly decreased in CCl4 treated HSF1KO mice. Pro-inflammatory cytokine, MCP1 was upregulated ($p<0.04$), whereas TGF-β was reduced ($p<0.05$) in CCl4 treated HSF1KO mice. While HSF1 DNA binding activity in the liver was increased early at 72hrs after acute CCl4 treatment, nuclear HSF1 and DNA binding was decreased in fibrotic livers after 6 weeks during increased hsp47 expression, suggesting HSF1 independent regulation of hsp47 expression. Our results reveal that deficiency of HSF1 exacerbates murine liver fibrosis by increasing pro-inflammatory cytokine MCP1 and profibrogenic genes. Further, induction of collagen-specific chaperone, hsp47 is independent of HSF1 during hepatic fibrosis. Our studies propose an important protective role for HSF1 during liver fibrosis suggesting its therapeutic potential.

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DC-SIGN Activation Alleviates Acute Lung Injury and Pulmonary Fibrosis in Mice

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Fibrosis is the progressive conversion of normal tissue to scar tissue, and is associated with 45% of deaths in the United States. A potential therapeutic that has shown great promise in multiple animal models and in Phase 1 and Phase 2 clinical trials is injections of the plasma glycoprotein Serum Amyloid P (SAP). SAP is a systemic regulator of the innate immune system, and belongs to an ancient family of proteins called pentraxins. SAP reduces neutrophil recruitment, alters monocyte

differentiation, and promotes anti-inflammatory macrophages to reduce inflammation and fibrosis. The closely related, but not glycosylated, pentraxin C-reactive protein (CRP) has a diametrically opposite effect and generally potentiates inflammation and fibrosis. Interestingly, both proteins bind the same Fcγ receptors (FcγR) with similar affinities. What causes the opposing effects of SAP and CRP is unknown. We addressed this question by testing the effect of SAP on FcγR deficient cells. To our surprise, contrary to the current model the FcγR were not necessary for the effects of SAP on neutrophils, monocytes, and macrophages. However, when SAP was desialylated with neuraminidase, the effects of SAP were largely abrogated, indicating that there exists a receptor that binds SAP in a glycosylation dependent manner. We found this receptor to be the human lectin receptor DC-SIGN (SIGN-R1 in mice) and showed that SAP but not CRP binds this receptor. DC-SIGN activation by a synthetic ligand or by anti-DC-SIGN antibodies was sufficient to mimic SAP effects on neutrophils, monocytes, and macrophages. We then mutated CRP (CRP A32N) to have a glycosylation similar to that of SAP, and examined the effects of CRP A32N on neutrophils, monocytes, and macrophages. CRP A32N was indistinguishable from SAP in our in vitro assays, further emphasizing the role of SAP glycosylation in allowing the immune system to distinguish SAP from CRP. In mice, we observed that a synthetic DC-SIGN ligand reduced neutrophil accumulation in a model of acute lung injury and, at 0.001 mg/kg, alleviated pulmonary fibrosis in an IL-10 dependent manner. We found DC-SIGN (SIGN-R1) on CD45+ cells and on mouse lung epithelial cells, and observed that SAP and the DC-SIGN ligand robustly potentiated IL-10 production from mouse epithelial cells. Together, our data suggest that SAP activates DC-SIGN to regulate the innate immune system differently from CRP, and that DC-SIGN is a potential target for therapeutics to treat fibrosis.

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HIV-1 Transgenic Rats Display Alterations in Gene Expression Associated with Aging in Liver

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Advance of combination of anti-retroviral therapy (cART) in last two decades has allowed life expectancy of individuals infected with human immunodeficiency virus (HIV) to approach that of the general population. In the United States, most of the HIV patients at the present time are 50 years of age or older. Clinically, growing older with HIV has been reported to be at risk of premature or accelerated aging. Aging is known to reduce the liver's ability to repair damages from inflammation caused by viruses including HIV, alcohol, or chemicals. However, little is known about how HIV and aging's combined effects on liver. The HIV-1 transgenic (HIV-1Tg) rat was created with a *gag*- and *pol*-deleted HIV-1 viral genome under the control of the LTR viral promoter, and it persistently expresses 7 of the 9 HIV-1 genes. This rodent model mimics HIV-infected patients on cART, who have controlled viral replication, but persistent HIV infection. We used this rodent model to study how aging with HIV may alter expression of the genes critical to major biological processes and risk factors for aging-related diseases in liver in hope to identify potential gene markers relevant to aging, HIV and aging with HIV. Four groups of animals, young F344 (1-mo. old), young HIV-1Tg (1-mo. old), old F344 (13-mo. old) and old HIV-1Tg (13-mo. old) were studied. Livers of each animal were collected. The expression of 84 aging related genes in the liver were determined by real-time PCR array using a rat aging commercial plate. Our data showed that little differences in expression profile of aging related genes between young F344 and HIV-1Tg rats. In contrast, there were profound differences between the two groups of aged animals. This aging related alteration could be attributed to aggravation by the persistent expression of HIV-1 proteins in the hepatic cells. In comparison to F344 rats, Vwa5a gene was down-regulated in both young and old HIV-1Tg rats. Thus, Vwa5a gene might be a potential biomarker of cellular senescence of HIV-1 infection. In comparison to the young animals, Bublb and Col1a1 genes were both down-regulated at similar level in the old HIV-1Tg and F344 rats, respectively. It is possible that these two genes are mainly related to common denominators of aging. Cdkn1c, Lsm5 and Lmnb1 were down-regulated mainly in old HIV-1Tg rats, but not in the F344

animals. Alteration of these three genes thus could be used as biomarkers for aging with HIV (Partially supported by NIH grants AA023172 and DA036175).

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Substrate Stiffness Modulates Endothelial Cell Functions Important In Neutrophil Adhesion And Migration

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Introduction: Lung dysfunction, as in COPD, ARDS and lung fibrosis, is often characterized by tissue remodeling, with a resultant change in substrate stiffness and tissue compliance. These lung diseases often involve neutrophil infiltration of lung tissue. Neutrophil adhesion to endothelial cells is an important first step in this process and is thought to be mediated by neutrophil binding to and exerting tractional forces on endothelial E-selectin, resulting in reorganization of the endothelial cell cytoskeleton. However, much of our understanding of endothelial cell function and endothelial-neutrophil interactions comes from experiments performed on high substrate stiffness such as polystyrene and glass. Substrate stiffness has been shown to affect cell signaling, tumor progression and metastasis, cytoskeletal organization and cell fate determination in a variety of systems. While it is known that changes in substrate stiffness occur in lung diseases, the effect of these changes on human pulmonary microvascular endothelial cell (HPMVEC) function at both physiologic ranges (2-8 kPa) and fibrotic ranges (25-50 kPa) and their contribution to disease progression and to modulation of inflammatory responses is poorly characterized.
Methods: The effect of substrate stiffness (2-50 kPa and polystyrene) on adhesion molecule expression by human pulmonary microvascular endothelial cells was determined in the presence and absence of the inflammatory mediator, TNF-alpha. Previous work in our lab has shown that cross-linking of E-selectin with antibody-coated magnetic beads increases the amount of E-selectin associated with the Triton-insoluble cell fraction, and subsequent pulling on the beads to mimic the tractional forces exerted by migrating neutrophils results in a decrease in this amount. We thus investigated the role of substrate stiffness in E-selectin-bound bead adhesion and pulling.
Results: Expression of the adhesion molecule ICAM-1 on HPMVECs increases with increasing substrate stiffness in both unstimulated and TNF-alpha-treated cells. E-selectin expression is also increased on polystyrene with TNF-alpha treatment. On soft (2 kPa) substrates compared to polystyrene, a greater amount of E-selectin associates with the Triton-insoluble fraction of HPMVECs after cross-linking with antibody-coated beads. After pulling, less E-selectin is associated with the Triton-insoluble fraction of cells on 2 kPa substrate compared to cells on polystyrene.
Conclusions: Expression of both E-selectin and ICAM-1 is less on HPMVECs grown on less stiff substrates that better mimic the pulmonary microvasculature than polystyrene. Strikingly, the fraction of E-selectin that associates with the Triton-insoluble cell fraction upon bead adhesion is greater on less stiff substrates, and pulling on the beads to mimic forces during neutrophil migration results in a greater reversal of this association. These results suggest that the underlying substrate stiffness can modulate both HPMVEC responses to inflammatory signals and to the cross-linking and pulling of adhesion molecules that occur during neutrophil adhesion and migration along endothelial cells during the process of neutrophil recruitment.

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ESAT-6 Exacerbates Inflammation and Fibrosis in a Multi-wall Carbon Nanotube MWCNT Model of Pulmonary Granulomatous Disease

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We have established a murine granuloma model (AJRCMB 2011, 45: 858) in which multiwall carbon nanotubes (MWCNT) elicit a granulomatous pathology markedly similar to that found in sarcoidosis, a prototypical human granulomatous disease. MWCNT-elicited granulomatous disease is chronic (granulomas persist up to 90 days), and

characterized by elevated pro-inflammatory cytokines together with T cell and macrophage recruitment – all traits found in sarcoidosis. ESAT-6 is an *Mycobacterium tuberculosis* secreted protein and T cells from patients with sarcoidosis have been reported to react to ESAT-6 peptides. We hypothesized that ESAT-6 might exacerbate granulomatous inflammation induced by MWCNT. Experiments were carried out in which MWCNT (100 µg) ± ESAT-6 peptide 14 [NNALQNLARTISEAG] (20 µg) were instilled into wild-type C57Bl/6 and macrophage-specific PPARγ KO mice. Control animals received instillations of vehicle (sham) or ESAT-6 alone. Animals were sacrificed after 60 days for analyses of granuloma incidence, fibrosis and bronchoalveolar lavage (BAL) cell expression of CCL2, MMP12, and osteopontin (OPN) mRNA. Semi-quantitative morphologic analysis indicated more abundant and larger granulomas (2-fold increase, p=0.03, n=6/group) in mice receiving MWCNT+ESAT-6 than in mice receiving only MWCNT. This effect was observed in both wild-type and PPARγ KO mice. Trichrome staining also revealed a greater extent of fibrosis in mice receiving MWCNT + ESAT-6 than mice receiving MWCNT alone. Mice receiving ESAT-6 alone had no granulomas or fibrosis and BAL cell cytokine expression did not differ from sham controls. BAL cell expression of CCL2, MMP12, and OPN was significantly higher in MWCNT + ESAT-6 mice than in mice receiving MWCNT alone or in sham controls ($p<0.05$). Findings indicated that ESAT-6 instillation exacerbated MWCNT-mediated granuloma formation as well as fibrosis. ESAT-6 also augmented BAL cell expression of the pro-inflammatory chemokine, CCL2, as well as the tissue remodeling factors, MMP12 and OPN. Data suggest that simultaneous exposure to ESAT-6 mycobacterial antigen and environmental MWCNT may worsen chronic granulomatous disease and fibrosis.

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Low-Grade Inflammatory Polarization of Innate Monocytes by Super-Low Dose Endotoxin

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Impaired wound healing often accompanies low-grade inflammatory conditions during which circulating levels of subclinical super-low dose endotoxin may persist. Monocyte polarization during chronic inflammation may deter effective wound repair. However, little is understood about the polarization of monocytes by super-low dose endotoxin. We observed that super-low dose endotoxin delays wound repair through the polarization of low-grade inflammatory monocytes. Super-low dose endotoxin preferentially programs a low-grade inflammatory monocyte state *in vitro* and *in vivo*, as represented by the elevated population of $CD11b^{+}Ly6C^{high}$ monocyte. Mechanistically, super-low dose endotoxin caused cellular stress, altered lysosome function and induced the transcription factor IRF5. TUDCA, a potent inhibitor of cellular stress, effectively blocked the monocyte polarization, and improved wound healing in mice injected with super-low dose endotoxin. Our data reveal the unique polarization of low-grade inflammatory monocytes by super-low dose endotoxin, its underlying mechanisms, and a novel intervention.

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RSV-Induced H3K4 Demethylase KDM5B Leads to Regulation of Dendritic Cell-Derived Innate Cytokines and Exacerbates Pathogenesis In Vivo

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Respiratory syncytial virus (RSV) infection can result in severe disease partially due to its ability to interfere with the initiation of Th1 responses targeting the production of type I interferons (IFN) and promoting a Th2 immune environment. Epigenetic modulation of gene transcription has been shown to be important in regulating inflammatory pathways. RSV-infected bone marrow-derived DCs (BMDCs) upregulated expression of *Kdm5b/Jarid1b* H3K4 demethylase. *Kdm5b*-specific siRNA inhibition in BMDC led to a 10-fold increase in IFN-b as well as increases in IL-6 and TNF-a compared to control-transfected cells.

The generation of *Kdm5b*$^{fl/fl}$-CD11c-Cre^{+} mice recapitulated the latter results during *in vitro* DC activation showing innate cytokine modulation. *In vivo*, infection of *Kdm5b*$^{fl/fl}$-CD11c-Cre^{+} mice with RSV resulted in higher production of IFN-γ and reduced IL-4 and IL-5 compared to littermate controls, with significantly decreased inflammation, IL-13, and mucus production in the lungs, and increase viral clearance. Sensitization with RSV-infected DCs into the airways of naïve mice led to an exacerbated response when mice were challenged with live RSV infection. When *Kdm5b* was blocked in DCs with siRNA or DCs from *Kdm5b*$^{fl/fl}$-CD11c-Cre^{+} mice were used, the exacerbated response was abrogated. Importantly, human monocyte-derived DCs treated with a chemical inhibitor for KDM5B resulted in increased innate cytokine levels as well as elicited decreased Th2 cytokines when co-cultured with RSV reactivated CD4^{+} T cells. These results suggest that KDM5B acts to repress type I IFN and other innate cytokines to promote an altered immune response following RSV infection that contributes to development of chronic disease.

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Low-Grade Inflammatory Programming of Innate Monocytes Through Disruption of Lysosome Function by Super-Low-Dose Endotoxin

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Altered innate immune homeostasis during low grade inflammation may lead to chronic non-resolving inflammatory diseases. However, the underlying mechanisms responsible for the disruption of monocyte homeostasis remain less understood. The Gram negative bacterial endotoxin, lipopolysaccharide (LPS), is a potent agent in the dynamic programming of monocyte homeostasis, with subclinical super-low dose LPS favoring the disruption of monocyte homeostasis and skewing of a low-grade inflammatory phenotype. The super-low level of LPS circulating in the blood, which is prevalent in the individuals with adverse health conditions or lifestyles, may be derived from leaky mucosal barriers or subclinical but sustained bacterial infection. In the present study, we treated bone marrow derived monocytes (BMDMs) with different doses of LPS (100 pg/ml – 1 μg/ml), and super low dose of LPS was sufficient to remarkably up-regulate IL-12 production. Interestingly, the induction of IL-12 reached the maximum at low LPS concentration (1 – 10 ng/ml), while the trend reversed as the concentration of LPS further increased. A similar trend was also observed in the CCR5 expression on monocytes/macrophages. Only super-low-dose LPS treatment resulted in the maximum expression of CCR5 on bone marrow cells cultured with M-CSF. The expression of Toll-interacting protein (Tollip), a key regulator of TLR4 pathway, was dramatically diminished in BMDMs after super-low-dose LPS treatment (50 pg/ml). Moreover, LPS treatment affected Tollip subcellular localization detected by confocal microscopy. Tollip co-localized with late endosomes/lysosomes maker, LAMP1, in resting cells, and translocated to mitochondria after treatment with 50 pg/ml LPS. To test whether disruption of lysosome fusion by super-low-dose LPS is mediated by Tollip clearance from late endosomes/lysosomes, we employed a Tollip mutant MEF cell line with a non-functional CUE domain. As in BMDMs, super-low-dose LPS treatment also caused translocation of Tollip from late endosomes/lysosomes to mitochondria in wild type (WT) MEFs. In contrast, Tollip translocation was blocked in mutant MEFs, since Tollip was retained in endosomes/lysosomes even in the presence of 50 pg/ml LPS. Starvation induced lysosome fusion with autophagosomes was interfered in WT MEFs after super-low-dose LPS treatment, demonstrated by separation of LAMP1 and autophagosome marker, LC3. However, this disruption was not observed in mutant MEFs with or without LPS treatment. These data showed that super-low-dose endotoxin preferentially skewed monocytes into a pro-inflammatory state, and resulted in potent inhibition of autophagosome-lysosome fusion that may account for the failure to resolve inflammation. Our work reveals the pathological impacts of super-low-dose endotoxemia and the critical role of Tollip in establishment of sustained inflammation in innate immune cells by super-low-dose LPS.

The IL-25/IL-25 Receptor Axis in B Cell Lymphomas of Germinal Center Origin

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Interleukin (IL)-25, also known as IL-17E, is structurally related to IL-17A but is the most divergent member of the IL-17 family. IL-25, that is produced by mucosal epithelial cells and different types of immune cells, has potent pro-inflammatory effects *in vitro* and *in vivo*, and promotes eosinophilia and type 2 immune responses. The heterodimeric receptor for IL-25 (IL-25R) is composed of the IL-17RA and IL-17RB subunits. So far, no information is available about the relationships between IL-25 and B cell malignancies. In this study, we have investigated the expression and function of IL-25 and IL-25R in primary neoplastic B cells from twenty four lymph node biopsies of patients with germinal center derived Non-Hodgkin B cell lymphomas, i.e. follicular, diffuse large B cell, and Burkitt lymphomas. The results obtained are the following, i) lymphoma cells expressed IL-25R, and IL-25 inhibited their proliferation, ii) IL-25 was expressed both in malignant and non-malignant cells in the tumor microenvironment, iii) IL-25 activated NF-kB signaling in lymphoma cells, and iv) exterted anti-tumor activity in a mouse model of follicular lymphoma. Tumor masses from IL-25 treated mice displayed necrotic-hemorragic areas associated with defective microvascular supply and reduced neoplastic cell proliferation. These histopathological findings were paralleled by the blunted expression of different po-angiogenic genes, the most prominent of the latter being VEGF-C. The data delineate a new anti-tumor role of IL-25 in germinal center-derived B cell lymphomas.

A New TLR Adaptor Pathway Linked to Inflammatory Cytokine Production From Macrophages

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The Toll-like receptor (TLR) family of pattern recognition receptors drive inflammatory cytokine production from macrophages and are intimately linked to chronic inflammatory disease processes and tumorigenesis. Here we describe a new adaptor pathway that mediates a sub-set of TLR4 responses upon activation of macrophages with lipopolysaccharide (LPS). TLRs are known to be tyrosine phosphorylated, but the mechanisms responsible and the biological consequences of this signalling event are poorly understood. We confirmed the importance of TLR4 tyrosine phosphorylation in mediating LPS-induced inflammatory cytokine production from macrophages, by showing that reconstitution of TLR4 knock-out primary macrophages with a TLR4 mutant in which a key tyrosine residue is mutated to phenylalanine results in normal cell surface TLR4 expression but defective LPS-induced cytokine production, as compared to reconstitution with wild type TLR4. We subsequently identified a novel cell surface protein that acts as a bridging adaptor by directly interacting with both TLR4 and tyrosine kinases to enable TLR4 tyrosine phosphorylation and downstream signalling responses. We used GST pull down assays, co-immunoprecipitation analysis and fluorescence polarization assays with recombinantly expressed proteins to confirm the interaction. Co-immunoprecipitation studies also showed that the TLR4-adaptor protein interaction is agonist-induced in cells. Furthermore, we have identified specific residues within the adaptor that are required for the interaction with the TLR4 TIR domain. Functionally, adaptor protein over-expression in macrophages enhanced LPS-induced TLR4 tyrosine phosphorylation, whilst silencing of it had the reverse effect. Similarly, gain- and loss-of-function approaches (retroviral over-expression and siRNA knock-down in primary macrophages) demonstrated that the adaptor is required for LPS-triggered signalling responses in macrophages, as

well as inducible production of a subset of pro-inflammatory cytokines (e.g. IL-6, IL-12p40). Remarkably, this pathway does not regulate several other LPS-inducible inflammatory cytokines. In summary, our studies have defined the molecular mechanism leading to LPS-mediated TLR4 tyrosine phosphorylation and downstream inflammatory cytokine production in macrophages. Our work reveals a new non-TIR-TIR mode of TLR-adaptor protein interaction in macrophages, and highlights that proximal TLR signalling events can impart remarkable specificity to downstream inflammatory cytokine production.

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Toxoplama gondii Interferes With Virus-Induced Induction of Inteferon-A in Human Plasmacytoid Dendritic Cells by Functional Mimicry of IL-10

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Plasmacytoid dendritic cells (pDC) are major producers of IFN-α, an antiviral cytokine involved in immunomodulation and anti-viral activity. The function and numbers of pDC are dysregulated in the context of HIV infection, and individuals with advanced HIV infection have increased susceptibility to opportunistic infections, including with *Toxoplasma gondii*. *T. gondii* is a life-threatening opportunistic infection in HIV-infected individuals, with areas of the world with high incidence of HIV also endemic for *T. gondii*. There is evidence from in vivo mouse studies that pDC in lymphoid tissue can become infected with *T. gondii*, thus making the interaction of pDC with the parasite and virus of significant interest. In this study, we investigated human pDC responses with virus (HSV and HIV) and *T. gondii* co-infection. Using flow cytometry and fluorescence microscopy, we determined that *T. gondii* invaded but did not induce IFN-α or TNF-α in human pDC, while both HSV and HIV induced IFN-α. However, with co-stimulation, *T. gondii* strongly inhibited both IFN-α and TNF-α produced in response to HSV and HIV, thus functionally inactivating pDC. Within the *T. gondii*-exposed pDC population, IFN-α production was inhibited only in cells infected by *T. gondii*, but not in *T. gondii* uninfected cells, indicating that the inhibition was acting *in cis*, whereas TNF-α was inhibited in the infected and partially in the uninfected pDC. *T. gondii* inhibited neither uptake of GFP-HSV nor localization of TLR-9 in $CD71^+$ endosomes in response to HSV. Using imaging flow cytometry, we found that virus-induced nuclear translocation of IRF7, required for IFN-α production in response to viruses, but not phosphorylation of the transcription factor, was abolished by the parasite. We previously demonstrated that IL-10 inhibits virus-induced IFN-a production by pDC. Similar to what we observed with *T. gondii* treatment, we now show that IL-10 treatment of pDC also inhibited IRF7 translocation, but not phosphorylation. Taken together, these data indicate that the block of the intracellular signaling cascade by *T. gondii* occurs downstream of TLR-9 recruitment and IRF7 phosphorylation but upstream of IRF7 translocation. The *T. gondii* virulence factor, ROP16 kinase, directly phosphorylates STAT3, further mimicking IL-10 anti-inflammatory signaling, which is mediated through STAT3. In pDC, phosphorylation of STAT3 was only seen in cells infected with *T. gondii*, ruling out a soluble mediator like IL-10 or IL-6 as responsible for the phosphorylation. Obstruction of IRF7 nuclear translocation and inhibition of IFN-α production by *T. gondii* was partially reversed by knocking out *T. gondii*-derived ROP16. These findings suggest a novel mechanism of inhibition of TLR signaling by *T. gondii* whereby the pathogen mimics IL-10 signaling and suggest potential negative consequences of HIV/*T. gondii* co-infection.

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Ablation of Host-Derived Semaphorin7A Decreases Tumor Progression in a Murine Model of Metastatic Breast Cancer

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We discovered that mammary tumor cells express high levels of axonal guidance molecule Semaphorin7A (SEMA7A). Tumor-derived SEMA7A induces monocytes to secrete pro-angiogenic chemokines to enhance tumor growth. Decreasing tumor-derived SEMA7A limits tumor growth and the production of angiogenic CXCL-

2/MIP-2 and VEGF-A by monocytes. Immune cells also express elevated levels of SEMA7A in tumors, however the role of stromal-derived SEMA7A in tumorigenesis is largely unknown. Recent reports describe hypoxia responsive elements in the SEMA7A promoter. It is well established that hypoxia enhances tumor growth by promoting angiogenesis and alternatively activating monocytes. We hypothesize that SEMA7A may be a hypoxia-inducible factor and that inhibition may deter the effects of hypoxia on tumor and immune cells. Following hypoxic stimuli, WT monocytes increased production of CXCL2/MIP-2, VEGF-A and Chitinase-3-like protein1 (CHI3L1). However, SEMA7A null monocytes failed to respond to hypoxia, which may be attributed to an impaired MAPK signaling response. Monocytes from SEMA KO tumor bearing mice showed a decreased production of angiogenic molecules. More importantly, we found that SEMA7A KO 4T1 mammary tumor bearing mice showed decreased tumor growth and metastasis compared to WT. Collectively, we show a role for stromal-derived SEMA7A in hypoxia-mediated mammary tumor progression. Understanding the role of SEMA7A may unravel tumor-host immune interactions in breast cancer.

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An Essential Role for Interferon-beta in the Induction of Interferon-Stimulated Gene Expression by LPS in Macrophages

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TLR agonists such as LPS and poly(I:C) induce expression of type I interferons such as IFN-α and -β by macrophages. However, the relative contribution of IFN-α versus IFN-β to the subsequent expression of IFN-stimulated genes (ISGs) has not been defined. To examine the role of IFN-β in the induction of ISGs by LPS, we compared the ability of LPS to induce ISGF3 activity and ISG expression in bone marrow-derived macrophages from wild-type (WT) and IFN-β gene (*Ifnb1*) knockout (KO) mice. We found that LPS treatment activated ISGF3 and induced expression of ISGs such as *Oas1*, *Mx1*, *Ddx58* (RIG-I) and *Ifih1* (MDA5) in WT macrophages but not in macrophages derived from *Ifnb1* KO or *Ifnar1* KO mice. The inability of LPS to induce activation of ISGF3 and ISG expression in *Ifnb1* KO macrophages correlated with the failure of LPS to induce activation of STAT1 and STAT2 in these cells. Consistent with these findings, LPS treatment also failed to induce ISG expression in bone marrow-derived macrophages from *Stat2* KO mice. Although activation of ISGF3 and induction of ISG expression by LPS was abrogated in *Ifnb1* and *Ifnar1* KO macrophages, activation of NF-κB and induction of NF-κB responsive genes such as *Tnf* (TNF-α) and *Il1b* (IL-1β) were unaffected by deletion of either the IFN-β or IFN-αR1 genes. These findings demonstrate that induction of ISGF3 activity and ISG expression by LPS is critically dependent on endogenous production of IFN-β and autocrine signaling through type I IFN receptors.

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Pellino-3 Promotes Endotoxin Tolerance and Acts as a Negative Regulator of TLR2 and TLR4 Signaling

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Development of endotoxin tolerance in macrophages during sepsis re-programs TLR4 signaling to inhibit pro-inflammatory cytokines without suppressing anti-inflammatory and anti-microbial mediators and protects the host from excessive inflammation and tissue damage. However, endotoxin tolerance renders septic patients immunocompromised and unable to control secondary infections. While previous studies have revealed the importance of several negative regulators of TLR signaling in endotoxin tolerance, the role of Pellino proteins has not been addressed. We show that induction of endotoxin tolerance in vivo in mice and in vitro in human monocytes, THP-1 and MonoMac-6 macrophages increases expression of Pellino-3. Overexpression of Pellino-3 in 293/TLR2 or 293/TLR4/MD-2 cells inhibited

TLR2/4-mediated activation of NF-κB and induction of IL-8 mRNA, while Pellino-3 ablation increased these responses. Pellino-3-deficient THP-1 cells had elevated TLR2/4-driven TNF-α, IL-6 mRNA and TLR4-driven CCL5 gene expression in response to TLR agonists and heat-killed Escherichia coli and Staphylococcus aureus, cytokines controlled by the MyD88- and TRIF-mediated pathways, respectively. In addition, deficiency in Pellino-3 slightly increased phagocytosis of heat-killed bacteria. Transfected Pellino-3 inhibited NF-κB activation driven by overexpression of MyD88, TRIF, IRAK1 and TBK1, TAK1 and TRAF6, and inhibited IRAK1 modifications and TBK1 phosphorylation. Finally, Pellino-3 ablation in THP-1 decreased the extent of endotoxin tolerization. Thus, Pellino-3 is involved in endotoxin tolerance and functions as a negative regulator of TLR2 and TLR4 signaling

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Biologic Significance of a Gonococcal Histone Deacetylase in Macrophage Infection

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Neisseria gonorrhoeae is a strict human pathogen that causes the sexually transmitted infection termed gonorrhea. We previously reported that *N. gonorrhoeae* can survive in macrophages where it up-regulates hepcidin, the master iron regulating peptide, expression to facilitate iron acquisition. We also observed that gonococcal infection suppresses the expression of host defense peptide LL-37 gene in human THP-1 macrophage-like cells and in peripheral human monocytes. However, the mechanism by which gonococci down-regulate host defense peptides expression is not known. The aim is to investigate the mechanism by which gonococcal infection in macrophages modulates host defense antimicrobial peptide (AMP) production for immune evasion to promote intracellular survival. Gonococci have a gene that encodes a histone deacetylase-like enzyme (GC-HDAC) that shares high 3D-homology to human HDAC2 and HDAC8. I employed computational modeling to predict GC-HDAC-like protein structure-function and found that it has an active catalytic pocket containing the highly conserved Zinc binding constellation, suggesting an HDAC-like activity. Indeed, HDAC inhibitors TSA and valproic acid can inhibit the growth of live gonococci. The GC-HDAC-like gene is present in all pathogenic *Neisseria* species and is expressed at all growth phases of gonococci strain FA19 assessed by quantitative RT-PCR. In eukaryotic cells, HDACs suppress gene expression by condensing chromatin packing that prevents transcription factors from binding to promoter regions. The hypothesis is that the GC-HDAC-like protein exerts epigenetic modifications on host histones to suppress LL-37 gene expression, which facilitates immune evasion and promotes intracellular survival. In order to determine the biologic significance of the HDAC-like protein, a GC-HDAC null mutation in gonococci strain FA19 was constructed and observed that the mutant has a growth defect that can be reversed by complementation. Macrophage infection assay was employed in vitro, and found that HDAC-deficient gonococci are killed more rapidly than wild-type gonococci in macrophage bactericidal assays. In contrast, wild type gonococci significantly reduced the expression of LL-37 and survived more in macrophages when compared to its HDAC-deficient isogenic mutant. To investigate lysine posttranslational modification in WT FA19 compared to HDAC-deficient gonococci, as well as in infected macrophages a global acetylome studies are under current investigations.

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Investigation of the Role of Complement Component C1q in Engulfment of Cancer Cells

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The metastasis and spread of cancer is due, in part, to a failure of the innate and adaptive arms of the immune system to detect transformed cells. Cancer cells are unique in that they have acquired cell surface markers characteristic of both live and apoptotic cells. C1q, an innate immune effector molecule, is required for engulfment of apoptotic cells in mice and humans. While C1q is required for the clearance of apoptotic cells, its role in the identification and clearance of cancer cells by phagocytes is unknown. Here we used a variety of models composed of macrophages and cancer cells to investigate C1q- dependent macrophage

engulfment of cancer cells, and we explored the mechanism behind this phenomenon. Initial experiments were performed with a hybrid system composed of primary mouse macrophages and human Jurkat cancer cells (a T-cell leukemia). Additionally, experiments were performed in a strict mouse model (primary mouse macrophages and the mouse EL-4 Lymphoma) and a strict human model (human monocyte-derived macrophages and human Jurkat cancer cells). C1q enhanced engulfment of cancer cells in all three systems. Our lab previously demonstrated that C1q upregulated expression of Mer tyrosine kinase and its ligand Gas6 to facilitate clearance of apoptotic cells. Mer deficient mice failed to respond to C1q with enhanced engulfment of cancer cells suggesting that residual apoptotic cells in cancer cell preparations may contribute to C1q-dependent enhanced engulfment. Future experiments are aimed at investigating the contribution of apoptotic cells to the observed C1q-dependent engulfment of cancer cells.

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Anti-Inflammatory Modulation of Macrophage Phenotype for Therapeutic Application in Implantation

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Currently, titanium-based biomedical devices are mainstream solution for implantation and organ replacement throughout the World due to low toxicity and biocompatibility of titanium alloys. Nevertheless, adverse effects such as peri-implantitis, aseptic loosening and osteolysis are sometimes observed upon implantation. Macrophages and their pro-inflammatory mediators are central players in implant-associated chronic inflammation. Local induction of anti-inflammatory macrophage phenotype is one of the strategies to minimize such adverse effects. However, long-term maintenance of anti-inflammatory state in macrophages is a challenging task due to plasticity of macrophage phenotype. In this study we characterized the effect of potent anti-inflammatory cytokine combination (M2 cocktail, M2ct) on cytokine production by human monocyte-derived macrophages in long-term culture and after re-polarization with pro-inflammatory stimuli. The results demonstrated that M2ct-stimulated macrophages sustained anti-inflammatory phenotype up to 12 days in *in vitro* culture even after stimulation with high dose of LPS. Moreover, after deprivation of cytokines from culture medium followed by challenge with LPS for 6 other days, TNFα release by M2ct-differentiated macrophages was strongly suppressed compared to prototypical M2 stimulator IL-4. Restoration of pro-inflammatory phenotype was possible only when macrophages were deprived of M2ct and stimulated with combination of IFNγ and LPS. Overall, this data demonstrate availability of potent anti-inflammatory cytokine combinations for local modulation of macrophage phenotype in case of implant-induced inflammatory complications.

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High Glucose Differentially Affects Expression of TLRs in Human Macrophages: Potential Mechanism of Chronic Inflammation in Diabetes

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Diabetic patients suffer from chronic inflammation regulated primarily by macrophages. M1 and M2 represent two major directions of macrophage functional polarisation. Toll-like receptors (TLR)-mediated response to exogenous and endogenous factors results in pro-inflammatory activation of macrophages.

Increased TLR expression in monocytes is associated with diabetic inflammation and complications. The aim of our study was to examine how elevated glucose levels affect expression of TLRs in M1 and M2.

Monocytes were isolated from healthy donors and cultured in serum-free medium in the presence of 5mM or 25mM glucose for 6 days. M1 and M2

differentiation was driven by IFN-γ and IL-4. Quantification of TLR mRNA expression was performed by RT-PCR and demonstrated that mRNA of TLR1, 2, 4, 6 and 8, but not TLR5 and 9, were expressed in M1 and M2. All identified TLRs were expressed on higher levels in M1 compared to M2 in low and high glucose conditions.

Increased glucose had a most pronounced stimulatory effect on the expression of TLR2 and less on TLR6. In the individual M1 cultures high glucose stimulated up to 8-fold increase of TLR2 and up to 28% increase of TLR6 expression. In M2, TLR2 expression was up to 6-fold higher in the presence of high glucose. High glucose had suppressive effect on the expression of TLR4 (in 5 of 8 donors up to 65%) in M1 and M2.

Comparative analysis of expression profiles of fat tissue samples and blood parameters from patients with metabolic syndrome revealed a negative correlation between TLR2 expression and Alanine transaminase and between TLR6 and HDL. TLR2 expression correlated positively with TNF-α plasma levels. Reduced TLR expression can be linked to the protective role of HDL in cardiovascular diseases.

We conclude that glucose regulates TLR2, TLR4 and TLR6 expression in a donor-specific way. Such individual responses suggest new glucose-mediated mechanism for the development of patient-specific inflammation-mediated complications in diabetes.

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Mechanism for the Lipopolysaccharide-Induced Release of High Mobility Group Nucleosome-Binding Domain-1 From Murine Macrophage Like RAW264.7

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Background: Alarmins are identified as endogenous mediators that have potent immune activating abilities. High mobility group nucleosomal binding domain-1 (HMGN1), a highly conserved, non-histone chromosomal protein, which binds to the inner side of the nucleosomal DNA, regulates the chromatin dynamics and transcriptional condition in the cells. Furthermore, HMGN1 behaves as a cytokine in the extracellular milieu by inducing the recruitment and maturation of antigen-presenting cells (dendritic cells) to enhance the Th1-type antigen-specific immune responses. Thus, HMGN1 is expected to act as an alarmin, when released into the extracellular milieu. In this study, we investigated the release mechanism of HMGN1 from macrophages using mouse macrophage-like RAW264.7 cells.

Methods: RAW264.7 cells were stimulated with bacterial lipopolysaccharide (100 ng/ml; *E.coli* O111:B4) in the absence or presence of a caspase 1 inhibitor YVAD-CHO or a necroptosis inhibitor Nec-1. Moreover, cell death was assessed by the release of lactate dehydrogenase and annexin V/propidium iodide-staining.

Results and Discussion: The results indicated that HMGN1 is released from LPS-stimulated RAW264.7 cells, accompanied with the release of lactate dehydrogenase. However, YVAD and Nec-1 did not change the LPS-induced HMGN1 and LDH release, suggesting that pyroptosis (caspase-1-acivated cell death) and necroptosis (programmed necrotic cell death) are unlikely involved in the release of HMGN1 from LPS-stimulated RAW264.7 cells. Moreover, flow cytometry indicated that LPS-stimulation did not essentially induce apoptosis but substantially augmented necrosis, as evidence by staining with annexin V/propidium iodide. Together these findings suggest that HMGN1 is extracellularly released from LPS-stimulated RAW264.7 macrophage-like cells, mainly accompanied with un-programmed necrotic cell death but not with pyroptosis, necroptosis and apoptosis.

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Pulmonary Mycobacterial Infection Increases Systemic Expression of Notch Ligand Delta-Like 4 on Myeloid Cells in Both Humans and Mice

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Although host-pathogen relationships have indicated that the hematopoietic system responds to infection that takes place at distal sites, our understanding of the systemic response to mycobacterial infection is limited. Our lab has previously demonstrated that mycobacterial antigens can elicit the expression of the Notch ligand delta-like 4 on the cell surface of myeloid antigen presenting cells via a TLR mediated pathway. Delta-like 4 is a multifunctional ligand that serves to induce a pro-survival program in T cells as well as alter cytokine production in favor if Th1 responses. We have recently discovered that this ligand is also upregulated on murine hematopoietic stem and progenitor cells in response to infection with BCG. Through studies in which we isolated delta-like 4 expressing stem cells and transferred them into congenic recipients we have discovered that the expression of delta-like 4 is maintained over short-term hematopoiesis. Analysis of human peripheral blood samples indicates that expression of delta-like 4 is elevated on CD14+ monocytes in the peripheral blood of individuals either actively or latently infected with mTB when compared to individuals that were treated for 6 months with antibiotics. In individuals with latent infection, delta-like 4 expression positively correlated with cytokine production from T cells when peripheral blood was cultured with multiple mycobacterial antigens. This correlation was absent in actively infected individuals. These findings suggest that delta-like 4 expression is altered during mycobacterial infection in both humans and mice. Furthermore, the expression of this ligand may alter the T cell immune response.

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The Impact of Maternal Obesity During Pregnancy on Neonatal Immunity

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Obesity during pregnancy is associated with several adverse health outcomes for the offspring that can persist into adulthood, notably a higher lifetime risk of developing obesity, asthma, diabetes, cardiovascular disease, and cancer, which lead to a higher all cause mortality rate compared to offspring of lean mother. These diseases have a significant inflammatory component, which suggest that maternal obesity impacts development and maturation of the neonatal immune system. This hypothesis is supported by murine studies demonstrating worse outcomes in models of autoimmunity and allergic sensitization in pups born to obese dams. More importantly, we have recently shown that monocytes and myeloid dendritic cells in human cord blood mononuclear cells (UCBMC) collected from babies born to obese mothers generate dampened responses following stimulation by TLR4 and TLR1&2 agonists compared to UCBMC collected from babies born to lean mothers. In order to uncover the molecular mechanisms underlying these functional changes we compared the transcriptome and DNA methylation patterns using RNA and Methyl Seq analysis of purified CD14 monocytes purified from UCBMC collected from babies born to obese or lean mothers. Our analysis identified 78 differentially expressed genes (DEGs, false discovery $p< 0.05$ and fold change (FC) >2) in monocytes from babies born to obese mothers, with 72 upregulated and 6 downregulated genes. Some of the notable DEGs include major histocompatibility complex II DQ beta 1 (HLA-DQB1; FC -11), ZBTB16 (FC= -8), Histone 1 member X (FC=6), and WNT5a (FC=663). These genes are known to participate in immune function (HLA-DQB1 and WNT5a), chromatin condensation (H1), and cell cycle progression (ZBTB16). We also identified 3008 genes that contained at least one differentially methylated cystosine (DMC), with 1011 hyper and 1997 hypomethylated genes. One of the most hypermethylated genes was SATB homeobox 1 (SATB1) and some of the greatly hypomethylated genes included Histone deacetylase 4 (HDAC4), histone lysine methyltransferase (SMYD3), forkhead box O1 (FOXO1), and Toll interacting protein (TOLLIP). In summary, our analysis shows that maternal obesity during pregnancy exerts significant impact on gene regulation and expression within the offspring's immune cells, which is likely to results in functional differences.

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mTORC1-TBK1 Interaction is Required for Macrophage Signaling and Activation

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Inflammation and anti-pathogen responses must be tightly regulated to ensure that host defense does not damage host tissues. The critical host defense kinase TBK1 (TANK-binding kinase 1) regulates type I interferon and inflammatory cytokine expression downstream of pathogen sensors including TLRs, RIG-I, MAVS, and cGAS. Mice lacking TBK1 have a paradoxical inflammatory phenotype even in the absence of a pathogenic challenge, and the regulation and function of TBK1 are incompletely understood. Specifically, the steps leading to TBK1 activation and control of its output magnitude are not known. In the setting of viral infection or TLR3 or 4 activation, TBK1 phosphorylates the transcription factors IRF3 and IRF7, but in other TLR contexts its outputs are unknown. In addition, TBK1 may in different contexts contribute to or inhibit NFκB activation. The embryonic lethality of TBK1 KO mice has hampered the analysis of TBK function in primary cells such as macrophages.

To understand the regulation of TBK1 activation, we used proteomic and biochemical methods to identify a physical interaction between the mammalian target of rapamycin complex 1 (mTORC1) and TBK1. We show that macrophages require mTOR activity for TBK1 transcriptional outputs in the setting of TLR3 or TLR4 stimulation. Deletion or inhibition of mTORC1 impairs activating phosphorylation of TBK1 in both the kinase domain and the coiled-coil domain, suggesting one or more intermediate kinases. Mutation of the mTOR-dependent phosphorylation sites impairs TBK1 activation downstream of TLR3 and TLR4. Finally, we use myeloid-specific deletion of TBK1 in mice to examine its role in macrophage activation and differentiation.

Macrophages must constantly process signals from their microenvironment to tune and direct inflammatory and anti-inflammatory functions. We define a novel interaction between the mTOR pathway, which regulates nutrient and growth signaling, and the TBK1 pathway, which regulates anti-viral and inflammatory signaling. As TBK1 both promotes and constrains anti-viral and inflammatory gene expression, the mTOR-TBK1 interaction is a critical point at which microenvironmental conditions may direct and regulate inflammation. Further understanding of the mTOR-TBK1 axis and the contribution of TBK1 to macrophage function will contribute to anti-inflammatory therapeutics.

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Hematopoietic Progenitor Cells are Secretory Cells That Influence Innate Immune Cell Phenotypes in Response to Staphylococcus aureus and TLR2 Ligands

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Hematopoietic progenitor cells reside in the bone marrow and give rise to mature leukocytes. They also play an active role in innate immune responses outside of the bone marrow. These progenitor cells are present in *Staphylococcus aureus* infected wounds in mice and can differentiate there to produce mature myeloid cells, a process in part dependent on TLR2. When stimulated with the TLR2 ligand Pam_3CSK_4 in vitro, progenitor cells produce significantly more Ly6G+ neutrophils and F4/80+ macrophages than vehicle control. This enhanced myeloid differentiation is prevented by the addition of indomethacin, a non-selective cyclooxygenase inhibitor. Indeed, it has been shown that hematopoietic stem and progenitor cells produce prostaglandin-E_2, which is responsible in part of myeloid differentiation within *S. aureus* infected wounds. In addition to secretion of prostaglandin-E_2, hematopoietic progenitors also secrete large amounts of the cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-10 (IL-10) when stimulated in vitro with Pam_3CSK_4. Lineage negative, c-kit+, Sca-1+ (LSK) hematopoietic progenitors extracted from *S. aureus* infected wounds and incubated ex vivo for 48 hours produce the same secretory profile. The secreted

cytokines influence the phenotype of monocytes, bead-enriched from murine bone marrow, when incubated with the hematopoietic progenitor cell supernatants in vitro. Supernatants from TLR2-stimulated hematopoietic progenitors resulted in significantly more CD11c+/CD11b-negative cells and significantly less CD11c+/CD11b-hi cells than supernatant from hematopoietic progenitors incubated with vehicle control. Thus, hematopoietic progenitor cell interaction with TLR2 ligands not only influences their differentiation, but also allows progenitor cells to influence innate cell phenotype via progenitor cell secretory products. This data provides further evidence of the important role of hematopoietic progenitor cells in the innate immune response to *S. aureus* infection.

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Temporally Defined miRNA Expression Patterns are Specific for Macrophage Polarization and Reveal Regulation of Soluble Vascular Endothelial Growth Factor Receptor 1 by miR-125a-5p

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Dynamic epigenetic mechanisms that regulate macrophage phenotypes following cytokine stimulation have not been completely defined. While macrophage microRNA (miRNA) expression *in vitro* after the addition of agonists has been reported, these studies focused on miRNAs expressed >12 hours to days after stimulation and at only one time point. However, early and temporal expression patterns of miRNA in macrophage phenotypes have not been defined. To address this gap, miRNA expression was determined over multiple time points during polarization. The temporal pattern of established macrophage phenotype markers confirmed macrophage polarization and revealed essential time points for studying miRNA expression. Elevated inflammatory cytokines (TNF-α, IL-1α, and IL-6) and nitric oxide levels in the cultures of IFN-γ + LPS stimulated macrophages confirmed M1 polarization. While increased arginase activity and *Irf4* transcription factor expression in IL-4 stimulated macrophages confirmed M2a polarization. Consistent with our previous studies, IL-4 treatment also resulted in a 7-fold decrease in vascular endothelial growth factor (VEGF) in the culture media compared to the media only (MO) control and M1 conditions. Although changes in macrophage phenotype marker levels could be detected as early as 30 minutes and as late as 24 hours, a robust response was evident for most markers by 3 hours of exposure to cytokines. Therefore, temporal miRNA expression patterns were explored at 0.5, 1, 3, and 24 hours following stimulation. Therein, many polarization-specific miRNAs were significantly changed by 3 hours and some expressed divergent patterns between M1 and M2a polarization conditions. One such miRNA, miR-125a was divergently expressed between the M1 (elevated) and the M2a (depressed) conditions. Consequently, inhibition of miR-125a-5p in MO and M1 cultures resulted in decreased VEGF with comparably low VEGF in M2a conditions. To determine the mechanism of the decreased VEGF, soluble VEGF receptor 1 (sVEGFR1 aka sFLT1) was measured. Inhibition of miR-125a-5p elevated sVEGFR1 and decreased VEGF in the culture media at 3 hours and 12 hours in the MO and M1 conditions. While in the M2a condition, inhibition of miR125a-5p resulted in increased sVEGFR1 at 3 hours and comparable values at 12 hours with controls. However, miR-125a-5p mimic did not alter sVEGFR1 in the culture media under any condition likely due to the high endogenous expression of miR-125a-5p in all macrophage cultures. As sVEGFR1 can sequester VEGF, we measured mRNA levels of VEGFR1 and VEGF. Inhibition of miR-125a-5p resulted in increased VEGFR1 with minimal changes in VEGF mRNA. A titration of the miR-125a-5p inhibitor across multiple doses established a dosage-dependent inverse relationship between sVEGFR1 expression and miR-125a-5p abundance. Thus, inhibition of miR-125a-5p in M1, M2a, and MO control conditions resulted in an indirect regulation of sVEGFR1 with resultant decreases in VEGF.

This approach has revealed the time course of macrophage polarization, identified divergent expression patterns of several polarization-specific miRNAs, and revealed miR-125a-5p as an indirect regulator of sVEGFR1 and extracellular VEGF levels. Angiogenesis is a crucial step in tumor growth and metastasis. VEGF has been shown to facilitate survival of existing vessels, contribute to vascular abnormalities (e.g. tortuousness and

hyperpermeability) that may impede effective delivery of antitumor compounds, and stimulate new vessel growth. Given that VEGF is a major regulator of vascular development during tumorigenesis, and inflammation, miR-125a-5p may be a potent target in macrophages for cancer therapeutics and lead to novel and effective treatments.

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Increased Th17 Response to γHerpesvirus Infection is Essential for the Development of Pneumonitis and Fibrosis in Mice Post Bone Marrow Transplantation

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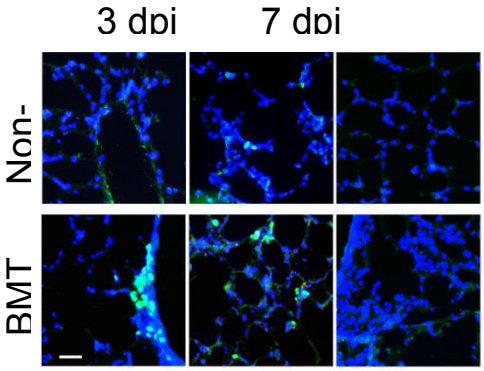
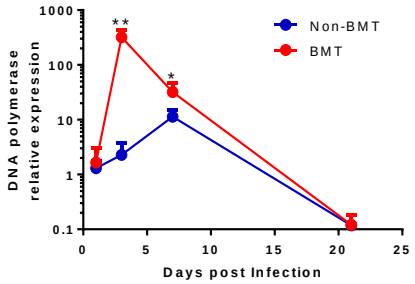
Hematopoietic stem cell transplantation (HSCT) efficacy is limited by numerous pulmonary complications. We developed a model of syngeneic bone marrow transplant (BMT) followed by infection with murine gamma herpesvirus (γHV-68) that results in pneumonitis and fibrosis. BMT mice experience increased early lytic replication comparing to non-BMT mice, but both establish viral latency by 21 days post infection (dpi). The BMT mice, but not non-BMT mice, developed pneumonitis and fibrosis at 21 dpi. Since no active viral replication was detected at that time, the pathological outcome in BMT mice mimics human "non-infectious" HSCT complications.

In this study, we aimed to investigate the mechanisms that underlie the development of pneumonitis and fibrosis in fully reconstituted BMT mice after γHV-68 infection. We found that CD4+ T cells in BMT mice are skewed towards IL-17A rather than IFN-γ production in response to γHV-68 infection. Transplantation of bone marrow from *Il-17a*$^{-/-}$ donors or treatment with anti-IL-17A neutralization antibodies at late stages attenuates pneumonitis and fibrosis in infected BMT mice, suggesting that hematopoietic-derived IL-17A is essential for development of pathology. IL-17A directly influences activation and extracellular matrix production by lung mesenchymal cells. To further understand how antigen presenting cells (APCs) in the lung direct CD4+ T cell differentiation, we enriched lung CD11c+ cells and examined their cytokine production and cellular function. We found that lung CD11c+ cells of BMT mice secrete more pro-T_H17 response cytokines TGF-β1, IL-23 and IL-6, and less T_H1 promoting cytokines IL-2 and IFN-γ, but not IL-12 in response to viral infection. Adoptive transfer of non-BMT lung CD11c-enriched cells restores robust T_H1 response and suppresses aberrant T_H17 response in BMT mice to improve lung pathology.

In conclusion, we found that lung APCs in BMT mice produced more pro-T_H17 cytokines and less critical pro-T_H1 cytokines in response to γHV-68 infection and cause CD4+ T cell differentiations to skew towards T_H17. The increased T_H17 response is essential for the development of pneumonitis and fibrosis likely by activating lung mesenchymal cells. Our data suggest "non-infectious" HSCT lung complications may reflect preceding viral infections and demonstrate that IL-17A neutralization may offer therapeutic advantage even after disease onset.





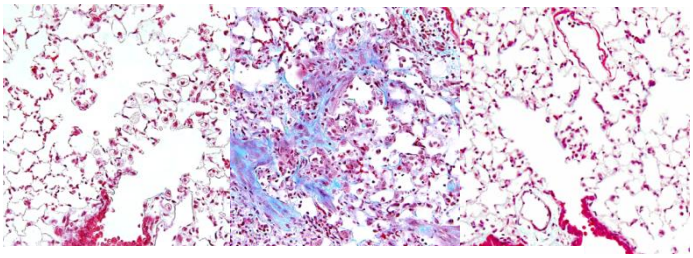


Figure 1

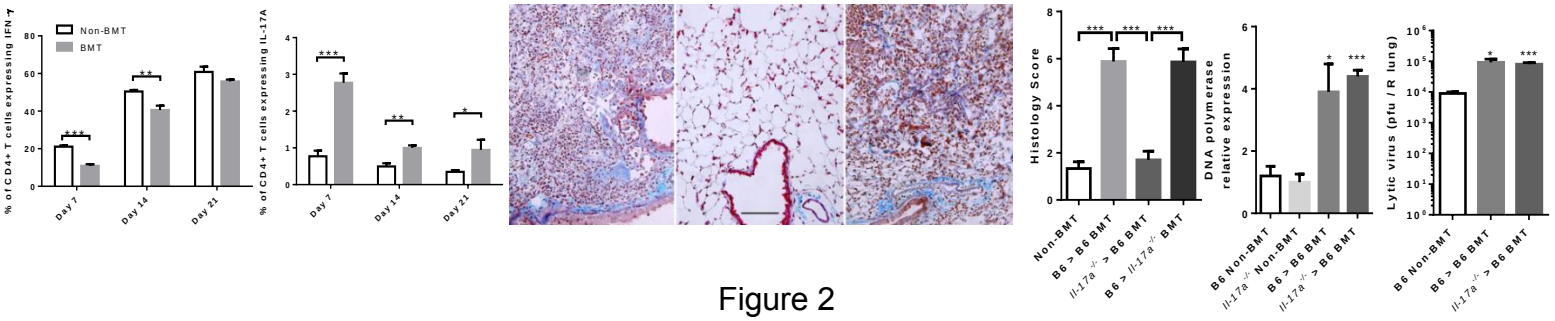


Figure 2

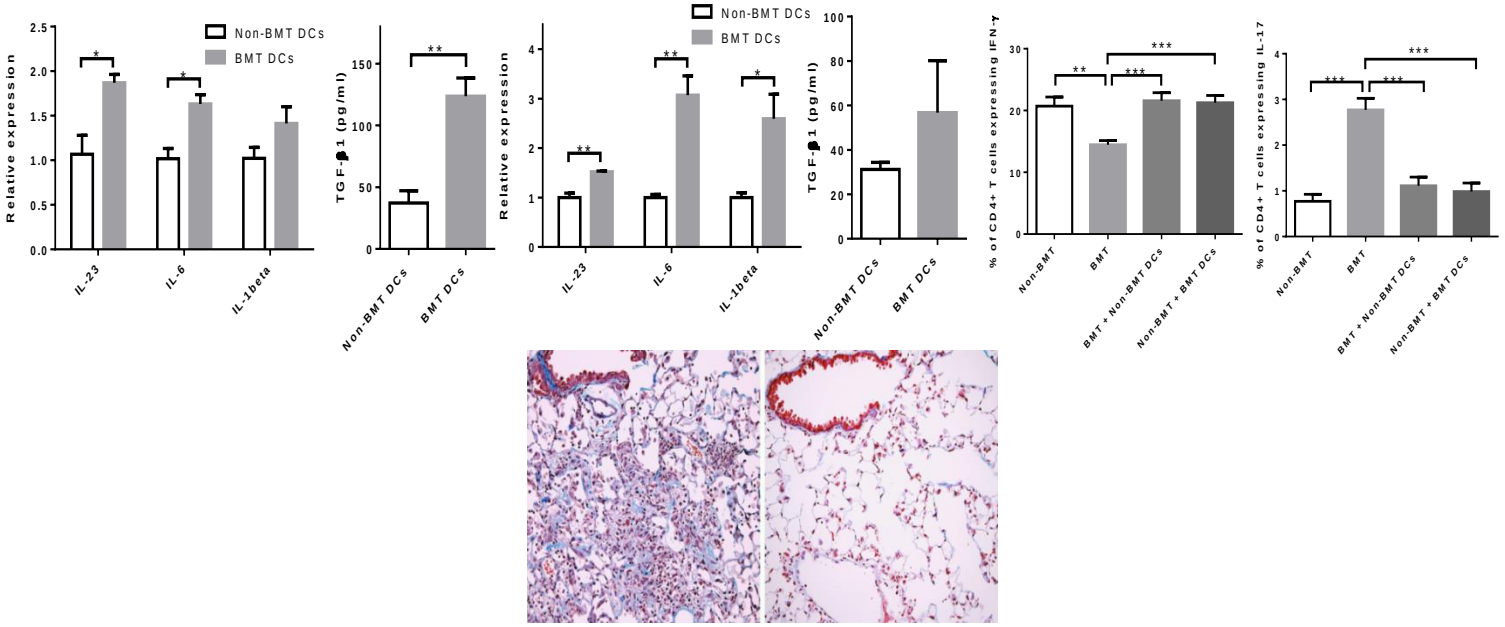


Figure 3

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Notch Mediated Regulatory T Cells Development is Maintained by an Epigenetic Mechanism

Hung-An Ting[1], Denise de Almeida Nagata[1], Matthew Schaller[1], Steven Kunkel[1], Ivan Maillard[2], Nicholas Lukacs[1]

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Notch signaling facilitates inducible Foxp3+ regulatory T cell (iTreg) differentiation. Epigenetic alterations, which include histone modifications, are critical in cell fate and differentiation decisions. Recent genome-wide studies demonstrated that iTreg have increased trimethylation on histone H3 at lysine 4 (H3K4me3) around the *Foxp3* loci, but the role of Notch signaling and its underlying epigenetic mechanisms remain unknown. Here we report that Delta-like ligand 4 (Dll4) increased H3K4me3 at the *Foxp3* promoter and conserved noncoding sequences (CNS) during iTreg differentiation. At the same time, Dll4/Notch signaling amplified SET and MYND domain containing protein 3 (Smyd3), a H3K4 di- and tri-methylase, expression *in vitro* during iTreg cell differentiation via RBPJk-mediated transcriptional activation. Dll4-treated iTreg cells have higher expression of CD25 (IL-2Ra) and an increased Foxp3+ population after IL-2 maintenance. The Foxp3 maintenance and H3K4me3 modification increases induced by Dll4 were both dependent on Smyd3. These results suggest that Notch ligand Dll4 enhance and maintain Foxp3+ Tregs through Smyd3-mediated epigenetic modification.

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Foxp3+ Regulatory T Cells Enhance Lung Epithelial Repair Following Acute Lung Injury

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Acute Respiratory Distress Syndrome (ARDS) causes significant morbidity and mortality each year and while the initial events in the pathogenesis of ARDS are well-defined there is a paucity of information regarding the mechanisms necessary for ARDS resolution. Repair of the alveolar epithelium after acute lung injury (ALI) is an essential component for resolution. $Foxp3^+$ Regulatory T cells (T_{regs}) have been demonstrated to be an important determinate of resolution in an experimental model of lung injury, and found to be present in patients with ARDS. Recently published data demonstrate that T_{regs} enhance alveolar epithelial proliferation after injury, and that T_{regs} directly promote type II alveolar epithelial cell (AT2) proliferation. Based on this data, we began to test the hypothesis that T_{regs} in the lung post injury produce growth factors which directly promote epithelial recovery. In the lung, growth factors such as fibroblast growth factor 7 (FGF-7), also known as keratinocyte growth factor (KGF), play an important role in lung repair. Immunoblot data confirm increased expression of KGF after intratracheal (IT) LPS-induced ALI; furthermore, preliminary data demonstrate a clear trend for adoptive transfer of T_{regs} into lymphocyte deficient *Rag-1*$^{-/-}$ mice to complement KGF levels back to WT concentrations after ALI. Moreover, isolated T_{regs} from *Foxp3*^{EGFP} mice post IT LPS-induced ALI exhibit a high level of transcriptional expression of *KGF* compared to $CD4^+$ lymphocyte controls.

Alveolar epithelial repair is a dynamic process, and additional study is needed to clarify the role of the specific cell types involved. Given the increase of T_{regs} in the lung after injury along with their expression of *KGF* during time points of resolution likely contribute to their epithelial reparative processes. Further work is ongoing to evaluate the role T_{reg}-expressed KGF plays in lung injury repair. These results will enhance understanding of lung resolution along with uncovering potential new targets to accelerate the recovery from ARDS.

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Rebalancing Tregs by Directly Targeting Epigenetic and Mitochondrial Processes During Sepsis

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Sirtuins are critical regulators of immunometabolic homeostasis. The loss of homeostasis occurs during the acute systemic inflammatory response to infection and can lead to immunometabolic dysfunction, organ failure, and death from sepsis. Sepsis accounts for 60-80% of deaths worldwide and currently has no mechanism based therapy to remedy this burden. We reported that SIRT1 remodels chromatin during sepsis to shift the pro-inflammatory response to a sustained hypo-inflammatory state, which maintains the imbalanced immune response. However, with treatment of the SIRT1 specific inhibitor EX-527, this imbalance is reversed, and in septic mice, survival is markedly improved. Recent studies suggest a role for SIRT1 in T regulatory cells (Tregs) in the adaptive immune response. To test the unifying hypothesis that SIRT1 activation also shifts the adaptive immune response from a T effector state to a T repressor state, we measured splenic CD4+ T effector cells (Teffs), CD8+ Teffs, and CD4+Foxp3+CD25+ Tregs in a sub-lethal cecal ligation and puncture mouse model 6 hours after administration of 10mg/kg EX-527. Here we demonstrate that Tregs are increased during murine post-acute sepsis. Concurrently, CD4+ Teffs are decreased. Treatment of septic mice during the hypo-inflammatory state with EX-527 significantly decreases splenic Tregs but does not significantly increase CD4+ Teffs. During this time, there are reciprocal changes in anti-inflammatory and proinflammatory cytokine production and circulation (e.g., IL-17 and IL-10). Additionally, we tested an agent distal to SIRT1 that directly acts on mitochondria to balance mitochondrial fueling. Our findings are similar to that of SIRT1 inhibition. We conclude that rebalancing the immunometabolic homeostasis

through either nuclear or mitochondrial processes may improve sepsis survival and that this molecular targeting bioenergy-based strategy might provide a new way to treat sepsis during its immunosuppressive phase.

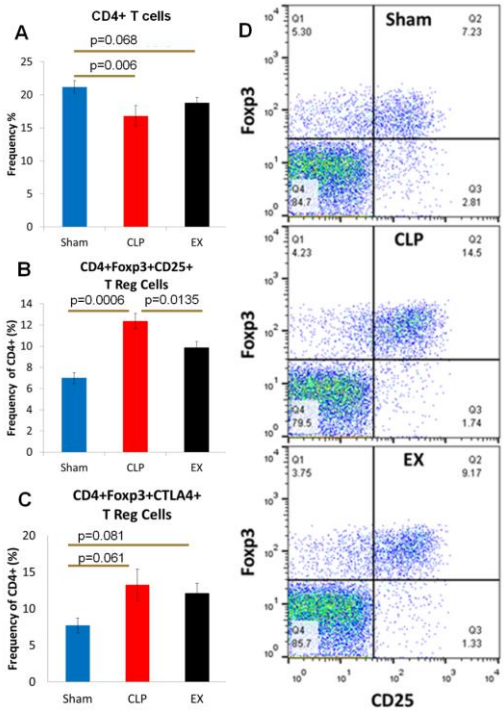

Figure 1 Blocking SIRT1 by EX reverses the increase in Tregs during sepsis.
Sham, CLP n=8; EX n=11 A) The percentage of splenic CD4+ T cells decreases during sepsis. The CD8+ T cells are not affected. B) In contrast, CD4+Foxp3+T regs increase during sepsis. Foxp3 is a key transcription factor controlling Treg development and function. C) The T regs also have an increase in CTLA4, a marker for suppressive potential (Sham, CLP n=3; EX n=4)

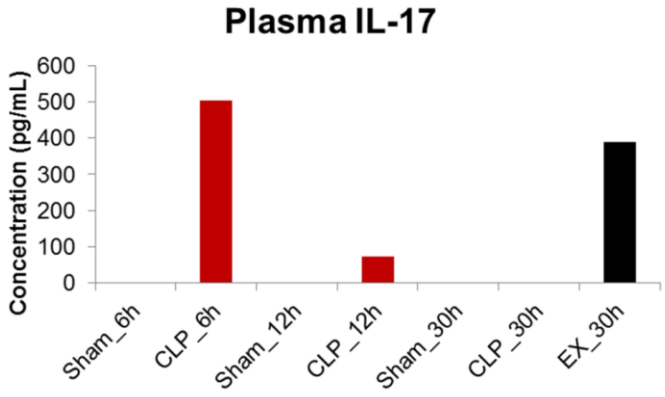

Figure 2 Blocking SIRT1 by EX reverses repression of IL-17 in plasma.
Pro-inflammatory cytokine IL-17 is elevated in plasma during early sepsis. As sepsis progresses, circulating IL-17 levels decrease. EX treatment restores IL-17 expression.

Gimap5 is Essential for GSK3β Inhibition During T Cell Activation Controlling the Transcriptional Program Required for Proliferation and Th17/Treg Differentiation

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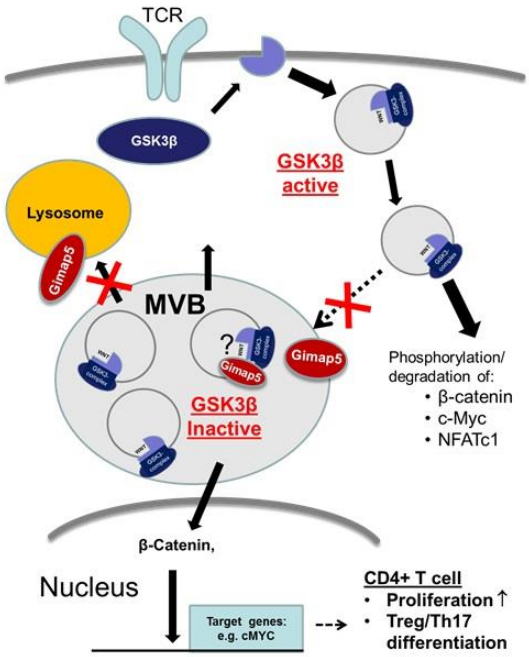
The GTPase of immunity-associated protein 5 (Gimap5) has been associated with lymphocyte survival and autoimmunity in rats, while polymorphisms in human GIMAP5 are associated with the development of asthma, allergic sensitization, type I diabetes and systemic lupus erythematosus. Previous studies reveal that Gimap5-deficient mice exhibit impaired T cell survival and develop severe early onset colitis that is $CD4^+$ T cell dependent and associated with a concomitant loss of Treg development and increased Th17 differentiation. Despite the important role of Gimap5 in lymphocyte survival and peripheral tolerance, the underlying mechanism(s) have remained unclear. We now provide critical new insight into the functional role of Gimap5 in T cells. Specifically, we report that Gimap5 is essential for the inhibition of glycogen Synthase Kinase-3 (GSK3) —a regulatory serine/threonine protein kinase whose inhibition is instrumental for the regulation of transcription factors such as c-Myc and NFATc1 required for T cell proliferation/differentiation. Therapeutic and genetic targeting of GSK3β completely reverses the pathology observed in Gimap5-deficient mice while normal lymphocyte survival is retained. Further studies suggest Gimap5 to control inhibition of GSK3 through a mechanism involving sequestration of GSK3 in multivesicular bodies and lysosomes. Our data provide important new insight into the regulatory pathways controlling the transcriptional program required for T cell proliferation/differentiation and may uncover novel strategies to therapeutically target lymphocytes in the context of a variety of (auto-) immune related diseases.



Gimap5; a critical regulator of GSK3 activity. *Upon TCR signaling, GSK3 is recruited to the membrane and internalized into endosomal vesicles. Subsequent translocation of GSK3β to Multi-Vesicular Bodies (MVBs) or lysosomes is required for inactivation of GSK3β. The sequestration of GSK3β in MVBs/lysosomes is impaired in T cells from Gimap5$^{sph/sph}$ mutant mice causing a failure to regulate transcription factors required for T cell proliferation and T cell differentiation and results in an increased Th17 and loss of Treg cells ultimately causing severe early onset colitis.*

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Integrative Studies on 3,3'-Diindolylmethane Triggered Regulation of Th17 and Treg Cells in Ameliorating Multiple Sclerosis Through Microbiome-Host Interplay

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Multiple sclerosis (MS) is considered the prototypic inflammatory disease of the central nervous system (CNS). This chronic neurodegenerative autoimmune disease affects over 400,000 people in the US and 200,000 worldwide. Current therapies for MS have harsh and prolonged side effects. Hence, there is need for more effective treatment modalities. The use of plant-derived indole, 3,3'-diindolylmethane (DIM) for MS has been explored but the precise mechanism of action has not been completely elucidated. We used experimental autoimmune encephalomyelitis (EAE), a murine model of MS, to explore the anti-inflammatory role of DIM. Epidemiological and biological studies have revealed the susceptibility of MS disease is dictated by an intricate interplay between genes and environmental factors and has turned the spotlight on gut commensal microbiota as a potential environmental risk factor for the disease. In this current study, we demonstrate that DIM mediates anti-inflammatory properties by promoting regulatory T cell (Tregs) differentiation while inhibiting Th17 cells. In addition, we evaluated the possible role of microbiome using 16S rRNA-based microbial community sequencing using fecal samples collected during the entire duration of the study. Our results demonstrated that treatment of EAE mice with DIM significantly reduced clinical symptoms and cellular infiltration with marked improvement of CNS tissue integrity and reduced demyelination which was also evident in histopathogical studies. In addition, DIM treatment of EAE mice led to a reduction in the percentage and absolute number of T cells particularly the CD4+ T cells infiltrating the CNS (spinal cord and brain). In addition, DIM promoted the generation of Tregs, while down-regulating the induction of Th17 cells both *in vivo* and *in vitro*. Further, DIM also proved highly effective in curtailing the overall severity of the disease which correlated with the alpha and beta diversity of the microbiota between naïve, diseased and treated groups. Our studies demonstrate that DIM treatment ameliorates EAE *via* the reciprocal induction of Th17 and Tregs thereby suppressing the aberrant autoimmune response in association with alterations in gut microflora.

(Supported by NIH grants P01AT003961, P20GM103641, R01AT006888, R01ES019313, R01MH094755, Veterans Affairs Merit Award BX001357) and University Grants Commission (UGC), Govt. of India (F.5-97/2014-IC-FD, DN: 6725).

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Notch Signaling Regulates T-cell Cytokine Expression in Response to MHV-68 Infection Following Bone Marrow Transplantation

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The Notch signaling pathway has been identified as an important mediator of T-cell cytokine responses in a variety of immunological maladies such as graft versus host disease, rheumatoid arthritis, and systemic lupus erythematosus. Using a genetically altered mouse model wherein CD4+ and CD8+ T-cells were ablated for all Notch signaling through expression of a dominant negative version of the Notch transcriptional regulator Mastermind Like Ligand (DNMAML) we have further identified Notch as an important mediator of cytokines during Murine Gammaherpesvirus 68 (MHV-68) infection following both syngeneic and allogeneic bone marrow transplantation (BMT). As with the human gammaherpes viruses, Epstein Barr virus (EBV) and Kaposis' sarcoma-associated herpesvirus (KSHV), MHV-68 infections in immunocompetent mice are self-limiting. Mice develop a mild pneumonitis when infected intra-nasally that is cleared by day 10 post infection after which permanent viral latency is maintained in the spleen and lymphnodes. Wild type syngeneic bone marrow chimeric mice infected with MHV-68, however, develop severe pneumonitis and pulmonary fibrosis by day 21 post infection driven in part by a switch from TH1 mediated immunity to TH17 mediated immunity. Expression of the Notch ligands DLL1, DLL4, Jagged1, and Jagged2 were markedly decreased in the lungs following BMT and MHV-68 infection. Further, DNMAML bone marrow chimeric mice infected for 21 days with MHV-68 developed increased fibrosis in comparison to wild type chimeras, and displayed elevated levels of IL-17 and IL-6 in the lungs as well as an expansion of IL17 producing CD4+ T-cells and a decrease in IFNγ producing T-cells. Similar to what was seen in Notch deficient animals, CD4+ T-cells isolated from the lungs and spleens of infected WT bone marrow transplanted mice displayed defects in Notch signaling resulting in decreased expression of the Notch target genes HES1, HES5, and HEY1 indicating that immune reconstitution following bone marrow transplantation alters T-cell associated Notch signaling. These alterations in Notch signaling also correlated with increased expression of IL-17 from CD4+ T-cells indicating that down-regulation of T-cell Notch signaling following viral infection may shift the immunologic balance from a protective TH1 response towards a pathogenic TH17 response.

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The Role of CCL2 in the Breast Cancer Tumor Microenvironment and Metastasis

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Background: Although many effective breast cancer treatments exist, metastasis remains a major problem. Increasing evidence suggests that leukocyte-associated chemokines may play an important role in tumor growth and metastasis. For instance, recent data shows that reduction of CCL2 diminishes the recruitment of inflammatory monocytes to the tumor microenvironment (TME) and inhibits metastatic seeding to lung and bone marrow. Therefore, our goal is to examine the impact of CCL2 on the TME and pre-metastatic niche (i.e., lung).

Methods: We are investigating the ability of CCL2 to entrain neutrophils. This examination includes *in vitro* assays using co-cultures of neutrophils isolated from FVB and BALB/c mice and tumors cells. These experiments will determine whether CCL2 enhance the capacity of neutrophils to kill tumor cells in cell cultures. We will also determine how delivery of this chemokine affects breast tumor growth and metastasis by treating mice possessing highly metastatic and poorly metastatic tumors with CCL2, a CCL2 receptor (CCR2) antagonist, or vehicle. Treatment will be delivered intranasally after tumor implantation and on days 7, 14 or 21, tumors, lungs, and regional lymph nodes will be harvested. Tumor size, number of metastases, and leukocyte infiltrate in both the tumor and the metastatic niche will be analyzed by FACS.

Results: Our preliminary studies demonstrate that naïve neutrophils isolated from FVB mice are capable of killing tumor cells *in vitro*. Our data

revealed that in a highly metastatic model, CCL2 increased tumor cell viability. However the addition of neutrophils overcame this effect. We have also found that CCL2 intranasal treatment caused only modest increases in CCL2 expression in BAL fluid and serum. Intranasal CCL2 did increase T and B cells in BAL and lung samples.

Conclusions & Future Directions: Our findings demonstrate that the addition of CCL2 *in vitro* enhances the activity of tumor entrained neutrophils and their ability to kill tumor cells. Ongoing studies are examining the effects of *in vivo* and *ex-vivo* delivery of CCL2 or a CCR2 antagonist on primary tumor growth and metastasis. We will also investigate how administration of CCL2 or a receptor antagonist will affect the presence or absence of leukocytes in the TME. These findings will determine whether the addition of CCL2 during treatment of the primary tumor will provide a mechanism for reducing the potential of future breast cancer metastases.

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Protein Kinase C delta (PKCδ) is Required for Human Neutrophil Migration during Acute Inflammation

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Neutrophil dysfunction plays an important role in sepsis-induced tissue injury through the release of proteases and oxygen radicals that damage host tissue. A key step in neutrophil-mediated tissue damage is the migration of activated neutrophils across the vascular endothelium. In the absence of specific pharmacologic therapies for this damage, we have identified Protein Kinase C delta (PKCδ) as a critical regulator of the inflammatory response. Previous studies indicated a role for PKCδ in regulating neutrophil migration but did not address specific mechanisms. We hypothesize that PKCδ is a critical regulator of neutrophils adhesion and migration on activated endothelium. To test this hypothesis, we investigated PKCδ inhibition using human umbilical vein endothelial cells (HUVECs) and human neutrophils during the inflammatory response under physiological flow conditions using our novel *in vivo* validated bioinspired microfluidic assay (bMFA). This assay has a microvascular network, reproduced from *in vivo* images, where the shear rate varies at different locations (15-500 1/sec). The vascular channels of the network are seeded with human endothelial cells while the tissue chamber is filled with a buffer or chemoattractant; the vascular channels and tissue chamber are connected by 3 μm wide gaps.

The assay were patterned on Polydimethylsiloxane (PDMS) using soft lithography. Microvascular networks were coated with fibronectin and HUVECs were cultured under shear flow. Confluent endothelial cells were pretreated with 10 U/mL of TNF for 4 h in the absence or presence of a PKCδ-TAT peptide inhibitor (5μM) and the tissue compartment was filled with either fMLP (β_2-integrin dependent) or IL-8 (β_2-integrin independent). Under physiologically relevant shear flow, the effect of PKCδ inhibition on adherence and migration were determined over 60 minutes. Adhesion and migration of neutrophils were quantified in the vascular channels of bMFA and near bifurcations which we have shown to be the focal point of adhesion *in vivo* and *in vitro*. Significant differences were determined using one or two-way ANOVA.

In agreement with our previous findings, our results indicate that adhesion of neutrophils to activated endothelium decreased with increasing shear rates and with the distance to the nearest bifurcation. PKCδ inhibition significantly reduced neutrophil adhesion in response to fMLP or IL-8 only in channels with the low shear rate flow and at bifurcations by 45±5% (p2-integrin dependent (fMLP), as compared to β_2-integrin independent (IL-8), neutrophil migration across endothelial cells.

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Phenotypic and Functional Characteristics of an HLA-DR+ Subset of Neutrophils in Brazilians with Cutaneous Leishmaniasis

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The vector-borne protozoan *Leishmania braziliensis* causes the chronic ulcerative skin disease cutaneous leishmaniasis (CL) in individuals living in endemic regions. In murine models, neutrophils (PMNs) are recruited to the site of infection minutes after parasite inoculation, but their role during chronic infection, and the role of PMNs in human disease, remain undefined. We hypothesized that PMNs from patients with active CL would exhibit different functional properties compared to healthy subjects. Despite the fact that CL is a localized disease, a subset of CL patients had circulating neutrophils expressing HLA-DR, a molecule thought to be restricted to professional antigen presenting cells. HLA-DR+ PMNs also expressed the co-stimulatory molecules CD80, CD86 and CD40. Recently described low-density PMNs contain a high percentage of HLA-DR+ PMNs. Sorted HLA-DR+ PMNs morphologically resembled conventional PMNs, and they were capable of phagocytosis and reactive oxidant generation. Nonetheless, PMNs from subjects with high proportions of HLA-DR+ PMNs promoted significant *in vitro* proliferation of T cells. Compared to conventional HLA-DR- PMNs, HLA-DR+ PMNs showed increased activation, degranulation, oxidant generation and phagocytosis of parasites and zymosan particles. Incubation of whole blood with inflammatory cytokines resulted in increased HLA-DR+ PMNs, suggesting a connection between neutrophil "priming" and upregulation of HLA-DR. These data suggest that CL causes expansion of a subset of HLA-DR+ PMNs that are primed for activation.

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How Neutrophils Avoid Traffic Jams at Sites of Inflammation

Daniel Irimia[1]
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That neutrophils move to inflammatory sites, die there, and are removed by monocytes/macrophages, is a common assumption that assigns neutrophils a passive role in the control of inflammation. How adequate numbers of neutrophils are recruited to inflammation sites is often considered the task of other cells of the immune system. Both insufficient and excessive neutrophil recruitment can be detrimental, favoring the spread of infection or triggering severe tissue damage, respectively. However, the fundamental rules that regulate the trafficking of neutrophils at sites of inflammation/infection are complex and consequently difficult to study using traditional *in vivo* models. To elucidate the neutrophil trafficking rules, we designed devices in which human neutrophils emerge directly from a droplet-size samples of whole blood and migrate towards chambers with chemoattractants and microbe-like particles. Inside these devices, human neutrophils could be monitored in detail, under precise control of the mechanical, biochemical, and microbe interactions conditions. We found that the number of neutrophils recruited by chemotaxis and departing by retrotaxis increases and stabilizes to dynamic equilibrium in the presence of chemoattractants alone. The migration of individual neutrophils ceases immediately after phagocytosis, altering the balance between chemotaxis and retrotaxis and increasing the number of neutrophils accumulating to the site. This number is proportional to the number of microbe-like particles in the chambers. Overall, autonomous neutrophil traffic regulation assures that a continuous supply of fresh neutrophils is available to infection sites, that the number of neutrophils accumulating is appropriate to the number of microbes, and that overpopulation is avoided during sterile inflammation.

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A Critical Concentration of Neutrophils and of CD8+T-cells Is Required to Reduce the Concentration of Bacteria, Virus Infected Cells, and Tumor Cells in vitro and in vivo.

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[1]*Columbia University, New York, NY*

Clinical and basic scientists have postulated for >75 years that neutrophil bactericidal activity is determined by the ratio of neutrophil to bacterial concentrations. In contrast, the studies reported here indicate the rate at which neutrophils kill bacteria in stirred suspensions and in fibrin gels in vitro, and in rabbit dermis in vivo is determined by the neutrophil concentration ([neutrophil]) and not by the neutrophil to bacterial ratio. In stirred suspensions, $>4 \times 10^5$ neutrophils/ml is required to reduce [bacterial] even when the neutrophil to bacterial ratio is 100:1. 4×10^5 neutrophils/ml is

very close to the concentration of neutrophils (5 neutrophils/ml) known to predispose humans to bacterial sepsis. We derived an equation ($b_t/b_0 = e^{-kpt + gt}$) (Eq. 1) that predicts the effect of changes in [bacterial] on neutrophil bactericidal activity (b_t/b_0, b_t = [bacterial] at time = t, b_0 = initial [bacterial], k = the second-order rate constant for bacterial killing = 2 x 10^{-8} ml/neutrophil/min, p = [neutrophil], g = the first-order rate constant for bacterial growth = 8 x 10^{-3}/min, and t = time in minutes. Only when p > g x k does [bacterial] decrease. We term p = g x k the Critical Neutrophil Concentration (CNC) (Li et al. P.N.A.S. 99, 8299-8294, 2002). Eq. 1 also describes neutrophil bacterial killing in three-dimensional fibrin matrices and in rabbit dermis. The CNC is 4 x 10^6 neutrophils/ml matrix in the presence of normal human serum and 1.6 x 10^7 neutrophils/ml matrix in C5-deficient serum. Application of this model to published data of others on killing of E. coli in rabbit dermis yielded CNCs of 4 - 8 x 10^6 neutrophils/ml dermis (Li et al., J. Exp.Med. 200, 613-624, 2004). Thus, in stirred suspensions and fibrin matrices in vitro, and in rabbit dermis in vivo, Eq. 1 fits the kinetics of bacterial killing by neutrophils and gives a lower limit (CNC) to the [neutrophil] required to decrease bacterial[.

Further studies (Budhu et al., J. Exp. Med. 207 (1): 223-235, 2010), show that Eq. 1 also enables us to calculate the Critical Concentration of tumor antigen-specific (Ag-Sp) mouse CD8+T-cells (CLC) required to hold constant the concentration of cognate antigen–expressing mouse melanoma cells in three dimensional collagen-fibrin matrices in vitro (CLC = 3.5 x 10^5 tumor antigen-specific CD8+T-cells/ml), and in established mouse melanomas in vivo (3 x 10^6 tumor antigen-specific CD8+T-cells/g tumor. These and other findings indicate that only 2-2.8% of in vitro or in vivo activated Ag-Sp CD8+T-cells are cytolytically active and that they account for all tumoricidal activity of the entire CD8+T-cell population. Ag-Sp CD8+T-cells kill cognate antigen-expressing tumor cells in three dimensional collagen-fibrin matrices with the same efficiency as they kill cognate antigen-expressing tumor cells in mouse spleen in vivo. By comparing the tumoricidal activity of Ag-Sp CD8+T-cells in these matrices with their tumoricidal activity in vivo, we can measure the immunosuppressive activity of the intra-tumoral environment.

Ectosomes from Human Neutrophils Laden with Staphylococcus aureus Induce a Pro-Inflammatory Response in Macrophages

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Diseases caused by *Staphylococcus aureus* (SA) typically manifest as skin infections but can also cause life-threatening diseases, including endocarditis and pneumonia. Initially, neutrophils (PMN) are the predominant cell type involved in the innate host defense against SA. During phagocytosis of SA, PMN release ectosomes, particles 100 to 1000 nanometers in size that bud off from the plasma membrane. We hypothesize that ectosomes arising from PMN during ingestion of SA amplify local inflammation and promote the exuberant tissue damage that typically accompanies staphylococcal infections. We used differential ultracentrifugation to isolate ectosomes from human PMN that had been fed SA for 20 minutes. As assessed by flow cytometry, ectosomes stained for the presence of lipids, as judged by the fluorescent probe PKH67, and the membrane protein CD66b. Immunoblotting of ectosomes detected the azurophilic granule protein myeloperoxidase as well as gp91*phox* and p22*phox*, components of the NADPH oxidase. In contrast, the SA proteins sortase A and protein A were not detected, suggesting that the ectosomes were host-derived and not from SA. Ectosomes stained positive for SYTO13 Green Fluorescent Nucleic Acid Stain, suggesting the presence of nuclear material, and DNA recovered from ectosomes migrated at greater than 12 kb when separated by agarose gel electrophoresis, suggesting it was eukaryotic in origin. To test the hypothesis that the ectosomes derived from PMN stimulated by SA were pro-inflammatory, we measured by ELISA the cytokine response of human monocyte-derived macrophages (MDM) treated with ectosomes or control agonists. Following 20 hours of incubation with ectosomes, MDM released pro-inflammatory cytokines IL1β and IL6 in a dose-dependent manner. Ectosomes also mediated cytotoxicity in MDM, as assessed by measurement of LDH released over time. Future studies will investigate the mechanism of cytotoxicity and how this may further propagate

inflammation. These data support the hypothesis that ectosomes from PMN that phagocytose SA exhibit pro-inflammatory properties and may thus contribute to the exuberant inflammation characteristic of staphylococcal disease.

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Sirtuin 2 Inhibition and Sepsis-induced Immunosuppression In *ob/ob* Mice.

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Introduction: Sepsis, the 11th leading cause of death, is responsible for over 200,000 deaths and $ 16 billion cost annually in the US alone. Sepsis is a dynamic disease with early (hyper-inflammatory) phase that quickly transitions to late (hypo-inflammatory: immunosuppression) phase. Obesity increases morbidity and resource utilization in already expensive care of sepsis patients. We have reported previously that the hypo-inflammatory phase in the lean mice is modulated via SIRT1 induction and SIRT1 inhibition during hypo-inflammatory phase of lean mice improves survival. However, the SIRT1-deficient-*ob/ob* mice exhibit a prolonged hypo-inflammatory phase without a significant induction of SIRT1; moreover SIRT1 inhibition during hypo-inflammatory phase of *ob/ob*-septic mice decreases survival. We observed that *ob/ob* septic mice induce SIRT2 expression instead. In the current project, we studied the role of SIRT2 in hypo-inflammatory response in *ob/ob* mice with sepsis.

Methods: Sepsis was induced using cecal ligation and puncture (CLP) in ob/ob mice. We studied leukocyte adhesion in small intestinal microcirculation as a marker for inflammation at different time-points post-CLP, with and without E. Coli lipopolysaccharide (LPS) restimulation (endotoxin tolerance) and mapped phases of sepsis. In 18-hour post-CLP (immunosuppression phase) group, we administered SIRT2 inhibitor and studied 1) endotoxin tolerance in small intestinal microcirculation 48 hours post-CLP and 2) 7-day survival. Lastly, we studied the effect of SIRT2 inhibition in endotoxin tolerant RAW264.7 cells to evaluate the mechanism of reversal of endotoxin tolerance *in vitro*.

Results: Phases of sepsis in *ob/ob* mice: We observed that the early hyper-responsive phase (endotoxin responsive) was quickly followed by hypo-responsive phase (endotoxin tolerant) within 12 hours post-CLP (**Figure 1**). The *ob/ob* mice remained endotoxin tolerant for the remainder of 7 days. We also observed increased SIRT2 expression in small intestinal tissue of *ob/ob* mice during hypo-inflammatory phase (not shown). SIRT2 inhibitor treatment during hypo-inflammatory phase endotoxin tolerance *in vivo*: As shown in **Figure 2**, while the vehicle treated mice remained endotoxin tolerant, SIRT2 inhibitor treated mice exhibited return of endotoxin responsiveness in small intestinal microcirculation. SIRT2 inhibition improves survival in ob/ob mice with sepsis: As shown in **Figure 3**, we observed that SIRT2 inhibitor treatment during hypo-inflammatory phase significantly improves survival in *ob/ob* mice. We also show that SIRT2 inhibition reverses tolerance in RAW264.7 cells *in vitro* and are currently studying the molecular mechanisms.

Conclusion: SIRT2 inhibition during hypo-inflammatory phase of sepsis in *ob/ob* mice reverses endotoxin tolerance and improves survival. SIRT2 inhibitors can be a potential therapeutic agent to treat sepsis during its hypo-inflammatory phase.

Figure 1

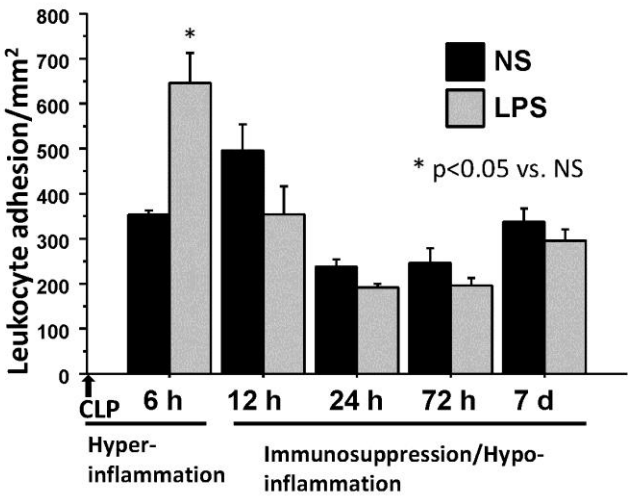


Figure 2

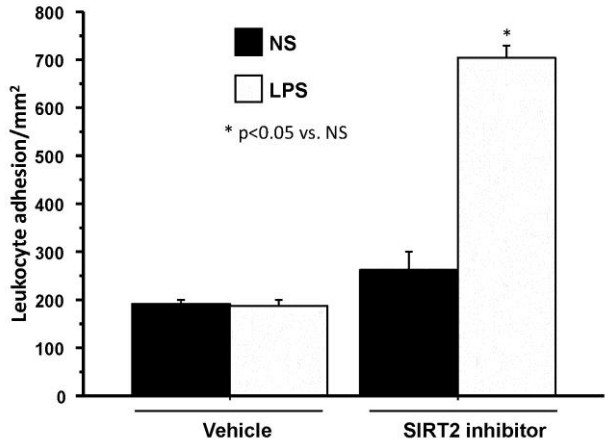
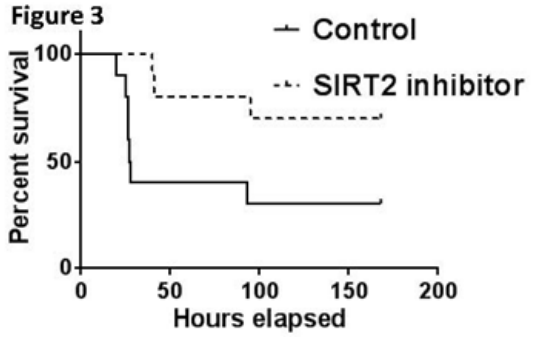


Figure 3



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Role of the Store-operated Calcium Entry Protein, STIM1, in Neutrophil Chemotaxis and Their Infiltration into Psoriasis-inflamed Skin

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Stromal interaction molecule 1 (STIM1) is a Ca2+ sensor protein that initiates store-operated calcium entry (SOCE). STIM1 is known to be involved in the chemoattractant signaling pathway for FPR1 in cell lines, but its role in in vivo functioning of neutrophils is unclear. Plaque-type psoriasis is a chronic inflammatory skin disorder associated with chemoattractants driving neutrophils into the epidermis. We investigated the involvement of STIM1 in neutrophil chemotaxis in vitro, as well as during chronic psoriatic inflammation. To this end, we used conditional knockout (KO) mice lacking STIM1 in cells of myeloid lineage (STIM1fl/fl LysM-cre). We demonstrate that STIM1 is required for chemotaxis because of multiple chemoattractants in mouse neutrophils in vitro. Using an imiquimod-induced psoriasis-like skin model, we show that KO mice had less neutrophil infiltration in the epidermis than controls, whereas neither chemoattractant production in the epidermis nor macrophage migration was decreased. KO mice displayed a more rapid reversal of the outward signs of psoriasis (plaques). Thus, KO of STIM1 impairs neutrophil contribution to psoriatic inflammation. Our data provide new insights to our understanding of how STIM1 orchestrates the cellular behavior underlying chemotaxis and illustrate the important role of SOCE in a disease-related pathologic model.

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Mechanisms of Neutrophil Apoptosis Inhibition by Francisella tularensis

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Francisella tularensis is a facultative intracellular bacterium, and tularemia is a rapidly progressive and potentially fatal pneumonic disease. As neutrophilia exacerbates disease whereas blockade of PMN influx into the lungs favors host survival, neutrophils contribute to tularemia progression rather than effective defense, but what accounts for this is poorly defined. We previously demonstrated that *F. tularensis* inhibits NADPH oxidase activity, escapes the phagosome, and replicates in PMN cytosol. Typically, phagocytosis accelerates PMN death. In marked contrast, we have shown that *F. tularensis* profoundly prolongs neutrophil lifespan, as indicated by inhibition of PS externalization and DNA fragmentation, as well as impaired activation of caspases-8, -9 and -3. PMN apoptosis is regulated at the level of gene expression, and our data demonstrate that *F. tularensis* markedly alters the neutrophil transcriptome, including 365 unique genes linked to apoptosis and cell survival. In particular, we show here that *F. tularensis* acts at multiple points to impair the intrinsic apoptosis pathway. First, BAX mRNA and protein are progressively down-regulated, and this correlates with sustained mitochondrial integrity and diminished release of the pro-apoptotic proteins cytochrome C, Smac, and Hrta2 to the cytosol. Second, full-length XIAP, which directly inhibits caspases-9 and -3, remained high for at

least 48 h post-infection. Third, the XIAP-degrading enzyme calpain appeared curtailed via *F. tularensis*-stimulated upregulation of CAST, which encodes the endogenous calpain inhibitor, calpastatin. As defects in PMN turnover favor progression to secondary necrosis and enhance tissue damage, our data begin to define molecular mechanisms to account for the profound accumulation of PMNs and necrotic tissue damage that occurs during tularemia. R-roscovitine is a cyclin-dependent kinase (CDK) inhibitor and apoptosis-inducing agent that has been used to treat several neutrophilic inflammatory disorders. We confirmed the ability of R-roscovitine to accelerate apoptosis of control PMNs, yet also show that its ability to induce death of *F. tularensis*-infected cells is curtailed, results that correlate with pathogen-induced up-regulation of the R-roscovitine targets and neutrophil pro-survival factors CDK2 and CDK5. Whether other pro-resolving agents may be useful for treatment of tularemia remains to be determined.

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Gelatinase Granules, not Generation of Reactive Oxygen Species, Participates in Human Neutrophil Killing of Environmental Mycobacteria

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The ubiquitous presence of mycobacteria in outdoor and indoor environments results in frequent human exposure, and yet the majority of Mycobacteria are non-pathogenic in the immunocompetent. To examine the ability of human neutrophils to inactivate nonpathogenic mycobacteria, we examined interactions between isolated primary human neutrophils and *Mycobacterium smegmatis*. Neutrophil killing assays indicated that around 60% of bacteria were incapable of forming colonies on agar within the first 30 minutes of infection, a value that increased to around 80% after 2 hours. Surprisingly, visualization of *M. smegmatis* after 2 hours of neutrophil exposure demonstrated that 80% of the bacteria were remaining impermeable to staining by Propidium Iodide. Generation of reactive oxygen species (ROS) was tested by quantifying the reduction of ferricytochrome C or dichlorofluorescein respectively. Despite the stimulation of both extracellular and intracellular ROS production by human serum-opsonized *M. smegmatis*, neutrophils whose NADPH oxidase complex was inhibited with diphenyleneiodium chloride (DPI) had a comparable amount of killing as uninhibited neutrophils. Measuring exocytosis of all neutrophil granules by either flow cytometry or ELISA showed that only the gelatinase granules were released, regardless of opsonization of *M. smegmatis*. Exocytosis of gelatinase granules was also seen in response to purified phosphoinositol capped lipoarabinomannan (PILAM), a mycobacterial wall component previously shown to be potent activator of other immune cells. These results suggest that gelatinase granule mobilization is the major mechanism by which neutrophils contribute to normal immunity to ubiquitous environmental mycobacteria.

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Role of Leukocyte ADAM17 in Sepsis Pathogenesis

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Sepsis is the result of an excessive inflammatory response during infection. It is the most expensive condition treated in US hospitals and its incidence continues to increase. A key feature of sepsis is that it promotes excessive cell dysfunction, which includes impaired neutrophil recruitment to sites of infection. During sepsis in animal models and patients, a number of adhesion molecules and receptors on the surface of circulating neutrophils important for their infiltration into infected tissue are greatly down-regulated in expression, and several of these are substrates of ADAM17. This membrane-associated metalloprotease cleaves cell surface proteins proximal to the cell surface, a process referred to as ectodomain shedding. We examined whether blocking the activity of ADAM17 in leukocytes during sepsis would enhance bacterial clearance. This was performed by using ADAM17 conditional knockout mice and selective ADAM17 inhibitors. We induced poly-microbial sepsis in ADAM17-null mice lacking ADAM17 in all leukocytes and control mice through cecal ligation and puncture. After sepsis induction, the ADAM17-null mice demonstrated

significantly reduced mortality. This corresponded with a marked increase in neutrophil recruitment in the peritoneal cavity, decreased bacteremia, and reduced systemic levels of various proinflammatory factors when compared to control mice. Taken together, our findings reveal that targeting ADAM17 during sepsis increases neutrophil infiltration into sites of infection, bacterial clearance, and survival. Therefore, ADAM17 may be a useful therapeutic target in the treatment and management of sepsis.

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Sonic Hedgehog Signaling in the Regulation of Hematopoietic Stem/Progenitor Cell Activation During the Granulopoietic Response to Escherichia coli Bacteremia

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In response to acute bacterial infection, bone marrow hematopoietic activity shifts toward enhancement of granulocyte development. This granulopoietic response is critical for increasing production of neutrophilic granulocytes to support host defense against invading pathogens. Previous studies from our group have shown that the initial stage of the granulopoietic response to bacterial infection is activation and reprogramming of hematopoietic stem/progenitor cells for granulocyte lineage commitment. At the present time, knowledge about the underlying cell signalling mechanisms remains scant. Hedgehog signaling has been reported to regulate stem/progenitor cell function during embryogenesis and in adulthood. This study evaluated the role of Sonic hedgehog (SHH) signaling in the regulation of hematopoietic precursor cell activation during the granulocytic response to bacteremia. In *in vivo* experiments, *E. coli* (E11775, ATCC, 1 x 10^6 and 5 x 10^7 CFUs/mouse) or saline was given to male Balb/c mice via penile vein injection. In a subset of animals, intravenous BrdU (1 mg/mouse) was administered 24 hours before the termination of each experiment. In *in vitro* experiments,isolated bone marrow cells were cultured without or with *E. coli* lipopolysaccharide (LPS, 20 ng/ml) stimulation in the absence and presence of specific mitogen-activated protein kinase kinase1/2 (MEK1/2) inhibitor PD98059 (25 μM) for 18 h. At both 24 and 48 h of bacteremia, the level of soluble SHH ligand in bone marrow elutes was significantly reduced as determined by ELISA. In contrast, the level of SHH protein in bone marrow cell lysates was markedly increased. These contrast changes suggest that the release of SHH ligand by bone marrow cells is inhibited and/or cell binding of soluble SHH ligand in hematopoietic niche environment is enhanced during systemic bacterial infection. Flow cytometric analysis also showed that SHH protein expression by bone marrow cells was significantly enhanced following bacteremia. This increase in SHH expression was regulated at the transcriptional level. Bone marrow SHH mRNA expression was significantly up-regulated at 12 and 24 h of bacteremia. Marrow lineage positive cells were the major cell type showing the increase in SHH expression. TLR4-ERK1/2 signaling mediated up-regulation of SHH expression by marrow cells. Inhibition of ERK activation with specific MEK1/2 inhibitor PD98059 blocked LPS-induced up-regulation of SHH expression by marrow cells in the in vitro culture system. Gli1 is a key component of the hedgehog pathway positioning downstream of SHH receptor activation. The baseline expression of Gli1 was high in hematopoietic stem/progenitor cells. In association with enhancement of SHH expression in the bone marrow, Gli1 mRNA expression by marrow cells was significantly up-regulated following bacteremia. Concomitantly, the expression of Gli1 protein by hematopoietic precursor cells was significantly increased following *E. coli* infection.This up-regulation of Gli1 expression was correlated with the increase in the proliferative activity of marrow hematopoietic precursor cells. Activation of cyclin D1 signaling appeared to be involved in Gli1-mediated hematopoietic precursor cell proliferation. Our data suggest that SHH signaling is activated in hematopoietic precursor cells during the host defense response to bacteremia, which may play an important role in mediating the activation and reprogramming of hematopoietic stem/progenitor cells.

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A Newly Appreciated Oral Pathogen, Filifactor alocis, Fails to Induce the Respiratory Burst of Human Neutrophils

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Almost 50% of adult Americans suffer from periodontitis which is a bacterially induced inflammation of the tissues that surround and support the tooth. Moreover, periodontal diseases are associated with serious systemic conditions, and studies have found positive correlations between periodontitis and cardiovascular disease, diabetes and rheumatoid arthritis. The accumulation of neutrophils, a critical component of the innate immune system, in the gingival crevice contributes to tissue damage. *Filifactor alocis* is a newly appreciated pathogen present in oral biofilms at periodontal disease sites. Studying the interactions between neutrophils and *F. alocis* will provide valuable information for delineating the role of this bacterium in periodontal disease and enhance our understanding of bacterial strategies to avoid leukocytes. The hypothesis that viable *F. alocis* modulates human neutrophil antimicrobial functions was tested. One of several antimicrobial mechanisms employed by the neutrophil is the production of superoxide within bacteria-containing phagosomes. Previous studies in our lab showed that human neutrophils challenged for 30 min with either non-opsonized or serum opsonized *F. alocis*, at a multiplicity of infection (MOI) of 10, failed to induce a robust respiratory burst response. Interestingly, neutrophils challenged for 30 min with the serum opsonized heat-killed organism, at an MOI of 10, elicited a 2.5 fold higher intracellular respiratory burst response compared to viable *F. alocis*. In addition, increasing the incubation time of neutrophils challenged with serum opsonized viable *F. alocis* to 60 and 120 min similarly failed to induce the respiratory burst response and no significant differences were observed among the three time points. To determine if the failure to induce the respiratory burst was mediated by secreted bacterial products; neutrophils were exposed to the *F. alocis* culture supernatant and the oxidative burst response was determined. The culture supernatant by itself did not induce a respiratory burst response; however it primed the *S. aureus*-stimulated response. The recruitment of specific granules to the phagosome assists in the accumulation of NADPH oxidase complexes because they contain approximately 60% of the membrane-bound subunits; therefore, a possible strategy to avoid/delay killing would be to hinder specific granule recruitment post-phagocytosis. After 30 min of bacteria challenge, only 35% of viable *F. alocis*-containing phagosomes were enriched for lactoferrin positive granules, compared to 66-70% of heat-killed *F. alocis* or *S. aureus*-containing phagosomes. Diverting granule exocytosis to the plasma membrane instead of the bacterial phagosome could prevent bacterial killing and contribute to the tissue damage characteristic of periodontitis. Viable serum opsonized *F. alocis* induced significant secretory vesicles and specific granule exocytosis; whereas the heat-killed bacteria significantly reduced secretory vesicle exocytosis but stimulated specific granule exocytosis to the same extent as the viable bacteria. These data demonstrate previously unexplored aspects of the new oral pathogen *F. alocis* and how this species modulates neutrophil function. By examining the differences between viable and heat-killed *F. alocis* and the effector molecules released by the pathogen during growth, we will begin to characterize *F. alocis'* role in the pathogenesis of periodontitis.

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Nrf2 Modulates Host Defense during S. pneumoniae pneumonia in Mice

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Introduction: Nrf2 is a transcription factor that regulates the transcriptional response to oxidative stress and plays critical protective roles in many organs. *S. pneumoniae* is the most common pathogen in community-acquired pneumonia. These studies tested the role of Nrf2 during *S. pneumoniae* pneumonia and identified Nrf2-dependent genes and pathways in lung tissue

and in lung neutrophils responding to this pathogen.

Methods: Nrf2 null (*Nfe2l2*$^{-/-}$) and wild type (WT) mice were studied at 6 and 24 h following instillation of *S. pneumoniae* or PBS. Neutrophil recruitment, edema, bacterial clearance and mortality were compared. Neutrophils were isolated from the lungs at 24 hrs after infection or PBS, and gene profiling was performed in lung tissue and lung neutrophils.

Results: At 6 h, the number of bacteria in the lungs was less, lung permeability was similar and fewer neutrophils were recruited in Nrf2 null compared to WT mice. mRNA profiling showed that 49 genes were differentially expressed in uninfected (PBS) Nrf2 null compared to WT mouse lungs. These results suggest that bacterial clearance is enhanced in Nrf2 null mice, perhaps due to compensatory defense mechanisms, resulting in less recruitment of neutrophils. At 24 h, the number of lavageable neutrophils was similar, the number of alveolar neutrophils was more variable and tending toward more, the number of neutrophils within the alveolo-capillary septae was greater, and the total number of lung neutrophils was greater in the Nrf2 null compared to WT mice, despite similar clearance of bacteria. At increasing inoculum numbers, mortality increased from 15% to 33% and 100% in Nrf2 null mice, whereas all WT mice survived. Gene profiling identified Nrf2-regulated genes in both lung tissue (35 genes) and lung neutrophils (24 genes) during *S. pneumoniae* pneumonia.

Conclusions: Nrf2 is required for host defense during *S. pneumoniae* pneumonia, as documented by the greatly increased mortality in Nrf2 null mice. Very acute (6 hr) and acute (24 hr) inflammatory responses depend on Nrf2 in different ways. The 49 differentially expressed genes in noninfected lungs may contribute to differences observed at the very early time. Neutrophils express Nrf2-dependent genes in both uninfected (PBS) and infected lungs. The greater numbers of neutrophils in the presence of similar numbers of bacteria may be due to neutrophils responding to more tissue damage at 24 h, resulting from the lack of Nrf2-dependent gene expression.

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Biological Significance of Binding and Activation of the Receptor for Advanced Glycation End-products by the Neutrophil-derived Protein CAP37 in Proliferative Retinopathies

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Cationic Antimicrobial Protein of 37 kDa (CAP37) is constitutively expressed in the azurophilic granules of human neutrophils and is inducible in other host cell types. This secreted multifunctional protein plays a significant role in host defense against infection and is associated with many inflammatory-mediated diseases. Recently, our lab identified CAP37 as a new ligand for the Receptor for Advanced Glycation End-products (RAGE). RAGE is a multiligand receptor of the immunoglobulin superfamily long implicated in inflammation and related diseases. The goal of our research was to determine the biological significance of the CAP37/RAGE interaction in retinal pigment epithelial (RPE) cells and delineate the domains of the CAP37 protein interacting with RAGE.

Purified CAP37 and RAGE were used to measure their interaction in vitro, using ELISA and far dot-blotting approaches. The semi-synthetic glycosaminoglycan ether (SAGE), a RAGE-specific inhibitor was used to reverse in vitro interactions. RPE cells constitute the outer blood-retina barrier of the eye. A human RPE cell line (ARPE-19) was used to test the biological effect of CAP37 on the barrier function of these cells by measuring their trans-epithelial electrical resistance (TEER) upon treatment with CAP37. SAGE was used to demonstrate RAGE-specific mediation of this effect. Synthetic bioactive peptides and analogs derived from the CAP37 protein were tested for interaction with RAGE by far dot-blotting, and SAGE was used to reverse these interactions.

Our results show that purified CAP37 binds to RAGE in vitro and that SAGE can reverse this

interaction. CAP37 decreases TEER in cultured ARPE-19 cells, through specific activation of RAGE as demonstrated by the inhibition of this effect by SAGE. Surprisingly, a highly antibacterial portion of CAP37, spanning amino acids 20 to 44, interacts minimally with RAGE by itself but its interaction is greatly enhanced by the addition of five arginine (5R) residues and/or addition of two minipegs (MP) to the peptide. Other bioactive peptides based on the CAP37 sequence did not interact with RAGE, either by themselves or with 5R and/or MP modifications.

These preliminary studies suggest that the blood-retina barrier breakdown can be mediated by CAP37 binding to RAGE. This event could be central in the pathogenesis of neovascular age-related macular degeneration and diabetic macular edema. More studies are ongoing to develop CAP37 peptide analogs as RAGE-specific antagonists for therapeutic applications in proliferative retinopathies.

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Investigation of mPGES-1 as a Novel Anti-Inflammatory Target in Equine Leukocytes

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Prostaglandin E_2 (PGE_2) is a potent inflammatory mediator in many equine diseases. PGE_2 synthesis at sites of injury aids in recruitment and activation of peripheral blood leukocytes (PBLs) such as neutrophils and monocytes. PBLs produce additional PGE_2 and exacerbate tissue damage and pain. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit synthesis of PGE_2 and other prostanoids by targeting cyclooxygenase (COX) enzymes, but can cause negative side effects due to nonselective prostanoid blockade. Thus, safer more selective anti-inflammatory targets are needed in horses. One promising target is microsomal prostaglandin E synthase-1 (mPGES-1), the terminal enzyme downstream of COX in the inducible PGE_2 synthesis cascade. The objective of this study was to investigate mPGES-1 as an anti-inflammatory target in equine leukocytes using an *in vitro* inflammatory model. Primary equine leukocytes were primed with GM-CSF, followed by LPS stimulation in the presence of an mPGES-1 inhibitor (MF63) or a nonselective or COX-2-selective inhibitor (indomethacin or NS-398, respectively). Leukocyte stimulation resulted in increased mPGES-1 and COX-2 mRNA expression, while constitutive enzymes cytosolic PGES (cPGES) and COX-1 remained unchanged. Interestingly, based on biochemical analysis mPGES-1 protein did not change following stimulation, while COX-2 protein significantly increased. PGE_2 secretion was significantly increased upon stimulation as measured via ELISA, and MF63 selectively inhibited PGE_2 synthesis only; indomethacin and NS-398 were nonselective and decreased PGE_2, TXA_2 and PGI_2. This indicates that mPGES-1 inhibition is a viable PGE_2-selective target in equine leukocytes. This knowledge could lead to safer, novel methods of controlling equine inflammatory diseases.

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The Oral Pathogen Filifactor alocis, Enhances Chemotactic Functions of Human Neutrophils

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Filifactor alocis is a gram-positive anaerobic rod which has emerged as an important periodontal pathogen. Neutrophils are a major component of the periodontal innate host response, and they are recruited in large numbers to the periodontal pocket. Neutrophils have the ability to detect signals from both intermediary and end-target chemoattractants. Interleukin (IL)-8 is an important intermediary chemotactic factor involved in the recruitment of neutrophils to the site of periodontal infection. The hypothesis that *F. alocis* challenge manipulates human neutrophil chemotaxis and cytokine release was tested. Human neutrophils were unstimulated or challenged with *F. alocis* (30 min, multiplicity of infection (MOI) 10) and their chemotactic activity

towards IL-8 was assessed using a transwell system. Transmigrated neutrophils were stained with the HEMA 3 stain set and visualized by light microscopy. Neutrophil chemotaxis induced by IL-8 was significantly enhanced when cells were challenged with *F. alocis* compared to non-infected cells. In addition, neutrophil migration towards buffer was significantly enhanced in *F. alocis*-challenged cells. The enhanced migration of *F. alocis*-challenged cells towards buffer was similar to the migration observed when the chemotactic gradient was eliminated; indicating that *F. alocis* induced a chemokinetic activity in human neutrophils. Similar to live bacteria, heat-killed *F. alocis* induced both chemotactic and chemokinetic activity of human neutrophils. To determine if the enhanced chemotaxis and chemokinesis was due to factors released from the bacteria-challenged neutrophils, supernatant was collected at 1-4-and 20 h post bacteria challenge, placed in the bottom of the transwells and unstimulated cell migration was evaluated. Significant neutrophil chemotaxis was observed only by the 20 h post-bacteria challenge supernatant. Additionally, IL-8 release from unstimulated and *F. alocis*-challenged cells at 1-4-20 h was measured by ELISA. Only a significant amount of IL-8 (average 3 ng/ml) was detected in the supernatant after 20 h post bacteria challenge. Previous studies in our laboratory showed that *F. alocis* (30 min, at MOI 10) induced significant secretory vesicles and specific granules exocytosis. To determine if neutrophil exocytosis participates in *F. alocis*- induced chemotactic and chemokinetic activity, neutrophils were pre-treatment with TAT-SNAP-23, a fusion protein known to block neutrophil exocytosis, followed by bacterial challenge. Blocking neutrophil degranulation significantly reduced the chemotaxis and chemokinesis induced by *F. alocis*. These results indicate that the chemotactic and chemokinetic activity of human neutrophils induced by *F. alocis* is not due to chemotactic factors released by the stimulated cells, but could be attributed to the cell activation and degranulation induced by the organism.

NETs (Neutrophil Extracellular Traps) Induce IL-1β Production by Macrophages in the Presence of Lipopolysaccharide

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Background: NETs are the network structures of extracellular fibers comprising of DNA, histone and granule proteins, which are released from neutrophils in response to bacterial or sterile stimuli. In addition to the well-described antimicrobial capability, NETs are reported to modulate the inflammatory reactions in the host. Importantly, NETs are reported to induce the IL-1β production by macrophages. Thus, in this study, we focused on the components of NETs involved in the IL-1β production by macrophages.

Methods: Mouse bone marrow-derived neutrophils were treated with LPS (lipopolysaccharide) to induce the NETs, and NETs were washed and recovered. Thereafter, mouse J774 macrophage-like cells were treated with NETs in the absence or presence of LPS (10 ng/ml) for 24 h, and the cell supernatants were used for the assay of IL-1β production and LDH (lactate dehydrogenase) release. Moreover, to clarify the components of NETs involved in the IL-1β production, J774 cells were incubated with NETs and LPS in the presence of serine protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride, AEBSF and a1-antitrypsin) and endonucleases (DNase I and micrococcal nuclease, MNase).

Results and conclusion: NETs significantly induced the IL-1β production in the presence but not in the absence of LPS without the LDH release (cell death). Interestingly, the NETs/LPS-induced IL-1β production was inhibited by Ac-YVAD-CHO (a caspase-1 inhibitor) and Ac-IETD-CHO (a caspase-8 inhibitor) as well as z-VAD-FMK (a pan-caspase inhibitor), confirming that caspase-1 and caspase-8 are involved in the processing of IL-1β in macrophages. Importantly, serine protease inhibitors (AEBSF and a1-antitrypsin) and endonucleases (DNase I and MNase) inhibited the NETs/LPS-induced IL-1β production. Together these observations indicate that both NETs and LPS are required for the induction of IL-1β production

by macrophages, and neutrophil granule-derived serine proteases (such as neutrophil elastase) and nuclear DNA are the essential components for triggering the NETs/LPS-induced IL-1β production by macrophages.

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Thrombospondin-1 is a Critical Mediator of Immune Complex Deposition Induced Acute Lung Injury

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Immune complex mediated acute lung injury (IC-ALI) is associated with decrease in pulmonary $GABA_BR$ expression and release of thrombospondin-1 (TSP-1), TNF-a and interleulin-1 receptor accessory protein (IL-1RAcP) in the bronchoalveolar lavage fluid (BALf). Administration of $GABA_BR$ agonist, baclofen (1 mg/kg) to rats after initiation of ALI preserved pulmonary $GABA_BR$ expression and blocked release of TSP-1 and IL-1RAcP in BALf and prevented lung damage. Pre-treatment of animals with $GABA_BR$ antagonist CGP52432 (1 mg/kg) prior to initiating ALI promoted release of TSP-1 and IL-1RAcP in the presence of baclofen. We hypothesized that blockade of TSP-1, a known pro-inflammatory protein released from damaged endothelial cells, would abrogate IG-IC-induced ALI. Animals were treated with CSVTCG (1 mg/Kg) peptide [inhibits TSP-1 interaction with CD36] or with anti-TSP-1 (0.2 mg/Kg) antibody [neutralizes TSP-1 in BALf] prior to initiating ALI and 4 h later animals were sacrificed. BALf and lung homogenates and lung tissue sections were collected. Blockade of TSP-1 activity after initiation of ALI decreased lung damage accessed by vascular leakage and histology. Similar to baclofen effects, blockade of TSP-1 activity inhibited release of TNF-a and IL-1RAcP in BALf after ALI, inhibited pulmonary NFkB activation, inhibited pulmonary lung apoptosis while promoting BAL neutrophil apoptosis. Stimulation of human neutrophils with TNF-a (2 ng/ml; 10 m) resulted in release of IL-1RAcP but not TSP-1 in cell supernatant while treatment of neutrophils with recombinant TSP-1 promoted release of IL-1RAcP in the cell supernatant. Thus, TSP-1 released in BALf after ALI may promote inflammation by stimulating release of pro-inflammatory IL-1RAcP from activated BAL neutrophils contributing to increased vascular permeability and lung damage associated with IG-IC induced ALI.

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Myristoylated Alanine Rich C Kinase Substrate (MARCKS) Is Involved in "Outside-in" β2-Integrin Function in Neutrophils

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$Beta_2$ integrins play an essential role in neutrophil recruitment to sites of tissue infection and inflammation and are a potential therapeutic target for drugs designed to modulate the response and/or function of neutrophils. Previous findings by our laboratory determined that β_2-integrin dependent neutrophil processes including adhesion and migration require the actin binding protein MARCKS (**m**yristoylated **a**lanine **r**ich **c** **k**inase **s**ubstrate), and that treatment of human neutrophils with the MARCKS-inhibitor peptide MANS (**m**yristoyl**a**ted **n**-terminal **s**equence) significantly attenuates these processes. In the current study, we further investigate the role of MARCKS in β_2-integrin signaling and activation in human neutrophils. We hypothesized that MARCKS function would be essential for one or more of the following aspects of β_2-integrin function: 1) signaling, 2) inside-out activation, 3) outside-in activation. We found that MANS peptide treatment of human neutrophils significantly inhibited β_2-integrin dependent-respiratory burst and Mn^{++} stimulated adhesion, but did not affect fMLF-stimulated upregulation of total or high-affinity β_2-integrins (CD18 and CD11b) as detected by flow cytometry. From these preliminary results we conclude that pharmacologic inhibition of MARCKS in human neutrophils significantly attenuates β_2-integrin signaling and outside-in

activation, but has no effect on β_2-integrin inside-out activation. These findings support the argument that MARCKS is a key regulator of β_2-integrin signaling in human neutrophils *in vitro*. Additional studies are needed to determine the potential for MARCKS inhibition as a method for modulating β_2-integrin dependent neutrophil responses that contribute to host injury and/or disease.

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Neutrophil Priming Occurs in Infants Undergoing Cardiopulmonary Bypass and Correlates with Post-Operative Acute Kidney Injury

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Infants with congenital heart disease frequently require cardiopulmonary bypass for surgical repair or palliation, which is known to cause systemic inflammation. A subset of infants who undergo bypass have a prolonged postoperative course with fluid overload and prolonged organ dysfunction including respiratory failure, acute kidney injury, and impaired cardiac output. These morbidities occur secondary to post-CPB inflammation with leukocyte activation as a major contributor to host inflammation. The goal of this study was to determine if neutrophil (PMN) priming occurs in infants with congenital heart disease undergoing cardiopulmonary bypass, and if PMN priming is predictive of post-operative morbidity. A prospective cohort study was performed in a tertiary care Pediatric Intensive Care Unit with post-operative cardiac intensive care patients. 38 patients were recruited, aged 5 days to 10 months, with congenital heart disease requiring cardiopulmonary bypass. Laboratory and clinical data were collected related to post-operative inflammation and morbidity including length of mechanical ventilation, inotrope scores, acute kidney injury and degree of anasarca. Operative data were also collected. Neutrophils were isolated from whole blood at 3 time points surrounding CPB and functionally studied for measures of neutrophil priming or pre-activation. Measures included priming of NADPH oxidase activity in response to fMLF as measured by chemiluminescence, and mobilization of intracellular protein stores to the cell surface as measured by flow cytometry. Priming of NADPH oxidase activity in response to fMLF occurred in 37% of infants 24 hours after undergoing CPB, as defined by an incremental increase in ROS generation pre to post CPB. Pre-operative steroid use is common practice for the neonatal population undergoing operative repair and steroids delayed priming of NADPH oxidase activity with a greater percentage of primers at 48 hours vs. 24 hours post-CPB. 50% of infants studied demonstrated enhanced mobilization of CD11b to the PMN surface post-CPB, another frequently used endpoint of neutrophil priming. Importantly, PMN priming was strongly associated with the development of acute kidney injury. Infants who displayed priming of NADPH oxidase activity in response to fMLF post bypass were 6.9 times more likely to develop AKI independent of gender, pre-operative steroid administration, or length of cardiopulmonary bypass. In conclusion, PMN priming occurs in a subset of infants undergoing CPB. Pre-operative steroids appear to delay neutrophil priming, not prevent it. AKI was significantly more frequent in those with PMN priming, independent of cardiopulmonary bypass time. The data from this pilot study suggest that neutrophil activation state could be utilized to predict inflammatory outcomes in patients undergoing cardiac surgery.

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The Effect of Methylprednisolone on Neutrophil Recruitment and Interferon-gamma Production in the Lungs after S. pneumoniae Pneumonia

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Rationale: Steroids have myriad effects on inflammation that differ by dosage, tissue and cell type. The effect of steroids on neutrophil recruitment to the lung and neutrophil function in the lung has not been clearly elucidated. We have previously shown that 24 hours after instillation of *S. pneumoniae* into the lungs of wild type mice, neutrophils are present and produce interferon-gamma. This interferon-gamma enhances bacterial clearance and modulates the expression of inflammatory mediators that regulate the resolution

or progression of pneumonia. Furthermore production of interferon-gamma by neutrophils requires functional NADPH oxidase. In exacerbations of chronic lung diseases like COPD, steroids are often used in conjunction with antibiotics without a clear understanding of their effect on host defense. In this project, we studied the effect of methylprednisolone on neutrophil recruitment to the lung and neutrophil production of interferon-gamma in both wild type (WT) and *Cybb*$^{-/-}$ mice. *Cybb*$^{-/-}$ mice are deficient in the gp91^{phox} component of NADPH oxidase and have no respiratory burst.

Methods: C57Bl/6 WT or *Cybb*$^{-/-}$ mice received methylprednisolone (4 mg/kg intraperitoneally) or sterile diluent. Immediately following, they were infected with *S. pneumoniae* (2.3 microliter/g body weight of bacterial suspension in PBS) by endotracheal instillation into the left lung or had an equal volume of sterile PBS instilled as a control. Approximately 24 hours later, the lungs were harvested and a single cell suspension of the left lung was generated. These cells were stained for Ly6G, to identify neutrophils, and intracellular interferon-gamma and analyzed by flow cytometry.

Results: Treatment with methylprednisolone resulted in a higher percentage of neutrophils in the lung digest of WT mice with *S. pneumoniae* pneumonia. A greater proportion of neutrophils in methylprednisolone-treated mice expressed interferon-gamma. These differences were small but statistically significant. However, the total number of neutrophils and total number of neutrophils expressing interferon-gamma was not significantly increased compared to mice not given methylprednisolone. Methylprednisolone-treated *Cybb*$^{-/-}$ mice had a significant increase in the number of neutrophils recruited to their lungs. However, *Cybb*$^{-/-}$ neutrophils had a complete defect in interferon-gamma production, and methylprednisolone did not repair this defect.

Conclusions: Our studies suggest that treatment with methylprednisolone prior to infection with *S. pneumoniae* increases the proportion of neutrophils expressing interferon-gamma. This effect could be an unexpected benefit of methylprednisolone. Methylprednisolone did not repair the defect in interferon-gamma induced by the absence of functional NADPH oxidase.

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Modulation of Neutrophil Reactive Oxygen Species Production by Staphylococcus aureus

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Neutrophils are the first line of defense after a pathogen has breached the epithelial barriers and unimpaired neutrophil functions are essential to fend off infections. *Staphylococcus aureus* (*S. aureus*) is a prevalent human pathogen that is able to withstand neutrophil killing, yet the mechanisms used by *S. aureus* to inhibit neutrophil bacterial clearance remain incompletely defined. The production of reactive oxygen species (ROS) is a vital neutrophil antimicrobial mechanism as shown by individuals suffering from chronic granulomatous disease (CGD) that have diminished ROS activity and consequently suffer from recurring bacterial infections. Herein, we show that *S. aureus* uses the SaeR/S two-component gene regulatory system to produce virulence factors that reduce neutrophil ROS production. Using ROS probes, we compare the temporal and overall production of neutrophil ROS production during exposure to the clinically relevant wild-type *S. aureus* LAC (USA300) and its isogenic LACΔ*saeR/S* mutant. Our results show that SaeR/S-regulated factors do not inhibit neutrophil superoxide production. However, the neutrophil oxidative burst is significantly reduced during exposure to wild-type LAC compared to LACΔ*saeR/S*. In addition, neutrophil hydrogen peroxide production is significantly reduced by SaeR/S-regulated factors. Consequently, the reduction in neutrophil hydrogen peroxide production results in decreased production of the highly antimicrobial agent hypochlorous acid/hypochlorite ion. These preliminary findings suggest a new evasion strategy used by *S. aureus* to diminish a vital neutrophil antimicrobial mechanism.

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CFTR Dysfunction in Myeloid Cells Causes Persistent Neutrophilic Inflammation in Cystic Fibrosis

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Persistent neutrophilic inflammation is the key characteristic of cystic fibrosis (CF) disease. Even though great progress has been achieved in understanding the basic CF defect, the molecular mechanism underlying this significant clinical manifestation remains unknown. Here we report a pivotal role of myeloid CFTR in control of neutrophilic inflammation. Myeloid CFTR-inactivated (Mye-Cftr-/-) and wild-type (WT) mice were challenged peritoneally with zymosan particles, creating a non-septic systemic inflammation. A lethal dose resulted in significantly higher mortality in Mye-Cftr-/- mice than in WT mice (37% vs 0%, n=15), indicating an intrinsic immune defect in protection against inflammatory shock in the myeloid CF mice. Further investigation using a sub-lethal dose demonstrated that at Day 2 both types of mice underwent acute inflammation by mobilizing comparable numbers of inflammatory cells (neutrophils and macrophages) to the peritoneal cavity, and had a comparable level of MIP-2, the major mouse neutrophil chemokine. However, Mye-Cftr-/- mice exhibited an impaired resolution of inflammation by sustaining neutrophil predominance at Day 4, while WT mice had transitioned to macrophage-prevalent inflammation. These data are consistent with our published lung inflammation data from the same myeloid CF mice (Ng et al., 2014, PLoS One; 9(9):e106813). Moreover, Mye-Cftr-/- mice had a significantly higher level of MIP-2 in the peritoneal fluid than WT mice at Day 4. *In vitro* investigation revealed that Mye-Cftr-/- neutrophils produced more MIP-2 than WT neutrophils (124 $\pm$ 36 pg/ml vs 79$\pm$ 55 pg/ml; $p<0.05$, n=5). Thus, myeloid CFTR function is indispensable to checking neutrophilic inflammation in normal host defense, and loss of such function in CF defines the long-observed, inordinate neutrophilic inflammation.

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Interferon-lambda (IFN-λ) Induces Signal Transduction and Gene Expression in Human Hepatocytes but not in Lymphocytes or Monocytes

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In this study, we compared the ability of interferon (IFN)-α and IFN-λ to induce signal transduction and gene expression in primary human hepatocytes, lymphocytes and monocytes. IFN-α drug products are widely used to treat chronic HCV infection; however, IFN-α therapy often induces hematologic toxicities as a result of the broad expression of IFN-α/β receptors (IFNAR) on many cell types, including most leukocytes. Recombinant IFN-λ1 is now being clinically tested as a potential alternative to IFN-α for treating chronic HCV infection. Although IFN-λ has been shown to be active on hepatoma cell lines such as HepG2 and Huh7, its ability to induce responses in primary human hepatocytes and/or leukocytes has not been examined. We found that IFN-λ induces activation of the Jak/STAT signaling pathway in murine and human hepatocytes, and the ability of IFN-λ to induce STAT activation correlates with induction of numerous IFN-stimulated genes (ISGs). Although the magnitude of ISG expression induced by IFN-λ in hepatocytes was generally lower than that induced by IFN-α, the repertoire of regulated genes was quite similar. Our findings demonstrate that although IFN-α and IFN-λ signal through distinct receptors, they induce expression of a common set of ISGs in hepatocytes. However, unlike IFN-α, IFN-λ did not induce STAT activation or ISG expression by lymphocytes or monocytes. This important functional difference may provide a clinical advantage for IFN-λ as a treatment for chronic HCV infection because it is less likely to induce the leukopenias that are often associated with the use of IFN-α drug products.

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The Neutrophil Protein CAP37 Disrupts the Amyloid Beta-RAGE Interaction

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Although the etiology of Alzheimer's disease (AD) is unknown, increased innate immune system activity has been established as an underlying factor. The cationic antimicrobial protein of 37 kDa (CAP37) is predominantly expressed in neutrophils where it serves as a host defense molecule and inflammatory mediator. In addition to its antimicrobial activity, CAP37 has strong immunomodulatory effects on monocytes, macrophages, and microglia. Cytoscape software, which reveals gene expression patterns, showed a positive correlation between expression of CAP37 and ligands for the receptor for advanced glycation end products (RAGE). RAGE is an inflammatory receptor that is increased in AD. Amyloid beta (Aβ) is one of the ligands of RAGE and is also an established hallmark of AD pathology. Stimulation of RAGE by Aβ has been shown to activate microglial cells leading to the increased production of pro-inflammatory cytokines and oxidative stress. Recent findings from our laboratory show that CAP37 is expressed in brains of AD patients. Our hypothesis is that CAP37 expressed in the brain parenchyma mediates signaling through RAGE in AD leading to neurotoxicity or neuroprotection.

Enzyme-linked immunosorbent assays (ELISAs) were performed to assess binding of CAP37 to RAGE and to Aβ. In addition, ELISAs were used to determine if CAP37 binding to Aβ competitively inhibited the binding of Aβ to RAGE. Since recent studies have revealed that CAP37 may have enzymatic activity, we investigated whether any observed binding inhibition was also due to CAP37 induced proteolytic degradation of Aβ, by performing matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF).

ELISAs revealed that CAP37 bound to RAGE and Aβ with K_d values in the nanomolar range. GM-0111, a sulfated glycosaminoglycan that blocks RAGE activation by its various ligands, significantly reduced CAP37 binding to RAGE, indicating specificity of the CAP37-RAGE interaction. CAP37 also significantly reduced the binding of Aβ to RAGE in a dose-dependent manner. MALDI-TOF results suggest that CAP37 may cleave the Aβ peptide. These findings demonstrate that CAP37 serves as a newly identified ligand for RAGE, capable of inhibiting the activation of RAGE by Aβ through its strong binding affinity to Aβ and/or RAGE. The proteolytic activity of CAP37 on Aβ may also be partially responsible for the disruption of the Aβ-RAGE interaction. We conclude that CAP37 may inhibit neurotoxicity by inhibiting Aβ signaling through RAGE. Altogether, these results could have important implications for designing AD therapeutics targeting innate immune system activity.

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Acute Simian Varicella Infection Causes Robust and Sustained Changes in Gene Expression in the Sensory Ganglia

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Varicella zoster virus (VZV) is a neurotropic alpha herpesvirus that causes varicella (chickenpox) during primary infection. VZV establishes latency in the sensory ganglia and can reactivate later in life to cause herpes zoster (shingles). The relationship between the virus and host during latency in the sensory ganglia is not well understood due to the limited access to specimens from humans during acute infection. However, intrabronchial inoculation of rhesus macaques with simian varicella virus (SVV), a homolog of VZV, recapitulates the hallmarks of VZV infection in humans. In this study, we used this animal model to characterize the host-pathogen interactions in the ganglia by

measuring both viral and host transcriptomes at days 3, 7, 10, 14 and 100-post infection. Here, we show both SVV viral DNA and transcripts are detected in the sensory ganglia as early as day 3-post infection, before establishing latency at day 7-post infection. Illumina RNA-Seq analysis shows that cessation of viral replication coincides with the development of a robust antiviral innate immune response in the ganglia. Finally, a significant number of genes that play a critical role in nervous system development and function remained down regulated into latency. These data demonstrate that SVV infection of the sensory ganglia results in profound and sustained changes in neuronal gene expression. These studies provide the first insights into the viral replication kinetics and host response in the sensory ganglia during acute varicella and enhance our understanding of the neurological complications caused by VZV.

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THC Treatment during Activation in Human Peripheral Blood Mononuclear Cells Suppresses HIV-1 Infection of CD4 T Lymphocytes

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Background. Δ^9-Tetrahydrocanabidiol (THC) mediates both the psychotropic and immunomodulatory effects of marijuana. Overall impact by marijuana as pro- or anti-inflammatory is poorly understood in humans, and remains a significant question particularly in the context of HIV-1 infection, which produces chronic innate activation and neurological complications despite suppressive therapy.

Methods. To investigate the consequences of THC exposure on immune modulation and HIV-1 infection of CD4 T lymphocytes, peripheral blood mononuclear cells (PBMC) were treated *ex vivo* with THC or ethanol vehicle control during PHA/IL-2 stimulation or at the time of exposure to replication competent, CCR5 using, HIV-1_{AD}. Supernatant levels of cytokines and HIV p24 antigen were measured by ELISA. Activation states and HIV receptor expression by T cells were monitored by flow cytometry. qPCR was utilized to measure mRNA expression of host cell viral restriction factors. Lymphocyte or monocyte populations were enriched by magnetic depletion.

Results. HIV-1 replication was significantly reduced by treatment during PHA/IL-2 stimulation prior to infection, but unaltered by THC treatment of PBMC at the time of infection. Reduced viral replication by THC treatment was independent of altered cell surface expression of CD4, CCR5, or CXCR4 intracellular viral restriction factors, distribution of T cell subsets (CD4+CD25+CD45RO+), or release of IL-4 (indicative of TH2) or IL-10 (indicative of Treg), but associated with elevated IFNγ, indicative of a TH1 response. In contrast to PBMC, THC treatment of enriched CD4 T lymphocytes during PHA/IL-2 stimulation reduced supernatant IFNγ levels by 60-90%, while THC treatment of monocytes increased supernatant IL-6 and TNFα by approximately 20%. To examine the contradictory effects of THC on cytokine expression and the potential influence of cell:cell interactions, enriched populations of isolated CD4 T cells or monocytes were treated with THC and PHA/IL-2, then combined at increasing ratios on the day of infection. THC treatment of enriched CD4 T lymphocytes had no impact on viral replication, while co-cultures of THC treated CD4 T lymphocytes and monocytes displayed reduced HIV-1 infection.

Conclusions. The effects by THC on inflammation are cell type and cytokine specific. HIV-1 infection is clearly reduced in the presence of THC-treated monocytes, underscoring an important role for non-HIV-1 target cells in viral infection of CD4 T cells. As legalization of marijuana for both recreational and medicinal purposes increases neurologic, inflammatory and virologic effects, impact of marijuana use by individuals living with HIV-1 requires investigation.

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IL-10 Mediated Neuroprotection Plays a Significant Role in the Immune Response to CNS Catheter Infection with S. epidermidis in Young Hosts

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Infants are at higher risk of *S. epidermidis* ventriculoperitoneal shunt infection. We hypothesized that this increased infection risk is due to alterations in the immature response that allow for infection. To evaluate this hypothesis, we adapted our previously published CNS catheter infection model to generate infection with *S. epidermidis* in 14 day old C57BL/6 mouse pups. Interestingly, in the young mice, the infection was more likely to spread from the catheter to the brain parenchyma than in older mice. Additionally, the younger pups had significantly lower levels of pro-inflammatory mediators CXCL1, CXCL2 and IL6, all of which play a role in recruiting immune cells to the site of infection. As expected, we found a decrease in the number of macrophages and neutrophils recruited to the brain tissue surrounding the catheter in pups as compared to adult mice by flow cytometry. In comparing wild type (WT) with IL-10 knockout (KO) pups, we found that IL-10 KO pups had significantly greater inflammation, as evidenced by increased pro-inflammatory chemokines such as IL-6 and CCL2 as well as increased influx of innate immune cells, including monocytes and macrophages. However, this increased pro-inflammatory response did not result in a decrease in bacterial burdens or improved clinical outcomes. The IL-10 KO pups experienced seizures (25%), not previously observed in our *S. epidermidis* infection model in either WT or IL-10 KO adults or WT pups, as well as significant abnormalities in nestlet building. We also found an IL-10 dependent increase in STAT3 phosphorylation in the brain tissues of young mice, which play a role in the neuroprotection mediated by IL-10 in this setting. In particular, this may be due to the role of STAT3 in decreasing inflammation from glia in response to infection, as our *in vitro* studies demonstrate an increase in neuron death following exposure to conditioned media from *S. epidermidis* biofilms and microglia, as opposed to *S. epidermidis* biofilm media alone. Collectively, these findings suggest that IL-10 plays a key role in the dampened inflammatory response to CNS catheter infections with *S. epidermidis* as a means of neuroprotection, and that IL-10-dependent, STAT3-mediated modulation of microglial inflammatory responses may be an important mechanism in this process. Better understanding of the immune mechanisms that place infants at higher risk of these infections can be used to guide future screening and adjunctive therapies.

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Metabolic Regulation of Tumor Infiltrating Lymphocytes

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There is a positive correlation between the accumulation of tumor infiltrating lymphocytes (TIL) in tumors and favorable clinical outcomes. However, TIL fail to eradicate tumor cells and prevent disease progression. While their failures have been attributed to their exhaustion (poor proliferation and cytokine production), the underlying molecular basis of this state is not fully understood. Generally, T cell activation induces massive clonal expansion, which requires robust duplication of proteins, lipids, and nucleic acids, which in turn requires rapid generation of cellular building blocks and energy. Activated T cells induce cellular signals that promote metabolic reprogramming to meet these increased bioenergetic and biosynthetic demands. This notion is supported by studies that have shown that glucose uptake and its metabolism via aerobic glycolysis (Warburg effect) are essential for generation and activity of effector T cells. Whether this essential metabolic reprogramming happens in TIL or not is unknown. Our studies have shown that TIL metabolically less active than acute effector CD8 effector T cells (CD8 Teff). On the other hand, TIL use glycolytic metabolism more than age-matched CD8 Teff do while they use similar level of oxidative phosphorylation metabolism (OXPHOS). These results suggest that TIL have metabolic profile that is distinct from acute and late CD8 Teff. Our investigation of glycolytic markers revealed that TIL express more PKM2 and take up more glucose

than age-matched CD8 Teff. The amount of glucose that TIL take up is similar to that of acute CD8 Teff, which express significantly higher level of glucose transporters (Glut1). These results suggest that TIL have glycolytic defect downstream of glucose uptake. In line with this, we have observed significant enhancement in TIL metabolic activity when we bypassed glycolysis by providing pyruvate. We did not observe deficiency in the expression of components of the PI3K/PDK1/Akt/mTOR/HIF1a signaling pathway (which has been reported to promote aerobic glycolysis) in TIL. In conclusion, glycolytic metabolism is suppressed in TIL although they express glycolytic machinery at similar level as acute CD8 Teff, which are highly glycolytic. Our preliminary study suggest that there is a progressive loss of glycolytic metabolism in TIL. Future studies will determine if promoting aerobic glycolysis in TIL can restore their proliferation and effector function.

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Metabolic Damage and Premature Thymus Aging Caused by Stromal Catalase Deficiency

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T lymphocytes are essential mediators of immunity produced by the thymus in proportion to mass. The thymus atrophies rapidly with age, resulting in diminished new T cell production. Decreased thymic output is compensated by duplication of existing cells, but results in progressive dominance by memory T cells, and decreased ability to respond to new pathogens or vaccines. We find that accelerated thymic atrophy results from stromal deficiency in the reducing enzyme catalase, leading to increased damage by reactive oxygen species (ROS) generated during aerobic metabolism. Genetic complementation of catalase diminished atrophy, as did chemical antioxidants, providing a mechanistic link between antioxidants, metabolism, and normal immune function. Progenitor lymphoblasts represent the primary source of thymic ROS, likely acting on stromal cells in trans. We propose that thymic atrophy represents a conventional aging process that is accelerated by stromal catalase deficiency in the context of an intensely metabolic lymphoid environment.

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Nuclear TRAF3 Inhibits CREB-mediated Survival and Metabolic Reprogramming in B Lymphocytes

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TRAF3 is an adaptor protein that negatively regulates signaling through CD40 and BAFF receptor in B lymphocytes. B cells isolated from B cell conditional TRAF3$^{-/-}$ mice display a remarkable pro-survival phenotype compared to wild type B cells, but the mechanism for this abnormal survival is poorly understood. We find that CREB protein expression and activity - but not mRNA - were increased in TRAF3$^{-/-}$ B cells. Inhibition of activity of CREB and its co-activator molecule CBP attenuated the survival of TRAF3$^{-/-}$ B cells. Immune precipitation revealed that TRAF3 associated with both CREB and CBP preferentially in the nucleus in B cells. The TRAF-C domain was identified as necessary and sufficient forTRAF3 nuclear localization. The human TRAF3 mutant LP1 isolated from a myeloma tumor and lacking a TRAF-C domain, failed to localize to the nucleus or associate with CREB. Guided by results of microarray analysis, we found that the proteins Glut1 and Hexokinase II, two CREB targets important for glucose metabolism, were elevated inTRAF3$^{-/-}$ B cells and associated with enhanced glucose uptake and broad metabolic reprogramming in Seahorse extracellular flux analysis. We are currently investigating the importance of TRAF3-regulated metabolic changes to B cell survival.

High Glucose Regulates Cytokines Responses in Human Macrophages

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Hyperglycaemia is a key factor of diabetic pathology. Macrophages are essential regulators of inflammation, that can be classified into two major types: classically (M1) and alternatively (M2) activated macrophages. Both populations play a role in diabetes. M1 are involved in the establishment and progression of insulin resistance and diabetes-associated inflammation, whereas M2 are involved in hyperglycaemia control. However, the effect of hyperglycaemia on differentiation and functional programming of macrophages is poorly understood. We established unique model system based on primary human monocyte-derived macrophages to examine the effects of hyperglycaemia on M1 and M2 differentiation. Monocytes were cultivated in the presence of 5mM and 25mM glucose for 6 days under stimulation with IFNgamma and IL4. In order to identify the effect of high glucose on the development of M1 and M2 phenotypes, cytokines were selected as key regulatory biomarkers. Using RT-PCR and ELISA the expression and release of TNFalpha and IL-1beta (M1 cytokines), and IL1Ra and CCL18 (M2 cytokines) were quantified. RT-PCR analysis revealed that high glucose induced mRNA of TNF-alpha, IL-1beta, and IL1Ra only in part of donors. However, in all analysed donors the increased secretion of all three cytokine release was demonstrated by ELISA. RT-PCR showed that high glucose supressed the M2 marker CCL18 mRNA levels, and this corresponded to the ELISA-measured suppression of CCL18 release. Our data suggest that individual profile of vascular complications can be caused by patient-specific cytokine profile induced by elevated glucose levels.

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Regulation of CD163 Expression in Human Macrophages in Diabetic Conditions and in Response to Implant Material

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Scavenging function of monocytes and macrophages is essential for the control of inflammatory reactions. The monocyte- and macrophage-specific scavenger receptor CD163 internalizes and degrades haemoglobin-haptoglobin complexes built due to intravascular haemolysis. This circumstance particularly occurs in inflammation, supporting the development of microvascular diabetic complications as well as failure in the installation of implants. The shedding of CD163 from the cell surface in inflammation leads to an impaired scavenging function and consequently increases the risk of vascular oxidative damage. The project aims to examine how high glucose conditions and contact with titanium affect the CD163-mediated scavenging of human primary differentially activated macrophages. $CD14^+$-monocytes were isolated from the peripheral blood of healthy donors by density gradient centrifugation and magnetic separation. M0 (non-stimulated), M1 (IFNgamma-stimulated) and M2 (IL-4-stimulated) macrophages were differentiated within 6 days in low (5mM) and high (25mM) glucose conditions. For the analysis of the effect of titanium on CD163 expression monocytes were seeded on titanium disks. CD163-mRNA expression was quantified by qRT-PCR, CD163 surface expression was analysed by flow cytometry. Increased glucose had a suppressing effect on CD163 mRNA expression in 8 out of 10 donors in M1; and in 7 out of 10 donors in M2. The suppressive effect of glucose was more pronounced in M2 compared to M1. Regarding surface expression, the effect of high glucose was donor-dependent in M0 and M2, whereas the expression of CD163 uniformly decreased in M1. The effect of titanium on the expression of CD163 was examined

in 12 individual monocytes cultures. Cultivation of M1 on titanium disks resulted in the stimulation of CD163 expression in 9 out of 12 cases; cultivation of M2 on titanium disks had a suppressive effect on CD163 expression in 10 out of 12 individual macrophage cultures. Our data suggest that the suppression of CD163 expression in macrophages by high glucose and titanium can contribute to the development of inflammation-mediated complications in diabetic patients and in patients with titanium implants.

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Dynamic Role of Toll-interacting Protein in the Inflammatory Response

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Toll-interacting protein (Tollip) is a novel modulator of the innate immune signaling process, with poorly understood mechanisms. We demonstrated that Tollip is critically involved in the fusion of lysosome with the autophagosome, contributing to the resolution of inflammation. On the other hand, Tollip may also modulate mitochondrial function. Thus, depending upon its subcellular location, Tollip may have distinct effects on innate immune cell activation. Its subcellular distribution and function may depend upon functional domains such as its C2 and CUE domain. In the current study, we tested the hypothesis that Tollip may be differentially involved in the expression of distinct sub-sets of inflammatory mediators depending upon its modification at C2 and CUE domain. To test this hypothesis, we employed WT, Tollip knockout or Tollip CUE mutant cells. Through RT-PCR analyses, we observed that LPS induced selected cytokines such as IL-12, IL-16, TNFα in WT murine embryonic fibroblasts (MEFs), but not in Tollip knockout MEFs. On the other hand, the expression levels of Interferon-induced protein with tetratricopeptide repeats (IFIT) were much higher in Tollip deficient MEFs. Intriguingly, MEFs harboring the Tollip CUE mutant showed significantly decreased mRNA levels of IL-12, IL-6, TNFα, as well as IFIT. Mechanistically, we observed that a key transcription factor Interferon regulatory factor 3 (IRF3) may be differentially modulated in WT, Tollip deficient, and Tollip mutant MEF cells. Taken together, our study reveals novel dynamics with regard to the modulation of differential cytokine expression by Tollip, potentially through its unique modulation of IRF3.

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High Levels of PGE2 in Bone Marrow Transplant Mice Impairs Autophagy in Alveolar Macrophages

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Bone marrow transplantation (BMT) is commonly used as a therapeutic strategy to treat different health disorders such as cancer. Although it benefits the host by restoring a damaged immune system with a healthy immune system, BMT recipients develop different complications in handling pulmonary pathogens. Previous studies have shown that immune and structural cells in the lungs of BMT recipients highly express cyclooxygenase (COX)-2, leading to overproduction of immunosuppressive prostaglandin E2 (PGE2) and may explain the susceptibility observed in BMT recipients to opportunistic pathogens such as *Pseudomonas aeruginosa*. Alveolar macrophages reside in the lower airways where they compose the first line of host defense against a wide array of pathogens. Previous studies have demonstrated that alveolar macrophages can target and clear pathogens such as *Pseudomonas aeruginosa* via autophagy, a mechanism by which pathogens are encapsulated in an autophagosome wherein the bacterial components are targeted for degradation. We have evidence that BMT recipient mouse alveolar macrophages harbor a deficiency in autophagy. To determine if PGE2 regulates autophagy in alveolar macrophages, we performed a series of in vitro and in vivo assays using a syngeneic BMT mouse model. Our results indicate impaired autophagy activity in alveolar macrophages in the presence of exogenous PGE2 as well as in the lung compartment of BMT mice. These observations highlight targeting the PGE2 signaling pathway as a method to restore immune activity in alveolar macrophages post-BMT.

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Complement Protein C1q Promotes Autophagy-Independent Survival of Macrophages during Clearance of Atherogenic Lipoproteins

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In the atherosclerotic lesion, macrophages are exposed to high levels of damaged low density lipoproteins (LDL). We have previously shown that complement component C1q recognizes and binds these oxidized (damaged) forms of LDL promotes their ingestion by macrophages, and downregulates pro-inflammatory cytokine production in vitro. C1q has also been shown to be protective in atherosclerosis in vivo. In these studies we are investigating additional potential protective mechanisms of C1q in macrophages during atherogenic lipoprotein clearance. An unbiased screen of the human monocyte derived macrophage transcriptome from macrophages ingesting damaged forms of LDL in the presence or absence of C1q highlighted a role for C1q in regulation of cell death. To investigate this further, we performed experiments in Raw264.7 macrophages to test the hypothesis that C1q improves survival in macrophages during atherogenic lipoprotein clearance. Survival assessment measured by an Alamar Blue assay showed that macrophages that had ingested either oxidized or acetylated lipoproteins in the presence of C1q had increased viability. To determine if autophagy was being activated by C1q in these macrophages, we performed immunoblots to measure relative levels of autophagosome protein LC3-II or sequestration protein p62 in the presence or absence of C1q. Levels of conversion of autophagosome protein LC3-I to LC3-II were unaltered in the presence of C1q, (average fold difference = 1.33 +/- 1.07, n=11). p62 protein levels were increased in macrophages by ingestion of oxLDL (average fold increase = 1.56 +/-0.66, p=0.01, paired students t-test, n=11), suggesting that oxLDL is stalling maturation of the autophagosome allowing for p62 accumulation. C1q modulation of p62 levels during ingestion of oxLDL were highly variable among experiments (average fold increase = 1.71 +/-1.23, n=11). When observed, the increase in p62 in the presence of C1q is likely due to a further reduction in autophagosome maturation however, rather than an increased synthesis of p62, since levels of p62 mRNA measured by quantitative RT-PCR were significantly reduced by C1q at 18 hours. These data suggest that C1q promotes macrophage survival during clearance of atherogenic lipoproteins, but the mechanism is independent of autophagy. Future studies will focus on identifying alternative pathways of survival including regulation of apoptosis.

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Disruption of the Polyisoprenyl Diphosphate Phosphatase 1 Gene Leads to Enhanced Bacterial Clearance in a Murine Model Of Pneumonia.

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Presqualene diphosphate (PSDP) is an intracellular lipid phosphate in leukocytes that controls the actions of key enzymes involved in cell activation. Recently, we demonstrated that polyisoprenyl diphosphate phosphatase 1 (PDP1), a lipid phosphate phosphatase that displays a distinct substrate preference for PSDP, can serve as a signaling nexus in human leukocytes. Exposing cells to soluble stimuli initiates protein kinase C dependent phosphorylation and activation of PDP1 with subsequent conversion of PSDP to its inactive monophosphate form, enabling cell activation. Here, in work in progress, we provide evidence that, similar to human PDP1, the murine PDP1 homologue displays a distinct substrate preference for PSDP. PDP1 deficient (PDP1-/-) animals were generated and assessed for their ability to clear a bacterial challenge in a murine model of pneumonia. At 2, 6, and 24 hours after intratracheal *E. coli* instillation, PDP1-/- mice relative to littermate controls displayed significantly lower CFUs in lung homogenates. Whole lung lavage of PDP1 -/- mice showed significant increases in alveolar macrophage numbers at baseline and 2

hours post-infection. Partial depletion of alveolar macrophages using chlodronate containing micelles demonstrated a positive correlation between alveolar macrophage numbers and bacterial clearance. Taken together, these data suggest that PDP1 is a pivotal signaling mechanism for cellular

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Effects of Extracorporeal Shock Wave Therapy on Gene Expression of Inflammatory Cytokines in Equine White Blood Cells

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Extracorporeal Shock Wave Therapy (ESWT) is high-energy sound waves transmitted to sections of the body. Extracorporeal Shock Wave Therapy was initially used for fragmentation of urinary stones in 1980s; since then it has been used therapeutically to accelerate the repair of tendon and ligament Injuries, stress fractures and non-unions. It has been reported that ESWT alters expression of some genes involved in angiogenesis, bone regeneration and inflammation. In present study, the effects of ESWT on expression of pro- and anti-inflammation cytokines were investigated. Extracorporeal Shock Wave Therapy was applied to 6 horses, blood samples were collected before and at different time points post ESWT; gene expression of both pro-and anti-inflammation cytokines was studied. The results showed that ESWT significantly increased the gene expression of anti-inflammation cytokines, interleukin 1 receptor antagonist (IL-1ra) and transforming growth factor, beta 1(TGF-β1). It suggested that ESWT exert anti-inflammatory effects through up-regulation of IL-1 ra and TGF-β1. Further investigation showed that IL-8 and COX-2 were also up-regulated. It indicated that ESWT also imitated inflammatory response in the early phase.

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Role of inflammasome dependent IL-1β in Hypersensitivity Pneumonitis

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Hypersensitivity Pneumonitis (HP) is an immune-mediated interstitial lung disease that develops following repeated exposure to a wide variety of inhaled environmental antigens. The disease is characterized by alveolitis, noncaseating granulomas and, in some patients, develops into a chronic form which is associated with fibrosis and emphysema. There are few therapeutic options for chronic HP; the mainstay of treatment is avoidance of the inciting agent and corticosteroids which have limited effects on outcome. We used the *Saccharopolyspora rectivirgula* (SR) mouse model of HP to determine the extent to which inflammasome activation and IL-1β production contribute to disease severity. WT mice exposed to SR for 3 times/week for 3 weeks exhibit increases in both IL-18 and IL-1β mRNA and protein. The canonical pathway for IL-1β production is through activation of the inflammasome and we measured an increase in mRNA expression of the inflammasome components NLRP3 and caspase 1 in the lungs of exposed mice. In vitro stimulation of bone marrow derived macrophages (BMDMs) with SR plus ATP resulted in an increase in IL-1β production suggesting SR is inducing pro-IL-1β expression. Pro-IL-1β production was dependent on TLRs 2 and 9 because BMDMs derived from *tlr2/9*$^{-/-}$ mice did not produce IL-1β following SR plus ATP stimulation. SR alone stimulated IL-1β production in BMDMs in the absence of ATP; this suggests that SR is capable of directly activating the inflammasome. IL-1β is functionally relevant during the disease because mice deficient in IL-1R signaling have decreased disease severity following repeated exposure to SR. WT and *il1R1*$^{-/-}$mice were exposed to SR for 3 weeks and the results demonstrated that *il1R1*$^{-/-}$mice had a decrease in alveolitis and the % of activated $CD4^+$ T cells in the BALF compared to WT mice. The decrease in cellular influx was accompanied by a decrease in mRNA expression of the pro-inflammatory cytokines TNF, IFNγ, IL-17, IL-18, CXCL9 and CXCL10. These results suggest that the IL-1/IL-1R

signaling pathway contribute to both neutrophil recruitment and IL-17 induction in HP.

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AIM2 Suppresses Colon Tumorigenesis by Interfering with DNA-PK-Dependent Akt Signaling

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The nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins are a family of cytosolic innate immune sensors that regulate inflammation. Upon stimulation by microbial components, several NLRs bind to the adaptor protein ASC and recruit caspase-1, forming the multi-protein complex known as the inflammasome. The inflammasome facilitates production of the proinflammatory cytokines IL-1 and IL-18, which provide a protective role in intestinal homeostasis as inflammasome-deficient mice (e.g., *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice) display increased intestinal inflammation and susceptibility to models of colitis and colon cancer. The NLR-like protein absent in melanoma 2 (AIM2) also forms an inflammasome and promotes IL-1b/IL-18 production, suggesting a similar protective role for AIM2 during colon tumorigenesis. Instead, we found that AIM2 protected against CAC by interfering with Akt activation by DNA-PK, but had little impact on inflammasome activation. Consistent with this finding, the impact of AIM2 was exerted on a non-bone marrow compartment, and AIM2 suppressed Akt activation and function in fibroblasts and colon organoid cultures *in vitro*. This AIM2-mediated suppression of Akt and Akt function was found to be DNA-PK dependent. Furthermore, AIM2 reduced Akt activation and tumor burden in the *APC*$^{min/+}$ model of sporadic colorectal cancer, and an Akt inhibitor nearly eliminated tumors in *Aim2*$^{-/-}$ mice. These combined results describe a novel mechanism by which AIM2 restricts tumorigenesis by interfering with DNA-PK-dependent Akt activation and implicates Akt inhibition as a therapy for AIM2-deficient human cancers.

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Understanding Host Responses Triggering Pathogen Virulence during Secondary *Staphylococcus aureus* Pneumonia

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Staphylococcus aureus (*S. aureus*) is one of the most prevalent bacterial infections and can cause infections in a wide variety of tissues. The innate ability of *S. aureus* to adapt to varied environments encountered during interaction with the human host is dependent on tightly controlled regulation of virulence accomplished by transcriptional regulatory networks, including two-component signal transduction systems. Previous work has established that many important *S. aureus* virulence factors are regulated by the SaeRS regulatory system and that this system is essential for evasion of human neutrophils, however, environmental factors that trigger SaeR/S and its impact on virulence in vivo is incompletely defined. Inasmuch as Influenza A infection greatly increases host-susceptibility to secondary pneumonia caused by *S. aureus*, in the current study we tested the hypothesis that immunological modulations in the lung environment caused by influenza A infection triggered increased virulence in the pathogen via the SaeR/S regulatory system. Using a murine model of bacterial superinfection we infected mice with influenza A/WSN/1933(H1N1) (750 PFU/50µL) followed by challenge with *S. aureus* (USA 300) ($5x10^8$ CFU/50mL) at day 6 post-influenza infection. Lung infections were initiated via aspiration from intranasal inoculation. Six-hours post- *S. aureus* challenge we compared both host and pathogen changes in superinfected mice to mice infected with either influenza A only or *S. aureus* only. Using RT-PCR and QuantiGene 2.0 transcriptional assays our preliminary results demonstrated an observed increase in gene expression in *saeR/S* and in SaeR-regulated virulence factors including PVL, *hlgA*, *hlgC*, and *lukA* as compared to gene expression in *S. aureus* isolated from mice infected with *S. aureus* only. Challenge with an isogenic *saeR/S* mutant strain (USA300Δ*saeRS*) confirmed virulence factors were regulated by SaeR/S in vivo. Since SaeR/S recognizes host factors, we are currently

investigating which host factor modulated by primary influenza induces SaeR/S leading to increased pathogenicity of *S. aureus*. Studies are focused on understanding the recruitment and modulation of the immune response during the influenza infection alone and early during secondary *S. aureus* infection. Preliminary findings demonstrated that *S. aureus* was responsible for increased neutrophil recruitment and primary influenza did not significantly alter the numbers of neutrophils recruited. Interestingly, influenza A infection alone caused recruitment of a CD11b low/mid, Ly6G(-), Ly6C(-) cell population that was drastically reduced in mice at 6 hours post-*S. aureus* challenge. Ongoing studies are further characterizing how this population modulates the host response early during *S. aureus* superinfection to identify the host changes potentially triggering activation of the SaeR/S virulence regulator. Taken together, our study suggests that specific host changes following influenza A infection triggers *saeRS*, stimulating the expression of *S. aureus* virulence genes resulting in increased morbidity and mortality seen in secondary *S. aureus* pneumonia.

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Macrophage-Specific Deletion of Peroxisome Proliferator-Activated Receptor-Gamma Exacerbates Inflammatory Immune Responses and Impairs Respiratory Function in a Mouse Model of Pulmonary Pseudomonas Aeruginosa Infection

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Alveolar macrophages normally reside in an anti-inflammatory state to limit excessive inflammation in the lung and prevent resulting impairment of respiratory function. They also play a pivotal role in response to infection by first phagocytosing pathogens and releasing pro-inflammatory molecules, and then resolving inflammation and clearing debris and apoptotic neutrophils. This is accomplished mostly by their capacity to shift from a pro-inflammatory to an anti-inflammatory phenotype. Peroxisome Proliferator-Activated Receptor-Gamma (PPARg) is a transcription factor critical for promoting resolution of the inflammatory response. In this study, we utilized macrophage-specific PPARg knockout mice (Mac PPARg KO) to assess the role of alveolar macrophage PPARg in regulating immune responses and respiratory function in the context of bacterial infection. Wild-type (C57Bl/6) or Mac PPARg KO mice were given intratracheal *Pseudomonas aeruginosa* (35,000 CFU) or sterile saline as a control. After 24 hours, plethysmography was performed to examine lung function, prior to euthanasia. Compared to wild-type mice with or without infection and saline treated Mac PPARg KO mice, infected Mac PPARg KO mice exhibited impaired respiratory function, with a 50% reduction in minute volume and breath rate ($p<0.05$), with a 5-fold increase in PenH ($p<0.05$). Lungs of infected Mac PPARg KO mice had drastically increased cellularity, interstitial thickening, and alveolar collapse, relative to other groups. Interleukin-6 levels were 40-fold higher in infected Mac PPARg KO ($p<0.05$), and expression of IL-6 and CXCL1 mRNAs were >300-fold higher ($p<0.01$), while TNFa and IL-1b mRNA expression levels were >30-fold higher ($p<0.01$), compared to infected wild-type mice. Furthermore, bacterial dissemination to the spleen was observed only in the infected Mac PPARg KO mice and not in infected wild-type mice. Together, these findings suggest the loss of PPARg in alveolar macrophages exacerbates pulmonary inflammation and reduces the ability to clear *Pseudomonas aeruginosa* infection. (Supported in part by NIH R01 AG018859 (EJK), R01 GM115257 (EJK), R15 ES022462 (MJT) and the Dr Ralph and Marian C Falk Medical Research Trust (EJK).

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Adhesive And Topological Cues Modulate Macrophage Phenotype Polarization

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Macrophages undergo continuous changes in function depending on signals in their microenvironment. During infection or inflammation, they polarize towards a pro-inflammatory phenotype and secrete cytokines and reactive species to combat infection. Under conditions of wound healing, these same cells

polarize towards a pro-healing phenotype and mediate processes that facilitate tissue repair. Despite a wealth of information known about how soluble factors including cytokines and chemokines influence immune cell function, little is known about how physical cues regulate their behavior. Our laboratory uses microfabrication tools combined with engineered biomaterials to control the physical environment of cells, and examine their effects on macrophage function. In our previous work, we found that geometry of adhesion, or cell shape, plays a critical role in regulating their polarization towards pro-inflammatory versus pro-healing states. Cells that are forced to elongate adopt a geometry similar to cells treated with pro-healing cytokines, and begin to express markers representative of pro-healing cells without the addition of exogenous cytokines. Furthermore, elongation protects cells from polarizing towards a pro-inflammatory phenotype even in the presence of soluble inflammatory stimuli. Using micromachined topographical surfaces, we found that macrophages sense 500 nm - 1 micron wide grooves and align along the direction of the grooves. Cell shape changes from surface topographies also led to an increase in expression of markers associated with a pro-healing phenotype. Mechanistically, abrogation of actin and myosin contractility using various pharmacological inhibitors prevented shape-induced changes in phenotype, suggesting an important role for the cytoskeleton in transducing shape signals to changes in biological behavior. Ongoing work seeks to further understand how physical cues presented by the extracellular matrix in healing tissues and biomaterial architecture influence macrophage function, and use this knowledge to develop improved biomaterials or tissue engineering strategies that promote wound healing.

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Tumor Associated Macrophages in Gaps of Ductal Tumor Structures Negatively Correlate with the Lymphatic Metastasis in Human Breast Cancer

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Tumor associated macrophages (TAM) in breast cancer animal models reveal phenotypic similarities with alternatively activated macrophages and can support tumor growth and metastasis. However, in several types of human cancers both positive and negative correlations between TAM and various parameters of tumor growth and spread were demonstrated. Up-to-date, limited information is available about intratumoral TAM heterogeneity and functional role of TAM subpopulations in tumor progression. The aim of our study was to examine if TAM presence in various morphological segments of human breast cancer correlated with clinical parameters. Patients with nonspecific invasive breast cancer T1-4N0-3M0 from Tomsk Cancer Research Institute were included in the study. CD68 and stabilin-1 expression in 5 different tumor segments (soft fibrous stroma; coarse fibrous stroma; areas of maximum stromal-and-parenchymal relationship; parenchymal elements and gaps of ductal tumor structures) were analyzed using immunohistochemistry and immunofluorescence/confocal microscopy. The expression of CD68 varied between analyzed segments. Soft fibrous stroma and areas of maximum stromal-and-parenchymal relationship showed highest expression of CD68 (79% of patients) while in areas with coarse fiber stroma expression of CD68 was found in 23% of patients. Inverse correlation of tumor size and expression of CD68 in gaps of tubular tumor structures was found ($R=-0.67$; $p=0.02$). Noteworthy, in case of the lymph node metastases the average score of CD68 expression in gaps of ductal tumor structures was lower (1.4 ± 0.5) compared to negative lymph nodes cases (3.1 ± 1.0; $F=10.9$; $p=0.007$). Confocal microscopy identified 3 phenotypes of TAM: $CD68^+$/stabilin-1^-; $CD68^+$/ stabilin-1^+ (over 50%); and $CD68^-$/ stabilin-1^+. However, expression of M2 marker stabilin-1 did not correlate with lymph node metastasis. We concluded that increased amount of CD68+ TAM in gaps of ductal tumor structures is protective against metastatic spread in regional lymph nodes and this effect is independent of M2 macrophage polarization.

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The Role of Glycoprotein A Repetitions Predominant (GARP) in Murine Regulatory T Cell Function

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Regulatory T cells (Tregs) are essential to the maintenance of immune homeostasis. Their suppressive function against T cell effector responses allows for the establishment of immunological tolerance and thus helps limit T cell–mediated diseases. One of the major mechanisms by which Tregs exert their suppressive function is via their production and surface expression of TGF-b. In recent studies it has been shown that TGF-β (in the form of latent associated protein, LAP) can be tethered to the surface of Tregs through association with the cell surface protein glycoprotein A repetitions predominant (GARP). Among hematopoietic cells, GARP expression is tightly restricted to Foxp3+ Tregs and LAP expression on activated Tregs is dependent on expression of GARP. On this basis it is understandable that inhibition of GARP has been shown to abrogate Treg suppression in most studies.

In the present study, we created a GARP-mCherry reporter mouse. The study of these mice confirmed that GARP is expressed and stored intracellularly in Foxp3+ Tregs under normal conditions and translocated to the cell surface where it binds LAP upon activation. Notably, while GARP is present on all natural Foxp3+ cells in the thymus, it is not present on TGF-b-induced Foxp3+ cells unless the inducing culture contains IL-2. In addition, the IL-2 inducing effect is reversed by inflammatory cytokines such as IL-6 and IL-27 that inhibit GARP expression to a somewhat greater degree than they inhibit Foxp3 expression. These regulatory effects of cytokines correlate with *in silico* analysis of the genomic regions flanking the GARP gene which were found to contain STAT3 and STAT5 binding sites.

Functional studies of GARP+ and GARP- Tregs revealed that both Treg populations have an equal capacity to inhibit CD4+ T cell proliferation *in vitro* and that such inhibition is not blocked by anti-GARP, ant-LAP or an inhibitor of TGF-b-receptor function. On the other hand, GARP+ but not GARP- cells induce CD4+ T cells to produce IL-17 when cultured with the latter in the presence of IL-6. Such induction was equal to that induced by TGF-b and IL-6 in the absence of Tregs. Thus, the inability to inhibit the suppressor capability of GARP+ cells with an agent that blocks TGF-b or TGF-b function was not due to the lack of ability of GARP+ cells to express or secrete TGF-b. It was more likely due to the fact that GARP+ (or GARP-) Tregs can suppress T cell proliferation *in vitro* by a mechanism that only partially depends on TGF-b. Whether this is true of Treg cell suppressor function *in vivo*, which has been closely linked to TGF-b-mediated effects, awaits further investigation. In any case these functional studies highlighted the fact that GARP+ Tregs can participate in T cell differentiation function.

Interestingly, induction of colonic inflammation by administration of Dextran Sodium Sulfate induced a 2-5-fold increase in the percentage of cells expressing Foxp3 but no significant increase in the percentage of cells expressing GARP. This again indicates that Foxp3 and GARP expression are independent and may depend on the cytokine milieu in an inflamed tissue. In addition, this suggests that certain Tregs may become functionally inactive due to lack of GARP expression.

Finally, we have taken advantage of the fact that we can isolate GARP-/Foxp3+ that do not express intracellular GARP (mCherry-) to study Treg gene expression profiles in the absence of GARP. We have found that GARP expression is indeed associated with the increased expression of proteins such as 4-1BB that is likely to affect Treg function. Thus, GARP may have functions prior to expression on the cell surface as a ligand for LAP.

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Cortisol-Treated Zebrafish Embryos Develop a Pro-Inflammatory Adult Phenotype with Reduced Regenerative Capacity

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Accumulating evidence indicates that chronic stress experienced early in life, even prenatally, can promote development of a pro-inflammatory phenotype predisposed to numerous physical and mental health problems and degenerative diseases of aging. One way that this is thought to occur is via developmental programming induced by glucocorticoid stress hormones. To investigate how such programming affects adult regenerative capacity, we treated zebrafish embryos with cortisol for the first five days of development, and examined the effects both during the treatment period and subsequently in adulthood. Treated larvae displayed elevated whole-body cortisol, glucocorticoid signaling, reactive oxygen species, and heart rate. Genome-wide gene expression and ontology enrichment analysis indicated that the cortisol-treated embryos significantly up-regulate processes associated with the immune system, while down-regulating processes associated with plasticity and regulation of inflammation. In adulthood the treated fish maintained elevated basal cortisol levels in the absence of exogenous cortisol, suggestive of a hyperactive stress system, and displayed constitutively higher expression of genes associated with glucocorticoid signaling, inflammation, and DNA damage response, as well as higher levels of DNA damage. In tailfin regeneration assays, adult fish derived from cortisol-treated embryos displayed increased incidence of defective regeneration, with altered patterns of neutrophil and macrophage infiltration, and aberrant expression of genes associated with glucocorticoid signaling, inflammation, and regeneration. These results are consistent with the idea that chronically elevated glucocorticoid signaling early in life directs development of a defensive, pro-inflammatory predisposition, at the expense of regenerative capacity.

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Mitochondrial Fueling and Bioenergy Paralysis Occur During the Monocyte Sepsis Response

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During the sepsis acute systemic inflammatory reaction monocytes rapidly change from a glycolytic and anabolic activator immune phenotype (M1-sepsis) to a lipolytic and catabolic repressor immune phenotype (M2-sepsis). This shift from anabolic activator to catabolic repressor monocyte often occurs within hours of sepsis onset and may reflect adaptation to an exaggerated proinflammatory response. Its molecular nature is poorly understood. Here, we report that the repressor M2-sepsis phenotype is metabolically and bioenergetically unresponsive/paralyzed to a second stress response. We used a human THP-1 monocyte cell model of sepsis, which trans-differentiates in vitro from an M-1 to M-2-sepsis phenotype after a strong TLR4 signal (1 ug/ml of LPS). From whole cell Seahorse SF24 respirometry and biochemical analyses, we observe that the ”paralyzed" repressor M-2-sepsis phenotype stimulated a second time with LPS is unable to increase oxygen consumption, generate reactive oxygen species (ROS), and lacks Spare Respiratory Capacity. It also is defective in glycolysis, glucose oxidation and lactic acid generation. Using isolated mitochondrial preparations, Complexes I, II, and III function normally when given artificial nutrients, but Complex IV cannot respire. Artificial ROS generation reverses nutrient and bioenergy paralysis. We conclude that redox pathways become inflexible in M2-sepsis repressor monocytes. Supported by NIH RO1 grants R01-GM102497 and RO1-AI065791 to cem, by and the Bioenergy Core of Wake Forest University Medical Center.

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The Society For Leukocyte Biology's 49th Annual Meeting With Joint Participation By Neutro2016

September 15-18, 2016 ■ University of Verona Congress Center ■ Verona, Italy

PROGRAM CHAIRS

Marco A. Cassatella (University of Verona)
Patrick P. McDonald (Université de Sherbrooke)

PLENARY TOPICS

Leukocytes in Host-Pathogen Interactions
Leukocytes in the Tumor Microenvironment
Metabolic and Autoimmune Diseases
Receptors, Signaling and Soluble Mediators

CONCURRENT SESSION TOPICS

Functional genomics
Myeloid Subsets
Cytokines in Inflammation and immunity
The expanding world of leukocytes
Neutrophil trafficking and effector functions
Emerging topics and methodologies in neutrophil biology
Advances in neutrophil differentiation and neutrophil-mediated inflammation resolution

INVITED SPEAKERS

Lee Ann Allen
Yasmine Belkaid
Triantafyllos Chavakis
Marco Colonna
Evgeniy Eruslanov
Muzlifah Haniffa
Clifford Lowell
Alberto Mantovani
Peter Murray
Gioacchino Natoli
Sergei Nedospasov
Sussan Nourshargh
Luke O'Neil
Giorgio Trinchieri
Veronique Witko-Sarsat
Arturo Zychlinsky

Visit **leukocytebiology.org** for the most up-to-date information!

PROGRAM - AT - A - GLANCE

SUNDAY, SEPTEMBER 27

7:30am - 6:00pm
Registration Desk Open
LOCATION: Grand Ballroom Foyer

8:00am – 1:00pm
Satellite Symposium on Alcohol and Immunology: Neuroimmunoendocrine Effects of Alcohol
LOCATION: Dogwood & Boxwood
(Pre-registration required)

11:00am – 12:30pm
Poster Talks: Highlighted Poster Session Presentations
LOCATION: Grand Ballroom 3

12:30 - 6:00pm
Exhibits
LOCATION: Grand Ballroom Foyer

1:30 - 3:00pm
Welcome and Plenary I- Autophagy, Cellular Stress and Innate Immunity
LOCATION: Grand Ballroom 4&5

3:00 - 4:30pm
SLB Presidential Nominees
LOCATION: Grand Ballroom 4&5

4:30 – 4:45pm
Coffee Break
LOCATION: Grand Ballroom Foyer

4:45 - 6:00pm
Senior Level Awards
LOCATION: Grand Ballroom 4&5

6:00 - 7:00pm
Opening Reception
LOCATION: Grand Ballroom Foyer

7:00 - 8:00pm
Keynote Lecture
LOCATION: Grand Ballroom 4&5
Bonazinga Winner, Sponsored by Accurate Chemical

8:00pm - 9:30 pm
Street Smarts of Science
LOCATION: Dogwood & Boxwood
(Pre-registration required)

MONDAY, SEPTEMBER 28

7:00 - 9:00am
Women and Diversity Special Session
LOCATION: Boxwood
(Pre-registration required)

8:00am - 5:00pm
Registration Desk Open
LOCATION: Grand Ballroom Foyer

8:00am - 5:00pm
Exhibits
LOCATION: Grand Ballroom Foyer

8:00 - 9:00am
Networking Breakfast
LOCATION: Breakfast provided in Lofton's Corner with additional seating in Grand Ballroom 1&2

9:00 - 10:30am
Plenary II - Epigenetics and Immunity
LOCATION: Grand Ballroom 4&5

10:30 - 11:00am
Coffee Break
LOCATION: Grand Ballroom Foyer

11:00am - 12:30pm
Concurrent Sessions

CS 1 - Lipid Mediator Regulation of Disease
LOCATION: Grand Ballroom 4&5

CS 2 - Immunologic Mechanisms of Vaccination
LOCATION: Grand Ballroom 3

12:30 - 2:00pm
Poster Session I - Lunch
LOCATION: Capital Ballroom

2:00 – 3:30pm
Plenary III - Microbiome and Mucosal Immunity
LOCATION: Grand Ballroom 4&5

3:30 - 4:00pm
Coffee Break
LOCATION: Grand Ballroom Foyer

4:00 - 5:30pm
Concurrent Sessions

CS 3- Inflammasomes and Inflammatory Disease
LOCATION: Grand Ballroom 4&5

CS 4- Fibrosis and Tissue Repair
LOCATION: Grand Ballroom 3

5:30 - 7:00pm
MTTG Session: "Career Transitions"
LOCATION: Boxwood
(Pre-registration required)

7:00 - 9:00pm
Social Mixer
LOCATION: Bahama Breeze

TUESDAY, SEPTEMBER 29

7:00 - 8:30am
Grant Writing Session Breakfast
LOCATION: Dogwood & Boxwood – breakfast provided.
(Pre-registration required)

8:00am - 4:30pm
Registration Desk Open
LOCATION: Grand Ballroom Foyer

8:00am - 4:30pm
Exhibits
LOCATION: Grand Ballroom Foyer

8:00 - 9:30am
Breakfast
LOCATION: Breakfast provided in Lofton's Corner

8:30 - 9:30am
Immunology in the News:
Special Breakfast Session, with Ian Crozier, Infectious Diseases Institute, Kampala, Uganda
LOCATION: Breakfast provided in Lofton's Corner. Presentation will be in Grand Ballroom 4&5

9:30 - 11:00am
Concurrent Sessions

CS 5 - DC and Macrophages in Tumorigenesis and Inflammation
LOCATION: Grand Ballroom 4&5

CS 6 - Treg/Th17 Balance in Inflammatory Disease
LOCATION: Grand Ballroom 3

11:00 - 11:15am
Coffee Break
LOCATION: Grand Ballroom Foyer

11:15 – 12:45pm
Concurrent Sessions

CS 7 - Neutrophils in Health and Disease
LOCATION: Grand Ballroom 4&5

CS 8 - Best of Journal of Leukocyte Biology
LOCATION: Grand Ballroom 3

12:45 - 2:15pm
Poster Session II - Lunch
LOCATION: Capital Ballroom

2:15pm
Member Business Meeting and Presidential Award Announcements
LOCATION: Grand Ballroom 4&5

2:30 - 4:00pm
Plenary IV - Neuro-Immune Interactions
LOCATION: Grand Ballroom 4&5

4:00 - 4:15pm
Coffee Break
LOCATION: Grand Ballroom Foyer

4:15 - 5:45pm
Plenary V - Metabolic Control of Immunity
LOCATION: Grand Ballroom 4&5

Follow the meeting on Twitter!

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