ABSTRACTS

1

AMPK Regulation of Leukocyte Functional Polarization

Jill Suttles
University of Louisville School of Medicine, Department of Micobiology and Immunology, Louisville, KY

In our studies of the function of fatty acid-binding proteins (FABPs) in leukocytes we found that expression of these lipid chaperones promotes inflammatory activity. Absence of FABP expression in macrophages and dendritic cells (DC) results in a polarized anti-inflammatory state and is accompanied by elevated activity of AMP-activated protein kinase (AMPK) a conserved serine/threonine kinase involved in the regulation of cellular energy status. An investigation of the contribution of AMPK to the reduced inflammatory potential of FABP-deficient cells revealed AMPK as an upstream regulator of an Akt/GSK3/CREB pathway that promotes expression of IL-10 while inhibiting the activity of NF-kB. In wild-type macrophages and DC, AMPK is activated by a variety of anti-inflammatory stimuli and is required for IL-10 induction of SOCS3 expression. AMPK-deficient macrophages and DC show enhanced proinflammatory cytokine expression and APC activity, as compared to wild-type cells, resulting in intensified promotion of Th1 and Th17 differentiation during antigen presentation. FABP-deficient CD4+ T cells display enhanced AMPK activity and are skewed towards development of a regulatory T cell (Treg) phenotype and refractory to Th17 differentiation. In contrast, AMPK-deficient CD4+ T cells resist TGF-beta stimulation of FoxP3 expression and Treg development and favor Th17 differentiation. Thus, AMPK activity in myeloid APC and CD4+T cells serves to attenuate innate and adaptive inflammatory responses, whereas FABP expression favors inflammatory activity. Thus, these proteins, known for their function in cellular metabolism, play opposing roles in the regulation of inflammatory responses. Supported by NIH R01 AI048850.

2

CCR2 in Monocyte and Hematopoietic Stem Cell Trafficking in Atherosclerosis and Inflammation

Izzy Charo
UCSF, Gladstone Inst of Cardiovascular Disease, San Francisco, CA

Chemokines are chemotactic cytokines that direct leukocyte trafficking to sites of inflammation and injury. This talk will provide background on the family of chemokines and their cognate receptors, and summarize results from genetic models in mice that suggest important roles for chemokines in atherosclerosis. Recent studies also suggest that CCR2, the receptor for MCP-1, plays an important role in trafficking of hematopoietic stem cells to sites of inflammation to mediate tissue repair.

3

Macrophages as a System

David A. Hume
The Roslin Institute, University of Edinburgh, Midlothian, Scotland, UK

Mononuclear phagocytes are a family of cells that participate in tissue remodelling during development, wound healing and tissue homeostasis. They are central to innate immunity, control subsequent acquired immune responses and contribute to the pathology of tissue injury and inflammation. With the escalation of genome-scale data derived from macrophages and related hematopoietic cell types, there is a growing need for an integrated resource that seeks to compile, organise and analyse our collective knowledge of macrophage biology. We have developed a community-driven web-based resource, www.macrophages.com, that aims to provide a portal onto various types of 'omics data to facilitate comparative genomic studies, promoter and transcriptional network analyses, models of macrophage pathways together with other information on these cells. Macrophages can express the large majority of protein-coding genes in the genome at a detectable level. These genes need to be expressed and regulated in a coordinated manner to generate a professional phagocyte and to modulate the behaviour of macrophages in health and disease. In this talk, I will discuss the various transcriptional regulatory networks that are involved in macrophage differentiation from progenitor cells, functional geneomics of macrophage-expressed genes and the coordinated regulation of a network of genes required to be an active phagocyte, the response of macrophages to lipopolysaccharide, and comparative analysis of the responses of mouse, human and pig macrophages.

4

Impact of Peripheral Infection and Inflammation on the Healthy and Diseased Brain

Hugh Perry
University of Southampton, School of Biological Sci, Experimental Neuropathology, Southhampton

Humoral and neural pathways mediate the communication between the peripheral immune system and the brain. These pathways signalling to the brain play an important role in the generation of metabolic and behavioural changes, so-called sickness behaviours, that help to protect us from the consequences of systemic infections. In healthy individuals the impact of systemic inflammation on the brain is part of our homeostasis and causes no damage to the brain. We have shown, however, that in animals with chronic neurodegeneration systemic inflammation may lead to exaggerated symptoms of sickness and accelerate the progression of neurodegenerative disease. The resident macrophages of the brain, the microglia ,appear to be primed by the ongoing neurodegeneration and give an exaggerated response to systemic inflammation. We have shown that in patients with Alzheimer's disease systemic inflammation and infections are associated with a more rapid cognitive decline and exacerbation of behavioural symptoms of the disease. These studies highlight the importance of understanding the molecular and cellular communication pathways between the immune system and the brain in health and disease.

ABSTRACTS

5

Impact of Innate Immunity and Toll-Like Receptor (TLR) Signaling during Central Nervous System Bacterial Infection

Tammy Kielian[1]
[1]*Univ of Nebraska Medical Ctr, Pathology and Microbiology, Omaha, NE*

Bacterial infections in the central nervous system (CNS) are typically associated with overt inflammation and edema that lead to parenchymal destruction, the extent of which is dictated by the time required for pathogen eradication. *Staphylococcus aureus* is a common etiologic agent of brain abscesses and possesses numerous virulence determinants that manipulate host immunity. Myeloid differentiation primary response gene 88 (MyD88), an adaptor utilized for Toll-like receptor (TLR)/IL-1R/IL-18R signaling, is essential for microglial recognition of *S. aureus* and the induction of CNS immunity in a mouse model of experimental brain abscess. Studies using radiation bone marrow chimera mice have demonstrated that MyD88 is required for CNS-intrinsic regulation of neuroinflammatory responses to *S. aureus*. Further evidence for the importance of MyD88/IL-1R signaling is demonstrated by the heightened sensitivity of IL-1R-deficient mice to CNS bacterial infection, which is accompanied by defects in peripheral immune cell recruitment and pathogen containment. Studies in mice deficient in various components of the NLRP3 inflammasome have further highlighted the critical role of IL-1 during CNS infection; however, unexpected roles for select inflammasome components have also been identified, suggesting that the inflammasome may exert functions that extend beyond its well recognized role in IL-1β processing. Collectively, these studies reveal important roles for MyD88 and the inflammasome in regulating innate immune responses during bacterial infection in the CNS parenchyma. This work was supported by the NIH National Institute of Neurological Disorders and Stroke (NINDS; R01 NS055385) to T.K.

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Good Cop, Bad Cop: Complement Proteins Uncover Novel Opportunities Modulating Neuroinflammation

Andrea J. Tenner, Maria I. Fonseca, Tracy Cole, Shu-Hui Chu, Marie E Benoit
University of California - Irvine, Department of Molecular Biology and Biochemistry, Irvine, CA

The role of the complement system in neurodegenerative disease progression is complex with strong evidence of both detrimental effects and protective effects in numerous models including animal models of Alzheimer's Disease. Evidence that cleavage of C5 plays a substantial detrimental role in AD progression has been provided by the demonstration of a decrease in pathology and the suppression of behavioral deficits in mouse models of AD treated with a CD88-specific C5a receptor antagonist, PMX205. Backcrossing the 3xTg AD mouse model to BUB, a mouse strain with substantially higher hemolytic complement activity, accelerated the development of pathology, while conversely, crossing a C5aR (CD88) knock out mouse to the "Arctic APP" AD mouse model, suppressed specific age- behavioral deficits. Previous studies have shown that both C3, likely due to its opsonic activity, and C1q, via its suppression of microglial production of inflammatory cytokines, trigger protective responses to A β challenge. In addition, C1q provides direct neuroprotection to primary neuronal cell cultures, via modulation of neuronal gene expression, in the absence of other complement proteins. These data suggest a novel therapeutic strategy combining the use of antagonists of the C5a receptor CD88 to specifically prevent C5a-induced proinflammatory, neurotoxic consequences in the brain, while stimulating C1q neuroprotective pathways to permit enhanced survival thereby slowing the progression of neurodegenerative diseases. Supported by NIH NS35144, AG 00538, and Alzheimer's Association.

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A System Analysis of Negative Feedback Control of Inflammation Reveals Surprising Specificities

Alexander Hoffmann
University of California San Diego Medical Center, Signaling Systems Lab, Dept of Chemistry, San Diego, CA

Immune responses demand not only rapid activation but also appropriate termination of signaling/transcriptional effectors. In fact, immune response signaling is highly dynamic and stimulus/pathogen-specific. Thus it is not surprising that an increasing number of negative feedback regulators are being identified, but it is often unclear whether they have overlapping function (representing fail-safe mechanisms) or specific functions. I will present my laboratory's combined kinetic modeling and experimental work to distinguish the functions of negative feedback regulators and show that their kinetic properties are key to understanding their physiological functions.

8

Translational Pharmacology of AVL-292, a Potent and Selective Inhibitor of B Cell Function

William Westlin
Avila Therapeutics, Waltham, MA

Bruton's tyrosine kinase (Btk) is a validated pharmaceutical target through human (XLA) and murine (xid) genetic mutations and preclinical genetic and pharmacological demonstrations of activity. Expression of Btk is limited to B lymphocytes, mast cells, and myeloid cells making it an attractive target for the development of targeted therapy in autoimmune diseases. A unique covalent probe technology has enabled translational pharmacology to define the precise relationship between dose, drug concentration, target site occupancy, and functional endpoints of activity and efficacy in preclinical cellular and in vivo model systems. Following this approach we have translated preclinical and clinical data using a highly selective inhibitor of Btk, AVL-292, into useful predictions of dose level and dosing frequency expected to derive therapeutic benefit in the clinical setting of autoimmune diseases.

9

Computational Models of Inflammation: Basic Insights and Translational Applications

Yoram Vodovotz
University of Pittsburgh School of Medicine, Department of Surgery, Immunology Computational and System, Pittsburgh, PA

Properly-regulated inflammation is central to homeostasis, but in adequate or overly-robust inflammation can lead to disease. Like many biological processes, inflammation and its various manifestations in disease are multi-dimensional. The advent of multiplexed platforms for gathering biological data, while providing an unprecedented level of detailed information about the dynamics of complex biological systems such as the inflammatory response, has paradoxically also flooded investigators with data they are often unable to use. Systems approaches, including data-driven and mechanistic computational modeling, have been used to decipher aspects of the inflammatory responses that characterize trauma/hemorrhage and sepsis. Through combined data-driven and mechanistic modeling based on Luminex™ datasets, computational models of acute inflammation in mice, rats, swine, and humans were generated. These studies suggest that acute inflammation goes awry when the positive feedback loop of inflammation → tissue damage/dysfunction → inflammation, driven by damage-associated molecular pattern molecules, fails to resolve under the influence of anti-inflammatory/pro-healing mediators. These systems-based insights may lead to novel, rationally-designed, and individualized therapies.

10

Toll-Like Receptors and NOD-Like Receptors: Signalling Inflammation and Infection

Luke O 'Neill
Trinity College, School of Biochemistry and Immunology, Dublin

In the field of inflammation research, the most important advances in the past 10 years has been in the uncovering of multiple pathways involved in innate immunity. The best characterised involve the Toll-like receptors (TLRs) and NOD-like receptors (NLRs. Genetic variation in several of these components has been linked to inflammatory diseases, notably in the TLR system and in the NLR protein Nalp3 and associated proteins. Work on knockout mice and the use of inhibitors continues to validate some of these proteins in disease. From work on Nalp3 there has also been a resurgance of interest in the IL1 system as a key driver of inflammation in diseases such as gout and diabetes (both Type I and Type II). For investigators interested in signal transduction, the area has proved very fruitful in terms of the discovery of new signalling pathways and processes. We now have a good understanding of the major components activated by TLRs, notably the TIR domain- containing adapters that initiate signalling following recruitment to TIR domains within the TLRs themselves, the IRAK family of protein kinases that are then recruited, and a series of ubiquitination and phosphorylation reactions that ultimately lead to the activation of transcription factors such as NF-kappaB and IRF family members. A role for metabolic processes including glycolysis in the regulation of signalling is also an emerging theme, suggesting that restoration of homeostasis after tissue injury and infection is a key goal of innate immunity. As we continue to unravel the molecular details of these processes, new therapeutic options will present themselves.

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Discovery of a Viral NLR Homolog That Inhibits the Inflammasome

Blossom Damania, Sean Gregory
UNC-Chapel Hill, Lineberger Cancer Center, Chapel Hill, NC

The nucleotide-binding and oligomerization, leucine-rich repeat (NLR) family of proteins sense microbial infections and activate the inflammasome, a multi-protein complex that promotes microbial clearance. Kaposi's sarcoma-associated herpesvirus (KSHV) is linked to several human malignancies. We report that KSHV Orf63 is a viral homolog of human NLRP1. Orf63 blocked NLRP1-dependent innate immune responses, including caspase-1 activation and processing of interleukin (IL)-1β and IL-18. KSHV Orf63 interacted with NLRP1, NLRP3, and NOD2. Inhibition of Orf63 expression resulted in increased expression of IL-1β during the KSHV lifecycle. Furthermore, inhibition of NLRP1 was necessary for efficient reactivation and generation of progeny virus. The viral homolog subverts the function of cellular NLRs, which suggests that modulation of NLR-mediated innate immunity is important for the life-long persistence of herpesviruses.

12

Neutrophils Drive MMP-8/-9 Gene Expression and Secretion in Tuberculosis by Complex Mechanisms

Catherine Ong, Paul Elkington, Shivani Singh, Jonathan S Friedland
Imperial College London, Infectious Diseases and Immunity

Introduction: Neutrophils are the main phagocytic cells recovered from lungs of patients infected with Tuberculosis (Tb). A major drive to tissue damage in Tb development is the matrix degrading phenotype in which the activity of matrix metalloproteinases (MMPs) is unopposed by their tissue inhibitors (TIMPs). We investigated key factors regulating neutrophil MMP secretion in Tb *in vitro* and in Tb patient samples. **Methods**: Neutrophils were infected with *Mycobacterium tuberculosis* (Mtb) or stimulated with conditioned media from Mtb infected monocytes (CoMTB). Analysis of MMP-8/-9 secretion and TIMP-1/-2 was by ELISA, Luminex array and zymography. MAP kinase and PI3-kinase signalling was studied by western blotting and chemical inhibition. NF-κB activity was assessed using Helenalin and SC-514. Gene expression of MMP-8/9 was investigated using qPCR. Neutrophil granule formation was assessed by confocal microscopy. Bronchoalveolar lavage samples from 34 well- characterized patients were analyzed. **Results**: Neutrophi l MMP-8/-9 secretion is upregulated with Mtb and is dependent on Tb multiplicity of infection. Secretion is independent of the MAP kinase and PI3 kinase pathways. CoMTB stimulated neutrophils resulted in a 2 and 3 fold up-regulation of MMP-8/-9 secretion respectively (both $p<0.001$). TIMP-2 but not TIMP-1 increased 2-fold ($p<0.001$). In CoMTB-driven network, both MAP kinase and PI3 kinase pathways regulate neutrophil MMP-8/-9 secretion

and gene expression. NF-kB regulates MMP-8/-9 in direct Tb and CoMTB stimulated neutrophils. Confocal microscopy showed colocalisation of early endosome marker Rab-5 with MMP-8 and -9. Elevated MMP-8 in bronchoalveolar lavage samples correlated with neutrophil lipocalin. **Conclusions**: Neutrophil MMP-8/-9 gene expression and secretion is upregulated by divergent mechanisms following direct effect by Tb or stimulation by monocyte-dependent Tb networks. The increased MMP/TIMP ratio will result in a proteolytic environment which may lead to patient morbidity associated with tissue destruction. **Funding** : Medical Research Council (Singapore and UK), UK National Institute of Health Research, Scadding Morriston Davies Fund.

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Divergent Roles for the FcRγ Chain in Inflammasome Activation

John R Janczy, [1]Sophie Joly, [2]Matthew Drescher, [3]Fayyaz Sutterwala[4]

[1]University of Iowa, Graduate Program in Immunology, Iowa City, IA; [2]University of Iowa, Inflammation Program, Iowa City, IA; [3]University of Iowa, Inflammation Program, Iowa City, IA; [4]Univ of Iowa, Iowa City, IA

The Nlrp3 inflammasome is a multi-protein complex that when assembled mediates the activation of caspase-1 with the subsequent secretion of the proinflammatory cytokines IL-1β and IL-18. A wide array of stimuli are capable of activating the Nlrp3 inflammasome including both endogenous danger signals and pathogens. Nlrp3 inflammasome activation by the fungal pathogen *Candida albicans* requires signaling through the CLR dectin-2 and its associated signaling component FcRγ. We sought to further characterize the role of FcRγ chain signaling in inflammasome activation using IgG immune complexes, which interact with FcγRs that also signal through the FcRγ chain. Paradoxically, we found that IgG immune complexes suppress macrophage Nlrp3 inflammasome activation in response to silica, ATP and *C. albicans* resulting in diminished IL-1 β secretion. This inhibition occurred at the level of caspase-1 activation and the production of pro-IL1 β was not inhibited by FcγR ligation. These data suggest that initiation of adaptive immunity and the generation of antigen specific antibodies may play a role in shutting off Nlrp3 inflammasome activity. Research supported by NIH grant R01 AI087630 (F.S.S.)

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On the Molecular Bases of IFNβ mRNA Expression in Human Neutrophils Transfected with Plasmidic DNA

Marco A Cassatella, [1]Nicola Tamassia, [2]Flavia Bazzoni[3]

[1]University of Verona, Dept of Pathology and Diagnostics, Verona/ Italy; [2]University of Verona, Pathology and Diagnostics, Verona/ Italy; [3]University of Verona, Pathology and Diagnostics, Verona/ Italy

In addition to their classical host defense functions, neutrophils also synthesize and secrete several cytokines and chemokines upon appropriate stimulatory conditions, for instance in response to LPS and other Toll-like receptors (TLRs) agonists. Even though human neutrophils express all known TLRs with the exception of TLR3, our knowledge on the TLR-dependent signaling components and the molecular mechanisms that, in these cells, regulate cytokine gene expression, particularly *via* TLR4, is still incomplete. While LPS is known to activate both myeloid differentiation factor-88 (MyD88)-dependent and MyD88-independent/TIR domain-containing adapter inducing IFN β (TRIF)-dependent cascades, we recently identified that neutrophils do not mobilize the MyD88-independent/TRIF-dependent signaling pathway downstream of TLR4. As a result, LPS-stimulated neutrophils are unable to transcribe IFN β since they do not activate TRAF family associated NF-κB binding kinase [TANK]-binding kinase-1 (TBK1), an interferon regulatory factor-3 (IRF3)-phosphorylating kinase, and, consequently, IRF3, a critical transcription factor for IFNβ induction. In light of a recent report identifying PKC ε as a crucial player for initiating the MyD88-independent/ TRIF-dependent pathway, and given that human neutrophils do not express PKCε , we attempted to restore the MyD88-independent pathway by overexpressing PKCε in neutrophils. By optimizing an electroporation method, we obtained very high transfection efficiency (more than 50 %) and also a remarkable PKCε expression in neutrophils. However, independently from the transgenic PKCε content, we observed that the simple electroporation of plasmidic DNA into neutrophils triggers a dramatic IFNβ mRNA expression that, however, resulted to be further increased by a subsequent LPS stimulation. Using Chromatin immunoprecipitation (ChIP) assays, we then observed that plasmidic DNA transfection induces the binding of IRF3 to the IFNβ promoter . By contrast, LPS stimulation in plasmidic DNA-transfected neutrophils did not increase the IRF3 binding but triggered NF-κB p50 and p65 binding to the IFNβ promoter. Interestingly, the latter activation was not detected when LPS was added to untransfected cells. Data suggest that human neutrophils are able to recognize microbial cytosolic DNA and, in turn, to promote the activation of IFNβ gene expression. Data also suggest that DNA recognition by neutrophils may cooperate with others PAMPs, such as TLR ligands, to modulate the innate immune response.

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Phospholipase D2 (PLD2) Is a Guanine Nucleotide Exchange Factor (GEF) for the Rac GTPase Necessary for Chemotaxis

Julian Gomez-Cambronero, [1]Mary C. Dinauer, [2]Hong-Juan Peng, [3]Karen Henkels, [4]Mahdu Mahankali[5]

[1]Wright State University School Medicine, Biochemistry and Molecular Biology, Dayton, OH; [2]Washington University School of Medicine, Department of Pediatrics, St. Louis, MO; [3]Washington University School of Medicine, Biochemistry and Molecular Biology, Dayton, OH; [4]Wright State University School Medicine, Biochemistry and Molecular Biology, Dayton, OH; [5]Wright State University School Medicine, Biochemistry and Molecular Biology, Dayton, OH

We have discovered for the first time that the enzyme phospholipase D2 (PLD2): (1) binds directly to the small GTPase Rac2 both in vitro and in vivo (by FRET); (2) PLD2 functions as a Guanine nucleotide Exchange Factor (GEF), since it switches Rac2 from the GDP-bound to the GTP-bound states. This effect is large enough to be meaningful (~70% decrease for GDP dissociation and 300%

increase for GTP association), the half-time is 6 min, and was enhanced with increasing PLD2 concentrations and compares favorably to other known Rho-family GEFs, such as Vav; (3) the protein-protein interaction is sufficient for the GEF function as it can be demonstrated in vitro with just the recombinant proteins and in the absence of phosphatidic acid (PA); (4) the GEF function of PLD2 is further enhanced (~ 25%) during the GTP binding step, if PA is included in the reaction mixture; (5) the PLD2-Rac2 protein-protein association involves residues 263-266 in PLD2 in the PH domain as one binding site, and N17 in Rac2 in the switch-1 region; (6) in living cells, silencing PLD2 leads to a Rac2 reduced activity; proving the crucial importance of PLD2 in Rac2 function; and (7) PLD-initiated Rac activation results in enhancement of cell adhesion, chemotaxis and phagocytosis of leukocytes. There are several known GEFs, but this is the first report of a GEF that is a phospholipase. We posit that the newly discovered function of PLD2 is advantageous for the cell in that it provides a direct mechanism of activating a GTPase in membrane structures, bringing spatially separated molecules together, ready for cell signaling. The grant HL056653 (J.G.-C.) from the National Institutes of Health has supported this work.

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Expression of Adipocyte/Macrophage-Fatty Acid Binding Protein Promotes Tumor Growth and Metastasis

Ashley S. Triplett, Bing Li, Jill Suttles

University of Louisville, Department of Microbiology and Immunology, Louisville, KY

It has been estimated that 15-20% of all cancer deaths in the U.S. are associated with obesity. It is well-established that obesity promotes low-grade chronic inflammation, however the mechanisms by which obesity-induced chronic inflammation may promote cancer development and progression are not well-defined. Fatty acid binding proteins (FABPs), which are intracellular lipid chaperones, regulate both metabolic and inflammatory pathways. Of the nine FABP family members, adipocyte/macrophage-FABP (A-FABP) has been found to be highly expressed in macrophages in both mice and humans and its expression is increased in response to a high-fat diet. In the present study we examined the influence of A-FABP expression on tumor growth and metastasis in mice under conditions of normal or high-fat feeding. Wild-type (WT) and A-FABP knockout (A-FABP KO) mice were placed on a normal or high-fat diet prior to the injection of Lewis Lung Carcinoma cells (LL/2). When fed a normal diet, LL/2 tumor metastasis was significantly reduced in A-FABP KO mice relative to WT mice, whereas tumor growth in A-FABP KO and WT mice was similar. However, a high fat diet resulted in a significant increase in both tumor growth and metastasis in WT, but not A-FABP KO mice. Western blot and RT-PCR analysis demonstrated that tumor-infiltrating macrophages isolated from A-FABP KO mice on a normal or high-fat diet have reduced pro-inflammatory cytokine production, NF-κB activation, and decreased expression of metastasis-promoting proteins, MMP-9 and MMP-12. Immunohistochemical analysis showed reduced expression of CD31 and VEGF in tumors from A-FABP KO mice on either diet compared to tumors from WT mice. Taken together, these data suggest that A-FABP contributes to tumor growth and metastasis and implicate A-FABP as a link between fat consumption and cancer progression.

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Oxygen Deprivation Leads to Phospholipid Bilayer Disruption and Phospholipid Scramblase 1 Upregulation in Endothelial Cells

Emily E. Archer Slone, Mary Roth, Ruth Welti, Sherry D. Fleming

Kansas State University, Department of Biology, Manhattan, KS

Ischemia, a lack of blood flow, initiates cellular injury which is exacerbated by reperfusion, the return of blood flow. Ischemia/reperfusion (IR) events, including heart attack and stroke, are a major human health concern. The mortality rate associated with IR of the intestine is currently 60 to 80%. This high mortality rate is due to: 1) the sensitivity of the intestine to IR, 2) systemic activation of the innate immune response resulting in damage to other organs, and 3) the lack of suitable therapeutic targets. Previous studies indicated involvement of a lipid or asymmetric change of the phospholipid bilayer in intestinal IR-induced pathology. Three classes of translocators are responsible for maintaining the asymmetry of the phospholipid bilayer. Scramblase proteins are ATP-independent, bi-directional transporters with relatively low specificity for lipid substrates. Each member of the scramblase class is localized to a specific cellular compartment, with phospholipid scramblase 1 (PLSCR1) at the plasma membrane. These characteristics support PLSCR1 activity as a likely mechanism for exposure of neoantigens for antibody recognition in our IR model. We hypothesized that similar to ischemia, hypoxia induces phospholipid bilayer disruption exposing neoantigens via PLSCR1 activity. Lipids from IR or hypoxia treatment were analyzed by mass spectrometry and a significant increase in free arachidonic acid was found compared to controls. Like the tissue, the mouse endothelial cell line, MS-1, showed an increase in arachidonic acid, Cox-2 transcription, and prostaglandin E_2 production following oxygen deprivation. Furthermore, recent studies indicate a significant increase in PLSCR1 transcription following hypoxia treatment. We conclude that hypoxic treatment of MS-1 cells induces similar lipid changes as IR treatment and that further investigation of PLSCR1 as a mechanism of exposing neoantigens is warranted.

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Class IA PI3K p110α Regulates Phagosome Maturation

Emily P. Thi, Ulrike E. Lambertz, Neil E. Reiner

University of British Columbia, Department of Medicine, Vancouver, BC

Among the three classes of phosphatidylinositol 3- kinases (PI3Ks), only the class III enzyme Vps34 has been shown to regulate phagosome maturation. In the course of studying phagosome maturation in THP-1 cells deficient in class IA PI3K p110α, we discovered that this PI3K isoform is required for vacuole maturation to progress beyond acquisition of Rab7 leading to delivery

of lysosomal markers. Phagosomes from PMA-differentiated THP-1 cells possessed the class IA product, $PI(3,4,5)P_3$, and immunoblotting of phagosome lysates showed the presence of p110α. Flow organellometry of phagosomes isolated from p110α knockdown cells indicated that although the levels of PI3P did not change as compared to control, a significant decrease in the levels of $PI(3,4,5)P_3$ was observed, and this correlated with a decrease in phagosomal recruitment of p110α. Examination of phagosome maturation markers present on phagosomes from p110α deficient cells revealed normal acquisition of Rab5, but a marked defect in acquisition of early endosomal antigen EEA-1, as well the lysosomal markers LAMP-1 and LAMP-2. Likewise, these cells were defective in phagosomal delivery of the lysosomal hydrolase, β-galactosidase. Despite lacking EEA-1 and lysosomal components, phagosomes from p110α deficient cells recruited normal levels of Rab7 as well as homotypic vacuole fusion and protein sorting (HOPs) components Vps41 and Vps16, and SNAREs Vti1p and VAMP7. The latter observations demonstrated that phagosomal Rab7 was active and capable of recruiting effectors involved in membrane fusion. Nevertheless, active Rab7 was not sufficient to bring about the delivery of lysosomal proteins and EEA-1 to the maturing vacuole, which are shown for the first time to be dependent on a class I PI3K. Thus, at least two classes of PI3K regulate phagosome maturation.

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MicroRNA-155 and HDAC11 Regulate LPS Sensitization in Kupffer Cells of Alcohol-Fed Mice

Shashi Bala, Jan Petrasek, Timea Csak, Donna Catalano, Karen Kodys, Gyongyi Szabo

UMass Medical School, Dept of Medicine, Worcester, MA

Background : Increased liver fat accumulation and inflammatory cytokine production are hallmarks of alcoholic liver disease (ALD). Sensitization of liver macrophages (Kupffer cells; KCs) to gut-derived LPS and increased TNFα production play a central role in the pathogenesis of ALD. Epigenetic regulation of TNFα production involves microRNAs (miRs) and histone deacetylases (HDACs) and recent studies suggest that alcohol has epigenetic regulatory effects. In this study, we evaluated the role of epigenetic modifiers such miRs and HDACs in the sensitization of KCs to LPS after chronic alcohol feeding. Methods: 8 week-old C57/Bl6 female mice were fed with 5% alcohol (Liber-DeCarli diet) for 4 weeks. Total liver and KCs were isolated and analyzed for miRs, HDACs and their target genes.

Results : We found that miR-155, which is a major regulator of TNFα production, was upregulated in total liver and isolated KCs of alcohol-fed (AF) mice. Moreover, its induction was amplified upon LPS treatment. Hence we reasoned that upregulation of miR-155 sensitizes KCs to LPS. To evaluate this hypothesis, we examined the expression of miR-155 and its target genes, SOCS1, SHIP1 and IRAKM that are negative regulators of LPS signaling. We found increased miR-155 and decreased expression of its target genes after alcohol feeding. MiR-155 exerts positive effect on TNFα and there was increased TNFα in KCs of AF mice, which was further amplified upon LPS treatment, suggesting a role of miR-155 in LPS sensitization. IL-10, an anti-inflammatory cytokine, was significantly decreased, whereas HDAC11, a negative regulator of IL-10, was significantly increased in KCs of AF mice. Upon LPS stimulation, HDAC11 was reduced and IL-10 was increased in KCs of pair-fed but not in AF mice. There was no significant change in HDAC3, suggesting alcohol specifically targets HDAC11 to shut down IL-10, which in turn increases pro-inflammatory cytokine production.

Conclusion : Collectively our results indicate that LPS sensitization in KCs after chronic alcohol consumption is governed by epigenetic modifications.

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SirT6 and SirT1 Coordinate a Switch between Macrophage Glucose and Fatty Acid Oxidation during the Acute Inflammatory Response

Tiefu Liu, Charles McCall

Wake Forest University School of Medicine, Molecular Medicine, Winston-Salem, NC

The acute inflammatory response induces gene-selective epigenetic reprogramming, shifts in cellular bioenergetics, and changes in metabolism. We recently reported that nicotinamide phosphoribosyl transferase (Nampt), NAD+ generation, and redox sensor SirT1 direct gene-specific epigenetic reprogramming of macrophages during the switch from the proinflammatory initiation phase to the adaptive phase of acute inflammation. Here, we hypothesized bioenergy sirtuin sensors also reprogram metabolism during the acute inflammatory response and discovered that SirT1 and SirT6 differentially coordinate a switch from glucose to fatty acid oxidation as energy sources. Using the THP1 human monocytic cell line, we observed that the TLR4-initiated responses rapidly enhanced glucose flux by inducing expression of glucose transporter Glut1; increased glycolysis by inducing expression of rate-limiting 6- phosphofructose kinase; and disrupted mitochondrial glucose oxidation by increasing pyruvate dehydrogenase kinase 1(PDK1) that inactivates pyruvate dehydrogenase (PDH). As TLR4 stimulated cells entered the adaptive phase, glucose and fatty acid flux and catabolism shifted by SirT6-specific repression of glucose flux and SirT1-dependent increases in fatty acid flux, transport into mitochondria and beta oxidation. We further found that sirtuin-regulated hypoxia inducing factor-1α (HIF1α) transcription factor and PPAR γ co-activator 1α (PGC1α) supported the metabolic switch. As support of principle in human inflammation, we observed increased fatty acid flux and β oxidation and reduced glucose flux and oxidation in sepsis blood leukocytes. We conclude that a bioenergy sensor command station of sirtuins integrates epigenetics and metabolism to direct the course of acute inflammation.

ABSTRACTS

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Smoking Altered DNA Methylation in Alveolar Macrophages Correlates with Gene Expression

Martha M. Monick, Rory Sears, Linda S. Powers, Alicia Gerke, Ihab Hassan, Thomas J. Gross, Robert A. Philibert
Univ of Iowa, Department of Internal Medicine, Iowa City, IA

Rationale: Cigarette smoking is a leading cause of both emphysema and lung cancer, yet the mechanism of how smoking drives disease is unclear. Alveolar macrophages are key players in the development of smoking-related diseases. One important, but little studied, regulator of macrophage function is epigenetic modification of gene expression.
Methods: Smokers with and without emphysema, and nonsmokers underwent bronchoscopy with bronchoalveolar lavage to obtain alveolar macrophages. DNA and RNA were isolated using the Qiagen DNeasy kit and MirVana kit methods, respectively. Genome wide methylation status of the samples was determined using Illumina Infinium 27K HumanMethylation array. Macrophage mRNA expression was analyzed using GeneChip Human Exon 1.0 ST Arrays.
Results: The distribution of methylation differed significantly with respect to smoking status. There is enrichment of differential methylation in genes from inflammatory pathways. Consistent with recent findings, significant methylation changes were particularly enriched in the areas flanking CpG islands. Analysis of matching gene expression data demonstrated a parallel enrichment for changes in inflammatory pathways. Of note, many genes with altered mRNA levels had reciprocal alterations in DNA methylation.
Conclusion: The alveolar macrophage data obtained from smokers, emphysema patients and nonsmokers demonstrates methylation changes that track with smoke exposure. The affected genes cluster in inflammation-linked pathways and a significant number of the genes with differential methylation also have altered gene expression. This discovery suggests epigenetic modification of alveolar macrophages contributes to smoking-related lung disease.
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Cigarette Smoke Decreases Dicer Protein Levels and Leads to an Accumulation of a High Molecular Weight Dicer Complex

Anna Reisetter, Linda S. Powers, Emma Nash, Anne Dickson, Mary E. Wilson, Karol Kremens, Alicia Gerke, Ihab Hassan, Thomas J. Gross, Martha M. Monick
University of Iowa, Department of Internal Medicine and VA Medical Center, Iowa City, IA

Rationale: Cigarette smoking is a leading cause of both emphysema and lung cancer, yet the mechanism of how smoking drives disease is unclear. Alveolar macrophages are key players in the development of smoking-related diseases. MicroRNAs have recently been found to regulate transcription of upwards of 30% of genes and may be involved in smoking altered gene expression. **Methods**: Smokers and nonsmokers underwent bronchoalveolar lavage for alveolar macrophages. RNA was isolated and microRNA expression analyzed using TLDA plates from ABI. Macrophage mRNA expression was analyzed using GeneChip Human Exon 1.0 ST Arrays and RT-PCR. For in vitro studies, alveolar macrophages and THP-1 cells were exposed to cigarette smoke extract, N-acetylcysteine and both in combination. Immunoblotting and immunoprecipitation were performed. **Results**: MicroRNA expression in smoker's compared to nonsmokers was significantly down regulated with up to a third of the expressed microRNAs down regulated in smokers. Data showed a defect in the maturation of the microRNAs from the primary transcript to the mature transcript. Dicer is a cytosolic nuclease involved in microRNA maturation. There was no difference in Dicer mRNA expression in smokers; there was a difference in Dicer protein expression. Western blotting of smoker compared to nonsmoker macrophage lysates showed both a decrease in Dicer expression and an accumulation of Dicer containing high molecular weight complexes in smoker cells. N-acetylcysteine appeared to attenuate the acute effect of smoking on Dicer, suggesting that cigarette smoke modifies dicer protein via oxidative stress. **Conclusion:** Smoking decreases Dicer levels in alveolar macrophages leading to decreased expression of a subset of microRNAs. This is a potential mechanism for the altered gene expression found in smoker's lungs. Funding: NIH R01 HL079901 and NIH RO1 HL096625 to M. M. and UL1RR024979 from the National Center for Research Resources (NCRR).

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Replenishing the Immune System in Health, Disease and Old Age

Paul W. Kincade
Oklahoma Medical Research Foundation, Immunobiology & Cancer Program, Oklahoma City, OK

We and others have found that flow cytometry can reveal heterogeneity in hematopoietic stem cells (HSC). Individual HSC can be biased such that some are very poor sources of lymphocytes as contrasted to ones with balanced outputs. Still other HSC are effective at generating B and T cells, but defective with respect to expansion. While complicating our understanding of immune system replenishment, heterogeneity provides adaptability as hematopoiesis can be dramatically altered during infections, influencing numbers and types of cells produced.
HSC diversity carries forward to later events, and similar appearing cells can arise from alternate differentiation pathways. In fact, new categories of lymphoid progenitors are still being discovered. Patterns of surface marker expression and transcription profiles, as well as results obtained with reporter mice, suggest that lymphopoietic cells are not closely synchronized. Loss of differentiation options is gradual, and ultimate fate can be established at different stages of lineage progression.
Hematopoietic cells are influenced by pathogen products, and we have studied their responses to Toll-like receptor ligands. Acute exposure activates HSC and mobilizes them to the periphery, while differentiation options for progenitors shift. Chronic, low-dose exposure causes durable HSC changes that include loss of self-renewal potential and myeloid bias. That is, the HSC become

selectively unable to replenish the immune system. Patterns of gene expression and cell surface phenotypes suggest similarities and differences between changes that occur during normal aging. We believe that persistent, low-grade infections could contribute to immunosenescence.

Textbook diagrams depict blood cell formation in terms of sequential, binary fate decisions, where the progeny of stem cells follow distinct, unidirectional paths. However, there is evidence that at least some early events may be reversible; raising the possibility that reprogramming occurs in some circumstances. Overall, a picture is emerging of a dynamic process that responds to infections and age.

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Expression and Function of IL-17A and IL-25 Receptors in B Cell Malignancies of Germinal Centre Origin

Vito Pistoia, [1]Elisa Ferretti, [2]Emanuela Ognio, [3]Emma Di Carlo[4]
[1]G. Gaslini Scientific Inst , Genova; [2]G. Gaslini Inst., Genova; [3]IST, Genova; [4]Dapartment of Pathology, Genova

Follicular lymphoma (FL), diffuse large cell lymphoma (DLCL) and Burkitt lymphoma (BL) are B cell malignancies originating from the germinal centre of secondary lymphoid organs. FL, the second B cell lymphoma in order of frequency, is common in adults, but rare in children and adolescents. FL is an indolent diseases highly responsive to chemotherapy but still incurable, highlighting the need for novel therapies. Interleukin-(IL)-17A and IL-17E (IL-25) are two cytokines belonging to the IL-17 family, associated with inflammation and allergy, respectively. IL-17A and IL-25 receptors are heterodimeric, i.e. composed of the subunits IL17RA/IL-17RC for IL-17A and IL-17RA/IL-1RB for IL-25. In this study we have investigated the expression and the *in vitro* and *in vivo* function of IL-17A and IL-25 receptors on neoplastic cells from patients with FL, DLCL and BL and from cell lines thereof. High expression of IL-17Ra and IL-17Rb in primary neoplastic cells (n=18) and cell lines (SUDHL-4, DOHH2, LY8, Raji and RAMOS) was detected by flow cytometry. To investigate the effects of IL-17A and IL-25 treatment on tumour growth, we injected SUDHL-4 cells sub-cutaneously into NOD/SCID mice. The mice were treated with 3 weekly doses of IL-17A (1 μg/doses), IL-25 (1 μg/doses) or PBS (controls) for 20 days. After this time mice were sacrificed, and tumor masses were collected for molecular, cellular and histological analyses . IL-17A induced a significant increase of tumour masses, while IL-25 inhibited tumour growth. Histological and immunohistochemical studies showed, compared to controls i) decreased cell proliferation in tumor masses from IL-25 treated mice; ii) enhanced vascular networks after IL-17A treatment, and defective angiogenesis after IL-25 treatment; iii) necrotic areas in masses from IL-25 treated mice. Moreover, molecular analysis by PCR array showed up-regulation of selected pro-angiogenic genes in tumors from IL-17A treated mice, and down-regulation of other pro-angiogenic genes in tumors from IL-25 treated mice. *In vitro* incubation of primary germinal centre lymphoma cells with IL-17A caused increased cell proliferation compared to controls, while IL-25 was uninfluential. No differences were observed in the proportion of apoptotic cells in IL-17A or IL-25 treated *vs* untreated cells. Intracellular flow cytometric analysis demonstrated that neoplastic B cells synthesized IL-17A. This study shows for the first time that IL-17A and IL-25 can modulate the growth of germinal-centre derived B cell lymphomas.

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SerpinB1 and Cathepsin G Regulate Neutrophil Homeostasis in a Cell-Autonomous and Caspase-Independent Death Pathway

Charaf Benarafa, Mathias Baumann
University of Bern, Theodor Kocher Institute, Bern, Switzerland

In response to danger signals, mature neutrophils are rapidly mobilized from the bone marrow (BM) reserve to the circulation and to injured tissues. This reserve is considerably reduced in serpinB1-/- mice due to a survival defect of mature neutrophils. SerpinB1 is expressed at high levels in neutrophils but also in non-hematopoietic cells and it inhibits neutrophil granule serine proteases (NSP), such as elastase (NE), cathepsin G (CG) and proteinase-3. Here, we show that mice lacking serpinB1 and active NSP due to deficiency in dipeptidyl peptidase I (serpinB1-/-.dppi-/-) had a normal neutrophil reserve. Deficiency in CG, but not NE, was also sufficient to fully restore a normal number of BM neutrophils in serpinB1-/- mice. Using BM chimera, we showed that serpinB1 deficiency in hematopoietic cells is necessary and sufficient to reproduce the BM neutropenia observed in serpinB1-/- mice. In addition, when irradiated GFP+ mice were reconstituted with a 1:1 mixture of serpinB1-/- (CD45.2) and wild type (CD45.1) hematopoietic cells, only a small percentage of the mature neutrophils were from the serpinB1-/- donor, whereas B cells of the two genotypes were found in equal proportions, indicating a cell autonomous survival advantage of neutrophils expressing serpinB1. Finally, spontaneous apoptosis of serpinB1-/- neutrophils in vitro was significantly increased compared to wild-type and this survival defect was rescued in serpinB1-/-.CG-/- mice in the presence (and absence) of caspase inhibitor Q-VD-oph. Collectively, these findings suggest that serpinB1 regulates a cell autonomous and caspase-independent neutrophil homeostatic death pathway mediated by cathepsin G.

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Age-Associated Changes in Monocyte/Macrophage Function Predispose the Elderly to Infectious Disease

Dawn M.E. Bowdish, Alicja Puchta, Chris P. Verschoor, Jennie Johnstone
McMaster University, Department of Pathology & Molecular Medicine, Hamilton, ON, Canada

Across the developed world the population is aging and as a result age-associated infections such as pneumonia occur with increasing frequency and cost. Although we know that the elderly have higher rates of infection, what little is known about age-associated changes in immune function (immunosenescence) focuses on changes in the adaptive immune system. In order to study age-associated changes in the innate immune response, particularly with regard

to the function of resident macrophages and recruited monocytes we have developed a unique aged mouse model of *Streptococcus pneumoniae* colonization of the upper respiratory tract (URT). Colonization of the nasopharynx by *S. pneumoniae*, which in healthy adults is generally asymptomatic and transient, is an essential pre-requisite to infection and is controlled in large part by resident and recruited macrophages. Using clinical isolates we have compared the ability of young (10-14wk) and aged mice (14mo, 20mo) to control bacterial colonization of the nasopharnyx. Aged mice have higher bacterial loads in the nasopharynx and develop fulminant pneumonia when colonized with *S. pneumoniae* strains that are rarely invasive in young mice. Translational studies in elderly patients indicate that rates of colonization are lower than in children and adults but that infection rates are higher, indicating that control of colonization is impaired. Multiple mechanisms appear to contribute to this loss of control including defects in leukocyte recruitment to the nasopharnyx due to skewed chemokine production and depletion of monocyte pools in the aged mice. In addition, recognition and cytokine responses to *S. pneumoniae* by human and murine macrophages are altered with age, but not necessarily suppressed, indicating that global immunosuppresion is not the primary reason for increased infection risk in the elderly and implying that changes in the microenvironment may contribute to changes in macrophage function.

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Hematopoietic Stem and Progenitor Cells Traffic to *S. aureus*-Infected Wounds Where They Proliferate and Differentiate along the Myeloid Lineage in a MyD88-Dependent Manner

Jennifer Granick,[1] Dori Borjesson,[1] Scott Simon[2]

[1]University of Calfifornia Davis, Vet Med: Pathology, Microbiology, Immunology, Davis, CA; [2]University of California Davis, Biomedical Engineering, Davis, CA

We have shown that lineage negative c-kit^{+} hematopoietic stem and progenitor cells (HSPC) home to the site of *Staphlyococcus aureus*-infected cutaneous wounds and there give rise to mature neutrophils (PMN). This response is physiologically important as antibody knock-down of HSPC resulted in a significant increase in wound bacterial burden, decrease in PMN number and increase in wound size. The initial inflammatory insult of wounding recruits HSPC in equal numbers to *S. aureus*-infected and saline sham-infected wounds 24 hours after the wounding event, but this influx is amplified in infected wounds on days 3 and 5 post-wounding. We observed a 3-fold increase in the number HSPC that traffic to wounds compared to saline sham-infected controls in C57Bl/6 mice on day 3 post-wounding. In contrast, mice lacking MyD88, the universal adaptor protein for toll-like receptors and the interleukin-1 family of receptors, there was no change in the number of HSPCs that home to a *S. aureus*-infected wound compared to saline controls. Similarly, ex vivo colony forming capacity of HSPCs collected from *S. aureus*-infected wounds is nearly twice that of those from saline controls in C57Bl/6 mice and expansion was absent in MyD88 knock-out mice, which was equivalent to that of C57Bl/6 saline sham controls. We conclude that *S. aureus* induces MyD88-dependent homing to and proliferation and differentiation of HSPCs at the site of cutaneous infection and this process is necessary for optimum wound resolution and healing.

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MicroRNA Control of DC Differentiation

Pieter J.M. Leenen,[1] Joey Riepsaame,[2] Sjaak N.J. Philipsen,[3] Joris Pothof[4]

[1]Department of Immunology, Erasmus MC, Rotterdam, The Netherlands; [2]Dept. Immunology, Erasmus MC, Rotterdam, The Netherlands; [3]Dept. Cell Biology and Genetics, Erasmus MC, Rotterdam, The Netherlands; [4]Dept. Cell Biology and Genetics, Erasmus MC, Rotterdam, The Netherlands

Dendritic cell (DC) differentiation is a complex process that requires tight control as DC are essential in immune homeostasis. To investigate the involvement of microRNAs in DC development, we profiled different *in vitro* generated DC subsets and identified differentially expressed microRNAs. Here, I highlight two microRNA-controlled targets: transcription factor Sp1 and M-CSF receptor (CD115). These molecules appear to be novel regulators of DC development. Sp1 is a target of miR-29a and miR-411. These microRNAs are down-regulated in the transition of DC precursors to immature DC in BM-derived DC development and this coincides with increased Sp1 mRNA and protein expression. Transient over-expression of miR-29a and miR-411, pharmacological inhibition of Sp1, or myeloid-specific ablation of Sp1 in a knockout mouse model all impaired GM-CSF-BMDC differentiation. In marked contrast, Flt3L-stimulated generation of conventional DC (cDC) and plasmacytoid DC (pDC) increased significantly when BM from Sp1-deficient mice was cultured. Also *in vivo*, development of Flt-3L-dependent DC was increased in these mice, as indicated by the highly elevated numbers of cDC and pDC in the spleen. These findings indicate that Sp1 is a newly identified transcription factor in DC development, which influences the balance between steady-state cDC and pDC vs. inflammatory monocyte-derived DC. M-CSFR is targeted by miR-155, miR-22 and miR-34a. Expression of these microRNAs was up-regulated in MHCII^{hi} mature BMDC and this mediated M-CSFR mRNA and protein down-regulation in both GM-CSF- and Flt3L-stimulated BMDC. Inhibition of the M-CSFR-targeting microRNAs *in vitro* not only resulted in sustained high level M-CSFR expression but also impaired LPS-induced DC maturation. In accordance, over-expression of M-CSFR in BMDC markedly inhibited development of MHCII^{hi} DC, indicating M-CSFR down-regulation as requirement for final DC maturation. Taken together, these results identify microRNAs as important regulators of crucial steps in DC development.

ABSTRACTS

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A STATus Report on Plasmacytoid Dendritic Cell Development

Stephanie S. Watowich,[1] Cliff Yang,[1] Eiji Esashi,[1] Huiyuan Zhang,[1] Hoainam Nguyen-Jackson,[1] Yong-Jun Liu,[1] Wllem Overwijk,[1] Ananda Goldrath,[1] Gustavo J. Martinez,[1] Haiyan Liu[2]
[1]University of Texas, MD Anderson Cancer Center, Department of Immunology, Houston, TX; [2]University of California, Department of Biological Sciences, San Diego, CA

The plasmacytoid dendritic cell (pDC) lineage was originally defined by its unique morphology and ability to produce massive quantities of type I interferons (IFNs) upon viral infection. PDCs undergo maturation to an antigen-presenting state and have important roles in anti-viral responses and immune tolerance. Moreover, pDC deregulation is linked with certain autoimmune disorders or cancer. Thus, understanding pDC developmental mechanisms is expected to reveal new approaches to control their production and function in a variety of human disease settings. Generation of pDCs is guided by cytokines and lineage-restricted transcription factors ; however, we lack insight into whether or how these pathways intersect to control pDC development. Using mice with genetic deletion of individual STAT genes, we uncovered signaling cascades regulating transcription factors with important roles in pDCs, as well as the consequences of STAT signaling on pDCs *in vivo*. We determined that granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibits pDC development by a STAT5-dependent mechanism. This involves STAT5-mediated suppression of *Irf8*, a positive regulator of pDCs, and induction of *Id2*, an inhibitor of the E-box protein E2-2 that directs pDC maturation *in vivo*. By contrast, STAT3 is critical for pDC homeostasis. STAT3 appears to operate in part by stimulating transcription of *Tcf4,* encoding E2-2, in response to FMS-like tyrosine kinase receptor 3 (Flt3) activation. Type I IFN signaling via STAT1 also regulates pDC generation when administered at doses achieved during acute viral infection, and has a cell autonomous role in accrual of Peyer's Patch pDCs under steady state conditions. IFN-alpha directly stimulates *Irf8* transcription via STAT1, which may contribute to promoting pDC development *in vivo*, yet IFN also suppresses *Tcf4* through as yet undefined mechanisms. The outcome of IFN-STAT1 signaling in pDCs is production of increased inflammatory cytokines and enhanced 17-producing Th cell generation upon TLR ligation, versus pDCs that mature under the guidance of Flt3-STAT3. Collectively, our data indicate that cytokine-activated STATs regulate the production and function of pDCs in part by controlling expression of transcription factors with important roles in DC development.

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The Complement Protein C1q Modulates Macrophage Activation and NLRP3 Inflammasome Activity during the Uptake of Apoptotic Cells

Marie E. Benoit, Elizabeth V. Clarke, Deborah A. Fraser, Pedro Morgado, Andrea J. Tenner
University of California Irvine, Department of Molecular Biology and Biochemistry, Irvine, CA

Deficiency in C1q, the recognition component of the classical complement cascade, leads to lupus-like auto-immune diseases characterized by auto-antibodies to self proteins and aberrant innate immune cell activation likely due to impaired clearance of apoptotic cells, which has been hypothesized to be in part due to the lack of C1q enhanced clearance of dying cells and suppression of the release of pro-inflammatory cytokines in LPS-stimulated monocytes and macrophages. Here we developed an autologous system using primary human lymphocytes and monocyte-derived macrophages (HMDMs) to characterize the effect of C1q on macrophage gene expression profiles during the uptake of apoptotic cells using whole genome microarray. C1q bound to early (EAL) and late (LAL) primary human apoptotic lymphocytes and enhanced their uptake by HMDMs. Second, C1q bound to EAL and LAL modulating expression of genes related to signal transduction and inflammation. Specifically, C1q induced type I interferons (IFNs) and IL-27, known to act sequentially to induce IL-10, an anti-inflammatory cytokine also up-regulated by C1q. Neutralization of type I IFNs partially prevented IL-27 induction by C1q. Finally, C1q decreased NLRP3 expression, increased POP1/ASC2 and NLRP12 expression, two negative regulators of inflammasome activity and inflammation, and suppressed caspase-1 dependent cleavage of IL-1β . In summary, C1q plays a role in containing the inflammatory response during removal of apoptotic cells and represents a potent immunoregulatory molecule of innate immune responses. This study identifies specific and novel molecular pathways induced by C1q to suppress macrophage inflammation and provides an initial understanding of the consequences of C1q-macrophage interactions in humans. Supported by NIH AI 41090 .

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TLR9 Targeted Treatment for the Immune Consequences of Radiation and Radiation Combined Injury

Veit Stoecklein, Akinori Osuka, James A. Lederer
Brigham and Women's Hospital, Harvard Medical School, Department of Surgery, Boston, MA

Nuclear events cause variable levels of radiation injury and combined trauma – referred to as radiation combined injury (RCI). Opportunistic infections and sepsis are a serious complication of radiation and RCI. We developed a mouse model to mimic RCI by exposing mice to radiation and near-simultaneous burn injury. In this study, this model was used to test the effectiveness of CpG oligodeoxynucleotides (ODNs), which are TLR-9 agonists, as treatments to enhance survival after secondary septic challenge from cecal ligation and puncture (CLP) following RCI. Mice were subjected to 1 Gy RCI and

treated on day 1 with a class A or B CpG ODN or saline. Seven days after RCI, mice underwent CLP to measure sepsis survival rates. Blood, spleens, and lymph nodes were prepared from CpG ODN treated and control mice to measure immune cell subsets by FACS. Cytokine profiling of spleen cell responses to LPS or anti-CD3 antibody was used to assess immune system phenotypes. Mice treated with a class B CpG (n=19 per group) had a slightly increased survival when compared to saline-treated controls (85% vs. 68%, p=0.21). However, mice treated with a class A CpG (n=21 per group) had a highly significant survival benefit (95% vs. 62%, p=0.0074). The results of immune system phenotyping studies showed that FoxP3+ regulatory T cells (Tregs) were significantly increased in mice treated with class A CpG ODN. Tregs were also slightly increased in class B CpG ODN-treated mice. Furthermore, LPS-induced IL-1beta, IL-6, IL-33 and TNFalpha were significantly decreased in the class A CpG group, but not the class B treated group. Thus, class A CpG ODN given early after RCI improves survival from secondary septic challenge in RCI. The changes seen in immune phenotype studies suggest that CpG ODN treatment enhances Tregs, which in turn may blunt the pathogenic response to sepsis in mice exposed to RCI.

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NADPH Oxidase 2 is Required for Normal Resolution of Systemic Inflammatory Responses

Laura C. Whitmore, Kelli L. Goss, Brieanna M. Hilkin, Erin M. Wahle, Francis J. Miller, Jessica G. Moreland

University of Iowa, Department of Pediatrics, Iowa City, IA

NADPH oxidase 2 (Nox2)-derived reactive oxygen species are critical for the effective killing of many pathogens by polymorphonuclear cells (PMNs). Patients with chronic granulomatous disease, who lack functional Nox2, are not only prone to serious infections, but also exhibit unexplained chronic inflammation, implicating a potential role for Nox2 in the resolution of inflammatory processes. Systemic inflammatory response syndrome (SIRS) is a clinical syndrome which includes multi-organ dysfunction and may occur as a component of bacterial sepsis, or in response to a sterile inflammatory stimulus. Using a murine model of sterile, generalized inflammation (intraperitoneal zymosan), we hypothesized that Nox2 would be necessary to resolve systemic inflammatory responses, and predicted that Nox2-deficient mice would exhibit excessive inflammation and mortality. A dramatic difference in mortality was observed between zymosan-injected $Nox2^{-/y}$ ($gp91^{phox-/y}$) and wildtype mice with 73% vs. 10% mortality, respectively, 3 days post-injection. Bronchoalveolar lavages (BALs) were performed to examine immune cell infiltration; whereas the wildtype mice BAL fluid had <1% PMNs, $Nox2^{-/y}$ mice BAL fluid had an average of 32% PMNs. Increased PMN infiltration and hemorrhage in the lungs of $Nox2^{-/y}$ mice was confirmed by histology. Similar levels of PMN recruitment to the peritoneum were observed, but PMNs from $Nox2^{-/y}$ mice exhibited increased surface expression of CD11b, an adhesion molecule, compared to wildtype, suggesting enhanced activation. Analysis of serum cytokines using a Bio-Plex Multi-Plex Assay indicated that several cytokines including IL-6, IL-10, IL-17, G-CSF and Eotaxin were substantially elevated in zymosan-injected $Nox2^{-/y}$ vs. wildtype. Considered in combination, these data are consistent with enhanced systemic inflammatory responses in mice lacking Nox2, implicating a requirement for Nox2 in inflammation resolution. We speculate that an absence of functional Nox2 may alter the interaction between the innate and adaptive immune responses skewing the T lymphocyte population toward subsets that promote inflammation. Funded by Carver Collaborative Grant.

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The Spatiotemporal Dynamics of Acetaminophen-Induced Liver Injury Lead to the Development of a Novel Lipid-Mediated Anti-inflammatory Therapeutic Strategy

Suraj J. Patel, Stefan Bohr, Kevin R. King, Arvin Iracheta-Vellve, Matthew Li, Martin L. Yarmush

Harvard Medical School, Massachusetts General Hospital, Boston, MA

Acetaminophen (APAP) hepatotoxicity is the most common cause of acute liver failure and is recognized as a significant public health problem. During APAP-induced hepatotoxicity, liver injury is caused by both the toxic drug metabolite and secondary innate immune activation. Unfortunately treatment remains limited to supportive care and transplantation, and specific medical therapies such as N-acetylcysteine (NAC) target only the initial toxic insult, and therefore depend on an extremely narrow temporal window for successful intervention. Here we show that APAP- induced hepatotoxicity is a biphasic injury, where the initial toxic insult is temporally followed by an overly exuberant neutrophil-mediated inflammatory response that both, amplifies the overall injury and limits recovery. Inhibiting neutrophil infiltration into the liver with resolvins, a recently identified group of endogenous lipid mediators that promote resolution during acute inflammation, protected against liver damage and successfully rescued mice from fatal APAP hepatotoxicity w hen administered as late as 12 hours after APAP challenge. This represents a 8-fold increase in the time-to-rescue window, compared to mainstay NAC therapy. Resolvin therapy limited systemic neutrophil motility, preventing their accumulation in the liver, and spatially confined the hepatocellular damage to only the toxic metabolite-injured cells, sparing the surrounding uninjured parenchyma. In summary, these findings reveal the spatiotemporal dynamics of liver injury during APAP toxicity, and demonstrate the feasibility of a new resolvin-based therapeutic strategy for rescuing from drug-induced hepatotoxicity by inhibiting the sterile inflammatory response.

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ABSTRACTS

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Leukocyte Adhesion and Signaling Under Flow

Rodger McEver
Oklahoma Medical Research Foundation, Cardiovascular Biology Research, Oklahoma City, OK

Circulating leukocytes emigrate into lymphoid organs or inflamed sites through sequential adhesive and signaling events. They tether to and roll on endothelial cells, then decelerate, arrest, and crawl into underlying tissues. Selectin-ligand interactions mediate tethering and rolling, whereas integrin-ligand interactions mediate deceleration, arrest, and crawling. L-selectin, expressed on leukocytes, binds to ligands on other leukocytes and some endothelial cells. P-selectin, expressed on activated platelets and endothelial cells, and E-selectin, expressed on activated endothelial cells, bind to ligands on leukocytes. Cell adhesion faces major kinetic and mechanical constraints in flowing blood. Bonds between adhesion molecules must form rapidly, and their lifetimes are affected by the forces applied by shear stress. Clustering of adhesion receptors in membrane domains modulates their functions. Engagement of selectin ligands on rolling neutrophils triggers a lipid raft-dependent signaling cascade that serially activates Src family kinases, Syk, Bruton's tyrosine kinase, and p38. These and other signals convert integrin $\alpha_L \beta_2$ to an extended but low-affinity conformation that slows rolling velocities. Signaling through G protein-coupled chemokine receptors activates integrins to an extended and high-affinity conformation that mediates arrest. These signaling pathways cooperate to maximize neutrophil recruitment to sites of inflammation. Dysregulated leukocyte adhesion contributes to inflammatory tissue damage in common diseases.

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Trafficking of Dendritic Cells into the Central Nervous System during Neuroinflammation

Pooja Jain,[1] Catherine Foss,[2] Divya Sagar,[1] Zafar K. Khan,[1] Martin Pomper,[2] Sharrón L. Manuel[1]
[1]Drexel University College Of Medicine, Doylestown, PA; [2]Johns Hopkins University, Baltimore, MD

In the healthy individual infiltration of lymphocyte into the central nervous system (CNS) is tightly controlled by the highly specialized blood-brain barrier (BBB). However, during neuroinflammation circulating lymphocytes readily cross the BBB and gain access to the CNS leading to edema, inflammation and demyelination. Interaction of circulating leukocytes with the endothelium of the BBB thus represents a critical step in the pathogenesis of neuroinflammatory diseases. To characterize the mechanisms of leukocyte trafficking, we have used cellular imaging as well as transmigration studies by utilizing an in vitro BBB model. Interaction of dendritic cells (DCs), monocytes, CD4 and CD8 T cells with primary human brain-derived microvascular endothelial cells was monitored at various time points. We have demonstrated that DCs (the most potent antigen presenting cells) exhibit a greater migratory potential compared to other immune cells tested during both steady state and upon activation with inflammatory mediators. The presence of chemokine CCL2 or MCP-1 facilitated transmigration of leukocytes with DCs again being the top responding cell type. We further proceeded to study the in vivo migration of DCs using non-invasive tools such as PET, optical and SPECT. We successfully traced peripherally injected exogenous DCs to the site of inflammation using PET as well as optical imaging by PSMA and luciferase (respectively) as probe. The ongoing studies are focused on tracing the path of radiolabeled DCs in the classic model of neuroinflammation (EAE) by 3-D SPECT imaging. Overall, these studies contribute to the growing importance of DCs with respect to the neuroinflammatory process due to neurotropic infectious pathogens or autoimmune phenomenon such as multiple sclerosis.

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Neutrophil Adhesion to Endothelial Cells Is Necessary for GM-CSF Induced Expression of Oncostatin M

Wafa Elbjeirami,[1] Clifton W. Smith[2]
[1]King Hussein Cancer Center, Pathology and Laboratory Medicine, Amman, Jordan; [2]Baylor College of Medicine, Pediatrics, Houston, TX

During and following transendothelial migration, neutrophils undergo a number of phenotypic changes resulting from encounters with endothelial-derived factors. This report uses an in vitro model with HUVEC and isolated human neutrophils to examine the effects of two locally-derived cytokines, granulocyte-macrophage stimulating factor (GM-CSF) and colony-stimulating factor (CSF) on oncostatin M (OSM) expression. Neutrophils contacting activated HUVEC expressed and released increased amounts of oncostatin M (OSM), a proinflammatory cytokine known to induce PMN adhesion and chemotaxis. Neutrophil transendothelial migration resulted in three fold higher OSM expression and protein levels compared to non-transmigrated cells. Addition of anti-GM-CSF neutralizing antibody reduced OSM expression level but anti-G-CSF was without effect. GM-CSF but not G-CSF protein addition to cultures of isolated neutrophils resulted in a significant increase in OSM protein secretion. However, inhibitionof $ß_2$ integrins by neutralizing antibody significantly reduced GM-CSF-induced OSM production indicating this phenomenon is adhesion dependent. Subcellular fractionation of neutrophil granules revealed a pre-existing pool of OSM with highest levels seen in MMP-9 rich population of granules with highest seen in gelatinase granules. Thus, cytokine-stimulated EC can produce sufficient quantities of GM-CSF to influence in an adhesion dependent manner the phenotypic characteristics of neutrophils resulting in the latter's transmigration. Both transmigration and adhesion phenomenon lead to increased production of OSM by neutrophils which then play a major role in inflammatory response.

ABSTRACTS

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CD93 Regulates Leukocyte Migration, Vascular Integrity and Complement Activation in Murine Peritonitis

Mallary C. Greenlee-Wacker*, Carlos Briseño*, Manuel Galvan*†, Gabriela Moriel*, Peter Velázquez *†, and Suzanne S. Bohlson*†

**Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame, Notre Dame, IN 46556†,Department of Microbiology & Immunology, Indiana University School of Medicine-South Bend, South Bend, IN 46617*

CD93 is emerging as a novel regulator of inflammation; however its exact molecular function is unknown. CD93 is expressed on cells involved in the inflammatory cascade, including myeloid and endothelial cells. Full length cell-associated CD93 and a soluble truncated form (sCD93) were elevated during thioglycollate-induced peritonitis. $CD93^{-/-}$ mice showed a 1.6 to 1.8-fold increase in leukocyte infiltration into the inflamed peritoneal cavity between 3 and 24 hours post injection of thioglycollate that returned to wildtype levels by 96 hours. Accompanying increased leukocyte recruitment, $CD93^{-/-}$ mice showed impaired vascular integrity, as demonstrated using fluorescence multi-photon intravital microscopy. $CD93^{-/-}$ mice also showed dysregulated C1q-hemolytic activity compared to wildtype controls. C1q-hemolytic activity in $CD93^{-/-}$ mice was 88% and 54% of wildtype activity at 0 and 3 hours post injection of thioglycollate, respectively. Leukocyte recruitment and C1q-hemolytic activity were restored to wildtype levels when CD93 was expressed on either hematopoietic or non-hematopoietic (radiation-resistant) cells in bone marrow chimeric mice. However, elevated levels of sCD93 in peritoneal lavage fluid were only observed when CD93 was expressed on non-hematopoietic cells. Since cell-associated CD93 was sufficient to restore a normal inflammatory response, these data suggest that cell-associated CD93, and not sCD93, regulates leukocyte recruitment and complement activation during murine peritonitis. To determine the mechanism by which cell-associated CD93 regulates complement activity we compared the complement regulatory activity of CD93 to its homologue, thrombomodulin (TM). Previous studies demonstrated that TM accelerates the inactivation of C3b into iC3b. We confirmed that TM promoted C3b inactivation on the surface of CHO-K1 cells; however, CD93 failed to promote the inactivation of C3b in this system. These data demonstrate that CD93 regulates leukocyte migration, vascular integrity and C1q hemolytic activity during peritonitis, and future efforts are aimed at identifying the molecular mechanism by which CD93 regulates these inflammatory functions.

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The Biology of Macrophage Colony-Stimulating Factor (CSF-1) and Its Receptor

David A. Hume

The Roslin Institute, University of Edinburgh, Midlothian, Scotland, UK

The receptor for macrophage colony-stimulating factor (csf1r) is expressed specifically in cells of the mononuclear phagocyte system. The receptor binds to two distinct ligands, CSF-1 and the recently-described IL34. This pattern is conserved across mammals and birds. The two ligands most likely bind to distinct parts of the receptor but have not yet been shown to have distinct biological functions. Although CSF-1 can promote monocytopoiesis in vivo, and macrophage proliferation in vitro, neither CSF-1 deficiency (in the op/op mouse) nor csf1r null mutation ablates circulating monocyte numbers. Mouse monocytes can be broadly subdivided based upon their expression of surface markers such as Ly6C. Administration of a high affinity antibody against the CSF1R in the mouse generated a time-dependent decline in the ly6C-negative population, with a corresponding increase in the ly6C-positive cells. In keeping with the view that ly6C-negative monocytes are the precursors of resident macrophages, the antibody treatment also caused a slow depletion of tissue macrophages in the majority of locations throughout the body, but had no effect on inflammation in a wide range of model systems. Hence, the main non-redundant function of CSF-1 in the mouse is to promote monocyte maturation towards a resident phenotype. These observations suggest alternative applications in the clinic. CSF-1 was cloned and expressed in the 1980s, but has not found a major clinical application. CSF-1 promotes expression in macrophages of genes involved in tissue repair, such as IGF-1 and urokinase plasminogen activator. Treatment of mice with CSF-1 can promote tissue repair in a range of model systems, including ischemia reperfusion injury and bone fracture. We are expressing CSF-1 molecules from multiple species to test alternative applications in tissue repair.

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Regulation of Innate Host Factors by *Mycobacterium avium* Perpetuates Survival in Human Macrophages

Nancy Vazquez,[1] Sofia Rekka,[1] Jan Orenstein,[2] Sharon M. Wahl[1]

[1]NIH, NIDCR, Bethesda; [2]George Washington University, Washington, DC

Mycobacterium avium complex *(MAC)* is an environmental microorganism that causes opportunistic infections (OI) in immunocompromised hosts, but rarely causes illness in healthy individuals. Absence of a properly functioning acquired immune response in HIV+ individuals allows *M. avium* progression and persistence within macrophages, particularly evident in HIV-1 and *M. avium* co-infected tissues, such as lymph nodes, intestines and lungs. Also, immune reconstitution inflammatory syndrome (IRIS) with a transient focal manifestation of MAC of variable duration after the initiation of antiretroviral therapy has been increasingly reported in HIV-1 infected individuals. Moreover, the use of TNFα blockers for the treatment of autoimmune

diseases has also led to an increased risk of infection/reactivation with various mycobacteria species. Macrophages are essential in controlling *M. avium* infection, but can become heavily infected in a situation of reduced $CD4^+$ IFN γ producing T cells as occurs in AIDS patients. While considered a consequence of deficiencies in IFN γ, it is still unclear how *M. avium* averts innate immunity. Infiltration and retention of macrophages in infected lymph nodes (LN) reflect active recruitment. *In vitro* infection of monocyte-derived macrophages with *M. avium* induces the transcription of IL-17, consistent with the presence of elevated IL-17 in co-infected LN despite the reduced number of T cells. Inhibition of the NFκB pathway suppressed mycobacteria-induced IL-17, likely blocking a TLR2 signal. Although not normally associated with macrophages, increased IL-21 gene expression by infected macrophages can perpetuate IFN unresponsiveness by sustaining suppressors of cytokine signaling (SOCS) expression. These data suggest that induction of the inflammatory cytokines IL-17 and IL-21 by *M. avium* in its macrophage hosts may, in part, enable the requisite critical mass of macrophages to harbor and propagate mycobacterial infections.

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Role of Galectin-3 in Classical and Alternative Macrophage Activation and Inflammatory Mediator Production during Acetaminophen-Induced Hepatotoxicity

Ana Cristina Dragomir,[1] Richard Sun,[1] Jeffrey D. Laskin,[2] Debra L Laskin[1]

[1]Rutgers University, Dept of Pharmacology & Toxicology, Piscataway, NJ; [2]UMDNJ-RWJ Medical School, Department of Envrn & Community Med, Piscataway, NJ

Accumulating evidence suggests that macrophages and mediators they release play a key role in hepatotoxicity induced by the analgesic, acetaminophen (APAP). However, their specific contribution to the pathogenic process appears to depend on their phenotype.In the present studies, we analyzed the role of the β-galactoside binding lectin, galectin-3 (Gal-3), in regulating macrophage activation phenotype and hepatotoxicity. Treatment of wild-type mice with a hepatotoxic dose of APAP (300 mg/kg, i.p.) resulted in a time-dependent induction in Gal-3 mRNA and protein expression in the liver, which reached a maximum after 48-72 hr. Immunohistochemistry indicated that Gal-3 was predominantly expressed by macrophages infiltrating into necrotic regions of the liver. To analyze the role of Gal-3 in APAP-induced hepatotoxicity, we used Gal-$3^{-/-}$ mice. In wild-type mice, APAP caused a time-dependent increase in serum transaminase levels, beginning within 6 hr. This was associated with histological evidence of centrilobular necrosis, which became prominent after 24-48 hr. These effects were significantly attenuated in Gal-$3^{-/-}$ mice. APAP-induced increases in expression of CD98, the macrophage receptor for Gal-3, as well as the classical macrophage activation markers, inducible nitric oxide synthase, interleukin-12, and the proinflammatory chemokines CCL3, CCL4, and CCL20 were also reduced. In contrast, expression of the alternative macrophage activation markers, Ym1 and Fizz-1 and the anti-inflammatory protein, galectin-1, was increased following APAP intoxication in Gal-$3^{-/-}$ mice, relative to wild type mice. This was correlated with increased hepatocyte proliferation and expression of proliferating cell nuclear antigen, markers of tissue repair. Taken together, these data provide support for the hypothesis that classically and alternatively activated macrophages play distinct roles in tissue injury and repair following APAP intoxication; moreover, Gal-3 plays an important role in regulating macrophage proinflammatory phenotype. Supported by NIH grants GM034310, ES004738, CA132624, AR055073 and ES005022.

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A Novel C1q-Dependent Pathway for Engulfment of Apoptotic Cells

Manuel D. Galvan[1,2], Bryanne D. Foreman[2], Suzanne S. Bohlson[1,2]

[1]Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame, South Bend, IN, *[2]Indiana University School of Medicine-South Bend, South Bend, IN*

Failure to efficiently clear apoptotic cells is linked to defects in development and autoimmunity. Complement component C1q is required for efficient clearance of apoptotic cells, and C1q deficiency is the strongest known susceptibility factor for autoimmunity associated with lupus. Despite the importance of C1q in apoptotic cell clearance, the mechanism by which C1q enhances apoptotic cell clearance is poorly understood. Here we describe a novel C1q-dependent engulfment mechanism for apoptotic cells. As shown previously, C1q directly enhanced phagocytosis of multiple target particles including antibody opsonized targets and mycobacteria, and engulfment was pertussis toxin sensitive. Prolonged macrophage exposure to C1q (4 or 18 hour incubation) specifically enhanced engulfment of apoptotic cells. The delayed C1q-dependent activation kinetic suggested a requirement for de novo protein synthesis, and thus macrophages were treated with cycloheximide to inhibit protein synthesis. Treatment with cycloheximide inhibited C1q-dependent engulfment of apoptotic cells but not C1q-dependent FcγR-mediated phagocytosis. The cycloheximide sensitive engulfment pathway was specific for C1q since the related collectin, MBL, failed to activate this pathway. Our results demonstrate an alternative C1q-dependent engulfment pathway whereby C1q elicits a macrophage phenotype specifically tailored for apoptotic cell clearance, and thus deficiency in C1q may contribute to lupus by altering macrophage protein expression required for effective apoptotic cell clearance. NIH R56 AI080877-07, IUSM Research Support Funds Grant, Eck Institute for Global Health

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Plasticity of Macrophage Phagocytic and Antimicrobial Programs

Dennis Montoya, Rosane M. Teles, Benjamin Ferguson, Robert L. Modlin

UCLA, Department of Medicine, Los Angeles, CA

A key issue in macrophage (Mφ) biology is the plasticity of differentiated Mφ . The rapid clearance of bacilli in leprosy skin lesions is associated with a conversion in Mφ program from the phagocytic to antimicrobial program. To investigate Mφ plasticity,

we utilized an *in vitro* model in which IL-10 or IL-15 induced the phagocytic or antimicrobial programs, respectively. Treatment of human monocytes with IL-10 for two days resulted in the induction of CD209, CD163, and scavenger receptors, promoting phagocytosis of mycobacteria and oxidized LDL (oxLDL), similar to Mφ found in the disseminated form of leprosy. To test plasticity, Mφ differentiated by IL-10 were then cultured with signals of the innate versus acquired immune response: IL-15 , toll-like receptor (TLR) 2/1 mycobacterial-derived ligand, and/or IFN-γ treatment for two more days. Only the innate and adaptive immune signals of TLR2/1 activation plus IFN- γ together , but not either signal alone, reprogrammed IL-10 derived Mφ to the antimicrobial phenotype. Furthermore, the TLR2/1 and IFN- γ stimulation induced the VDR antimicrobial pathway and decreased phagocytic uptake of oxLDL. IL-15 derived Mφ subsequently cultured with IL-10 converted from the antimicrobial to phagocytic phenotype and induced phagocytic uptake. These data demonstrate plasticity in Mφ phenotype and functional programs and provide potential therapeutic intervention for infectious disease.

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The Self Renewing, Multipotent CD8+ Memory Stem Cells

Luca Gattinoni,[1] Enrico Lugli,[2] Mario Roederer,[2] Nicholas P. Restifo[2]

[1]NIH, NCI, Tumor Immunology, Bethesda, MD; [2]CCR, NCI, NIH, Bethesda, MD

Immunological memory is thought to depend upon a stem cell-like, self-renewing population of lymphocytes capable of differentiating into effector cells in response to antigen re-exposure. Mouse studies have recently revealed the existence of a long-lived self-renewing $CD8^+$ memory population with the multipotency to generate and sustain all memory and effector subsets. These T cells termed "memory stem cell" (T_{SCM}) were found within the phenotypically naïve-like T cell compartment. We have now identified human T_{SCM}. *In vivo* these resting cells have a largely naïve phenotype but express high levels of CD95, IL-2Rβ , CXCR3, and LFA-1. T_{SCM} exhibit numerous functional attributes distinctive of memory cells, including a history of multiple replications and the ability to rapidly acquire effector functions upon antigen re-challenge and proliferate in response to homeostatic cytokines. Compared to conventional memory populations, T_{SCM} show enormous expansion capability and more efficiently reconstituted immunodeficient hosts and mediated superior anti-tumor responses in a humanized mouse model. T_{SCM} also occur in nonhuman primates and have a similar phenotypic and functional profile to their human counterparts. Consistent with their early differentiation stage, T_{SCM} are localized primarily to the lymph nodes, and virtually absent from mucosal tissues. Immuno-monitoring of T cell responses during SIV infection revealed that T_{SCM} have enhanced self-renewal and survival compared to conventional memory T cells. The identification of T_{SCM} population is of direct relevance to the design of novel vaccines and T-cell therapies.

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Plasmin Triggers Chemotaxis of Monocyte-Derived Dendritic Cells through an Akt2-Dependent Pathway and Promotes a T Helper Type 1 Response

Thomas Simmet, Tatiana Syrovets, Xuehua Li, Felicitas Genze

University of Ulm, Inst. of Pharmacol. of Nat. Prod. & Clin. Pharmacol, Ulm, Germany

Pivotal roles have been proposed for dendritic cells (DCs) and the adaptive immune response in atherosclerosis. Using immunohistochemistry we found that both, plasmin and DCs, are abundant in human atherosclerotic lesions, where they colocalize. Hence, we investigated whether plasmin might alter the function of monocyte-derived DCs.

Indeed, plasmin was a potent activator of human DCs in vitro. Stimulation of immature DCs with plasmin, but not with catalytically inactivated plasmin, elicited actin polymerization and a chemotactic cell migration in Transwell chambers comparable to that triggered by the standard chemoattractant FMLP. Plasmin-stimulated DCs showed rapid activation of Akt, ERK1/2 and p38 MAP kinases, followed by phosphorylation of the regulatory myosin light chain and chemotaxis. DCs express Akt1 and Akt2, yet plasmin activated exclusively Akt2 via a p38 MAPK-dependent pathway. Knockdown of Akt2, but not of Akt1 by shRNA inhibited the plasmin-induced ERK1/2 activation and the chemotaxis. Moreover, plasmin-stimulated DCs expressed IL-12 and induced polarization of CD4+ T cells towards the IFN-γ-producing, proinflammatory Th1 phenotype. At variance to the chemotactic response, the polarization of CD4+ T cells was independent of Akt activation. Consistent with a role for DCs and the adaptive immune response in atherogenesis, we demonstrate that DCs in human atherosclerotic vessels exhibit activated Akt.

These data point to a novel link between protease activity and the adaptive immune response, which may be of particular relevance in atherogenesis. Thus, plasmin generation in the atherosclerotic vessel wall might contribute to accumulation of DCs, activation of the adaptive immune response and aggravation of atherosclerosis.

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CCR7/CCL19 Promotes S1P1 Expression via ERK5

Charlotte M. Vines,[1] Laura A. Shannon,[1] Megan Roth,[2] Charles A. Bill,[1] Psachal A. Calloway[1]

[1]Univ of Kansas Med Ctr, Department of Microbiology, Kansas City, KS; [2]KUMC, Department of Pharmacology, Kansas City, KS

The goal of these studies is to determine the role of C-C Chemokine receptor 7 (CCR7) in regulating the expression of a signaling cascade. These signaling events ultimately control the migration of T cells during an immune response. CCR7 is used by naive T cells to control their chemotaxis into the lymph nodes in response to gradients of CCL21. Within the lymph nodes naive T cells bind cognate MHCs on activated dendritic cells. These activated DCs express high levels of CCL19. We hypothesized that prolonged exposure of naive T cells to CCL19 regulates signaling to control expression of the sphingosine 1 phosphate 1 (S1P1) receptor. The

S1P1 then promotes egress from the lymph nodes within 72 hours. We have found that stimulation of the human HuT78 lymphoma line, and murine T cells led to up regulation of extracellular regulated kinase 5, a transcription factor for Kruppel like factor 2 within 72 hours. In turn, the T cells up regulated KLF2, which is a transcription factor that is required for expression of S1P1. The increased expression of S1P1 was determined by measuring migration to of the T cells to S1P. Loss of ERK5 in ERK5 $^{flox/flox}$-Lck-Cre mice, led to loss of migration via S1P1. We conclude that CCR7 not only promotes migration to the lymph nodes, but also controls the signaling events the lead to exit from the lymph nodes.

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The Role of Aryl Hydrocarbon Receptor in IL-23 Dependent Release of IL-22 Following Ethanol Exposure and Burn Injury

Juan L. Rendon, Xiaoling Li, Mashkoor A. Choudhry
Loyola University Medical Center, Stritch School of Medicine, Maywood, IL

Interleukin (IL)-22, a cytokine released by T lymphocytes, is crucial in regulating mucosal immunity. Our laboratory has demonstrated that acute ethanol (EtOH) exposure prior to burn injury results in suppressed intestinal T cell immunity, increased gut permeability and enhanced bacterial translocation. Furthermore, we found a decrease in Peyer's patch (PP) IL-22 release. Within the gut, IL-22 contributes to epithelial cell regeneration/proliferation and antimicrobial peptide release. IL-23, released by mucosal dendritic cells, is indispensible in the expression of IL-22. Recently, the aryl hydrocarbon receptor (AHR), a cytosolic transcription factor, has also been implicated in the regulation of IL-22. The present study examined whether restitution of IL-23 prevents the decrease in PP IL-22 release following EtOH and burn injury and whether this is dependent on AHR. Male mice, ~25 g, were gavaged with EtOH (2.9 mg/kg) to achieve blood EtOH levels of 90-100 mg/dL prior to receiving a 12-15% total body surface area full thickness burn. Animals were sacrificed one day post injury and PP were processed for single cell suspensions. Mixed cells (2 x 10^6 cells/mL) were cultured in the presence of plate bound anti-CD3 (5 μg/mL) and soluble anti-CD28 (1 μg/mL) +/- recombinant IL-23 (10 ng/mL) +/- AHR inhibitor (10 μM) for 48 hrs. Following culture conditions, supernatants were harvested and analyzed for IL-22 levels, by ELISA. When combined with EtOH exposure, burn injury results in a 74% decrease in IL-22 ($p<0.001$) release, as compared to sham injury. Restitution of IL-23 successfully restores IL-22 release (6 fold increase, $p<0.01$). Moreover, IL-23 dependent restoration of IL-22 is inhibited in the presence of an AHR inhibitor. Together these data suggest that IL-23 modulation of IL-22 is dependent on AHR. (Supported by NIH R01AA015731(MAC), F30AA020167(JLR), T32AA013527(EJK), the Loyola SSOM MD/PhD Program and the Dr. Ralph and Marian C. Falk Trust)

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Myeloid Cell Dysfunction in Septic Shock: A Novel Regulatory Role for B and T Lymphocyte Attenuator

Nicholas Shubin, Chun-Shiang Chung, Sean Monaghan, Daithi Heffernan, Alfred Ayala
Brown University/Rhode Island Hospital, Department of Surgery, Providence, RI

B and T Lymphocyte Attenuator (BTLA) is a recently characterized immuno-regulatory receptor expressed on leukocytes that is capable of inhibiting cellular proliferation and activation when ligated. Considering other studies evaluating similar co-inhibitory receptors, including PD-1 and CTLA-4, found that these receptors contributed to septic morbidity/ mortality, our initial hypothesis was that BTLA also effects sepsis progression by inhibiting activation and/or effector function of the leukocytes it's expressed on. In human studies, we observed that the number of $BTLA^+$ and herpes virus entry $mediator^+$ ($HVEM^+$) cells (the only known ligand for BTLA) were increased significantly on the peripheral blood lymphocytes and monocytes, but not granulocytes of septic ICU patients. Subsequently, using a model of septic shock in mice (CLP; cecal ligation and puncture that produced ~70-80% mortality by 48h), we found that BTLA was induced on macrophages ($F4/80^+$) and $CD11c^+$ cells in the peripheral blood and peritoneum. We also noted that BTLA -/- CLP mice were significantly protected from septic mortality, multiple organ damage as well as peripheral lymphoid organ apoptosis, while their peritoneal bacterial burden and inflammatory cytokine induction (TNF-alpha & IL-10) was reduced at 24h post-CLP. To the extent that BTLA contributes to the above septic morbidity by impairing innate cell population(s) effector function, we found that *ex vivo* macrophages from CLP BTLA -/- mice secreted less inflammatory cytokines then CLP WT mouse cells. In addition, we also observed that myeloid-derived suppressor cells (MDSC) ($Gr1^+$, $CD11b^+$), macrophages and neutrophils ($Gr1^+$) were poorly recruited to the peritoneum 24h post-CLP in the BTLA -/- mice. Together, we document not only that BTLA expression is a potentially important marker/ mediator of septic morbidity in humans and mice, which results, in part, from its effects on innate myeloid cell function/ recruitment.

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Cell Surface Trafficking of TLR1 is Differentially Regulated by the ER Chaperones PRAT4A and PRAT4B

Bryan E. Hart, Richard Tapping
University of Illinois, Department of Microbiology, Urbana, IL

Toll-like receptors are pattern recognition receptors that provide a first line of host defense against invading pathogens. Subcellular localization and trafficking is vital for proper recognition of conserved molecular patterns by their respective TLRs. For example, TLR1, 2, 6, and 10 are displayed on the plasma membrane, where they are best available to recognize cell wall components of invading bacterial and fungal pathogens. We have recently identified a frequent single nucleotide polymorphism of TLR1 (I602S) which greatly inhibits trafficking of the receptor to the cell surface (Johnson, CJ, J Immunol 178, 7520). The aberrant surface display of the TLR1 602S variant is associated with a

marked inability of cells to respond to soluble TLR1/2 agonists, but not to particulate agonists. While TLR1 602S is ER-localized in resting cells, this variant can traffic to phagosomes containing *M. avium*. Mutagenesis of TLR1 amino acid sequence and examination of TLR9/1 chimeras has revealed a trafficking motif required for TLR1 surface expression, which is interrupted by the 602S polymorphism. An examination of ER-resident TLR chaperones, PRAT4A and PRAT4B, reveals differential roles in the regulation of TLR1 surface trafficking. Increased PRAT4A expression induces the surface expression of both TLR1 variants. Conversely, overexpression of PRAT4B reduces surface display of TLR1 602I, mimicking the aberrant phenotype of TLR1 602S. This result suggests that the PRAT4B chaperone has an inhibitory effect on TLR1 trafficking. Indeed, co-immunopreciptiation shows that TLR1 602S has a stronger interaction with PRAT4B than TLR1 602I. Moreover, knockdown of PRAT4B expression rescues cell surface expression of TLR1 602S. Collectively, these results provide a mechanistic explanation for the differential trafficking of TLR1 I602S variants.

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Glycyrrhetinic Acid Attenuates HER2 Tyrosine Kinase Activity

David J. Fast, John F. Rebhun, Louise Schneider, Jeffrey D. Scholten

Access Business Group, Department of Analytical Svs, Ada, MI

Licorice root compounds have been used for thousands of years for numerous medical complaints including stomach upset, sore throat, and arthritis. Glycyrrhizin, a triterpene saponin glycoside found in licorice presents anti-viral, anti-cancer, and anti-inflammatory properties; however, the root mechanism of action of glycyrrhizin remains unclear. In certain cellular systems, glycyrrhizin attenuates membrane-receptor-mediated inflammatory responses. Here we describe another mechanism by which glycyrrhizin impacts cellular activity and may explain some of its additional effects. During routine screening assays for anti-inflammatory and anti-proliferative ingredients it was noticed that derivatives of glycyrrhizin inhibited NFκB and p42/p44 MAPK/ERK (mitogen activated protein kinase) activation. Subsequent assays determined that glycyrrhizin (50 μg/ml) blocked the phosphorylation of EGFR (epidermal growth factor receptor) by ~50% without interring with the binding of Fluor-labeled EGF to its receptor. Glycyrrhizin and its aglycone derivative glycyrrhetinic acid directly inhibited recombinant ErbB2 tyrosine kinase activity using a EGFR target peptide with an IC50 of 70 and 40 μg/ml respectively. Further work is necessary to delineate the impact of tyrosine kinase inhibition on the NFκB stimulated pathways.

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The Role of cAMP in the Induction of IL-10 by *Lippia sidoides*

David J. Fast, John F. Rebhun, Arun Rajgopal, Jeffrey D. Scholten

Access Business Group, Department of Analytical Svs, Ada, MI

Lippia sidoides is commonly used in Brazilian folk medicine due to its anti-inflammatory properties. We have found that an extract of Lippia inhibits activation of NFκB using a luciferase reporter cell assay but activates CREB. Activation of CREB by Lippia may be due to its ability to inhibit phosphodiesterase. Since activation of the cAMP pathway is known to downregulate expression of proinflammatory cytokines such as TNF-α and IL-8 while upregulatiing expression of the anti-inflammatory IL-10, we were interested in whether Lippia could induce IL-10 expression. Treatment of vitamin D differentiated THP-1 cells with Lippia alone had little effect on expression of IL-10. Forskolin, an activator of adenyl cyclase, and 8-Br-cAMP, a cell permeable cAMP analog had only modest effects on IL-10 production. However, when Lippia was combined with forskolin or 8-Br-cAMP, a significant increase in IL-10 release was seen. This effect was even greater in the presence of TLR agonists such as LPS or Pam3Csk4. Finally, thymol and carvacrol, phytochemicals known to found in Lippia did not elicit the IL-10 inducing effect either alone or in the presence of forskolin. In conclusion, IL-10 production by human macrophages is increased under conditions which elevate cellular cAMP levels and is augmented in response to TLR engagement.

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The Tug-of-War between Dendritic Cells and Human Chronic Viruses

Pooja Jain

Drexel University College of Medicine, Department of Microbiology and Immunology, Doylestown, PA

The human immune system is under constant challenge from many viruses, some of which the body is successfully able to clear. Other viruses have evolved to escape the host immune responses and thus persist, leading to the development of chronic diseases. Dendritic cells (DCs) are professional antigen presenting cells that play a major role in both innate and adaptive immunity against different pathogens. For the past few years our efforts have been focused on exploring the participation of DCs in the pathogenesis of HTLV-1, HIV-1 and HCV. We observed previously that depletion of DCs in CD11c-DTR transgenic mice enhanced the susceptibility to cell-free HTLV-1 infection. We further performed the host-pathogen interaction studies utilizing Flt3 ligand derived murine bone marrow DCs (FL-DCs). First, the kinetics of viral entry, proviral integration, and expression of the viral protein Tax was established and then effects of cell-free HTLV-1 was examined on these cells. Phenotypically, FL-DCs demonstrated activation and produced an array of proinflammatory cytokines as well as IFN-α. Virus-matured FL-DCs also stimulated proliferation of autologous $CD3^+$ T cells and IFN- γ production. Gene expression studies revealed upregulation of interferon-stimulated genes, most cytokines, and transcription factors but a distinct downregulation

of many chemokines. Overall, these results highlight the critical interaction of DCs with a human chronic virus important for the early immune responses.

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Indenoquinoxaline Derivatives Are Potent Inhibitors of JNK Family Protein Kinases

Mark T. Quinn,[1] Igor A. Schepetkin,[1] Liliya N. Kirpotina,[1] Mark A. Jutila,[1] Andrei I. Khlebnikov[2]

[1]Montana State Univ, Department of Immunology & Infectious Diseases, Bozeman, MT; [2]Altai State Technical University, Chemistry, Barnaul, Russia

The activating protein 1 (AP-1) and nuclear factor-κB (NF-κB) transcription factors regulate many important physiological and pathological processes, including innate inflammatory responses. Using a cell-based phenotypic reporter gene assay, we identified a unique series of tetracyclic indenoquinoxaline derivatives that inhibited lipopolysaccharide (LPS)-induced NF-κB/AP-1 activation. Compound IQ-1 (11H-indeno[1,2-b]quinoxalin-11-one oxime) was found to be a potent non-cytotoxic inhibitor of interleukin (IL)-1α, IL-1β, IL-6, IL-10, tumor necrosis factor α, interferon γ, granulocyte-macrophage colony-stimulating factor, prostaglandin E_2, and nitric oxide production in human monocytic cells and primary mononuclear cells. Three additional potent inhibitors of cytokine production were identified through further screening of IQ-1 analogs. Screening of over 120 protein kinases and subsequent analysis revealed that analog IQ-4 (11H-indeno[1,2-b]quinoxalin-11-one-O-(2-furoyl)oxime) was a specific inhibitor of the c-Jun N-terminal kinase (JNK) family, with preference for JNK3. This compound, as well as IQ-1 and two additional indenoquinoxalines, were high-affinity JNK inhibitors with nanomolar binding affinity. Furthermore, molecular docking studies suggested that the active indenoquinoxalines form hydrogen bonds with Asn152 and Gln155 in the ATP-binding pocket of JNK3. These amino acids are not conserved in other MAP kinases and hence may contribute to the selectivity for JNKs over other kinases. Thus, these indenoquinoxaline derivatives represent specific small-molecule modulators for mechanistic studies of JNKs, as well as a potential leads for the development of anti-inflammatory drugs.

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Sphingosine Analog AAL-R Increases Dendritic Cell Responses upon TLR7 Stimulation

Bumsuk Hahm, Young-Jin Seo, Curtis J. Pritzl, Madhuvanthi Vijayan

University of Missouri-Columbia, Department of Surgery and Molecular Microbiology & Immunology, Columbia, MO

Sphingosine analogs display immune suppressive activities and thus have therapeutic potential for the treatment of autoimmune diseases. In this study, we determined the role of a sphingosine analog, AAL-R, in the host dendritic cell (DC) responses upon the stimulation of toll-like receptors (TLRs). AAL-R inhibited DC maturation in response to TLR3 or TLR4 activation, representing its known immunosuppressive action. In contrast, AAL-R increased TLR7-mediated DC responses by elevating the level of MHC-I molecule, co-stimulatory molecules, and type I interferon (IFN), and by enhancing DC capacity to induce antigen-specific CD8 T cell proliferation. Further, AAL-R increased the phenotypic maturation and functionality of DCs infected with lymphocytic choriomeningitis virus (LCMV). Underlying molecular mechanism involves type I IFN signaling, since AAL-R failed to increase the response of type I IFN receptor-deficient DCs to the TLR7 stimulation or LCMV infection. Thus, our data indicate that AAL-R's regulatory action is strongly affected by the form of pathogenic molecular patterns and is stimulatory when DCs are treated with a TLR7 agonist or infected by LCMV. These findings enhance our understanding of sphingosine regulation of host immune system in particular upon pathogenic infections.

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Innate Immune Activation Controls Early *Staphylococcus aureus* Biofilm Infection

Mark L Hanke,[1] Sam Sanderson,[2] Tammy Kielian[3]

[1]University of Nebraska Medical Center, Department of Pathology and Microbiology, Omaha, NE; [2]University of Nebraska Medical Center, School of Allied Health Professions, Omaha, NE; [3]Univ of Nebraska Medical Ctr, Omaha, NE

Nosocomial infections represent the second most common type of health care-associated infection, affecting approximately 300,000-500,000 patients each year. *S. aureus* is a major cause of surgical site infections leading to significant morbidity and mortality in the modern health care system. In the presence of a foreign body, such as medical implants, the likelihood of infection is increased and the threshold for colonization is drastically lower. *S. aureus* has a predilection towards biofilm growth on artificial surfaces, which are usually recalcitrant to antibiotic therapy. Further complicating the issue of biofilm eradication stems from recent studies from our laboratory demonstrating that *S. aureus* biofilms evade macrophage effector mechanisms typically associated with clearance of planktonic bacteria. To overcome this immune evasion, we have examined the efficacy of EP67, a conformationally-biased, response-selective agonist of human $C5a_{65-74}$ that invokes host innate and acquired immune responses. Prior studies have established that EP67 (YSFKDMP(MeL)aR) preferentially elicits cytokine production from C5a receptor (C5aR)-bearing macrophages and dendritic cells without any effects on C5aR-bearing neutrophils. Here we have utilized a mouse model of catheter-associated biofilm infection to demonstrate that prophylactic administration of EP67 limits *S. aureus* biofilm formation. EP67-treated mice displayed significant reductions in bacterial burdens on both infected catheters and within surrounding tissues at days 3 and 14 post-infection when compared to animals exposed to the inactive, scrambled sequence of EP67 or a vehicle control. Furthermore, macrophage infiltrates associated with *S. aureus* biofilms were increased compared to vehicle-treated mice. Several inflammatory mediators were

significantly elevated in tissues surrounding biofilm infections in EP67-treated animals, which may account for the observed anti-microbial effects of the peptide. Collectively, these findings demonstrate that EP67 is capable of beneficially enhancing the host immune response to *S. aureus* biofilm infections. Supported by P01 AI083211 Project 4 to T.K.

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IL-1b but not IL-18 Release is Regulated by the NLRP3 Inflammasome and P2X7R in Microglia

Richa Hanamsagar,[1] Victor Torres,[2] Tammy Kielian[3]

[1]Universtiy of Nebraska Medical Center, Department of Pharmacology and Experimental Neuroscience, Omaha, NE; [2]Joan and Joel Smilow Research Center, New York, NY; [3]Univ of Nebraska Medical Ctr, Omaha, NE

Staphylococcus aureus (*S. aureus*) infection in the central nervous system (CNS) leads to abscess formation typified by widespread inflammation and necrosis. Microglia are the resident macrophage population in the CNS parenchyma and represent an important component of the innate immune response against invading pathogens such as *S. aureus*. The NLRP3 inflammasome is a protein complex responsible for processing the proinflammatory cytokines IL-1β and IL-18 into their mature forms following exposure to both pathogen- and danger-associated molecular patterns. Although previous studies from our laboratory have established that microglia are capable of recognizing *S. aureus*, the molecular machinery responsible for cytokine release remains to be determined. The objective of this study was to examine the functional role of the NLRP3 inflammasome and its adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruiting domain (ASC), in eliciting IL-1β and IL-18 release by microglia following *S. aureus* exposure. Primary microglia isolated from NLRP3 or ASC knockout (KO) mice were treated with various live *S. aureus* or isogenic hemolysin mutant strains, since recent evidence suggests that purified hemolysins can trigger inflammasome activation. IL-1β production was significantly attenuated in both NLRP3 and ASC KO microglia following exposure to live *S. aureus*, whereas surprisingly, IL-18 levels were not affected. IL-1β release was also attenuated in WT microglia following exposure to *S. aureus* α- and γ-hemolysin mutants; however, residual IL-1β was still detected, suggesting the involvement of alternative toxins for maximal cytokine induction in response to live bacteria. We also established that microglial NLRP3 inflammasome activation was dependent on extracellular ATP since IL-1β production was significantly attenuated by the P2X7 receptor inhibitor, AZ11645373. Overall, our results demonstrate that the NRLP3 inflammasome and P2X7R play critical roles in the selective production of IL-1β, but not IL-18, by microglia following *S. aureus* exposure.

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Interaction of gp96 with Fcγ Receptor I Regulates the Survival of *Escherichia coli* K1 in Macrophages

Rahul Mittal, Nemani V. Prasadarao

Children's Hospital Los Angeles, Department of Infectious Diseases, Los Angeles, CA

Escherichia coli K1 is one of the most common pathogens causing neonatal meningitis associated with high mortality and morbidity. Lack of understanding of the pathogenesis of the disease has hindered the development of effective preventive strategies. Our previous studies have shown that *E. coli* expressing outer membrane protein A (OmpA) survives and multiplies in macrophages by upregulating the expression of FcγRI (CD64), which acts as receptor for bacterial entry into cells. Our hypothesis is that *E. coli* manipulates the macrophage function via OmpA interaction with CD64 during the initial stages of infection to evade immune responses. Here, we set out to elucidate how CD64 interaction with OmpA regulates *E. coli* survival in macrophages. Since gp96 has been shown to play an important role in regulating phagocytic ability of macrophages, we examined gp96 and CD64 expression as well as phosphorylation of PKC-α and Filamin A in RAW 264.7 macrophages infected with OmpA+ and OmpA- *E. coli*. Immunoprecipitation of macrophage cell lysates obtained after infection with anti-CD64 antibody followed by Western blotting with anti-gp96 antibody revealed that CD64 interacts with gp96. In addition, we observed that the phosphorylation of PKC-α and upregulation of Filamin A expression in macrophages infected with OmpA+ *E. coli*. Silencing of gp96 or Filamin A expression using siRNA or blocking PKC-α by chemical inhibitors downregulated the expression of CD64, and thereby the survival of *E. coli* in macrophages. These results demonstrate that gp96 and CD64 interaction is critical for the survival of *E. coli* in macrophages. Therefore, targeting OmpA and gp96/CD64 interacting domains by peptidomimetics or small molecules to prevent the survival of *E. coli* in macrophages may be a therapeutic strategy for preventing neonatal meningitis.

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Stress-Induced Increases in Bacterial Killing Are Dependent upon the Intestinal Microbiota

Rebecca Allen, William Lafuse, John F. Sheridan, Michael Bailey

The Ohio State University, Columbus, OH

Exposure to social stressors is known to prime the innate immune system for enhanced reactivity to inflammatory stimuli, but the mechanisms by which stressor exposure can enhance immune activity are not well-defined. In mice, exposure to a stressor called social disruption (SDR) increases circulating cytokines and primes splenic macrophages for an enhanced capacity to kill *Escherichia coli,* primarilythrough an increased production of the highly microbicidal compound peroxynitrite. Previous results demonstrate that the intestinal microbiota are necessary for the SDR-induced increase in circulating cytokines. This study tested the hypothesis that the microbiota are also necessary for the stressor-induced increase in the ability of splenic macrophages to kill *E.coli*. To test

this hypothesis, mice were exposed to the SDR stressor involving repeated defeat by an aggressor mouse. Following SDR, splenic macrophages were cultured with *E. coli*, and the number of bacteria phagocytosed and killed was enumerated. In addition, peroxynitrite production was assessed using a fluorescent reporter dye, 123-DHR. When commensal bacterial populations were reduced through daily oral administration of a non-absorbable antibiotic cocktail, SDR exposure failed to enhance the killing of *E. coli*. Moreover, peroxynitrite levels were significantly lower in the antibiotic treated mice compared to the vehicle treated mice exposed to SDR. To confirm the importance of the microbiota, we exposed germ free mice, i.e., completely sterile mice, as well as conventionalized germ-free mice, i.e., germ-free mice colonized by donor stool, to the stressor. Again, SDR failed to increase peroxynitrite production and bacterial killing in splenic macrophages from germ-free mice, but SDR-exposed conventionalized germ-free mice produced both significantly more peroxynitrite and killed more *E. coli*. These data demonstrate that the microbiota are necessary for SDR to enhance bacterial killing and peroxynitrite production by splenic macrophages. Future studies will determine the mechanisms by which the microbiota can enhance splenic macrophage activity.

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Carbon Nanoparticle Lung Damage: HMGB1, Pattern Recognition Receptors and Cytokines

Betty L. Herndon,[1] Monisha Das,[2] Elisabet Nalvarte,[3] Agostino Molteni,[4] Tim Quinn,[5] Eugene Fibuch[6]

[1]UMKC School of Medicine, Med, Basic Medical Science, Kansas City, MO; [2]Univ. MO Kansas City School of Med., Pulmonary-Critical Care, Kansas City, MO; [3]UMKC School of Medicine, Kansas City, MO; [4]UMKC School of Med, Pathology, Kansas City, MO; [5]UMKC School of Medicine, Kansas City, MO; [6]UMKC School of Medicine, Anesthesiology, Kansas City, MO

A recent report and a review on sterile cell injury/repair contain different points of view on the involvement of the high mobility group box protein 1 (HMGB1) in cytokine release associated with sterile damage [1,2]. This report addresses the topic using in vivo, ex vivo and in vitro exposure of a rat model to single wall carbon nanoparticles (CN). An increased potential for human lung exposure to CN exists, in both occupational settings and consumer products. CN are known to produce cellular cytotoxicity and lung inflammation in animals, but the mechanisms are incompletely understood. We hypothesized that, on pulmonary exposure, the initial cytotoxic effect of CN is the cellular release of damage-associated molecular pattern recognition molecules, specifically the high mobility group box 1 (HMGB1) protein. HMGB1, extracellularly, exerts its actions as a ligand for pattern recognition receptor (PRR) activation in target cells. Single doses of 0.025 mg CN (tested endotoxin free) dispersed in 0.050 mL pulmonary surfactant were intratracheally instilled into rat airways (n=45). Rat bronchoalveolar lavage fluid (BAL), pleural fluid (PF), lungs and sera were harvested at 0.5h, 3 h, 24h, 1 week or 4 weeks after CN. Centrifugal cytology was analyzed on the BALs and PFs which were also measured (ELISA) for HMGB1 and cytokines (IL-6 and IL10). Pulmonary histology was examined. BALs were further used to activate PRRs in RAW Blue cells, stably transfected lung macrophages expressing the secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-*k*B and AP-1 activation. A positive linear correlation was seen in HMGB1 released *vs.* cell injury (total protein) in rats exposed to CN for the first week. This response was accompanied by the appearance of inflammatory cells in lung parenchyma, BAL and PF and translocation of CN via macrophage migration from the airways to the mesothelial lining. The HMGB1-containing BAL induced significant expression of SEAP in the cell supernatants, highest at 24 hr. Control wells with purified swine HMGB1 protein activated RAW blue cells in a dose-dependent manner. The cytokines IL-10 and IL-6, known to respond to HMGB1-receptor complexes, were elevated at two times ($p=0.04$). The inflammatory IL-6 predominated early and the non-inflammatory IL-10 predominated late. Both responded at times when RAGE was the predominant HMGB1 receptor (flow cytometry) and HMGB1 was elevated outside the cell. The results show that the nuclear chaperone HMGB1 is rapidly released from pulmonary cells following sterile i.t. CN administration suggesting a toxic effect of CN on pulmonary tissue with expression at different times of both inflammatory and homeostatic cytokines. The data further suggest a role for HMGB1 in the cytotoxicity and inflammatory effects of CN through activation of cellular PRRs. [1] Kratochwill K et al, *Am. J. Pathol* 178: 1544-55, 2011; [2] Tsan M-F, *J. Leukocyte Biol*. 89:847-54, 2011

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Differential Activation of Mast Cells by Different Strains of Influenza A Virus

Joshua J. Obar,[1] Amy Graham,[1] Julianne Zickovich,[1] Carly Grant,[1] Peter Palese[2]

[1]Montana State University, Department of Immunology & Infectious Diseases, Bozeman, MT; [2]Mount Sinai Sch Med, Microbiology, New York, NY

Influenza A virus is one of the most common seasonal respiratory infections. In the United States, influenza virus infection leads to ~36,000 influenza-related deaths and ~1.7 million hospitalizations each year. Significant pathology after influenza virus infection is caused by the host immune response; thus, understanding the innate immune response during influenza virus infection is critical for identifying mechanisms of influenza virus-mediated lung pathology. The lung is protected from pathogens by alveolar epithelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells. Typically, the role of the mast cell has been overlooked in influenza immunopathogenesis. In mice that lack functional mast cells (B6.Cg-$Kit^{W\text{-}sh}$), pathological immune responses are altered depending on the influenza strain, and we found that both the A/WSN/33 and A/PR/8/34 influenza viruses generate significant immunopathology in C57BL/6 mice, but only the pathology induced by A/WSN/33 was mast cell dependent. B6.Cg-$Kit^{W\text{-}sh}$ mice could be complemented using C57BL/6-derived bone marrow mast cells, thus indicating the defect was within the mast cell compartment. *In vivo*, both A/WSN/33 and A/PR/8/34 induced robust neutrophil recruitment to the BALF by day 4 post-

infection; however, neutrophil recruitment was mast cell dependent only during A/WSN/33 infection. Neutrophil recruitment and inflammatory cytokine production both correlated with increased lung permeability and pathology, as well as systemic disease severity, as measured by weight loss. To assess why inflammatory response to the A/WSN/33, but not the A/PR/8/34, strain of influenza A virus was mast cell dependent, we next examined the ability of each to activate *in vitro*-derived bone marrow mast cells. We found that that A/WSN/33, but not A/PR/8/34, influenza virus activated bone marrow-derived mast cells to produce cytokines and degranulate. Importantly, a recombinant A/PR/8/34 virus that expresses the hemagglutinin (HA) glycoprotein from A/WSN/33 instead of its own HA could induce cytokine production from bone marrow-derived mast cells, demonstrating an important role for the HA glycoprotein in dictating mast cell activation. Next, we assessed the signaling pathways and pattern-recognition receptor(s) (PRR) important for mast cell activation during A/WSN/33 infection. Mast cell activation by influenza A virus was dependent on Syk and p38. Although the PRR important in mast cell activation remains elusive, it is not a C-type lectin receptor and is Dap12, FcγrI, MyD88, and Caspase-1 independent. Thus, we have identified a unique inflammatory cascade, which could be targeted to limit morbidity and mortality observed following infection with certain influenza virus strains. *This work is supported by NIH grant P20 RR-020185 and K22 AI091647.*

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Evasion of Inflammasome Activation by *Francisella tularensis* LVS

Ann M. Janowski,[1] Tyler Ulland,[2] Blake W. Buchan,[3] Bradley D. Jones,[3] Fayyaz Sutterwala[1]

[1]University of Iowa, Iowa City, IA; [2]Univ of Iowa, Dept of Inflammation Prog, Iowa City, IA; [3]University of Iowa, Microbiology, Iowa City, IA

Francisella tularensis is a Gram-negative bacteria and the causative agent of the disease tularemia. Escape of *F. tularensis* from the phagosome into the cytosol of the macrophage triggers the activation of the AIM2 inflammasome. Activation of the AIM2 inflammasome results in autocatalytic cleavage of caspase-1, resulting in the processing and secretion of IL-1β and IL-18, which plays a crucial role in innate immune responses against *F. tularensis.* We have identified the *5,10-methyltetrahydrofolate synthetase* gene as being important for *F. tularensis* live vaccine strain (LVS) virulence. Infection of mice *in vivo* with a *F. tularensis* LVS *5,10-methyltetrahydrofolate synthetase* mutant resulted in increased survival compared to mice infected with wild-type LVS. The *5,10-methyltetrahydrofolate synthetase* mutant also induced increased inflammasome-dependent IL-1β and IL-18 secretion and cytotoxicity in macrophages *in vitro* . In contrast, infection of macrophages with a *F. tularensis* LVS *pseudouridine synthase* mutant resulted in diminished IL-1β and IL-18 secretion from macrophages *in vitro* compared to macrophages infected with wild-type LVS. In addition the *pseudouridine synthase* mutant was not attenuated upon *in vivo* infection. Taken together these findings suggest that inhibition of inflammasome activation is an important virulence strategy for *F. tularensis* LVS.

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TLT2 Promotes Neutrophil Activation and Chemotaxis via Intrinsic and Extrinsic Mechanisms

Louis B. Justement, Kimberly A. Thomas, Matthew M. Halpert, R. G. King

University of Alabama at Birmingham , Department of Microbiology, Birmingham, AL

TLT2 is a member of the TREM locus, a cluster of genes encoding eight receptors in mice and humans. Of these, four are direct homologues, Trem-1, Trem-2, Trem like transcript (TLT)-1, and TLT2. TLT2 is unique among these receptors as it does not contain structural motifs that promote phosphotyrosine based signal transduction and is expressed by macrophages, neutrophils, and B lymphocytes in mouse and human; thus TLT2 is the only member of the TREM family expressed by cells of both the adaptive and innate immune systems. Ligation of TLT2 on murine neutrophils results in enhanced generation of reactive oxygen species (ROS) in response to stimulation with the formylated peptide FMLP. This potentiation of ROS production is similar in magnitude, but differs kinetically from the observed priming effects of LPS. Additionally, TLT2 engagement potentiates other cellular responses to FMLP, including degranulation and chemotaxis. Ligation of TLT2 enhances the migratory response of neutrophils to the murine chemokines KC, and MIP-2, their human homologue IL-8, as well as to the activated complement component C5a. The cellular responses to GM-CSF, LPS, and FcR ligation were unaltered, however, suggesting that TLT2 specifically potentiates the response of neutrophils to signals derived from G protein-coupled receptors (GPCRs). Administration of anti-TLT2 antibodies in mice results in an increase in the number of neutrophils that accumulate in response to croton oil, a nonspecific inflammatory mediator, supporting a role for TLT2 in enhancing neutrophil transmigration into inflamed tissue. Additionally, competitive adoptive transfer experiments demonstrate a specific enhancement in accumulation of anti-TLT2 mAb-treated neutrophils in experimentally induced lung inflammation. Collectively these results demonstrate that TLT2 engagement specifically potentiates the response of neutrophils to GPCRs resulting in increased cellular responses to signals derived from these receptors including transmigration into sites of inflammation and enhanced antimicrobial activity. Macrophages, like neutrophils, express TLT2, and its ligation on these cells induces the production of numerous factors independent of secondary stimuli, as evidenced by ex vivo treatment of both resident peritoneal and alveolar macrophage with anti-TLT2 mAb. This phenomenon is recapitulated in vivo following injection of anti-TLT2 mAbs resulting in the production of chemokines and growth factors. Ultimately, this results, in enhanced neutrophil influx supporting the conclusion that TLT2 plays an important protective role in normal biology. Thus, TLT2 may be involved in an intricate feed-forward loop wherein it is important for both driving the production of factors that recruit neutrophils to sites of infection/inflammation, as well as enhancing the response of neutrophils towards chemokines and bacterial products that signal

via GPCRs.

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Systemic Effects of Burn Injury on Epithelial Defense Mechanisms in Distal Sites

Jennifer K. Plichta, Steve Droho, Parita Patel, Richard L. Gamelli, Katherine Radek

Loyola University Medical Center, Department of Surgery/BSTI, Maywood, IL

Burn patients are highly susceptible to infection, likely related to an impaired global epithelial barrier. Antimicrobial peptides (AMPs), such as cathelicidin (CRAMP) and beta-defensins (i.e. BD1 and BD14), maintain epithelial barriers by eliciting direct antimicrobial and chemotactic responses. In parallel, lipids serve as both a physical and chemical barrier. Here, we assessed cutaneous barrier recovery and AMP production in the skin, lungs, and bladder of male C57Bl/6 mice receiving a 15% scald burn (10 seconds, 93ºC) or sham water bath. Unburned, distal skin was subjected to barrier disruption via tape stripping to assess barrier recovery by transepidermal water loss (TEWL) and analysis of lipid synthesis enzyme gene expression. AMP gene expression in non-burned skin, lungs, and bladder were also determined. Burned skin exhibited increased TEWL 24 hours post-burn (sham 12 vs. burn 50, p=0.0003) and a decrease in the lipid synthesis enzyme, HMG-CoA reductase (HMGCR) (sham 1 vs. burn 0.295, p=0.019). The distal, non-burned skin demonstrated a 50% attenuation of increased TEWL at 24 hours following barrier disruption (p=0.04) normally required for barrier restoration. HMGCR gene expression was similarly reduced in non-burned skin. BD14 (sham 1 vs. burn 0.316, p=0.036) and the kallikreins responsible for epidermal AMP processing (i.e. KLK5 and KLK7) were also reduced in distal skin after 24 hours (KLK5 p=0.022, KLK7 p=0.014). By 96 hours post-burn, reduced gene expression of CRAMP and continued suppression of KLK5 was observed in distal skin. In lungs, both CRAMP (sham 1 vs. burn 19.5, p=0.029) and BD14 (sham 1 vs. burn 2.3, p=0.077) were increased by 96 hours post-burn, while only BD1 was increased in the bladder (sham 1 vs. burn 1.96, p=0.0629). In conclusion, burn injury prolongs barrier dysfunction and AMP perturbations in distal tissues, which likely increases the incidence of infections in burn patients and requires further scrutiny.

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A Specific Isoform of Histone Deacetylase 7 Promotes TLR4-Mediated Inflammatory Responses in Macrophages

Matthew J. Sweet, Melanie R. Shakespear, Greg Kelly, Praveer Gupta, Larisa Labzin, Kate Schroder, Katharine M. Irvine, Nabilah Ahmad Kamal, David P. Fairlie

University of Queensland, Institute for Molecular Bioscience, Brisbane, Qld, Australia

In humans, the 18 histone deacetylase (HDAC) genes are grouped into distinct classes: class I HDACs (HDAC1, 2, 3, 8), class IIa HDACs (HDAC4, 5, 7, 9), class IIb HDACs (HDAC6, 10), class III HDACs (SIRT1-7) and a single class IV HDAC (HDAC11).HDACs deacetylate lysine residues on histone proteins and were first characterized as regulators of chromatin architecture. It is now clear that these enzymes modify a wide array of non-histone substrates to control diverse cellular processes including signal transduction, transcription factor activation, and protein trafficking/secretion. Broad-spectrum HDAC inhibitors were originally developed as anti-cancer drugs, but several of these compounds are also therapeutic in animal models of inflammatory diseases including sepsis, inflammatory bowel disease and rheumatoid arthritis. This implies that some HDACs have pro-inflammatory functions, but the identities of such HDACs, as well as the molecular mechanisms by which they act, are poorly understood. Here we show that TLR4-inducible expression of a sub-set of inflammatory genes in primary mouse and human macrophages was dependent on HDAC activity. An alternatively spliced isoform of HDAC7 that lacks the first 22 amino acids (HDAC7-s) promoted TLR4-dependent inflammatory responses in macrophages. Neither full length HDAC7 nor other class IIa HDACs had this activity. HDAC7-s acted by modulating activity of the HIF-1alpha transcription factor, a key pro-inflammatory transcription factor driving myeloid-dependent inflammation. A novel HDAC7-selective inhibitor reduced TLR4-inducible inflammatory mediator production from macrophages, suggesting that HDAC7-s represents a candidate inflammatory disease target. In summary, we have identified a specific HDAC that promotes TLR responses, and have provided further evidence that lysine acetylation/deacetylation is a key molecular switch modulating signal transduction and cellular activation.

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Inducible-Nitric-Oxide-Synthase (iNOS) Is Generally Required for Mouse Ig Isotype Switching Recombination

Pyeung-Hyeun Kim, Mi-Ra Lee, Goo-young Seo

Kangwon National University, Department of Molecular Bioscience, Chuncheon, Republic of Korea

The IgA antibody plays an important role in protecting mucosal surfaces against pathogens. It has recently been shown that nitric oxide (NO) plays a critical role in mouse IgA synthesis. In the present study, we further characterized inducible-nitric-oxide-synthase-deficient (iNOS $^{-/-}$) mice in the context of Ig expression. The amount of IgA in fecal pellets was substantially diminished in iNOS $^{-/-}$ mice and was paralleled by a decrease in IgA production by Peyer's patch cells. Interestingly, the amount of all IgG subisotypes, as well as IgA, was substantially diminished in sera and in cultured spleen B cells from iNOS $^{-/-}$ mice. Moreover, the synthesis of TGFβ1-inducible IgA and IgG2b in iNOS$^{-/-}$ mice was also lower than that in WT mice. However, levels of Ig germ-line transcripts, and expression of TGF-β receptor type II (TβRII) and BAFF/APRIL, were comparable between iNOS$^{-/-}$ and WT mice. Expression of activation-induced cytidine deaminase (AID) was diminished in iNOS$^{-/-}$ B cells, but restored by a NO donor, SNAP. These results indicate that iNOS regulates Ig isotype switching events at the level of AID gene expression.

ABSTRACTS

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Smad3 and ERK-CREB Mediate Activin A-Induced APRIL Expression in Mouse Macrophages

Goo-young Seo, Hwa-Joung Lee, Jae-Hee Kim, Mi-Ra Lee, Pyeung-Hyeun Kim

Kangwon National University, Department of Molecular Bioscience, Chuncheon, Republic of Korea

A proliferation-inducing ligand (APRIL) is primarily expressed by macrophages and dendritic cells, and stimulates B cell proliferation, differentiation, survival, and Ig production. In the present study, we investigated the role and signaling mechanisms of activin A in APRIL expression by mouse macrophages. Activin A markedly enhanced APRIL expression in mouse macrophages at both the transcriptional and protein levels. Overexpression of dominant-negative (DN)-Smad3 and SB431542 abrogated activin-induced APRIL transcription. Furthermore, activin A induced Smad3 phosphorylation. These results indicate that activin A enhances APRIL expression through both activin receptor-like kinase 4 (ALK4) and Smad3. In a subsequent analysis of activin A signaling, it was found that PD98059, an extracellular signal-related kinase (ERK) inhibitor, eliminated activin A-induced APRIL expression. On the other hand, overexpression of cAMP responsive element-binding protein (CREB), a molecule downstream of ERK, augmented activin A-induced APRIL expression, and this effect could be abolished by PD98059. This finding that activin A induces ERK and CREB phosphorylation suggests that ERK and CREB act as intermediates in APRIL expression. Taken together, these results demonstrate that activin A can enhance APRIL expression through two different pathways, Smad3 and ERK/CREB.

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Microparticle-Associated Tissue Factor Activity Is Reduced by Inhibiting Complement Factor 5 in *Neisseria meningitidis*-Exposed Whole Blood

Reidun Øvstebø,[1] Anne Pharo,[2] Anne-Marie S. Trøseid,[3] Marit Hellum,[3] Peter Kierulf,[1] Petter Brandtzaeg,[4] Tom E. Mollnes,[5] Carola Henriksson[1]

[1]Oslo University Hospital, Ullevål, Department of Medical Biochemistry, Oslo; [2]Institute of Immunology, Oslo , Oslo; [3]Blood Cell Research Unit, Oslo; [4]Oslo University Hospital, Department of Pediatrics, Oslo; [5]Institute of Immunology; Rikshopsitalet, Rikshospitalet University Hospital, Oslo

Objectives: *Neisseria meningitidis (Nm)* may cause fulminant meningococcal sepsis with massive activation of the coagulation and complement cascades. Bacterial cell wall molecules, particularly lipopolysaccharides (LPS), induce Tissue Factor (TF) expression on monocytes and monocyte-derived microparticles (MPs). Using *Nm*-exposed whole blood, we investigated the interplay between the complement and the hemostatic system with special focus on TF. The complement cascade was challenged by Soliris®, a potent Mab inhibiting C5, and cell-associated TF mRNA, MP-associated TF-activity and the terminal C5b-9 complex (TCC) were measured. Material and methods: Heparinized whole blood (10 U/mL), from healthy donors, was exposed to 10^8 Nm/mL for 4hours (37°C, constant rolling) in the absence or presence of the complement inhibitor (Soliris® 100 μg/mL). Then, 500 μl whole blood was added to 1.38 mL of PAXgene® solution and stored frozen until qRT-PCR (TF- and β_2 M-mRNAs). For analysis of TCC, plasma was obtained from whole blood by centrifugation, (2000xg, 15 min, RT), EDTA added and plasma stored frozen until analysis. To measure MP-associated TF- activity, plasma aliquotes (1 mL) was further centrifuged (17000xg, 30 min, RT), 850 μl removed and the remaining MP-enriched pellets were washed twice (17000xg, 30 min, RT) with NaCl/Owren/albumin /citrate solution before subjected to a clot formation assay. Results: In whole blood exposed to *Nm*, Soliris® reduced the coagulopatic response by down-regulating TF mRNA from 113 to 29 fold changes (n=3, p=0.052) all relative to whole blood not exposed to *Nm*. MP-associated TF-dependent procoagulant activity was reduced with approximately 50% (n=7, p=0.033). Presence of Soliris® effectively inhibited the complement system, reducing the levels of TCC from 36.2 to 2.2 AU/mL (n=4, p= 0. 005). Conclusions: In whole blood exposed to *Nm*, the complement and hemostatic systems are interplayers. Complement inhibition of C5 (Soliris® 100 μg/mL) decreases cell-associated TF mRNA and MP-associated TF-dependent procoagulant activity.

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CC Chemokines and Alcoholic Liver Injury: MCP-1/CCL2 Links Hepatic Inflammatory Response and Triglyceride Accumulation

Pranoti Mandrekar, Aditya V. Ambade, Donna Catalano

University of Massachusetts School of Medicine, Department of Medicine, Worcester, MA

CC chemokines are secreted by various liver cell types and influence disease progression and/or resolution. Amongst known CC chemokines, serum and liver of patients with alcoholic hepatitis exhibited highest CCL2 or monocytechemoattractant protein 1 (MCP1) levels. Alcoholic liver disease (ALD) is characterized by steatosis or triglyceride accumulation and elevated inflammatory cytokines induced in the liver by prolonged alcohol exposure. The functional role of CC chemokines in ALD remains elusive. Here we determined whether MCP1, a CC-chemokine contributes to alcohol-induced liver injury. Wild-type (WT) and MCP1-deficient (KO) C57/BL6 mice received Lieber-de-Carli diet with 5% v/v ethanol or isocaloric liquid control diet for 5 weeks. Parameters of liver injury, steatosis and pro-inflammatory responses were studied. Alcohol feeding significantly increased serum ALT in WT mice with increased MCP1 in serum and liver tissue as compared to pair fed WT mice. In contrast, MCP1 KO mice fed with alcohol showed no significant increase in serum ALT compared to pair-fed KO controls. Hepatic steatosis and triglyceride levels were significantly elevated in alcohol fed WT but inhibited in MCP1 KO mice. Expression of liver pro-inflammatory cytokines TNFα, IL-1β and IL-6 was induced in alcohol-fed WT mice but inhibited in MCP1 KO, independent of NFκB activation. Increased expression and DNA binding of fatty acid regulators, peroxisomeproliferator-activated receptor, PPARα and PPARγ, was accompanied by induction of PPARα target genes, acylCoAoxidase (ACOX) and carnitinepalmitoyl transferase-1 (CPT-1) in livers of alcohol-fed MCP1 KO mice compared to alcohol fed WT mice.In vitro assays reveal an inhibitory effect of MCP1 on PPARα mRNA and PPRE

binding. In conclusion, MCP1 deficiency protects mice against alcoholic liver injury by inhibition of pro-inflammatory cytokines and induction of PPARα target genes related to fatty acid oxidation, linking inflammatory chemokines to hepatic lipid metabolism. (Supported by NIAAA grant AA017545)

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Aberrant Host Defense against *Leishmania major* in the Absence of Secretory Leukocyte Protease Inhibitor (SLPI)

Nancy McCartney-Francis, Wenwen Jin, George McGrady, Sharon M. Wahl

NIDCR/NIH, OIIB, Bethesda, MD

Resistance to intracellular pathogens such as *Leishmania major* relies on the development of Th1 cellular immune responses wherein coincident upregulation of IFN-γ and inducible NO synthase (iNOS) mediate parasite killing and resolution of infection. Critical to successful host responses are suppressive mechanisms to limit tissue damage. SLPI, a potent epithelial-derived serine protease inhibitor with anti-microbial and anti-inflammatory functions, has been implicated in innate host defense. SLPI is induced by *L. major*and increased SLPI expression is evident within lesions following local infection with *L. major*. In contrast to the self-resolving infection in C57Bl/6 wildtype (WT) mice, SLPI-deficient ($SLPI^{-/-}$)mice develop a strong Th1 response to *L. major*, yet fail to control infection and develop destructive nonhealing lesions with systemic spread of parasites. Because SLPI is both produced by macrophages and antagonizes macrophage function, we examined the contribution of macrophage polarization to the defective host response in the absence of SLPI. Biogel-elicited peritoneal macrophages from SLPI null and WT mice were first primed overnight with either IFN-γ or IL-4 to generate classically activated M1 macrophages or alternatively activated M2 macrophages, respectively. Following infection with *L. major*, $SLPI^{-/-}$ M1 macrophages expressed elevated levels of iNOS RNA as compared to WT M1 macrophages, suggesting an increased capacity to kill parasites. In contrast, arginase was more highly expressed in WT M2 macrophages as compared to $SLPI^{-/-}$ M2 macrophages and expression declined following *L. major* infection. However, when we examined the M1/M2 responses in vivo following *L. major* infection we found that both IFN-γ and iNOS were persistently overexpressed in chronic lesions of $SLPI^{-/-}$ mice, but concomitantly IL-4 and arginase also remained elevated as compared to WT. These studies suggest that convergence of M1 and M2 macrophage responses may influence the outcome of innate host defense against intracellular parasites and SLPI is critical for coordinating resistance to chronic leishmaniasis.

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An ENU Mutant with Unusually High Representation of the Memory-Like CD44hi Subset within CD8 T Cells

John Kung, Mei-Ling Chang

Academia Sinica, Institute of Molecular Biology, Taipei, Taiwan

Ethyl-nitrosourea (ENU) was used to treat C57BL/6 (B6) male mice, followed by a 3-generation breeding scheme to generate G3 mice that were screened for abnormal marker expression by multi-parameter flow cytometry. Particular attention was given to the relative ratios of naïve to memory type based on the intensity of CD44 expression. Altered ratio of naïve to memory type T cells is potentially indicative of dysfunctional mechanism that govern memory T cell generation. One identified mutant from pedigree 079 (P-079) was characterized by unusually high representation of $CD44^{hi}$ cells within $CD8^{+}$ T cells under SPF housing conditions. Heritability testing revealed an autosomal dominant mode of transmission. Analysis of (B6 mutant x BALB/c)F2 mice by STR (short tandem repeat) mapping indicated linkage of the mutant phenotype to the 159 to 164Mb region on chromosome 2. Matings between $CD44^{hi}$ phenodiviant mice did not yield homozygous mutant mice, indicating homozygosity at the mutant locus causes lethality. DNA sequencing of genes within the 158 to 164Mb region of Ch 2 is now in progress. The P-079 ENU mutant mouse may be a valuable model in which to understand transcriptional regulation of the CD44 gene expression and whether $CD44^{hi}$ expression is causally related to T cell activation and/or memory generation.

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Analysis of ER-Hoxb8 Immortalized Cell Lines Revealed a Role of Calcium-Binding Proteins S100A8 and S100A9 for Phagocyte Dynamics

Christoph O. Spiekermann,[1] Christina Erhardt,[2] Georg Haecker,[3] Susanne Kirschnek,[3] Johannes Roth,[2] Hans Haecker,[4] Thomas Vogl[1]

[1]University of Muenster, Institute of Immunology, Muenster; [2]University of Muenster, Institute of Molecular Virology, Muenster; [3]University of Freiburg, Department of Microbiology and Hygiene, Freiburg; [4]St. Jude Children's Research Hospital, Department of Infectious Diseases, Memphis, TN

Background: S100A8 and S100A9 are calcium-binding proteins highly expressed in monocytes and neutrophils. Both are released by activated phagocytes during various inflammatory diseases. Complexes of both proteins (known as calprotectin) act as extracellular danger signals, able to activate Toll-like Receptor-4 (TLR-4) expressing cells. However, in spite of extensive research on these calcium-binding proteins, their intracellular role with regard to the function of phagocytes is still unclear. **Materials and Methods**: We immortalized progenitor cells of S100A9 deficient and wild-type mice (strain C57Bl/6) by transfection of the ER-Hoxb8 gene (Wang et al., 2006, Nat. Meth. 3, pp 287-293). The immortalized cell lines differentiate into macrophages or neutrophils depending on the cell culture conditions. To investigate the potential of these cell lines as alternative for primary cells we analyzed the expression pattern of various receptors by FACS as well as the expression of both S100-proteins by RT-PCR and western-blot. Functional analyses were carried out concerning transmigration, phagocytosis and respiratory burst. Dependent on stimulus, release of S100A8 and S100A9 was quantified by ELISA. **Results**: Expression of F4/80 or CD11b during differentiation of ER-Hoxb8 macrophages and Gr-1 on ER-Hoxb8 neutrophils is in good correlation to isolated primary cells. Furthermore ER-Hoxb8 cells adhere and migrate normally and show phagocytosis and respiratory burst comparable to BMDM (bone marrow derived macrophages). In ER-Hoxb8 monocytes/

macrophages S100A8 and S100A9 are highly expressed on day 2 and decrease during maturation to macrophages in accordance to what is observed for BMDM. In ER-Hoxb8 neutrophils a continuous increase of S100A8/A9 expression up to 4 days was observed also in accordance to BM-derived neutrophils. Furthermore we could show that knock-out of the S100A9 gene influences dynamic processes of phagocytes like transmigration and phagocytic activity. **Conclusion**: Analyzing ER-Hoxb8-imortalized cell lines we identified an intracellular role of S100A8/A9 for phagocyte dynamics.

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Nlrp3 Inflammasome Activation Is through Mitochondrial Dysfunction and Independent of Reactive Oxygen Species

Qiong He,[1] Shankar S. Iyer,[2] Alicia K. Olivier,[3] Vickie Knepper-Adrian,[2] William M. Nauseef,[2] Suzanne L. Cassel,[2] Fayyaz S. Sutterwala[2]

[1]University of Iowa, Graduate Program in Immunology, Iowa City, IA; [2]University of Iowa, Inflammation Program and Department of Medicine , Iowa City, IA; [3]University of Iowa, Department of Pathology, Iowa City, IA

Activation of the Nlrp3 inflammasome occurs in response to numerous agonists, although the actual mechanism by which activation occurs has not been determined. Previously described activators of the Nlrp3 inflammasome have been shown to induce the generation of mitochondrial reactive oxygen species (ROS); in addition, pharmacological blockade of ROS inhibits the activation of the Nlrp3 inflammasome leading to a model in which mitochondrial ROS is a required upstream mediator for Nlrp3 inflammasome activation. Here we show that the oxazolidinone antibiotic linezolid results in both the Nlrp3-dependent *in vitro* release of the proinflammatory cytokine IL-1β and *in vivo* neutrophilic influx following its intraperitoneal administration and that Nlrp3 deficiency protects animals from linezolid-induced bone marrow toxicity. Importantly, linezolid is unique amongst Nlrp3 activators in that its ability to activate the Nlrp3 inflammasome is independent of ROS. Instead, the common pathway shared by linezolid's activation of the Nlrp3 inflammasome and the ROS-dependent activators is disruption of the mitochondrial membrane potential. This novel finding demonstrates that ROS generation is not the canonical activator of Nlrp3 but rather an intermediary step that can lead to the mitochondrial perturbation that is critical for Nlrp3 inflammasome activation.

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Mechanisms of TNFα-Induced Priming of Human Neutrophils

Silvia M. Uriarte,[1] Madhavi J. Rane,[2] Richard A. Ward, [3] Kenneth R. McLeish[4]

[1]University of Louisville, Molecular Signalling Group, Louisville, KY; [2]University of Louisville, Dept of Medicine & Neuphrology, Louisville, KY; [3]University of Louisville, Louisville, KY; [4]Univ of Louisville, Dept of Med, Louisville, KY

Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), prime the respiratory burst of human neutrophils. Both granule exocytosis and phosphorylation of cytosolic NADPH oxidase components have been implicated in this priming; however, their relative importance has not been established. We generated a fusion protein containing the TAT cell-penetrating sequence and a SNARE domain of SNAP-23 (TAT-SNAP-23) which inhibited granule exocytosis in a concentration-dependent manner. Priming of isolated human neutrophils was measured as the increase in fMLF-stimulated superoxide release following incubation with TNFα 2 ng/ml for 10 min. Pre-treatment with TAT-SNAP-23 induced a dose-dependent inhibition of secretory vesicle, gelatinase, and specific granule exocytosis and of TNFα-induced priming of fMLF-stimulated superoxide release, without altering superoxide release by unprimed cells. Comparison of the dose-dependent inhibition curves showed that the reduction in secretory vesicle and gelatinase granule exocytosis was linearly related to the reduction in TNFα-induced priming, while there was no direct relationship between priming and specific granule exocytosis. Inhibition of Pin1 cis-trans prolyl isomerase activity resulted in a significant 75% reduction in TNFα-induced priming. Inhibition of Pin1 had no significant effect on secretory vesicle exocytosis, while specific granule exocytosis was significantly inhibited by 47%. Inhibition of exocytosis by TAT-SNAP-23 reduced $p47^{phox}$ translocation to the plasma membrane in TNFα-primed, fMLF-stimulated neutrophils. We conclude that both exocytosis of secretory vesicles and gelatinase granules and Pin1 activity are necessary for $p47^{phox}$ translocation to the plasma membrane and priming of respiratory burst activity.

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C1q Activates Distinct Signaling Mechanisms in Human Monocytes, Macrophages and Immature Dendritic Cells

Elizabeth V. Clarke, Deborah A. Fraser, Marie E. Benoit, Andrea J. Tenner

University of California Irvine, Department of Molecular Biology and Biochemistry, Irvine, CA

Previous studies by our lab and others have highlighted a role for C1q in directing phagocytic and cytokine responses in human monocytes, human monocyte-derived macrophages (HMDM) and immature dendritic cells (iDC). Although, in general, C1q directs these phagocytic cells towards a more limited immune response by inducing anti-inflammatory cytokine IL-10, we observed differences in cytokine responses in relation to the differentiation state of these cells, and the particle ingested. These studies aim to determine if different phagocytes employ common signaling mechanisms in response to C1q and whether these correlate with the observed cytokine responses.

Candidate transcription factors / pathways which may be involved—including NFkB, ERK/CREB and Jak/STAT—were identified by promoter analysis of C1q modulated genes, as measured by microarray analysis. Experiments were performed to assess the activation of these transcription factors by C1q, either immobilized on a plate in monocytes, HMDM and iDC or bound to autologous apoptotic lymphocytes in HMDM using flow cytometric and Western blot analysis.

Phosphorylation of CREB, previously shown to be enhanced in monocytes adhered to immobilized C1q, was not enhanced

in HMDM or iDC or by C1q on apoptotic cells in monocytes, macrophages, or iDCs. However, NFkB p50p50 was enhanced in HMDM interacting with immobilized C1q, similar to our previously reported enhancement in monocytes. C1q-bound to late apoptotic cells also modulated expression of HMDM genes involved in the Jak/Stat pathway as measured by microarray analysis and increased STAT1 phosphorylation in HMDM relative to levels observed after ingestion of late apoptotic cells without C1q.
These data, consistent with increased levels of IL-10 in C1q-treated phagocytes, begin to define the molecular pathways by which C1q effects a regulatory or anti-inflammatory environment in phagocytes and further demonstrates the importance of differentiation/polarization state of the macrophage in response to environmental stimuli.
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The Pivotal Role of Glutathione Reductase in Host Defense against Gram-Negative Bacteria

Yusen Liu, Jing Yan, Xiaomei Meng, Lyn M. Wencket, Katherine Lintner, Leif D. Nelin, Bernadatte Chen, Charles V. Smith, Lynetter K. Rogers
Children's Research Institute at Nationwide Children's Hospital, The Ohio State University, Department of Pediatrics and Center for Perinatal Research, Columbus, OH

Glutathione reductase (Gsr) is an enzyme that reduces glutathione disulfide to the sulfhydryl form, a major cellular antioxidant. Oxidative burst plays an important role in pathogen killing and initiation of inflammation. Proper redox regulation is crucial for balancing effective pathogen elimination and host preservation. We tested the hypothesis that Gsr plays a critical role in host defense by comparing wildtype (WT) and Gsr- mice after E. coli infection. Compared to WT mice, Gsr- mice exhibited substantially higher mortality, associated with greater bacterial burden, cytokine storm, and striking histological abnormalities. Surprisingly, Gsr- mice displayed increased resistance to LPS. While Gsr- mice exhibited defects in phagocyte mobilization, there appeared no substantial defects in either cell signaling or cytokine production in Gsr- macrophages. The role of Gsr in phagocytic oxidative burst was assessed by both flow cytometry and bioluminescence imaging. The oxidative burst in WT neutrophils after PMA stimulation was sustained for more than 30 min, while oxidative burst was very transient in Gsr- cells (<5 min). Likewise, oxidative burst after E. coli infection was also significantly weaker in the Gsr- neutrophils. Thus, Gsr plays a critical role in host defense against bacterial infection by regulating various immune functions, including facilitation of bacterial killing via sustaining oxidative burst in phagocytes, through maintenance of a delicate redox balance.

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Hypoxic Peritoneal and Splenic B1a B Cell Subsets Exhibit Different Phenotypes

Kaori Knights, Sherry Fleming
Kansas State University, Department of Biology, Manhattan, KS

During tissue injury, the hypoxic local environment exposes neo-antigens recognized by naturally occurring antibodies (NAb). The NAb repertoire possesses limited diversity with broad specificity allowing for early recognition of pathogens. However, the poly-reactivity of NAb, produced by B1a B-cells, may lead to self-reactivity and autoimmunity. B1a B-cells express CD5, secrete IL-10, and participate in the innate immune response. The B1a B-cell phenotype recently was sub-divided into peritoneal B1a B-cells which express IgM, CD11b, and Siglec-G and splenic B1a B-cells which express IgG and CD43. B-cells migrate to hypoxic environments and play a role in the inflammatory response. However, the direct effect of hypoxia on B1a B-cells after migration is currently not well understood. We hypothesized that hypoxia may alter the B1a B-cell phenotype and NAb response differentially based on location. After 2 – 24 hours of normoxia or hypoxia, we compared phenotype and activity of two B1a B-cell lines representing different subsets. Wehi-231 cells resemble peritoneal B1a B-cells and LK 35.2 cells resemble splenic B1a B-cells. We determined mitochondrial activity, cell viability, nitric oxide (NO) production, antibody secretion, and mRNA and protein expression of B1a related markers. Although hypoxia did not alter antibody production in either cell line, each expressed a distinct response to hypoxia. Although mitochondrial reductases remained unchanged, hypoxic LK 35.2 cells grew slower, failed to produce NO and expressed decreased B1a B-cell markers based on mRNA and protein expression. In contrast, following hypoxia, peritoneal-like Wehi-231 cells produced significantly less mitochondrial reductase and NO, while the growth rate and B1a B-cell markers remained unchanged. Together, these data suggest that the B1a B-cell response to hypoxia and tissue injury are distinct for peritoneal and splenic subsets of B1a B-cells.

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TLRs 2 and 9 Modulate Innate Immune Cell Activation in Hypersensitivity Pneumonitis

Kelly J. Andrews,[1] Hossam Abdelsamed,[1] Ae-Kyung Yi,[2] Elizabeth A. Fitzpatrick[3]
[1]Department of Molecular Sciences, [2]Department of Pediatrics, [3]Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center Memphis, TN

Hypersensitivity pneumonitis (HP) is an interstitial lung disease that develops following repeated exposure to environmental antigens. The disease is characterized by alveolitis, granuloma formation, and in some patients, fibrosis. Using the *Saccharopolyspora rectivirgula* (SR) mouse model of HP, previous studies in our lab demonstrated that the acute response to SR was dependent on MyD88 signaling pathways. Mice that are genetically deficient in MyD88 did not respond with neutrophil recruitment or pro-inflammatory cytokine production following one SR exposure. The goal of this study was to determine whether the MyD88-dependent TLRs 2 and 9 are responsible for innate immune cell activation during HP. We intranasally exposed C57Bl/6, TLR2$^{-/-}$, TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice with SR and measured neutrophil recruitment. Our results demonstrate that TLR2/9$^{-/-}$ mice have a reduction in neutrophil recruitment and MIP-2 compared to WT

mice throughout the disease. Additionally, there is a decrease in the pro-inflammatory cytokines IL-6 and TNF in the bronchoalveolar lavage fluid of TLR2/9$^{-/-}$ mice compared to WT mice. The decrease in inflammation was not due to an increase in IL-10 production since TLR2/9$^{-/-}$ mice did not express increased levels of IL-10 mRNA. Interstitial macrophages isolated from the TLR2/9$^{-/-}$ mice exposed to SR did not upregulate MHC II or the co-stimulatory molecules CD40 or CD80 to the same extent as WT macrophages. The decrease in IL-6 production was accompanied by a decrease in the % of Th17 cells in the lungs of TLR2/9$^{-/-}$ mice. The decreased Th17 response was not due to a switch to Th1, Th2, or Treg response since we did not detect increases in these populations. The results from these studies demonstrate that TLRs 2 and 9 contribute to recognition of SR and play a role in the development of HP. This work was supported by the National Institutes of Health (HL084172).

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Smoking Increases Soluble FLT1 (VEGF Receptor) Expression in Alveolar Macrophages

Linda S. Powers, Emma Nash, Anna Reisetter, Alicia Gerke, Thomas J. Gross, Martha M. Monick

University of Iowa, Department of Internal Medicine, Iowa City, IA

Rationale: Alveolar macrophages are key players in the development of smoking-related diseases. Gene expression data in smoker's macrophages shows significant up regulation of the VEGF receptor (FLT1). The FLT1 gene yields both a full length protein (a membrane bound tyrosine kinase receptor) or a soluble variant containing the extra-cellular domain of FLT1. The soluble FLT1 serves as a decoy receptor, inhibiting VEGF signaling. In this study we examined the effect of smoking on FLT gene expression in alveolar macrophages. **Methods:** Smokers and nonsmokers underwent bronchoalveolar lavage to obtain alveolar macrophages. RNA was isolated and mRNA expression analyzed using GeneChip Human Exon 1.0 ST arrays. Gene expression validation and analysis of sFLT1 transcripts were performed by real time PCR with primers specific for the membrane receptor form of FLT1 and its soluble transcriptional variants. **Results:** FLT1 expression was 3 fold greater in smokers alveolar macrophages with a p value of <0.0001. When we examined the transcript variants expressed from the FLT1 promoter, we found up regulation of the full length transcript (variant 1, approximately twofold) in smoker's macrophages. We also found differential up regulation of a soluble variant (variant 2, between four and eleven fold). To confirm up regulation of the soluble form, we analyzed smoker macrophage supernatants and found a significant increase in released soluble FLT1. **Conclusion:** Chronic exposure to cigarette smoke in long-term smokers results in increased expression of FLT1 and in particular increased expression of the soluble variant. This data suggests that an anti-angiogenic microenvironment is established in smoker lungs via macrophage production of soluble FLT1. The role this plays in smoking-related disease is still to be determined. Funding: NIH R01 HL079901, NIH RO1 HL096625 to M. M. and Grant Number UL1RR024979 from the National Center for Research Resources (NCRR).

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TLR4-Driven Autophagy in Hepatocytes Involves LC3 and p62 Colocalization

Christine Chen, Patricia Loughran, Melanie J. Scott, Timothy R. Billiar.

University of Pittsburgh Medical Center, Department of Surgery, Pittsburgh, PA

Introduction: Autophagy is a selective degradation pathway and impairment of autophagy has been associated with liver injury. TLR4-stimulation by LPS has been shown to upregulate autophagy in macrophages and hepatocytes although the exact mechanism linking TLR4 and autophagy remains elusive. The objective of this study was to determine whether LPS-stimulated autophagy in hepatocytes follows a classical autophagy pathway. **Methods**: Primary C57BL/6 mouse hepatocytes were treated with LPS (1 ug/mL) up to 24h. Whole cell lysates were collected and immunoblotted for LC3, p62/SQSTM1, Nrf2, and Beclin-1. Cells were also transfected with GFP-LC3BII and immunostained with anti-p62 antibody. P62 mRNA levels were measured using RT-PCR. **Results**: LC3-puncta formation significantly increased together with LC3-colocalization with p62 by 4h after LPS and was maximal at 16h. P62 mRNA and protein levels also increased with LPS treatment at 4h, but returned to baseline after 8h. Nuclear levels of Nrf2, the transcription factor that upregulates p62, were upregulated early up to 4h. LC3BII and Beclin-1 levels remained constant throughout the time course. **Conclusion**: TLR4-stimulation by LPS resulted in p62 and LC3 interaction in hepatocytes, which may be driven by TLR4-dependent activation of Nrf2 independent of the classical autophagy pathway.

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Role of the NLRP3 Inflammasome in the Immune Response to Vaccines

Shruti Wilson, Fayyaz Sutterwala, Suzanne L. Cassel.

University of Iowa Hospitals & Clinics, Department of Immunology, Iowa City, IA

Humoral immunodeficiencies occur in half of all primary immunodeficiency cases, however, disease etiopathogenesis largely remains unknown. Evaluation of the functional antibody status includes measurement of IgG titers to vaccine antigens, including to the alum-containing tetanus vaccine. Alum's mechanism of action remains unknown, however, this adjuvant is a known NLRP3 inflammasome activator. The NLRP3 inflammasome refers to a macromolecular complex composed of NLRP3, a member of the cytoplasmic Nod-like receptors. Inflammasome activation results in the production of the inflammatory cytokine IL-1β. We hypothesize that alum in commercial vaccines stimulates an immune response through NLRP3 inflammasome activation and that the defective antibody response to alum-containing vaccines seen in some patients is due to a defect in the innate immune response to the adjuvant used. In mouse models we determined that wildtype peritoneal macrophages primed with LPS and stimulated with alum-containing vaccines resulted in IL-1β secretion. This was dependent on the NLRP3 inflammasome, as macrophages deficient in NLRP3 and ASC exhibited impaired IL-1β release.

Similarly, the human response to alum-containing vaccine also activates the inflammasome pathway, as IL-1β release was observed when peripheral blood mononuclear or whole blood cells were LPS primed and stimulated with tetanus vaccine. Through our immunodeficiency clinic database, we identified patients with a defective response to the tetanus vaccine for assessment of NLRP3 inflammasome competency. Blood samples were drawn after written informed consent was obtained from patients. Heparinized whole blood samples were LPS primed and stimulated with NLRP3 activators (alum and tetanus vaccine) and as a control an AIM2 inflammasome activator (Poly dA:dT), followed by IL-1β measurement. In order to identify defects specific to the inflammasome, an induction index was calculated, which is the level of responsiveness to NLRP3 activators compared to the responsiveness to the AIM2 inflammasome activator. The standard induction index determined thus far for healthy control subjects is 2.305 with a standard deviation of 0.70. Through our ongoing screen we aim to identify patients with impaired NLRP3 activity, and to determine if this is associated with genetic polymorphisms in inflammasome components leading to a loss of inflammasome function.

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Caspase-1 Is Liver Protective after Hemorrhagic Shock in Mice Independent of Nalp3 Inflammasome

Qian Sun, Melanie J. Scott, Timothy R. Billiar.
University of Pittsburgh, Department of Surgery, Pittsburgh, PA

Activation of the inflammasome by reactive oxygen species (ROS) and Damage Associated Molecular Patterns (DAMPs) produced during ischemia/reperfusion and hemorrhagic shock leads to maturation of caspase-1, which cleaves pro-inflammatory cytokines (IL-1β, IL-18) and causes their subsequent release. However, our previous data showed that caspase-1 -/- mice had more liver damage and higher pro-inflammatory cytokine levels after hemorrhagic shock compared with WT. In this study we investigated whether this effect is mediated by Nalp3 inflammasome, which has been shown to activate caspase-1 induced by ROS. **Methods:** Caspase-1-/-, Nalp3-/- and C57BL/6 (WT) mice underwent sham surgery (femoral artery cannulation only) or HS (1.5h hemorrhagic shock + 0h, 1.5h, 4.5h and 24h resuscitation) (n=5-7/experimental group). Whole liver lysates were collected and immunoblotted for caspase-1. ALT, AST and Plasma cytokines levels were measured. **Results** : Caspase-1 is activated in a time dependent manner in the liver of WT mice after HS, but not in caspase-1 -/- mice, suggesting inflammasome activation. Caspase-1 -/- mice showed significantly more liver damage at 4.5h after HS than the WT mice, as well as higher levels of pro-inflammatory cytokines IL-6 and MCP-1. However, caspase-1 is also activated in Nalp3 -/- mice shown by western blot and circulating IL-18 levels. In addition, Nalp3-/- mice had similar levels of liver damage as the WT mice, suggesting activation and the protective effect of caspase-1 is not mediated by Nalp3 inflammasome. **Conclusion:** Caspase-1 is protective in the liver after hemorrhagic shock independent of Nalp3 inflammasome, through a mechanism that has yet to be identified. Elucidating the protective effect of caspase-1 may help understand the regulation of inflammation after trauma and hemorrhage.

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Regulation of the Inflammatory Response: Enhancing Neutrophil Infiltration under Chronic Inflammatory Conditions

Zhen Bian[1], Yanan Guo[1], Binh Ha[1], Ke Zen[2], Yuan Liu[1]
[1]*Georgia State University, Atlanta, GA;* [2]*NanJing University, NanJing, P.R. China*

Neutrophil (polymorphonuclear leukocytes, PMN) infiltration is a central component of innate immunity, but is also a major cause of inflammation-related tissue damage. For this reason, PMN infiltration must be tightly controlled. Using zymosan-induced peritonitis as an in vivo PMN infiltration model, we show that PMN response and infiltration were significantly enhanced in mice under inflammatory conditions including colitis and diabetes when compared with healthy control mice. Adoptive transfer of leukocytes from mice with inflammation into healthy recipients, or the other way around, and then inducing peritonitis demonstrated that both circulating PMN and tissue microenvironments including tissue macrophages were altered during chronic inflammation and that they collectively promoted PMN infiltration. Detailed analyses of dextran sulfate sodium (DSS)-elicited colitis revealed that enhanced PMN infiltration occurred only in the post-acute/chronic stage of inflammation and was associated with markedly increased IL-17A in serum, suggesting an involvement of activated TH-17 cells in the global modulation. Suppressing IL-17A function by a neutralization antibody eliminated the enhancement of PMN infiltration and IL-6 production and prevented severe tissue damage in DSS-treated mice. In summary, this study shows that inflammation at the chronic stage produces feedback signals that enhance PMN infiltration and promote the inflammatory reaction. This work was supported, in part, by NIH DK62894 (YL) and a Research Scholar Grant from the American Cancer Society (YL).

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3-(1*H*-Indol-3-yl)-2-[3-(4-nitrophenyl)ureido]propanamide Derivatives are Agonists of Human Formyl Peptide Receptor 2

Mark T. Quinn,[1] Liliya N. Kirpotina,[1] Igor A. Schepetkin,[1] Marcello Leopoldo[2]
[1]*Montana State University, Department of Immunology & Infectious Diseases, Bozeman, MT;* [2]*Università degli Studi di Bari "A. Moro, Farmaco-Chimico, Bari, Italy*

N-formyl peptide receptor (FPR1) and FPR2 are G protein-coupledreceptors (GPCR) involved in host defense and sensing cellular dysfunction. Previously we found that antagonists of gastrin-releasing peptide/neuromedin B receptors (BB_1/BB_2), PD168368 and PD176252, were potent mixed FPR1/FPR2 agonists [Mol. Pharm. (2011) 79: 77-90]. In the present studies, we screened 13 structural derivatives of PD176252 for their ability to activate human neutrophils and HL-60 cells transfected with human FPR1 or FPR2. While none of the compounds had BB_2 antagonist activity, five of the compounds stimulated Ca^{2+} flux in HL-60 cells expressing FPR2, but not in HL-60 cells expressing FPR1, suggesting they were selective for FPR2. The most potent compounds EMY-96
[(*R*)3-(1*H*-indol-3-yl)-2-(3-(4-nitrophenyl)ureido)-N-((1-(pyridin-

2-yl)cyclohexyl) methyl)propanamide] and its *S*-isomer ML-16 induced Ca^{2+} flux with EC_{50} values in the low micromolar range. Furthermore, pretreatment of FPR2/HL-60 cells with specific the FPR2 antagonist WRW4 prevented Ca^{2+} flux activated by EMY-96 and ML-16. Interestingly, neither EMY-96 nor ML-16 was able to induce Ca^{2+} flux in human neutrophils; however, EMY-96 desensitized human neutrophils and FPR2/HL-60 cells to subsequent activation by the hexapeptide WKYMVM. In addition, all five active compounds dose-dependently stimulated human neutrophil chemotaxis. Lastly, these compounds induced β-arrestin binding to FPR2. Thus, these 3-(1*H*-Indol-3-yl)-2-[3-(4-nitrophenyl)ureido] propanamide derivatives represent unique FPR2 agonists and exhibit novel properties in human neutrophils and FPR-transfected HL-60 cells. This work was supported in part by National Institutes of Health grant P20 RR-020185, National Institutes of Health contract HHSN266200400009C, an equipment grant from the M.J. Murdock Charitable Trust, and the Montana State University Agricultural Experimental Station.

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TLR10 Is a Global Suppressor of TLR-Induced Pro-inflammatory Gene Activation

Song Jiang, Xinyan Li, Richard Tapping
University of Illinois, Department of Microbiology, Urbana, IL

Through direct sensing of bacterial, fungal or viral components, Toll-like receptors (TLRs) activate intracellular signaling events that drive the cellular expression and release of immune mediators. These activation events not only induce inflammatory processes, but also initiate and orchestrate the longer term protective responses of the adaptive immune system. Despite a decade of research and tens of thousands of publications on TLRs, the biologic function of TLR10 has remained unknown. In efforts to uncover a signaling function for TLR10, we generated vector control and stable TLR10-expressing cells by retroviral transduction of the human myelomonocytic U937 cell line. To determine the effect of TLR10 on TLR-mediated activation, we performed a quantitative PCR array experiment which surveyed 84 proinflammatory cytokines, chemokines, transcription factors and acute phase proteins in our U937 cell lines following lipopeptide activation of TLR2. These data showed that the stimulation of RNA message for a number of the inflammatory genes, including IFN-beta and IL-6, was significantly reduced in TLR10-expressing cells compared to vector control cells. No effect on cell viability was observed in any of the cell lines either before or after stimulation. Surprisingly, TNF-alpha, IP-10 and IL-6 release induced by a variety of TLR agonists, including those for TLR2, TLR3 and TLR4, were also greatly suppressed in the TLR10-U937 line but remained robust in the vector control U937 cells, showing that the suppressive effect of TLR10 extends to other members of the TLR subfamily. Transient transfection of HEK cells shows that TLR10 inhibits MyD88-induced NF-kB activation as well as TRIF-induced activation of an IFN-beta promoter. These results suggest that human TLR10 acts to regulate pro-inflammatory signaling and cytokine production and suggest that TLR10 is unique among TLRs as a global suppressor of TLR-induced activation.

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CXCR1 and CXCR2 Couple to Distinct G-Protein Couple Receptor Kinase to Modulate Leukocyte Functions

Micheler R. Richardson, Katherine Haynes
North Carolina Central University, JLC-BBRI Cancer Research, Durham, NC

The Interleukin-8 (IL8/CXCL8) receptors, CXCR1 and CXCR2, couple to Gi to induce leukocyte recruitment and activation at sites of inflammation and infection. Upon activation by CXCL8, both receptors become phosphorylated and desensitized. In this study we investigate the role of specific G-protein couple receptor kinase (GRK) in CXCR1 and CXCR2-mediated cellular functions. To that end, small interfering RNA (siRNA) was used to inhibit GRK 2, 3, 5 and 6 expressions in RBL-2H3 cell stably expressing CXCR1 or CXCR2. The data demonstrate that inhibition of GRK6 increased CXCR2-, but not CXCR1, mediated phosphoinositide (PI) hydrolysis (~30%) and β-hexosaminidase release (~40%). GRK6 inhibition also increased CXCR2 resistance to phosphorylation, desensitization and internalization. In contrast, suppression of GRK2 enhanced CXCR1-mediated PI hydrolysis (~20%) and β-hexosaminidase release (~35%) with no significant effect in CXCR2-mediated functions. Peritoneal neutrophils from mice deficient in GRK6 ($GRK6^{-/-}$) also displayed increased CXCR2-mediated G-protein activation and receptor resistance to internalization. Taken together, the results indicate that, upon interaction CXCL8, CXCR1 and CXCR2 complex with distinct GRK to mediate and regulate inflammatory responses. Supported by NIH/NIAID AI-38910

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A Protective Role for Inflammasome Activation Following Injury

James A. Lederer, Akinori Osuka, Veit Stoecklein, Marc Hanschen
Harvard Medical School , Brigham and Women's Hospital, Department of Surgery, Boston, MA

The inflammasome is activated in response to pathogen or endogenous danger signals and acts as an initiator as well as a mediator of inflammatory reactions. In this study, we wished to identify whether the inflammasome is activated *in vivo* by injury. And if so, we wanted to characterize the kinetics, the immune cell distribution, and the functional impact of inflammasome activation on the injury response. Since caspase-1 activation is the final product of inflammasome pathway activation, we used cleaved caspase-1 p10 and p20 as a measure for inflammasome activation in cells. We first developed a procedure to stain for caspase-1 p10 and p20 by flow cytometry (FACS) in LPS + ATP stimulated spleen cells. This method for measuring caspase-1 activation was validated using FLICA, a fluorescently-tagged specific binding reagent for activated caspase-1. Once validated by *in vitro* studies, we measured caspase-1 activation by FACS in immune cell subsets prepared from the lymph nodes and spleens of sham- or burn-injured mice at different time points. Lastly, the functional significance of inflammasome activation following burn injury was tested in mice treated with the specific caspase-1 inhibitor, AC-YVAD-

CMK. The results of *in vitro* studies indicated that ATP and LPS stimulation induced significant caspase-1 activation in dendritic cells, macrophages, and NK cells. This approach also revealed caspase-1 activation in CD4 and CD8 T cells as well as B cells. We then measured caspase-1 activation in cells prepared from the lymph nodes and spleens of sham- or burn-injured mice. Significant caspase-1 activation was detected in macrophages and dendritic cells by 4 hours after injury and peaked by day 1 after injury. FLICA staining confirmed that caspase-1 activation occurred in these cells at 1 day after injury. We also found significant injury-induced caspase-1 activation in NK cells, CD4 T cells, and B cells, but CD8 T cells did not demonstrate caspase-1 activation. Surprisingly, we found that blocking caspase-1 activation with AC-YVAD-CMK *in vivo* caused significantly higher mortality in burn-injured mice ($p<0.01$). Taken together, these findings document that injury induces inflammasome activation in many immune cell subsets, but primarily in macrophages and that inflammasome activation plays a protective role in the host response to severe injury.

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Dendritic Cells Take the Center Stage in Human Aging: Role in Chronic Inflammation Immune Dysfunction

Anshu Agrawal, Sudhir Gupta
School of Medicine, University of California, Irvine, Irvina, CA

Dendritic cells (DCs) are the major antigen presenting cells of the body that are critical for generation of immunity and maintenance of tolerance. Advancing age has a profound affect on dendritic cell functions. DCs from aged display a higher basal level of activation and secrete pro-inflammatory cytokines leading to chronic inflammation. An increased response to self antigens such as mammalian DNA contributes further to inflammation, and autoimmunity during aging. In contrast, DCs from aged subjects are impaired in their ability to mount effective immune responses against foreign antigens such as influenza virus, which includes reduced Interferon secretion and impaired capacity to prime T cell responses. The mechanisms underlying the altered DC functions in aged humans are not well understood. We have previously shown that NFκB and PI-3 kinase signaling pathways are altered in aged DCs. Affymetrix gene analysis of DCs from aged and young subjects revealed upregulated PTEN, which may be responsible for impaired PI-3 kinase signaling pathway. Studies using chromatin-immunoprecipitation (CHIP-IP) with histone antibodies (H3K4, H3K9) demonstrate increased levels of suppressive histones, which suggest chromatin remodeling may also be a key player in altering the response of DCs with age.Age-associated alterations at the genetic as well as chromatin remodeling may therefore be responsible for the changes in the function of DCs from aged subjects.

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Evidence for Two CRIB Domains in Phospholipase D2 (PLD2) That the Enzyme Uses to Specifically Bind to the Small GTPase Rac2 during Chemotaxis

Julian G. Cambronero,[1] Mary C. Dinauer,[2] Hong-Juan Peng,[1] Karen Henkels,[1] Mahdu Mahankali[1]
[1]Wright State University School Medicine, Department of Biochemistry and Molecular Biology, Dayton, OH; [2]Washington University School of Medicine, Department of Pediatrics, Saint Louis, MO

Phospholipase D (PLD) and small GTPases are vital to cell signaling during inflammation. We report that the Rac2 and the PLD2 isoforms exist in the cell as a lipase-GTPase complex that enables the two proteins to elicit their respective functionalities, such as adhesion and chemotaxis. A strong association between the two molecules was demonstrated by co-immunoprecipitation, which was confirmed in living cells by FRET with CFP-Rac2 and YFP-PLD2 fluorescent chimeras. We have identified the amino acids in PLD2 that define a specific binding site to Rac2. This site is composed of two CRIB (Cdc42-and Rac-Interactive Binding) motifs that we have named "CRIB-1" and "CRIB-2", in and around the PH domain in PLD2. Deletion mutants PLD2-ΔCRIB-1/2 negate co-immunoprecipitation with Rac2 and diminish the FRET signal in living cells. The PLD2-Rac2 association was further confirmed in vitro using affinity purified, recombinant proteins. Binding was saturable with an apparent Kd of 3 nM and was diminished with PLD2-ΔCRIB mutants. Further, PLD2 bound more efficiently to Rac2-GTP than to Rac2-GDP or to a GDP-constitutive Rac2-N17F mutant. Increasing concentrations of recombinant Rac2 in vitro and in vivo during cell adhesion or chemotaxis inhibit PLD. We propose that in activated cells, PLD2 affects Rac2 in an initial positive feedback, but as Rac2-GTP accumulates in the cell, this constitutes a "termination signal" leading to PLD2 inactivation. Supported by the grant HL056653 (J.G.-C.) from the National Institutes of Health.

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MPLA Induced Neutrophil Recruitment Protects against Severe Sepsis

Shalaka Metkar, Jack Silver, Sanna M. Goyert
City University of New York, Sophie Davis School of Biomedical Education, New York, NY

Previous studies have shown that CD14-deficient mice are resistant to severe infection with clinical isolates of *E. coli*; resistance is accompanied by early neutrophil recruitment to the site of infection and an enhanced ability to clear the bacteria. In contrast, normal mice do not display early neutrophil recruitment, leading to bacterial dissemination, severe sepsis and increased mortality. Differential neutrophil recruitment in normal and CD14-/- mice is also observed in response to smooth LPS, the form of LPS that is predominant in various pathogenic strains of *E. coli*. However, unlike the response to LPS or *E. coli*, normal mice show early neutrophil recruitment when treated with various truncated LPS derivatives, including MPLA, a monophosphorylated derivative of LPS, Lipid A, and Ra LPS, a rough form of LPS lacking the

polysaccharide O-antigen. All of these truncated molecules induce low levels of the neutrophil-attracting chemokines MIP-2 and KC at the site of infection, but fail to induce significant levels of systemic chemokines that appear to block neutrophil transmigration from the circulation in response to smooth LPS or *E. coli*. MPLA-induced early chemokines leading to neutrophil recruitment protect normal mice against severe *E. coli* infections.

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NOX2-Dependent Surface Translocation of BLT1 Is Essential for Exocytotic Degranulation of Human Mast Cells and Eosinophils Induced by LTB4

Deulle Min, Myeong Heon Shin

College of Medicine, Yonsei University, Seoul, Korea

Mast cells are known to play a key role in mucosal immune responses by releasing various inflammatory mediators including histamine, cysteinyl leukotriens, cytokines and chemokines. Human eosinophils are an important immune cells for allergic diseases. Leukotriene B_4 (LTB_4) is a potent activator and chemoattractant and is implicated in several inflammatory and allergic diseases via BLT1 receptor. In this study, we hypothesized LTB_4 can causes immune responses in human mast cells and eosinophils. Therefore, we first observed LTB_4 induced immune responses, such as degranulation, intracellular ROS generation, migration, $p47^{phox}$ phosphorylation and NOX2 activation in HMC-1 cells. In addition, pretreatment with DPI and apocynin, NADPH oxidase inhibitors, strongly suppressed the degranulation and ROS generation induced by LTB_4. Thereafter, we confirmed the expression of BLT1 and BLT2, receptors for LTB_4, by surface and intracellular expression of HMC-1 cells in resting and LTB_4-stimulated state by FACS analysis. As a result, BLT1 receptor was translocated from cytosol to membrane when stimulated with LTB_4. We pretreated HMC-1 cells and eosinophils with Src-family tyrosine kinase inhibitor, PP1, before reaction with LTB_4. BLT1 translocation induced by LTB_4 was inhibited by pretreatment of inhibitors of Src-family tyrosine kinase. Also, BLT1 traslocation was not induced by NOX2 siRNA transfection in HMC-1 cells. Thereafter, we examined the effect of BLT1 antagonist, U75302, on immune responses of HMC-1 cells induced by LTB_4. Consequently, pretreatment of BLT1 antagonist showed the strong inhibitory effect on degranulation, ROS generation, migration, and NOX2 activation induced by LTB_4. Our data collectively demonstrate that LTB_4 can induce the NOX2-dependent surface translocation of BLT1 is very critical for a degranulation in human mast cells and eosinophils.

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Crystallization and Ligand-Binding Studies of Human CD14

Stacy Kelley,[1] Richard Tapping,[2] Satish Nair[1]

[1]Department of Biochemistry, [2]Department of Microbiology, University of Illinois at Urbana-Champaign

Myeloid differentiation antigen, CD14, is a lipid shuttling receptor responsible for binding acylated ligands present in bacterial pathogens and delivering them to two of the innate immune system's Toll-like receptor (TLR) complexes. TLR ligand binding activates pro-inflammatory signaling cascades. In the case of Gram-negative bacteria, CD14 accepts lipopolysaccharide (LPS) from lipopolysaccharide binding protein (LBP) and shuttles it to the TLR4 complex, allowing the immune system to detect picomolar levels of LPS. CD14 also shuttles other bacterially derived tri- and di-acylated lipoproteins to members of the TLR2 subfamily. The structure of mouse CD14 without a bound ligand was previously solved by Jie-Oh Lee's group (JBC, 2005, 280: 11347). Human and mouse CD14 share 65% AA similarity and a predicted N-terminal ligand binding pocket. Our work focuses on structure determination of human CD14 with or without a bound ligand, and characterization of CD14 ligand binding. Two human and one mouse soluble CD14 constructs have been expressed as Fc-fusion proteins in HEK293F cells and purified after cleavage of the Fc tag. Previous initial crystallization screens that resulted in 6.7 Å have been further optimized to 4.0 Å. An ELISA based IL-8 assay has confirmed the biological activity of the proteins. Native PAGE gel shifts and ForteBio's Octet optical biosensor platform has enabled the detection of multiple CD14-ligand interactions.

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CD14 and LBP Independently Deliver The Triacylated Lipopeptide Pam3CSK4 to TLR2 and TLR1 but Are Not Part of the Final Ternary Signaling Complex

Diana Rose E. Ranoa,[1] Stacy L. Kelley,[2] Richard I. Tapping[1]

[1]Department of Microbiology, [2]Department of Biochemistry, University of Illinois at Urbana-Champaign

The TLR2 subfamily of Toll-like receptors plays a pivotal role in the recognition of a variety of microbial cell wall components such as lipoproteins, lipoteichoic acids, and lipoarabinomannans. In response to microbial agonists, TLR2 forms heterodimeric signaling complexes either with TLR1, 6 or 10 resulting in inflammatory responses. Crystal structure analysis reveals that a triacylated lipopeptide is coordinately bound by TLR1 and TLR2 to form a trimeric signaling complex (Jin, MS, Cell 130, 1071). LBP and soluble CD14 are two lipid binding serum proteins that have been shown to enhance TLR2-induced cell activation. However, their role in inducing stable TLR2-TLR1-Pam_3CSK_4 ternary complex formation has not been previously explored. To investigate the role of LBP and CD14 in ternary complex formation we have performed biophysical studies using functional soluble forms of human CD14, LBP and the extracellular domains of TLR1 and TLR2. Size exclusion chromatography experiments reveal that the interaction between TLR1 and TLR2 requires incubation with Pam_3CSK_4. Moreover, the addition of substoichiometric amounts of either soluble CD14 or LBP to this reaction significantly enhances TLR2-TLR1-Pam_3CSK_4 ternary complex formation. Western blot analyses of the eluted fractions from the size exclusion column show that LBP or CD14 act to disaggregate lipopeptides in solution in preparation for delivery, however, neither LBP nor CD14 are physically associated with the final TLR2-TLR1-Pam_3CSK_4 ternary complex. Together, our results show that either LBP or CD14 can drive ternary complex formation by catalytically delivering the triacylated lipopeptide Pam_3CSK_4. These findings define a critical role for LBP and CD14 in TLR2 signaling with implications for improved drugs designed to target this receptor system.

ABSTRACTS

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The Role of Exosomal RNA in *Leishmania* Pathogenesis

Ulrike E. Lambertz,[1] Mariana E. Oviedo Ovando,[2] Peter Unrau,[2] Neil E. Reiner[1]
[1]University of British Columbia, Department of Medicine, Vancouver, BC; [2]Simon Fraser University, Department of Molecular Biology & Biochemistry, Burnaby, BC

A major hallmark of infection with leishmania is the persistence of organisms within host macrophages brought about by manipulation of cellular functions. Evidence has suggested that impairment of macrophage microbicidal functions is linked to export of leishmania-derived virulence factors into host cytosol. Consistent with this, our group recently demonstrated that leishmania secrete known and candidate protein virulence factors via exosomes. Importantly, exosomes released from leishmania were taken up by macrophages and had immunomodulatory effects on host myeloid cells.

It has also been shown that exosomes released from mammalian cells contain RNA molecules. Moreover, virus infected cells were observed to release exosomes containing virally encoded small regulatory RNAs, which mediate posttranscriptional gene silencing in target cells. Based on these findings, we hypothesized that leishmania exosomes, in addition to proteins, may also contain parasite-derived RNA and that these molecules contribute to leishmania pathogenesis. Indeed, using radiolabeling, gel electrophoresis as well as Agilent Bioanalyzer analysis, we have shown that leishmania exosomes do in fact contain RNA. Interestingly, the size range of exosomal RNA was considerably narrower than that of total leishmania RNA, with the majority of sequences being smaller than 200 nt. Furthermore, by Sanger sequencing we identified a common 12 nt motif in 80% of the RNA sequences found in exosomes. We are currently characterizing leishmania exosomal RNA by deep sequencing. Future studies will focus on investigating the effects of leishmania exosomal RNA on host cells.

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Circulating Hematopoietic Progenitor Cells from Peripheral Blood of Pigs as a Potential in vitro Model to Test Efficacy of Radiation Countermeasures

Maria Moroni, Barbara Ngudiankama, Mark Whitnall
Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD

The Hoechts negative side population (SP) is highly enriched in long-term culture initiating cells and in primitive hematopoietic cells. We have identified in the peripheral blood of Gottingen minipigs a SP and were able to culture GM-, GEMM-colony forming units (CFUs) and burst-forming unit erythroids (BFU-Es) from isolated PBMCs seeded on Methocult-H. These cells were less differentiated than circulating PBMCs and had higher expression of CD117, a marker of porcine hematopoietic progenitor cells. Bone marrow-residing and proliferating progenitor cells are radiosensitive, and their number and mitotic activity are highly reduced by relatively small doses of radiation; after bone marrow recovery, functionality of the immune system remains imbalanced. We used circulating hematopoietic progenitor cells isolated from PBMCs as an *in vitro* model to study the effect of radiation on progenitor cells. We found that not only the number and the size of CFUs (colony forming units) were severely diminished 3 hours after irradiation; colonies also lost the ability to differentiate and underwent a shift towards the GM lineage. Colonies from peripheral blood irradiated *in vitro* or *in vivo* showed a long-lasting G0/G1 cell cycle block. We tested the ability of G-CSF, the standard cytokine for treatment of the hematopoietic syndrome, to restore irradiated progenitor cells. G-CSF was able not only to reverse the cell cycle block and restore proliferation, but also to induce differentiation into GEMM-CFUs. Our results show the protective action of G-CSF may extend beyond mobilization and proliferation of neutrophils. Peripheral blood, irradiated *in vivo* or *in vitro*, provides an easily accessible source of circulating progenitor cells that could be used for preliminary countermeasure drug efficacy testing *in vitro*. This work was supported by funding from NIAID OD-0505-01 and AFRRI RBB2DG.

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Evaluation of a Water-Soluble Folate-Everolimus Conjugate (EC0565) and its Folate Receptor-Specific Anti-inflammatory Mechanism

Yingjuan J. Lu,[1] Nikki Parker,[1] Torian Stinnette,[1] Vicky Cross,[1] Elaine Westrick,[1] Kristin Wollak,[1] Patrick J. Klein,[1] Mark A. Gehrke,[2] Iontcho R.Vlahov,[1] Christopher P. Leamon[1]
[1]Endocyte, Inc., West Lafayette, IN; [2]Upsher-Smith Laboratories, Inc, Maple Grove, MN

Folate receptor (FR) has been identified as a promising target for anti-macrophage therapies. In the present study we investigated EC0565, a water-soluble folate conjugate of everolimus, as a FR-specific inhibitor of the *mammalian target of rapamycin* (mTOR).

Using various sources of FR-expressing macrophages, we confirmed that EC0565 inhibited mTOR signaling and cell proliferation in a FR-specific manner. Under standard culture conditions, EC0565 did not appear to be cytotoxic, but it could arrest proliferating macrophages in the G0/G1 phase of cell cycle under serum-starved conditions. Despite its tendency to form micelles at high concentrations, EC0565 is 100% bioavailable when administered subcutaneously. In thioglycollate-dosed rats, EC0565 treatment yielded FR-specific inhibition of proliferating cell nuclear antigen expressed by the thioglycollate-stimulated peritoneal cells. In rats with adjuvant-induced arthritis, biweekly treatment of EC0565 effectively reduced paw edema and splenomegaly; and regardless of its micellar nature, EC0565 demonstrated *in vivo* target specificity via the FR. EC0565 was also found to be more potent than orally-administered everolimus and subcutaneously-delivered etanercept. Further, EC0565 was found more active than oral everolimus in rats with experimentally induced uveitis.

In conclusion, EC0565 is the first high FR-binding mTOR inhibitor that exhibits FR-specific anti-inflammatory activities both *in vitro* and *in vivo*. Our data suggest that folate-targeted mTOR inhibition may be an effective way of suppressing inflammatory responses in activated macrophages accumulated in sites of chronic inflammation.

ABSTRACTS

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Immunotherapy Using LPS-Induced Endotoxin Tolerant Dendritic Cells to Treat Experimental Autoimmune Encephalomyelitis

Fang Zhou

Thomas Jefferson University, Dept of Neurology, Philadelphia, PA

LPS produced by Gram-negative bacteria induces endotoxin tolerance and suppresses inflammatory responses *in vivo*. However, the mechanisms are poorly understood. Here we show that LPS modulates phenotypes of bone marrow-derived dendritic cells (DCs) and down-regulates OX40 ligand expressed on CD11c+ DCs. In addition, LPS-treated DCs suppress OX40 expression and secretion of cytokines produced by Th17 cells. Our data also demonstrated that i.v. transfer of LPS-treated DCs blocks EAE development and down-regulates expression of IL-21, IL-22, IFN-γ and IL-10 in Th17 cells. These results suggest that LPS-induced endotoxin-tolerant DCs may inhibit activity of Th17 cells via down-regulation of cytokine production in Th17 cells and then affect EAE development *in vivo*. Our results reveal a potential mechanism of LPS-induced endotoxin tolerance mediated by DCs and a possibility of using LPS-treated tolerant DCs to treat autoimmune diseases such as MS/EAE.

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Osteopontin Enhances HIV-1 Replication and Is Increased in the Brain and Cerebrospinal Fluid of HIV-Infected Individuals

Amanda M. Brown,[1] Tanzeem Islam,[1] Karen Marder,[2] Robert Adams,[1] Sujata Nerle,[1] Masiray Kamara,[1] Caitlin Eger,[1] Bruce Cohen,[3] Giovanni Schifitto,[4] Justin C. McArthur,[1] Ned Sacktor,[5] Carlos A. Pardo[1]

[1]Johns Hopkins University, School of Medicine, Department of Neurology, Baltimore, MD; [2]Columbia University, New York, NY; [3]Northwestern University, Chicago, IL; [4]University of Rochester, Rochester, NY; [5]Johns Hopkins Bayview Medical Center, Baltimore, MD

The prevalence of neurocognitive dysfunction associated with HIV-1, now known as HIV-associated neurocognitive disorder (HAND), continues to increase despite the use of combination anti-retroviral therapy. Prominent features of HAND are sustained systemic and central nervous system inflammation and immune activation. Importantly neurons are not infected by HIV and indirect mechanisms play a key role in the disorder. Central to HAND is the role of macrophages both HIV-infected and uninfected that secrete soluble factors, many of which are neurotoxic and immunomodulatory in nature. In order to develop more effective treatments, a better understanding of the molecular mechanisms by which key soluble factors propagate neurodegenerative processes is needed. In this regard we identified osteopontin (OPN, *SPP1*, gene), a cytokine-like factor, using subtractive hybridization, as an upregulated gene in HIV-infected primary human monocyte-derived macrophages. Knockdown of OPN in primary macrophages significantly impaired HIV-1 replication. Using a surrogate cell culture system in which OPN was expressed ectopically, OPN significantly enhanced HIV infectivity and replication. A role for OPN signaling through a NF-κB-dependent pathway was found as a significant increase in the degradation of the NF-κB inhibitor, IκBα and an increase in the nuclear-to-cytoplasmic ratio of NF-κB were found in HIV-infected cells expressing OPN compared to controls. In addition, enhanced HIV LTR promoter activity stimulated by OPN could be blocked by mutation of the NF-κB binding domain. Interestingly, our in vitro findings correlated with increased OPN levels quantified in tissue samples. OPN levels were significantly higher in HIV-infected individuals both with and without neurocognitive disorder compared to levels in the cerebrospinal fluid (CSF) from normal and multiple sclerosis controls. In HIV-infected individuals with moderate to severe cognitive impairment OPN levels were highest. Moreover in brain tissue from HIV-infected individuals with cognitive disorder compared to those without impairment, OPN was significantly elevated. Collectively, these data suggest that HIV-1 replication is stimulated by OPN and that high levels of OPN are present in the central nervous system of HIV-infected individuals, reflecting ongoing inflammatory processes at this site despite anti-HIV therapy. We thank the Margaret Q. Landenberger, The Cooke Family and Campbell Foundations as well as the NIH (NS44807 (JCM) and P30MH075673 (JCM) for funding this work.

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Residual NADPH Oxidase and Survival in Chronic Granulomatous Disease*

Douglas B. Kuhns,[1] W. Gregory Alvord,[2] Theo Heller,[3] Jordan J. Feld,[4] Kristen M. Pike,[5] Beatriz E. Marciano,[6] Gulbu Uzel,[6] Suk See DeRavin,[7] Debra A. Long Priel,[1] Benjamin P. Soule,[7] Kol A. Zarember,[7] Harry L. Malech,[7] Steven M. Holland,[6] John I. Gallin[7]

[1]SAIC-Frederick, Inc., NCI Frederick, Clinical Services Program, App Dev Res Directorate, Frederick, MD; [2]Data Management Services, NCI Frederick, Biostatistical Consulting Division, Frederick, MD; [3]NIDDK/NIH, Liver Diseases Branch, Bethesda, MD; [4]Toronto Western Hospital, University Health Network, Liver Centre, Toronto; [5]SAIC-Frederick, Inc., NCI Frederick, Laboratory of Molecular Technology, Frederick, MD; [6]NIAID/NIH, Laboratory of Clinical Infectious Diseases, NIAID, Bethesda, MD; [7]NIAID/NIH, Laboratory of Host Defenses, Bethesda, MD

Background Failure to generate phagocyte-derived superoxide and related reactive oxygen intermediates (ROI) is the major defect in chronic granulomatous disease (CGD), causing recurrent infections and granulomatous complications. CGD is caused by missense, nonsense, frameshift, splice, or deletion mutations in the genes for $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ (autosomal CGD), or $gp91^{phox}$ (X-linked CGD), which result in variable production of neutrophil-derived ROI. We hypothesized that residual ROI production might be linked to survival in patients with CGD. **Methods** We assessed the risks of illness and death among 287 patients with CGD from 244 kindreds. Residual ROI production was measured with the use of superoxide-dependent ferricytochrome c reduction and flow cytometry with dihydrorhodamine oxidation assays. Expression of NADPH oxidase component protein was detected by means of immunoblotting, and the affected genes were sequenced to identify causal mutations. **Results** Survival of patients with CGD was

strongly associated with residual ROI production as a continuous variable, independently of the specific gene affected. Patients with mutations in $p47^{phox}$ and most missense mutations in $gp91^{phox}$ (with the exception of missense mutations in the nucleotide-binding and heme-binding domains) had more residual ROI production than patients with nonsense, frameshift, splice, or deletion mutations in $gp91^{phox}$. After adolescence, mortality curves diverged according to the extent of residual ROI production. **Conclusions** Patients with CGD and modest residual production of ROI have significantly less severe illness and a greater likelihood of long-term survival than patients with little residual ROI production. The production of residual ROI is predicted by the specific NADPH oxidase mutation, regardless of the specific gene affected, and it is a predictor of survival in patients with CGD.

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Resistant Mice Are Made Vulnerable to *Citrobacter rodentium*-induced Colitis When Exposed to Prolonged Restraint

Amy Hufnagle,[1] Jeff Galley,[1] Nicola M. Parry,[2] Michael Bailey[1]
[1]The Ohio State University, Department of Oral Biology, Columbus, OH; [2]Massachusetts Institute of Technology, Cambridge, MA

Citrobacter rodentium , a murine homologue of enteropathogenic *E. coli*, has been shown to colonize all strains of mice, however disease progression in different murine strains can vary widely from subclinical symptoms to fatal infection. Strains that develop subclinical symptoms are considered resistant to *C. rodentium*-induced colitis while mice that succumb to *C. rodentium* infection are deemed susceptible. Susceptible strains develop severe colitis due to an overzealous host Th1 response. This study was designed to test the hypothesis that exposure to prolonged restraint will cause the resistant CD-1 mouse to become highly susceptible to infectious colitis when infected with *C. rodentium* during stressor exposure. To test this hypothesis, male CD-1 mice were subjected to overnight restraint on 7 consecutive nights. Mice were orally challenged with *C. rodentium* immediately following the first night of stressor exposure. Mice were sacrificed on days 1, 3, 6, and 12 post-infection (p.i.). Results indicated that stressor exposure significantly increased the pathogen load in the colon on days 6 and 12 p.i. Infections with *C. rodentium* are usually confined to the colon due to its non-invasive nature. However, stressor exposure was able to significantly increase, not only the likelihood of dissemination (25% of controls vs. 87% of stressed mice), but also the amount of pathogen found in each spleen, which was over 10 times higher in the stressed mice compared to controls. Overall colitis, including an assessment of hyperplasia, dysplasia, edema, epithelial defects, and inflammation was also significantly increased in stressed mice on day 12 p.i. It is likely that the chemokine CCL2, the proinflammatory cytokine TNF-α, as well as iNOS were involved in the increased colitis scores as their mRNA production were all significantly increased by stressor exposure on day 12 p.i. Together, these data illustrate the deleterious effects of prolonged stressor exposure during gastrointestinal infection.

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***Lactobacillus reuteri* Reduces the Increased Gut Inflammation Found in Stressor Exposed C57BL/6 Mice Challenged with *Citrobacter rodentium*.**

Jeff Galley,[1] Amy Hufnagle,[1] Nicola M Parry,[2] Mark Lyte,[3] Michael Bailey[1]
[1]The Ohio State University, Department of Oral Biology, Columbus, OH; [2]Massachusetts Institute of Technology, Cambridge, MA; [3]Texas Tech University Health Sciences Center, School of Pharmacy, Lubbock, TX

The inflammatory bowel diseases, as well as irritable bowel syndrome, are often exacerbated during periods of psychological stress. The mechanisms by which stress enhances colonic inflammation are not well understood, but could involve disrupted homeostatic interactions between the mucosal immune system and intestinal microbiota. In a previous study involving the use of 454 pyrosequencing, we found that exposing mice to a social stressor significantly reduced the relative abundance of colonic tissue-associated bacteria in the genus *Lactobacillus*, and in particular, *Lactobacillus reuteri*. Because *L. reuteri* has been shown to mediate mucosal immunity and to protect the host against pathogenic invasion, we tested whether administering *L. reuteri* would prevent stressor-induced enhancement of colonic infection. Mice were subjected to a repeated social defeat stressor, called social disrupt ion, prior to and during infection with *Citrobacter rodentium*, a murine pathogen that is homologous to enteropathogenic *Escherichia coli*. Mice exposed to the stressor had higher levels of colonization by the pathogen, higher levels of colonic Tumor Necrosis Factor-α (TNF-α) and Inducible Nitric Oxide Synthase (iNOS) mRNA, as well as an increased colitis score (an evaluation of cellular infiltration, edema, hyperplasia, dysplasia, and epithelial defects) on Day 12 post-*C. rodentium* challenge. When the mice were fed back *L. reuteri* after every cycle of the stressor, the effects of the stressor on colonic inflammation and histopathologic colitis were abrogated. The reduction in colonic inflammation occurred even though the *C. rodentium* burden was unaffected, indicating that *L. reuteri* acts through an anti-inflammatory means rather than an anti-bacterial one. These data support the hypothesis that stressor-induced disruptions of gut microbiota, specifically of *L. reuteri*, exacerbate colonic inflammation during pathogen exposure. Maintaining *L. reuteri* levels through oral feed-back prevented the stressor-induced increase in colonic inflammation, demonstrating the importance of *L. reuteri* in regulating the inflammatory response in the colon.

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ABSTRACTS

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β-Arrestin1: An Important Regulator of Inflammation and Bacterial Clearance in Septic Peritonitis

Deepika Sharma,[1] Nandhakumar Packiriswamy,[2] Narayanan Parameswaran[2]

[1]Department of Microbiology and Molecular Genetics, [2]Department of Physiology, Michigan State University, East Lansing, MI

β-arrestins, initially considered as being adaptor proteins in G-protein signaling termination, have lately found a niche of their own; acting as scaffold proteins in modulation of MAPK and NF-KB pathway, receptor internalization through clathrin-coated pits, chemotaxis and apoptosis. β-arrestins have also been shown to be important positive and negative regulators of inflammation. The aim of the current study is to delineate the role of β-arrestin1 (βArr1) in polymicrobial sepsis via cecal ligation and puncture (CLP), a clinically relevant model of sepsis. We performed CLP on wild type (WT), $\beta Arr1^{-/-}$ and $\beta Arr1^{+/-}$ mice and examined the local and systemic increase in inflammatory mediators, cellular infiltration and bacterial load. At 6 hrs post-CLP, peritoneal and serum levels of IL-6, IL-10, MCP-1 and TNF-α were significantly enhanced in $\beta Arr1^{-/-}$ mice compared to WT mice. Interestingly, $\beta Arr1^{+/-}$ mice had enhanced IL-6 and IL-10 production only in the peritoneum fluid suggesting that β-arrestin1 likely regulates inflammation in a gene-dose dependent and site-specific fashion. The number of infiltrating monocytes, neutrophils and macrophages in the peritoneum were comparable in all three genotypes demonstrating that the enhanced inflammatory response is not due to differential infiltration of immune cells. Contrary to enhanced production of inflammatory mediators, bacterial clearance was impaired in the $\beta Arr1^{-/-}$ mice with higher bacterial load in blood and peritoneum at 32 hrs post CLP, compared to the WT mice. The $\beta Arr1^{+/-}$ mice, however, had bacterial load comparable to the WT mice. This decreased capacity for clearing infection in the $\beta Arr1^{-/-}$ mice was also not due to difference in peritoneal infiltration or number of circulating immune cells, since neutrophil numbers in blood were higher and monocyte numbers comparable to wild type in $\beta Arr1^{-/-}$ mice. Together, these data suggest that inflammatory response and bacterial clearance are differentially regulated by the gene dose of β-arrestin-1 following septic peritonitis.

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Role of Leukocyte ADAM17 in Regulating Inflammation during Sepsis

Chunmei Long

University of Minnesota, Dept of Comparative Biomedical Science, St. Paul, MN

Inflammation is the body's initial response to infection, which can be harmful when excessive, as exemplified in sepsis inflammatory syndromes. Ectodomain shedding is a proteolytic process that directs both instantaneous and prolonged alterations in the activity of various cytokines, cytokine receptors, and adhesion molecules, and ADAM17 is a key membrane metalloprotease involved in this process. At this time, very little is currently known about the *in vivo* function of ADAM17 in regulating inflammation during infection. In this study, we generated *Adam17* gene targeted mice (ADAM17-null mice) in which only the leukocytes lacked functional ADAM17, and then examined its role in the inflammatory and host responses during peritoneal sepsis. ADAM17-null mice showed significantly increased survival and bacterial clearance during sepsis, which was associated with a reduction in systemic proinflammatory cytokine levels and bacterial burden. An underlying mechanism accounting for the enhanced host response in ADAM17-null mice is a very rapid yet transitory infiltration of neutrophils into the peritoneal cavity of ADAM17-null mice when compared with control mice. We are currently examining the molecular processes that underpin the accelerated recruitment of neutrophils in ADAM17 null mice. Overall, our study provides the first direct evidence of the instrumental *in vivo* role of leukocyte ADAM17 in modulating inflammation and host resistance during sepsis.

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Cysteine Protease in Soluble Amoebic Protein Derived from *Entamoeba histolytica* Induces Augmentation of Adhesion in Human Mast Cells

Seong Hoon Kim, Deulle Min, Kyeong Ah Kim, Young Ah Lee, Myeong Heon Shin

Yonsei University College of Medicine, Department of Environmental Medical Biology, Seoul, Korea

Entamoeba histolytica is an enteric tissue-invasive protozoan parasite that causes amoebic colitis and occasionally liver abscess in humans. Mast cells are tissue-resident immune cells to play an important role in regulation of tissue inflammation responses. However, information regarding *E. histolytica* component-induced inflammation responses of mast cells is limited. In this study, we investigated whether adhesion capacity of mast cells can be affected by stimulation of *E. histolytica*. When Human mast cell line (HMC-1) cells were stimulated by soluble form of soluble amoebic protein (SAP), adhesion of HMC-1 cells was significantly increased compared to results for cells stimulated with medium alone. In addition, when HMC-1 cells were stimulated by immobilized form of SAP, adhesion of HMC-1 cells was significantly increased compared to results for cells stimulated with immobilized form of bovine serum albumin. In contrast, intracellular ROS generation and degranulation in HMC-1 cells was not induced by stimulation of SAP. Next, we investigated what factor(s) in the SAP can trigger augmentation of adhesion in HMC-1 cells. Adhesion of mast cells induced by soluble form of SAP was remarkably inhibited by pretreatment of SAP with heat (100 °C for 10 min). Immobilized SAP-induced mast cell adhesion was also attenuated by pretreatment with heat. Considering the fact that cysteine protease (CP) is a key virulence factor of *E. histolytica* in tissue invasion, we therefore investigate whether amoeba-derived CP can be involved in adhesion of mast cells induced by SAP. When SAP was pretreated with cysteine protease inhibitor E-64c, SAP-induced adhesion response was strongly inhibited. Papain, representative cysteine protease, also induced augmentation of adhesion in HMC-1 cells. All together, these results suggest that *E. histolytica*-derived CP may be a crucial factor for SAP-induced adhesion in HMC-1 cells. Our result shows that CP derived from *E. histolytica* induces

mast cell-mediated tissue inflammatory responses during human amoebiasis.

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Neutrophil Apoptosis Is Associated with Loss of Signal Regulatory Protein alpha (SIRPalpha) from the Cell Surface

Per-Arne Oldenborg, Asa Stenberg, Janove Sehlin
Umea University, Department of Integrative Medical Biology, Umea, Sweden

Neutrophils play a major role in the development of inflammatory diseases. During inflammation, large numbers of neutrophils are recruited from the blood and subsequently undergo apoptosis, which involves changes in the cell surface expression of a number of receptors. Neutrophils express the immunoglobulin superfamily-member signal regulatory protein alpha (SIRPα), which is a receptor involved in regulating cell adhesion and migration. Here we investigated whether neutrophil expression of SIRPα was affected during apoptosis. We found that apoptotic neutrophils lost SIRPα from their cell surface with kinetics similar to the loss of CD16. The vast majority of neutrophils with reduced SIRPα also expressed phosphatidylserine on their surface, and loss of the receptor was dependent on the activation of caspases. Specific inhibition of MMP3 or MMP8 prevented a substantial fraction of receptor loss, suggesting that proteolytic mechanisms were involved. In addition, SIRPα was also found on smaller membrane vesicles released during apoptosis. Our data suggest that neutrophils reduce their SIRPα expression during apoptosis, which may be part of the functional down-regulation seen in apoptotic neutrophils.

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The Regulation of Methionine Sulfoxide Reductases in *S. aureus* Ingested by Human Neutrophils

Yun Yun Pang,[1] Jeff M. Boyd,[2] Alexander R. Horswill,[3] William M. Nauseef[1]
[1]University of Iowa College of Medicine, Inflammation Program, Department of Medicine, Iowa City, IA; [2]Rutgers, The State University of New Jersey, Department of Biochemistry and Microbiology, New Brunswick, NJ; [3]University of Iowa, Department of Microbiology, Iowa City, IA

S.aureus (*SA*) colonizes greater than 30% of the US population. In the host, polymorphonuclear neutrophils (PMN) form the first line of defense against *SA*. PMN are rapidly recruited to the site of infection, where they ingest and kill bacteria. Failure to clear *SA* can lead to infections that in the most severe cases can be fatal.
To understand how *SA* evades the host immune responses we studied methionine sulfoxide reductases (Msr); these enzymes repair oxidized methionines and have been reported to be important in defense against oxidant damage in both prokaryotes and eukaryotes. We investigated whether *SA* upregulated the expression of the genes encoding these enzymes on being ingested by PMN and whether this helped *SA* to survive within PMN phagosomes.
We fed serum-opsonized *SA* to PMN *in vitro* and at the chosen time points recovered the bacteria and assessed their expression of *msr* by real-time PCR. The contribution of these enzymes to the survival of *SA* was assessed by comparing the viability of recovered bacteria deficient in *msr* to the isogenic wildtype organism.
We found that *SA* sensed changes in their local environment on being ingested by PMN. There was rapid induction of *msr* expression. *SA* deficient in *msr* trended towards being more susceptible to killing by PMN. This suggested that *SA* may exploit Msr to improve their chances of survival within the hostile environment of PMN phagosomes.
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Sepsis Induced Potentiation of Peritoneal Macrophage Migration is Mitigated by PD-1 Gene Deficiency

Alfred Ayala, Ye Sul Kim, Arnaldo Carreria, Gwendolyn Elphick, Nicholas Shubin, Chun-Shiang Chung
Rhode Island Hospital/Brown University, Department of Surgery, Providence, RI

We have previously shown that the expression of T-cell toleragen, Program Death (PD)-1, on MØs during sepsis appears to be involved in septic morbidity/ mortality. However, PD-1's effect on phagocyte function(s) has not been extensively described. Here we have used the murine septic model of cecal ligation and puncture (CLP) to determine if sepsis alters *ex vivo* peritoneal MØ migration/motility, cell spreading/ shape and/or phagocytic functions. What we found was that CLP induced a marked increase in ex vivo migration/motility and an increased cell spreading, but this was lost in the absence of PD-1. Subsequent, *in vitro* cell culture, studies documented in the mouse MØ cell line, J774, that when cells where incubated for 90 mins with PD-1 blocking antibodies (but not control Isotype) that this led to aggregation of cytoskeletal protein alpha-actinin and F-actin. Further, *ex vivo* experiments looking at alpha-actinin and F-actin staining in MØ, derived from Sham or CLP mice, illustrated that a similar punctate/ aggregational pattern (along with cell spreading) was evident on the cells from wild-type CLP mice but was lost PD-1 -/- CLP mouse MØ. Alternatively, phagocytic activity was inversely affected when compared to the changes in migration and cell spreading, i.e., CLP induced suppressed MØ phagocytosis in wild-type mice but this response was lost in PD-1 -/- CLP animals. Finally, we observed that using blocking anti-PD-1 antibodies, a non-selective phosphatase inhibitor and a selective Rap1 inhibitor that fMLP-induced migration by J774 cells could be markedly attenuated. Taken together, we believe the data support a role for PD-1 in mediating aspects of macrophage function, such as migration and phagocytic capacity, outside of those related to the induction of classic T-cell tolerance. (Supported by funds from NIH GM46354 & HL73525)

ABSTRACTS

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Baclofen, a γ-Amino Butyric Acid B Receptor 2 Agonist Ameliorates Immune Complex Mediated Acute Lung Injury by Altering Neutrophil Activation

Madhavi J. Rane, Kenneth Ro McLeish, Jon B. Klein, Shunying Jin

University of Louisville, Department of Medicine & Neuphrology, Louisville, KY

We have previously demonstrated that the γ -amino butyric acid B receptor ($GABA_BR$) potentially plays a significant role in the inflammatory response and neutrophil-dependent ischemia-reperfusion injury in the brain. In contrast, $GABA_BR$ agonists, such as baclofen, have been shown to have anti-inflammatory effects in the kidney. The presence of $GABA_BR2$, GAD65/67, GABA transporter are documented in the lung but its role in regulating acute lung injury is not documented. Moreover, recently we determined that γ -aminobutyric acid (GABA), the GABA transporter, and $GABA_B$ receptors are present in neutrophil granules. Since the $GABA_BR$ signaling machinery is present in the lungs and in neutrophils, we chose to determine the cross-talk between these pathways in the setting of neutrophil-mediated model of acute lung injury (ALI). Rats were subjected to immune complex deposition model (IgG-IC) of acute lung injury (ALI). Two hr after initiation of ALI, rats were untreated or treated with 1 mg/Kg of baclofen for 2 additional hr and then sacrificed. Lungs were harvested to generate lung tissue lysates and lung tissue sections. Bronchoalveolar lavage (BAL) fluids (BALf) were spun down to separate BAL supernatants from BAL cells (>90% polymorphonuclear leukocytes, PMNs). BAL cells and lung tissue lysates were subjected to appropriate immunoblot analysis and lung tissue sections were subjected to H&E, naphthol AS-D chloroacetate esterase (NACE), myeloperoxide, and TUNEL, staining. BAL supernatants were immunoblotted for TNF-α . In the IgG-IC model of acute lung injury (ALI), our data indicates an anti-inflammatory role for baclofen. ALI augmented vascular leakage, neutrophil influx, lung edema and hemorrhage, increased lung caspase-3 activity and decreased lung $GABA_BR2$ expression. Baclofen treatment inhibited ALI-induced release of pro-inflammatory cytokine TNF-α in BALf, reduced vascular leakage and induced BAL PMN apoptosis. Concomitant with this baclofen treatment inhibited caspase-3 cleavage in the lung tissue, increased $GABA_BR2$ expression in the lung, and reduced lung damage. Considered together, these data indicate that $GABA_B$ receptors in the lung and BAL PMNs cross-talk to attenuate ALI, but the precise mechanism of this protection is currently unknown.Baclofen treatment may serve as a therapeutic option to control neutrophil-mediated inflammatory pathologies. Funding Source: NIAID

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Investigation of the Protective Role of the TLR1 I602S Polymorphism in Mycobacterial Disease

Bryan E. Hart, Richard I. Tapping

University of Illinois, Department of Microbiology and College of Medicine, Urbanna, IL

We have recently identified I602S as a frequent single nucleotide polymorphism of TLR1 which greatly inhibits trafficking of the receptor to the cell surface, confers hyporesponsiveness to TLR1 agonists, and protects against the mycobacterial diseases leprosy and tuberculosis. The fact that homozygous TLR1 602S individuals, who lack cell surface expression of TLR1, are protected from mycobacterial disease seems counterintuitive to the protective role that TLRs are thought to play in host defense. However, other published studies have suggested that mycobacteria subvert the TLR system to their advantage. For example, mycobacterial-derived TLR1 agonists have been shown to inhibit IFN-gamma induced macrophage activation events normally required for effective host defense against intracellular bacteria. During our exploration of the TLR1 I602S polymorphism we have found that primary human monocytes and macrophages from TLR1 602S homozygotes are resistant to the mycobacterial abrogation of surface activation markers normally upregulated by IFNγ. This differential resistance between TLR1 602S and TLR1 602I is observed upon stimulation with soluble membrane fractions of mycobacteria but not with whole mycobacterial cells. This is consistent with the observation that both receptor variants are recruited to phagosomes containing whole mycobacteria. In addition, previous studies have shown that Mycobacteria utilize TLR1 signaling to induce expression of host arginase-1 which depletes cellular levels of arginine required for macrophage antimicrobial oxidative burst via iNOS. Our results show that TLR1 602S homozygotes resist the mycobacterial-dependent induction of host arginase-1 when challenged with soluble mycobacterial-derived agonists but not with whole mycobacteria. Taken together, these results show that soluble mycobacterial products, perhaps released from granulomas, preferentially disarm incoming monocytes of TLR1 602I individuals versus individuals homozygous for TLR1 602S who lack cell surface expression of TLR1. We believe this mechanism may provide a rational explanation for the protective role of the TLR1 602S variant in mycobacterial disease.

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Self-Limiting CD4 T Cell Immunity Induced by EBV Oncogene N-LMP1

Kai-ping Chow,[1] Chen Chang[2]

[1]Chang-Gung University, Microbiology and Immunology, Taoyuan; [2]Academic Sinica, Institute of Biomedical Sciences, Taipei

N-LMP1 is a variant of Epstein-Barr Virus (EBV)-encoded oncogene latent membrane protein 1 (LMP1) identified in Taiwan. Although it is considered as a promoter in the development of nasopharyngeal carcinoma, the immunogenicity of N-LMP1 is unclear. The purpose of this study was to characterize the immunogenicity of N-LMP1 and the relationship between the immunogenicity and the in vivo tumor progression. To achieve our goal, the immunogenicity was assessed by the ability of N-LMP1 to induce tumor rejection in a transplantable tumor model in the immunocompetent host. The mechanism of tumor rejection was further analyzed by in vivo depletion and adoptive transfer of CD4T and CD8T cells in normal and SCID mice, respectively. As the development of new blood vessels is critical for rapid tumor cell expansion and metastasis, the impact of the vaccination upon tumor angiogenic dynamics was then evaluated by series of non-invasive dynamic contrast-enhanced magnetic resonance imaging

(DCE-MRI) and further confirmed by quantitative RT-PCR. The results demonstrated that only N-LMP1, but not the non-N-LMP1, expressor induced tumor rejection. And the tumor rejection was abolished only when CD4T cells were depleted from normal mice. While CD4T cells alone were sufficient to protect SCID host against N-LMP1 tumor, SCID mice received IFNg$^{-/-}$ CD4T cells failed to reject the tumor, suggesting that IFNg mediates the anti-N-LMP1 CD4T cell response. Furthermore, the development of tumor vasculature measured by DCE-MRI and quantitative RT-PCR of beta 3 and tie2, the markers of active neovascularization, was shown blocked after vaccination. In conclusion, these studies reveal that N-LMP1 is immunogenic, which may induce CD4T cell-mediated angiogenic blockade to self-limit tumor progrression.

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IL-12 Modulates NF-κB p50 Over-expression in Tumor-Associated and Tumor-Infiltrating Macrophages

Courtney J. George, Katharine Hobbing, Jill Suttles, Robert D. Stout

University of Louisville School of Medicine, Department of Microbiology and Immunology, Louisville, KY

In established tumors, cells of the myeloid lineage, including macrophages, are vital components of the strong immunosuppression observed within the tumor microenvironment. Several signaling pathways have been implicated in the immunosuppressive phenotype seen in macrophages in the tumor microenvironment. The NF-κB signaling pathway in macrophages has been shown to be key in initiation, maintenance, and promotion of tumors. The NF-κB family member, p50, has been reported to be over-expressed in tumor associated and infiltrating macrophages. This over-expression is thought to promote p50 homodimer formation that, in the nucleus, represses key inflammatory processes, establishing an immunosuppressive state in the macrophages. We have demonstrated that IL-12 treatment in vivo transiently renders tumor associated and tumor infiltrating macrophages immunocompetent and that this phenotypic change in macrophages contributes to IL-12's anti-tumor effects. Whether IL-12 induces the observed changes in macrophage phenotype directly or indirectly is uncertain. Recent findings in our laboratory demonstrate that macrophages in normal and tumor-bearing mice can respond directly to IL-12 as evidence by modulation of several signaling pathways. Our laboratory has observed that IL-12's impact on tumor-associated macrophages is due, in part to down-regulation of p50 over-expression. Our results indicate that while IL-12 treatment of control macrophages does not affect total p50 expression, IL-12 treatment of macrophages from 4T1 tumor-bearing animals, reduces the abnormally high levels of p50. Our results provide novel insights into the mechanism by which IL-12 may directly impact the functional phenotype of macrophages within the tumor microenvironment.

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A Polychromatic Antibody Cocktail to Investigate Phenotypic and Functional Markers on Human Myeloid and Plasmacytoid Dendritic Cells

Pooja Jain,[1] Michael R. Betts,[2] George Makedonas,[2] Sharrón L. Manuel[1]

[1]*Drexel University College of Medicine, Doylestown, PA;* [2]*University of Pennsylvania , Philadelphia, PA*

Antigen presentation is a critical feature of adaptive immunity and essential in self versus nonself discrimination as well as in stimulating immune responses against pathogens. Dendritic cells (DCs) are considered to be the most potent antigen-presenting cells and have long been recognized as key regulators of the immune system. Because DCs are such crucial cells of the immune system, an extensive assessment of their quality in infected individuals is critical. Therefore, we have standardized a unique DC cocktail containing 12 different functional DC markers using an LSR II polychromatic flow cytometer. To this point, we have tested our newly standardized cocktail on the fresh, frozen, and cultured peripheral blood mononuclear cell (PBMCs) as well as on monocyte-derived DCs (MDDCs), a widely used surrogate system for primary blood DCs. This study emphasizes the practicality of utilizing frozen versus fresh cells, especially in rare disease states, wherein obtaining fresh material is virtually impossible. Furthermore, we focus on the feasibility of PBMCs compared to whole blood as starting material. To our knowledge this is the first comprehensive DC phenotyping protocol that uses an extensive list of functional markers (>8). Collectively, these investigations possess great potential to enable investigators to perform immune cell monitoring and develop diagnostic and therapeutic strategies.

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Maintenance of the Co-stimulatory Ability of Dendritic Cells Can Partially Compensate for Diminished Differentiation and Antigen Processing Capability Following Treatment with CD47

Trisha Macfarlane, Sanjukta Bandyopadyhay, Carol L. Miller-Graziano

University of Rochester Medical Center, Department of Surgery Research, Rochester, NY

Thrombospondin-1 triggering of its constitutive inhibitory receptor CD47 on monocytes (MO) suppresses their subsequent immunostimulatory activity. The mechanisms for this inhibition are unclear, as CD47 triggering also directly inhibits T cells, affects MO antigen processing, and results in differentiation of dysfunctional dendritic cells (DC). DC co-stimulatory and co-inhibitory activation of T cells could also be affected in the CD47 MO-derived DCs. Here we assess how CD47 triggering impacts MO-DC differentiation and individual DC functions. Isolated human peripheral blood monocytes were cultured for 6 days in the presence of 1250 IU/ml IL-4 and 1500 IU/ml GM-CSF ± 15 μg/ml αhCD47. The resulting numbers of differentiated DCs were determined by CD1a

expression. The ratio of co-inhibitory to co-stimulatory receptors expressed on these CD1a+ DCs was analysed by flow cytometry and DC antigen processing dysfunctions by DQ ovalbumin fluorescent cleavage. DC STAT6 translocation to the nucleus was assessed using Amnis ImageStream technology. DCs were co-cultured with autologous T cells stimulated with immobilized αCD3 to determine if CD47 triggering during MO-DC differentiation altered the DC costimulatory activation of T cells in the presence and absence of additional CD47. Results: CD47 triggering inhibits MO-DC differentiation by decreasing STAT6 activation, decreasing the resulting DC numbers. Additionally, DCs differentiated in the presence of CD47 have deficient antigen processing capabilities. The ratio of co-inhibitory to co-stimulatory receptors increased on the CD47 treated DCs from 1.1:1 to 2.4:1. However, this ratio change was insufficient to prevent the DCs co-stimulation capabilities to rescue T cells from the direct inhibitory effects of CD47 in T cell-DC co-cultures. In summary, MO-DC differentiation and antigen processing are diminished by pre-treating MO with CD47, but there is no effect on their co-stimulation for autologous T cells, and DCs differentiated with αCD47 still prevent αCD47 direct inhibition of T cell TCR (αCD3) induced activation. (NIH 3R01-GM036214-22A2S1)

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Defective Bone Marrow Erythropoiesis Following Burn Trauma Is Improved by Propranolol Treatment

Kuzhali Muthumalaiappan,[1] Andrea Szilagyi,[1] John Halerz,[1] Li Ke He,[1] Richard L. Gamelli,[2] Ravi Shankar[1]

[1]Loyola University Medical Center, Burn & Shock Trauma Inst, Maywood, IL; [2]Dean-Stritch School of Medicine, MD Facs, Maywood, IL

Anemia in severe burns accounts for >50% of transfusions unrelated to surgical blood loss. Administration of exogenous erythropoietin (EPO) in burn patients did not prevent anemia. We have recently found that resistance to EPO in burn mice, is due to progressive decrease in EPO responsive bone marrow (BM) erythroid precursors with a temporal redistribution in the BM erythroid and myeloid compartments. We have previously established that catecholamines accelerated BM monocytopoiesis in burn sepsis and that hemopoietic stem cells (LSK) express beta-adrenergic receptors (AR). Therefore, we hypothesized that beta-AR blockade following burn will improve BM erythropoiesis by shifting the hemopoietic commitment toward megakaryocyte erythrocyte progenitors (MEP). **Method:** $B_6D_2F_1$ mice were randomized into sham (S) and Burn (B) groups. B received a 15% TBSA scald burn. Starting on post burn day#1 (PBD), both groups were reassigned to vehicle or propranolol (B+P) [4mg/kg body wt.] treatments, administered once daily for 6 days. On PBD#7, bilateral femurs were harvested and total BM eluted. The MEP fraction was identified with a combination of mAbs ($Lin^{neg}IL7R^{neg}Sca1^{neg}cKit^{hi}CD34^{neg}Fc\gamma R^{neg}$) by flow cytometry. Reticulocytes and erythroid precursors were identified by flow cytometry based on$CD71^+$ expressions and high or lowforward scatter of the $Ter119^+$ fraction. **Results:** Burn resulted in a significant decrease in the number of BM MEP fraction, which was recovered by propranolol (MEP: S = $97\pm8X10^3$, B = $*44\pm1X10^3$, B+P = $80\pm10X10^3$, * $p<0.05$ vs. S and B+P). Similarly, burn-induced deficits in the number of BM reticulocytes and erythroid precursors were increased significantly with propranolol treatment (Reticulocytes: S = $42\pm6X10^5$, B = $*15\pm3.5X10^5$, B+P = $40\pm3.5X10^5$; Erythroid precursors: S = $35\pm3X10^5$, B = $*14\pm3X10^5$, B+P = $30\pm3.5X10^5$, * $p<0.05$ vs. S and B+P). **Conclusion:** Our data suggest that beta-AR blockade shifts the hemopoietic commitment toward MEPs thereby improving BM erythroid precursors lost post burn.

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Phenotypic Duality of Myeloid Cells within the Peritoneal Inflammatory Response to Implanted Foreign Material

Jane E. Mooney,[1] Barbara E. Rolfe,[1] Kim M. Summers,[2] Milena M. Gongora,[3] Sean M. Grimmond,[3] Julie H. Campbell,[1] David Hume[2]

[1]University of Queensland, Australian Institute for Bioengineering & Nanotechnology, Brisbane; [2]The Roslin Institute and R(D)SVS, Edinburgh; [3]University of Queensland, Institute for Molecular Bioscience, Brisbane

The implantation of foreign material in the peritoneal cavity initiates an inflammatory response in which bone marrow-derived cells are recruited to the implant. The majority of these cells have monocyte/macrophage morphology. Over time, a multi-layered capsule of tissue comprised predominantly of myofibroblasts encapsulates the foreign material, effectively shielding it from the immune system. The transgenic MacGreen mouse, which expresses EGFP under the control of the *cfms (Csf1r)* promoter, was used to study the time-dependent accumulation of myeloid cells in this response. Monocytes and neutrophils in the inflammatory infiltrate and tissue capsules were identified by variable expression levels of EGFP, Ly6C and F4/80. $EGFP^+$ $Ly6C^+$ subsets were identified at days 2 and 4 post-implantation; over time (days 7-14), Ly6C expression was down-regulated, concomitant with up-regulation of F4/80, indicative of monocyte-to-macrophage maturation. Morphological analysis of FACS-sorted $EGFP^+$ subsets from the day 28 tissue capsule identified monocyte/macrophages and neutrophils in the $EGFP^{lo}$ subset, and macrophages, multinucleated giant cells, and other large elongated cells, within the $EGFP^{hi}$ fraction. Co-expression of EGFP and myofibroblast marker alpha-smooth muscle actin was also demonstrated, increasing from 11.13 ± 0.67% at day 14 to 50.77 ± 12.85% of total cells at day 28. Using BioLayout *Express*^{3D}, clusters of co-expressed genes associated with the $EGFP^{hi}$ cells from inflammatory infiltrate and tissue capsules over a 28-day time course were identified. The *Acta2* gene (encoding alpha-smooth muscle actin) was found within a cluster of 407 probes that were expressed in early infiltrating cells. This gene cluster also included *S100a4* (fibroblast-specific protein-1), *Cnn2* (calponin2, actin binding protein), and *Tnni1* (skeletal muscle troponin) as well as myeloid related genes *S100a8* and *S100a9*, and monocyte markers *Ly6c1,Ccr2* and *Cx3cr1*. The co-expression of genes associated with both mesenchymal and myeloid lineages suggests phenotypic duality of cells within the peritoneal inflammatory response to implanted foreign material.

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ABSTRACTS

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CD16-Negative CD121b-Positive Regulatory Monocytes Control T Cell-Induced Colitis in vivo

Georg Varga,[1] Jan M. Ehrchen,[2] Athanasios Tsianakas,[2] Matthias Ross,[3] Andreas Luegering,[3] Kirsten Roebrock,[1] Nadine Nippe,[2] Anne Brockhausen,[2] Johannes Roth,[1] Cord Sunderkoetter[2]
[1]University Hospital Muenster, Institute of Immunology, Muenster, Germany; [2]University Hospital Muenster, Department of Dermatology, Muenster, Germany; [3]University Hospital Muenster, Department of Medicine B, Muenster, Germany

Monocytes play a pivotal role not only in propagation, but also in resolution of inflammation. We showed that glucocorticoids (GC) induce regulatory monocytes (Mreg) with a distinct and stable phenotype in vitro and in mice *in vivo*.
Murine Mregs resemble Myeloid-derived suppressor cells (MDSC) and interact with cells of the adaptive immune system, notably CD8+ T cells to suppress their activation. We now were interested to characterize murine Mregs in more detail and to extend examination of their regulatory potential also on CD4+ T cells *in vitro* and *in vivo*. We found that Fc Rg III (CD16) is down-regulated while immuno-regulatory molecules like IL-1R type II (CD121b), IL-4Rα (CD124) and IL-10 are up-regulated. We then showed that Mreg confer regulation to cells of the adaptive immune system, especially to T cells: CD4+ and CD8+ effector T cells are diminished in their capacity to proliferate and to produce pro- inflammatory cytokines IFN γ and IL-17 when they have contact to Mregs *in vitro*. In vivo in a murine colitis model transfer of Mreg leads to resolution of inflammation in mice suffering from T-cell-mediated colitis. Furthermore, Mreg use CD124 to confer their anti-inflammatory properties *in vivo* and thereby regulate production of IFN γ and IL-17 from T-cells. In conclusion, GC generate regulatory monocytes that are capable to control ongoing T cell responses in vitro and in vivo, and thus have a high potential to become valuable tools in treatment of inflammatory diseases.

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Regulation of Myeloid APC Activity by AMP-Activated Protein Kinase

Kelly C. Carroll, Bing Li, Jill Suttles
University of Louisville, Department of Microbiology and Immunology, Louisville, KY

AMP-activated protein kinase, AMPK, is a serine/threonine kinase that regulates energy homeostasis and metabolic stress in eukaryotes. Previous published work from our laboratory demonstrated a role of AMPK as a negative regulator of macrophage inflammatory activity. With use of AMPK-deficient mice, we have further demonstrated that absence of AMPK expression in both dendritic cells (DC) and macrophages results in heightened inflammatory function and an enhanced capacity for antigen presentation. DC generated from AMPK-deficient mice express elevated baseline and LPS-induced expression of co-stimulatory and adhesion molecules and have enhanced phagocytic activity as compared to wild type cells. Production of the proinflammatory cytokine IL-6 by AMPK-deficient DC in response to both LPS and CD40 stimulation is elevated as compared to wild type cells. LPS stimulation of AMPK-deficient DC also resulted in decreased production of IL-10. As a consequence, AMPK-deficient APC are much stronger promoters of IFN-γαμμα and IL-17 production by CD4+ T cells during antigen presentation. Overall our data indicate that AMPK expression in macrophages and DC serves to attenuate inflammatory responses, including by diversion of TLR and CD40-mediated signaling pathways. This work was supported by National Institutes of Health Grant AI048850.

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Elevated AMPK Activity in Fatty Acid-Binding Protein-Deficient Macrophages: Evidence for AMPK Maintenance of an IL-10 Autocrine Loop

Yanfang Zhu,[1] Duygu Sag,[2] Jill Suttles[1]
[1]University of Louisville, Department of Microbiology and Immunology, Louisville, KY; [2]La Jolla Institute for Allergy & Immunology, La Jolla, CA

We reported previously that macrophages deficient for expression of adipocyte/macrophage fatty acid-binding protein (A-FABP) are polarized to an anti-inflammatory state. In macrophages, A-FABP serves as a negative regulator of the activity of peroxisome proliferator activated receptor y , PPARy . Thus, A-FABP-deficient (A-FABP-/-) macrophages have elevated PPARy activity which is accompanied by reduced NF-κB activity and inflammatory cytokine production, and increased production of IL-10. In an investigation of the signaling pathways contributing to maintenance of this anti-inflammatory phenotype we noted consistently elevated levels of active (phosphorylated) AMP-activated protein kinase (AMPK) in A-FABP-/- macrophages. We have identified AMPK as a negative regulator of macrophage inflammatory activity that is activated by anti-inflammatory cytokine stimuli, including IL-10, which suggested that the constitutively elevated AMPK activity in FABP-/- macrophages may be a result of autocrine IL-10 activity. We demonstrate that IL-10 activation of a PI-3K/Akt/CREB pathway is impaired in AMPK-deficient macrophages and elevated in macrophages expressing a constitutively active AMPK. In addition, AMPK expression was found to be required for optimal IL-10-mediated activation of STAT3 and induction of expression of suppressor of cytokine signaling 3 (SOCS3). In sum, our data indicate that A-FABP regulation of PPAR ψ activity in macrophages serves as a control point for an IL-10/AMPK autocrine loop that upregulates anti-inflammatory gene expression. Thus, low or absent A-FABP expression will activate this pathway to sustain an anti-inflammatory phenotype, whereas elevated A-FABP expression, such as occurs as a result of high fat intake, will suppress this pathway.
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ABSTRACTS

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TNF-alpha Involvement in Alveolar Bone Repair Process in Mice

Gustavo P. Garlet,[1] Andreia E. Vieira,[1] Carlos E. Repeke,[1] Elcia M. Silveira,[1] Carolina F. Francisconi,[1] Ana Paula F. Trombone,[2] Gerson F. Assis,[1] Rumio Taga[1]

[1]School of Dentistry of Bauru FOB/USP, Dept of Biological Sciences, Bauru / SaoPaulo; [2]Instituto Lauro de Souza Lima - ILSL, Bauru

While cytokines have been implicated in the pathogenesis of chronic inflammatory bone diseases, its role in bone repair process remains unknown. The objective of this study was to characterize the role of pro-inflammatory cytokine TNF-alpha in alveolar bone healing process after tooth extraction in TNFp55KO strain compared to C57Bl/6 (WT) mice. Following the extraction of the right upper incisor, the maxilla containing the remaining alveolus was collected 0, 7, 14, 21, 28 and 42 days after extraction for histomorphometric and molecular (RealTimePCR) analysis. The results demonstrated that in WT mice the initial formation of clot was followed by the transient appearance of foci of inflammatory infiltrate and a granulation tissue, gradually infiltrated by vessels and substituted by bone. Interestingly, in WT mice, TNF-alpha mRNA was detected with an initial peak at 7 days, followed by a decreasing expression over time and a second but smaller peak at 28 and 42 days. Bone markers expression was found to support the histological findings, with an initial high expression of COL-I, followed by a marked expression of CBFA-1 and ALP, and subsequently by higher levels of OCN and then by PHEX. TNFp55KO strain presented increased counts of inflammatory cells in healing area, despite of a slight decrease in the expression of the chemokines CXCL1, CCL-2 and CCL3. TNFp55KO also presented a delay in angiogenesis and osteogenesis processes, associated with a longer lasting granulation tissue and higher proportion of osteoclasts evidenced during the periods of the repair process. Molecular analysis of bone markers demonstrated that delayed angiogenesis in TNFp55KO strain was associated with increased COL-I mRNA levels and decreased CBFA-1, ALP, OCN and PHEX expression, while the expression of osteoclastogenic marker RANKL was not significantly modulated by the absence of TNFp55. The results presented here demonstrate that the absence of TNF-alpha interferes in alveolar bone repair through mechanisms that involve the modulation of inflammatory cell migration and osteogenic markers expression during the course of alveolar bone repair. Supported by FAPESP.

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Exploratory Analysis of Prototypical T Helper Cytokines as Biomarkers of Clinical Leprosy and Reactional States

Ana Paula F. Trombone,[1] Patricia S. Rosa,[1] C. Guidella ,[1] Luciana R.V. Fachin,[1] Eliane A. Silva,[1] Somei Ura,[1] Cleverson T. Soares[1], Gustavo Garlet,[2] Andrea F.F. Belone[1]

[1]Instituto Lauro de Souza Lima - ILSL, Pathology Section, Bauru / SaoPaulo; [2]School of Dentistry of Bauru FOB/USP, Dept of Biological Sci, Bauru / SaoPaulo

Mycobacterium leprae . Leprosy comprises a broad spectrum of clinical forms, namely tuberculoid (TT), borderline tuberculoid(BT), borderline borderline (BB) borderline lepromatous (BL) and lepromatous (LL) forms, characterized by clinical, histopathological and immunological features. In immunological terms, TT comprises a Th1-associated pole where cell mediated immunity to *M. leprae* is associated with few lesions and bacilli, while the opposing LL pole is characterized by Th2 immune response with several lesions and greater bacillary load. However, the clinical scenario is quite more complex, since the BT, BB and BL forms present intermediate characteristics (with a progressive reduction in cellular responses and increased bacillary load), as well the type 1 (reverse reaction, RR) and 2 (erythema nodosum leprosum, ENH) reactions whose immunological characteristics can be variable, being the Th1/Th2 archetype insufficient to fully explain disease pathogenesis. Therefore, we analyzed the serum levels of Th (Th1, Th2, Th17, Treg) prototypical cytokines looking for the identification of biomarkers with potential to diagnostically distinguish the different leprosy forms. The serum levels of IFN-γ, IL-4, IL-10, IL-6, TGF-β, IL-22 and IL-17 were evaluated in 72 leprosy patients (10TT, 10BT, 12BB, 8BL, 9LL, 10RR and 13ENH) and healthy controls (n=10) by means of ELISA, and analyzed initially as potential leprosy markers and subsequently as leprosy forms and reactional states markers. Our results demonstrate that IFN-γ, IL-4 and IL-17 were not present in detectable levels in the serum of both controls and patients, irrespective of its leprosy form or reactional state. The frequency of IL-10, IL-6 and IL-22 detection was significantly higher in patients than in controls, while TGF- β was detected in all the samples from both groups, but in similar levels. When the cytokine levels were compared within the leprosy subgroups, TGF-β and IL-6 were found to discriminate typical forms and reactional states, being the levels of both TGF-β and IL-6 higher in reactional patients. When all the subgroups were compared, no significant differences were observed. Taken together our results suggest that TGF-β and IL-6 are potential biomarkers to distinguish between leprosy forms and reactional states, but a larger sample must be evaluated in order to confirm this findings and to expand the investigation to the clinical subgroups.

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Pathogenic Free-Living *Naegleria fowleri* Induces ROS-Dependent Necroptosis in Jurkat T Cells via Toll-Like Receptor 2 and 4

Kyoung Ju Song

Yonsei University, Department of Environmental Medical Biology, Seoul, Korea

Naegleria fowleri, a free-living amoebae, is the causative pathogen of primary amoebic meningoencephalitis in humans and experimental mice. Upon host invasion, *N. fowleri* is capable of destroying tissues and host cells through lytic necrosis. However, the signaling mechanism by which *N. fowleri* induces host cell death via necroptosis is unknown. Electron microscopy indicated that incubation of Jurkat T cells with live *N. fowleri* trophozoites induced necrotic morphology of the Jurkat T cells. *N. fowleri* also induced cytoskeletal protein cleavage, extensive poly (ADP-ribose) polymerase hydrolysis, and lactate dehydrogenase (LDH) release. Although no activation of caspase-3 was observed in Jurkat T cells co-incubated with amoebae, intracellular reactive oxygen species

(ROS) were strongly generated by NADPH oxidase (NOX), but not mitochondria. Pretreated cells with necroptosis inhibitor necrostatin-1 or NOX inhibitor diphenyleneiodonium chloride (DPI) strongly inhibited amoeba-induced ROS generation and host cell death, whereas pan-caspase inhibitor z-VAD-fmk did not. *N. fowleri* secretory products (NfSP) strongly induced intracellular ROS generation and cell death. Necroptotic effects of NfSP were effectively inhibited by pretreating NfSP with proteinase K. Moreover, NfSP induced LDH release and intracellular ROS accumulation was inhibited by pretreating Jurkat T cells with DPI or necrostatin-1. Furthermore, blocking TLR2 or 4 mediated signaling by pretreatment of Jurkat T cells with specific Ab significantly prevented the NfSP induced ROS generation and cell death. These results suggest that *N. fowleri* induce necroptosis in Jurkat T cells via TLR2 and 4 mediated ROS-dependent signaling.

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Alteration of Gut Microbiome by House Dust from Homes with Pets Alters Pulmonary Immune Responses and Attenuates Allergic Disease

Tine Demoor[1], Susan V. Lynch[2], Christine C. Johnson[3], Kei Fujimura[2], Dennis R. Ownby[4], Edward M. Zoratti[3], Homer A. Boushey[2], Susan L. Havstad[3], Kevin R. Bobbitt[3], Kimberly J Woodcroft[3], Marcus Rauch[2], Aaron A. Berlin[1], Ganesa Wegienka[3], and Nicholas W. Lukacs[1]

[1]*University of Michigan Medical School,* [2]*UCSF Medical Center,* [3]*Henry Ford Hospital and Health System, and* [4]*Georgia Health Sciences University*

Atopic asthma is more common in westernized countries and its prevalence has increased greatly during the last century and has been linked to the Hygiene Hypothesis. Interestingly, exposure to pets during infancy correlates with a lower risk of developing asthma during later life: pets have been associated to the microbiome in dust, it is well known that soil and dust are ingested by children and alterations in gut microbiome have been associated with asthma development. Using 2 different mouse models of allergic asthma (cockroach antigen – CRA and ovalbumin-OVA), we set out to study pulmonary immune responses after oral exposure with house dust collected from homes with pets. In the CRA-model, dust exposure significantly decreased the Th2 cytokine protein expression (IL-4, IL-5, IL-13) in restimulated lymph nodes. Correspondingly, dust-treated animals had significantly reduced total serum IgE levels in response to allergen. Moreover, these mice showed reduced mucus hypersecretion in the lung, by PAS staining and QRT-PCR for gob5. Using a T cell transfer model of ovalbumin-induced inflammation, dust-exposed mice had lower numbers of DO11.10 T-cells in the lymph nodes and attenuation of T-cell cytokine responses (IL-4, IFNγ) of restimulated lymph node cells. Interestingly, Phylochip™ analysis of the cecal content of these mice revealed that the dust treatment had altered the microbiome in favor of Lactobacillus species. Altogether these data suggest that short-term oral exposure of dust from homes with pets alters the gut microbiome and strongly implicate this event in the attenuation of pulmonary allergen-induced immune responses.

LATE-BREAKING ABSTRACTS

LB1

Transmigration of Neutrophils through Cerebrovascular Endothelium Induces a Neurotoxic Phenotype

Charlotte M. Allen, Emmanuel Pinteaux, Nancy J. Rothwell, Stuart Allan
University of Manchester, Faculty of Life Sciences, Manchester, UK

Neuronal damage as a result of cerebral ischaemia is exacerbated by a subsequent inflammatory response triggered within the brain. An important contributor to the inflammatory response to brain injury is the blood brain barrier (BBB). The BBB consists primarily of an endothelial monolayer with characteristic tight junctions, which normally maintain low permeability. Transport across the BBB is selective and highly controlled, but BBB integrity is disrupted after cerebral ischaemia. Neutrophils are recruited to the activated endothelium where they transmigrate into the brain parenchyma contributing further to the neuronal injury.

In this study, we tested the hypothesis that neutrophils that have migrated across the BBB acquire neurotoxic properties, using an *in vitro* model of BBB consisting of primary murine brain endothelial cells (MBEC) grown on Transwell® inserts. MBECs were activated with the cytokine interleukin-1β (IL-1β, 100ng/ml) to induce the migration of primary murine neutrophils. Migrated neutrophils were subsequently collected and their phenotype and neurotoxic properties assessed.

Through the use of flow cytometry the purity and viability of these neutrophils were determined through tandem cell surface and intracellular labelling. The sustained viability of transmigrated neutrophils in comparison to the naïve population was confirmed with a significant increase in the anti-apoptotic marker phosphorylated-Akt ($p<0.05$). The direct addition of transmigrated neutrophils onto primary, murine, cortical neuronal cultures resulted in a significant increase (>35%) in neuronal cell death ($p<0.05$). This neurotoxic phenotype of transmigrated neutrophils could be attributed to a soluble factor present in conditioned medium. This was confirmed through trypsin digestion and heat inactivation. Tumour necrosis factor-alpha (TNFα) or IL-1α also induced neutrophil migration and the acquisition of a neurotoxic phenotype.

The identification and better understanding of this neurotoxic mechanism of transmigrated neutrophils could lead to the discovery of new therapeutic targets for the treatment of cerebrovascular inflammation in brain disorders.

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LB2

IL-1R Signaling Is Essential for Host Defense during Acute Bacterial Brain Abscess Formation

Juan Xiong, Tammy Kielian
University of Nebraska Medical Center, Department of Pathology and Microbiology, Omaha, NE

Staphylococcus aureus (S. aureus) is a common etiologic agent of bacterial brain abscess. We have previously established that a vigorous IL-1 response is elicited immediately after *S. aureus* infection and MyD88, a central adaptor for Toll-like receptor/IL-1R/IL-18R signaling is essential for survival and proinflammatory mediator release. However, the role of IL-1R activity in host defense during brain abscess development remains unclear. IL-1R KO mice were more susceptible to intracerebral *S. aureus* infection, as demonstrated by enhanced mortality rates and significantly higher bacterial burdens compared to WT animals. Loss of IL-1R signaling also led to a dampening of cytokine and chemokine expression, which resulted in a significant reduction in neutrophil and macrophage infiltrates into the brain. However, examination of primary microglia from IL-1R KO mice revealed that proinflammatory mediator secretion and phagocytosis was equivalent between both IL-1R KO and WT microglia. Overall, these results demonstrate that IL-1R signaling plays a pivotal role in the genesis of protective immune responses during the acute stage of brain abscess development through *S. aureus* containment, cytokine/chemokine production, and peripheral immune cell recruitment.

LB3

Sustained Self-Activation of NK Cells in vitro and in vivo by Expression of IL-12/shh-C Fusion Proteins

Lining Zhu, Xianzhong Yu, Yanzhang Wei
Clemson University, Department of Biological Sciences, Clemson, SC

Objective: In the present study, the fusion protein IL-12/mouse sonic hedgehog C-terminal domain (shh-C) would, once produced from transfected NK cells, goes through autocatalytic processing to form cholesterol modified IL-12 molecules and stays in the vicinity of or bind back to those producing cells. This would establish an autocrine loop of IL-12 for the activated NK cells and reduce the need of IL-2 administration. **Methods**: EGFP and IL-12 sequences were fused in frame with mouse sonic hedgehog C-terminal domain (Shh-C) to form eGFP/shh-C and IL-12/shh-C fusions. IL-12 without shh-C tail was used as control. Lentiviral particles were produced with a vector system from System Biosciences. Subsequently, cell surface markers, cell proliferation rates, and IFN-gamma secretion of NK cells transduced with different viral particles were analyzed with flow cytometry, alamar blue cell proliferation assay, and cytokine ELISA respectively. For *in vivo* studies, C57BL/6 mice received transduced NK cells or NK cells transduced with different lentiviral particles intravenously without IL-2 support 3 days after receiving tail vein injection of B16 melanoma tumor cells. Lung tissues were analyzed to determine the infiltration of modified NK cells into the tumor microenvironment **Results**: The transduction rates of the three (eGFP/shh-C, IL-12, IL-12/shh-C) lentivirus-transduced NK cell populations 5 days after infection were ranging from 20% to 32% when MOI was 1, and were maintained within this range at least for up to 10 days after transduction. EGFP/shh-C, IL-12, and IL-12/shh-C lentivirus infected NK cells were observed to have significantly increased Mac-1 expressions (25%, 27%, and 18% respectively). Perforin production in IL-12/shh-C infected NK cells was doubled compared to that in untreated NK cells. When given 10-fold less support of IL-2, NK cells with IL-12/shh-C transgene can reach the same proliferation rate with eGFP/shh-C transduced NK cells with 10-fold more IL-2 addition, suggesting that the fusion protein decreased the requirement of NK cells for

IL-2. The amounts of IFN-gamma in the supernatant of IL-12/shh-C virus transduced NK cells 5 days and 7 days after transduction were 40% (p=0.008) and 48% (p=0.00045) higher respectively compared to the untreated group. Immunofluorescent staining in the lung tissue slides from B16-bearing mice confirmed that donor NK cells successfully infiltrated into lung tissues. Plasma of tumor injected mice collected 7 days and 14 days after adoptive transfer of IL-12/shh-C infected NK cells contained remarkably elevated level of IFN-gamma and TNF-alpha, compared to that of B16 only group. **Conclusion**: The IL-12/shh-C domain fusion protein could function, once autoprocessed to form cholesterol anchored IL-12, to maintain the activated status of NK cells *in vitro*. In addition, IL-12/shh-C virus transduced NK cells successfully infiltrated into B16 tumor nodules in lung tissues.

LB4

Autophagy in RSV-Infected DC Modulates CD4+ T Cell Cytokine Production in vitro

Michelle Reed, Susan Morris, Nicholas W. Lukacs
University of Michigan, Department of Pathology, Ann Arbor, MI

Severe infection in infancy with respiratory syncytial virus (RSV) is a known risk factor in the subsequent development of chronic pulmonary disease, while subsequent RSV exposure may produce bronchiolitis and pneumonia in susceptible individuals. During pulmonary viral infection, resident and recruited dendritic cells (DC) recognize and transport viral antigens to the lymph node, present antigen to T cells, produce cytokines and express costimulatory molecules to activate an appropriate adaptive immune response. Previous studies have suggested that macroautophagy, an intracellular process facilitating delivery of cytoplasmic constituents to endosomes for degradation, functions in intracellular pathogen surveillance and pathogen delivery for processing and presentation by DC.

These studies investigated a role for autophagy in DC maturation during infection with RSV, and examined the impact of autophagy blockade on the priming of CD4+ T cells. RSV infection of mouse bone marrow-derived dendritic cells (BMDC) induced autophagosome formation, while blockade of autophagy through 3-methyladenine (3MA) treatment or culture from haploinsufficient Beclin-1 +/- mice decreased expression of costimulatory molecules and pro-inflammatory cytokines. Inhibition of autophagy in RSV-infected BMDC further resulted in enhanced Th2 cytokine secretion and decreased IFN γ expression in CD4+ lymph node T cells isolated from RSV-sensitized mice. Similarly, *in vitro* production of Th2 cytokines by CD4+ ovalbumin-reactive T cells was augmented and IFN γ production diminished by co-culture with RSV-infected Beclin-1 +/- BMDC, or wild-type BMDC treated with 3MA. These results provide evidence that virally-induced autophagy in DC plays an important role in host response to RSV infection by facilitating the appropriate differentiation of CD4+ T cells.

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LB5

Adenosine Promotes Alternative Macrophage Activation via A2A and A2B Receptors

Balazs Csoka,[1] Balazs Koscso,[1] Zoltan H. Nemeth,[2] Peter J. Murray,[3] Zsolt Selmeczy,[1] Gyorgy Hasko[1],
[1]UMDNJ-NJMS, Newark, NJ; [2]Morristown Memorial Hospital, Morristown, NJ ; [3]St. Jude Children's Research Hospital, Memphis, TN

Classical macrophage activation proceeds following treatment with the T helper 1 cytokine interferon-γ or lipopolysaccharide and results in the production of pro-inflammatory mediators. Alternative macrophage activation is induced by exposure to the T helper 2 cytokines interleukin-4 and IL-13, and leads to the induction of genes that promote the resolution of inflammation. Adenosine is an endogenous immunoregulatory molecule that has been implicated in suppressing the pro-inflammatory responses of classically activated macrophages; however, the role of adenosine in governing alternative macrophage activation has not been studied. We show here that adenosine treatment of IL-4- or IL-13-activated macrophages augments the expression of alternative macrophage markers arginase-1, tissue inhibitor of matrix metalloproteinase-1, and macrophage galactose-type C-type lectin. The stimulatory effect of adenosine on alternative macrophage activation required both A_{2A} and A_{2B} receptors. Of the transcription factors previously known to drive alternative macrophage activation, CCAAT-enhancer-binding protein β was required, while cAMP response element-binding protein and signal transducer and activator of transcription 6 were dispensable in mediating the enhancing effect of adenosine. In addition, adenosine augmented alternative macrophage activation by stimulating p38 mitogen activated protein kinase. We propose that adenosine receptor activation suppresses inflammation and promotes tissue restitution, in part, by enhancing alternative macrophage activation.

LB6

IRF3 Polymorphisms in SJL/J Mice Induce Different Innate Anti-Theiler's Virus Immune Responses in Macrophages Compared with IRF3 from B10.S Mice

Thomas M. Petro,[1] Tyler C. Moore,[2] Katherine Bush,[3] Fahd M. Al-Salleeh,[4] Deborah M. Brown[2]
[1]University of Nebraska Medical Center, Oral Biology, Lincoln, NE;[2]University of Nebraska, School of Biological Sciences, Lincoln, NE; [3]University of Nebraska Medical Center, College of Dentistry, Lincoln, NE;[4] University of Nebraska Medical Center, Department of Surgical Specialties, Lincoln, NE

Inexplicably, certain viral infections persist in the host, an outcome that can ultimately cause disease. In a well-known animal model of viral persistence leading to disease, Theiler's murine encephalomyelitis virus (TMEV) infects macrophages of SJL/J (H-2s) mice establishing persistent infections leading to demyelinating autoimmune-like disease. In contrast macrophages from B10.S (H-2s) mice clear TMEV, which prevents subsequent disease. Because activation of the transcription factor IRF3 induces IFNβ, ISG56, and apoptosis for viral clearance, but also disease-causing cytokines, such as IL6, our central hypothesis is that differences

in IRF3 contributes to the differences in TMEV persistence and disease between these two strains of mice. Here we identify three single nucleotide polymorphisms in the IRF3 gene of SJL/J versus B10.S mice that are predicted to change single amino acids located in the DNA-binding, nuclear-localization, and autoinhibitory domains of IRF3. To test the anti-viral properties of these two IRF3s, SJL-IRF3 and B10S-IRF3 expression vectors were transfected into RAW264.7 macrophage cells before infection with TMEV. SJL-IRF3 expression in RAW264.7 cells responding to TMEV increased IL-6, ISG56, IFN β and TMEV replication as measured by RT-PCR compared with B10S-IRF3 expression. In contrast, B10S-IRF3 expression in RAW264.7 cells augmented pro-apoptotic DEVD cleavage caspase activation and western blot Bax expression compared with SJL-IRF3 expression. Interestingly, RAW264.7 cells expressing SJL-IRF3 exhibited lower IL-6, ISG56, and IFNβ in response to polyIC, a TLR3 agonist, compared with cells expressing B10S-IRF3. To correlate these findings with responses in the TMEV-model, we found that primary macrophages from SJL/J mice responding to TMEV infection expressed higher levels of IL-6 mRNA and TMEV RNA at 24 h compared with macrophages from B10.S mice. However, the 0-24 h accumulated secretion of IL-6 protein as measured by ELISA was higher from B10.S compared with SJL/J macrophages. Therefore IRF3 polymorphisms contribute to some of the critical events leading to viral persistence and altered cytokine expression in the TMEV model.

LB7

Lack of Early Mammalian Cell Immune Response during Exposure to Manufactured Nanoparticles

Azam F. Tayabali, Kathy C. Nguyen

Health Canada, Healthy Environments and Consumer Safety Branch, Ottawa, ON

Nanoparticles (NPs) are defined to be between 1-100 nanometers in at least one dimension. Their small size increases their surface area to volume ratio and alters their chemico-physical characteristics. These properties give them novel attributes that are useful in industrial and consumer applications (eg. therapeutics, cosmetics, food preservation/packaging, environmental remediation). However, the safety of many NPs has not been clearly evaluated, and much of the toxicity research is conflicting in its conclusions. In an effort towards clarifying the potential early effects of NP exposure, select NPs (cadmium telluride quantum dots, nano-polystyrene, nanosilver and nanogold) were exposed to J774A.1 macrophage and HT29 epithelial cells, and their supernatants were harvested at intervals between 2h and 24h. Cytotoxicity measured by MTT bioreduction was greatest for quantum dots, followed by nanosilver, and negligible for nano-polystyrene and nanogold. Neutrophil chemoattractants KC and IL-8 and cytokines including IL-1β, IL-6, RANTES, and TNF-α, as well as extracellular nitric oxide from supernatants were relatively unchanged during exposures to all NPs. However, when sub-cytotoxic concentrations of quantum dots were used in pre-exposures, followed by exposure to *Pseudomonas aeruginosa*, cytotoxicity was elevated by as much as 22%. Also, at sub-cytotoxic levels of quantum dots, some but not all inflammatory mediators (nitric oxide, TNF-α, KC and IL-8) were suppressed by up to 5-fold. These data suggest that although NPs do not primarily cause changes in levels of immune mediators, they may cause immunosuppression of the normal response to infection.

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LB8

Interleukin-4 Alters TLR Signaling Pathways during Endotoxin Challenge

Candice M. Brown,[1] Ashley N. Church,[2] Charles E. McCall[3]

[1]Wake Forest School of Medicine, Molecular Medicine, Winston Salem;[2]Wake Forest School of Medicine, Internal Medicine/ Molecular Medicine, Winston Salem, NC;[3]Wake Forest School of Medicine, Molecular Medicine, Winston Salem, NC

Macrophage and monocyte polarization toward classical (M1) or alternative (M2) pathways is a defining feature of the innate immune response. Activation of Toll-like receptor (TLR) signaling pathways via endotoxin challenge initiates a pro-inflammatory (M1) cascade accompanied by phase shifts in bioenergy, epigenetic reprogramming, and metabolism designed to combat infection. Recent studies suggest endotoxin tolerance is also characterized by activation of anti-inflammatory (M2) signaling cascades. To test whether activation of M2 signaling cascades prior to endotoxin stimulation modifies prototypical M1 pro-inflammatory responses, we primed human monocytes with interleukin-4 (IL-4), which induces M2 activation pathways, prior to endotoxin challenge. THP-1 cells, a well-established model of human sepsis, were stimulated with lipopolysaccharide (LPS, 1μg/ml) in the absence and presence of IL-4 (20 ng/ml) priming for 24h followed by assessment of cell phenotype and protein levels at 0h, 1h, 4h, and 24h following LPS challenge. Flow cytometry was used to assess the effect of IL-4 on monocyte/macrophage polarization. Our results showed that 24h LPS stimulation alone induces a CD14+/CD16+/CD36+/CX3CR1- cell population; in contrast, IL-4 priming induces a large phenotypically distinct cell population that is CD14-/CD16-/CD36-/CX3CR1-. To determine whether IL-4 also influences expression of the bioenergy sensor, Sirtuin 1 (SIRT1), we found that LPS stimulation steadily increases SIRT1 protein levels over 24h; in contrast, IL-4 priming induces high SIRT1 protein levels in cells that are sustained over 24h of LPS challenge. These results demonstrate that IL-4 induces a signaling program that closely resembles endotoxin tolerance by inducing an M2 phenotype that is coupled with increased SIRT1 protein levels and sustained in the face of endotoxin challenge.

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LB9

The Temporal and Regional Profile of Leukocyte Chemokine Expression and Consequent Leukocyte Infiltration into Rat Brain Following Soman-Induced Status Epilepticus

Erik A. Johnson, Thuy L. Dao, Michelle A. Guignet, Claire E. Geddes, Andrew I. Koemeter-Cox, Robert K. Kan

USAMRICD, Department of Pharmacology, Abeerdeen Proving Ground, MD

Soman (GD), a potent organophosphorus acetylcholinesterase inhibitor, can cause prolonged status epilepticus (SE) activity that damages specific regions of the brain. This pathological process includes a neuroinflammatory response where leukocyte recruitment is a prominent feature. The purpose of this study is to characterize the regional and temporal increases in chemotactic signals, neutrophil infiltration and macrophage appearance in the brain using the rat GD neuroprotection model developed at USAMRICD. Protein levels of chemokines that attract leukocytes (i.e. CXCL1, MCP-1 and MIP-1α) were quantified using multiplex bead immunoassays up to 72 hours following SE onset in multiple damaged brain regions. These inflammatory factors were then localized to specific neural cell types within the injured brain. Lastly, neutrophil infiltration and the appearance of macrophages were quantified and correlated to the expression of these chemokines. Concentrations of CXCL1, MCP-1 and MIP-1α were significantly increased after seizure onset and positively correlated with either neutrophil infiltration or macrophage appearance. Lastly, the neural cell expression of these factors originated from neurons, endothelial cells and microglia as shown by fluorescent immunohistochemistry. These data show the time course of chemotactic signals that originate from specific neural cell types injured by GD-induced SE. The induction of this inflammatory response may play a key role in the progressive secondary brain pathology observed following GD-induced SE. Disclaimer: The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division.

LB10

Role of the NLRP3 Inflammasome in the Generation of Th17 Adaptive Immune Responses

Jeffrey J. Sadler,[1] Shruti Wilson,[2] Kahyong Goh,[1] Fayyaz S. Sutterwala,[1] Suzanne L. Cassel[3]

[1]University of Iowa, Inflammation Program, Iowa City, IA; [2]University of Iowa Hospitals & Clinics, Department of Immunology, Iowa City, IA; [3]University of Iowa, Inflammation Program & Department of Medicine, Iowa City, IA

Upon activation NLRP3 along with the adaptor molecule ASC and the cysteine protease caspase-1 forms a multiprotein complex, called an inflammasome, which facilitates the activation of caspase-1 with the subsequent processing of pro-IL-1β and pro-IL-18 into their active secreted forms. Although the role of NLRP3 in response to pathogen and danger associated molecular patterns is well documented, its role in shaping adaptive immune responses to these insults remains unclear. Of particular interest is the influence of NLRP3 inflammasome generated IL-1β on the differentiation of T helper cells into Th17 effector cells. To explore this we used a mouse collagen-induced arthritis model; both NLRP3- and ASC-deficient mice had significantly less severe disease compared to wild-type (WT) mice. Consistent with this, ex-vivo CD4+ T cell restimulation with collagen resulted in diminished IL-17 secretion in both NLRP3- and ASC- deficient mice. This defect in IL-17 production in NLRP3- and ASC-deficient mice was recapitulated using WT and knockout mice carrying the chicken ovalbumin (OVA)-specific TcR transgene (OT-II) that were immunized with complete Freund's adjuvant (CFA) and ovalbumin. Adoptive transfer of WT OT-II CD4+ T cells into NLRP3-/- mice followed by immunization with CFA/OVA resulted in defective IL-17 generation, whereas IL-17 production remained intact when NLRP3-/- OT-II CD4+ T cells were transferred into WT mice. These data suggest that the NLRP3 inflammasome is critical to the fate determination of T helper cells to Th17, but the requirement is not intrinsic to the CD4+ T cell itself. We hypothesize this requirement for NLRP3 lies in the antigen presenting cell, where activation results in the generation of
IL-1β, known to support the differentiation of CD4+ T cells to Th17 effector cells.
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LB11

PGE2 Upregulates Expression and Activity of TLRs in Macrophages in an EP4-Dependent Manner

Adam R. Streit, Nicholas J. Zarkadis, Alice Rutatangwa, Ronald R. Bean, Alexander Shnyra

Kansas City University of Medicine and Biosciences, Department of Pharmacology and Microbiology, Kansas City, MO

Toll-like receptors (TLRs) expressed by Mϕ play a key role in innate and adaptive immunity. Recognition of distinct pathogen-associated molecules by TLRs triggers NF-κ B signaling pathway that culminates in activation of proinflammatory transcriptional programs controlling function of M ϕ. Tumor-associated M ϕ (TAMs) may also be activated in a TLR-dependent manner resulting in local inflammatory milieu. Although TAMs activities are tightly regulated by the constituents of tumor microenvironment, selective tumor-derived mediators controlling expression of TLRs have yet to be identified. Overexpression of Cox-2 has been shown in various tumors where it correlates with poor prognosis. PGE_2 is well known to exhibit immunosuppressive effects on M ϕ. However, it remains to be determined how each PGE_2 receptor subtypes, EP2 or EP4, regulates activities of TAMs in tumors expressing Cox-2. For this purpose, we studied PGE_2-regulated expression of TLRs and their functional activities in wild-type human M ϕ ($EP2^+/EP4^+$) and M ϕ with low expression of EP2 ($EP2^{low}$) due to stable transfection with a shRNA lentiviral vector plasmid effectively inhibiting EP2 expression. In addition, both $EP2^+/EP4^+$ and $EP2^{low}$ M ϕ were stably transfected with an NF-κB/AP-1-inducible reporter gene coding for secreted embryonic alkaline phosphatase (SEAP). Our results show that exposure to PGE_2 (50 ng/ml) strongly upregulated transcriptional expression of TLR1, TLR2, TLR4, and TLR6 in $EP2^{low}$ M ϕ but not in $EP2^+/EP4^+$ M ϕ. Functional activity of each upregulated TLR in $EP2^{low}$ M ϕ were further confirmed by treating the cells with selective agonist tripalmitoylated lipopeptide

Pam3CSK4 (TLR1/TLR2), heat-killed *Listeria monocytogenes* (TLR2), *E. coli* K12 LPS (TLR4) and synthetic lipoprotein Pam2CGDPKHPKSF (TLR6) by using cDNA array and assessing TLR-induced activation of the reporter gene. Collectively, our data suggest that selective EP2 antagonists used for treatment of Cox-2-expressing tumors may potentiate pro-inflammatory properties of TAMs by upregulating expression and activities of TLRs.

LB12

Local versus Systemic Inflammation As Contributing Factors to AD-Like Neuropathology in the APP/PS1 Mouse Model

Brenda C. Salumbides, Yosef Koronyo, Julia Sheyn, Michael Pham, Keith L. Black, Maya Koronyo-Hamaoui
Cedars Sinai Medical Center, Department of Neurosurgery, Los Angeles, CA

Abnormalities detected in PBMCs from AD patients as compared to age-matched healthy controls support a role for immune responses in the progression and treatment for Alzheimer's disease (AD). Preclinical studies have shown that boosting a peripheral immune response via weekly glatiramer acetate (GA) immunization improves cognitive performance, restricts pathology, regulates cerebral inflammation, associated with AD in $APP_{SWE}/PS1_{\Delta E9}$ transgenic (ADtg) mice. Robust response to GA vaccination in treated ADtg mice is likely to act through several mechanisms aside from T-cell mediated activity such as increased cerebral recruitment of protective monocytes. We examined the beneficial effects of combination immunotherapy using GA with or without systemic enrichment with $CD115^+$ monocytes. Specifically, we investigated the effects of adoptive transfer of inflammatory monocyte subsets introduced to the peripheral blood including alterations in cytokine-chemokine profiles in immunized versus saline-treated control ADtg mice. We found increased spontaneous monocyte cerebral infiltration surrounding active amyloid lesions in the brains of GA-immunized and monocyte-treated ADtg mice compared to controls. In all immunized groups, we obtained decreased levels of soluble and insoluble amyloid burden, reduced astrogliosis, and substantial attenuation of cognitive decline. Assessment of brain tissue from immunized ADtg mice shows increased local IL-10 and MMP-9 levels, which we attribute to immune-modulatory activity mostly of infiltrating monocytes, but also resident microglia, within the microenvironment. CCL2/MCP-1 levels were also elevated in ADtg mice receiving $CD115^+$ monocytes. Thesestudies identify immune modulatory activity by $CD115^+$ monocytes, independent and in combination with GA, as mediators of disease progression in the APP/PS1 mouse model.

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LB13

EP2 Signaling Pathway Controls Immunoediting of Macrophages by PGE2-Producing HCA-7 Colon Carcinoma Cells

Nicholas J. Zarkadis, Alice K. Rutatangwa, Ronald R. Bean, Adam R. Streit, Alexander Shnyra
Kansas City University of Medicine and Biosciences, Department of Pharmacology and Microbiology, Kansas City, MO

Background: Tumor microenvironment exhibits potent immunosuppressive effects on cells of the myeloid lineage. Tumor-associated macrophages (TAMs) exhibit an "M2-like" phenotype of alternatively-activated macrophages ($IL\text{-}12^{low}$, $IL\text{-}23^{low}$, $IL\text{-}10^{high}$) that is believed to promote tumor growth and metastasis. Among various tumor-derived constituents, PGE2 may contribute to immunoediting of TAMs although it is not clear whether EP2 or EP4 subtype of PGE2 receptor is involved.
Methods: We generated human macrophages (Mϕ) with low expression of EP2 ($EP2^{low}$) achieved by a stable transfection with a lentiviral vector plasmid encoding EP2-specific shRNA. TAMs were generated *in vitro* by co-culturing of wild-type ($EP2^+/EP4^+$) and $EP2^{low}$ Mϕ with PGE2-producing HCA-7 colon carcinoma cells. TAMs obtained were then isolated by using anti-CD14 Magnetic beads. Transcriptional activation of genes in $EP2^+/EP4^+$ and $EP2^{low}$ TAMs was analyzed using RT^2 Profiler™ PCR Array.
Results: We found that wild-type $EP2^+/EP4^+$ TAMs exhibited transcriptional activation of genes coding for M2-type cytokines, chemokines and other immunoregulatory molecules. In contrast, $EP2^{low}$ TAMs expressed a mixed M1/M2 phenotype. Furthermore, LPS-induced activation of wild-type $EP2^+/EP4^+$ TAMs resulted in defective expression of M1-type cytokines and chemokines as compared to those expressed LPS-challenged Mϕ. In $EP2^{low}$ TAM, however, LPS induced strong expression of genes coding for cytokines and chemokines prototypic for classically-activated Mϕ. The results obtained were further validated by a quantitative RT-PCR and Cytokine/Chemokine Protein Array.
Conclusion: Our results suggest that EP2 signaling pathway may play a key role in PGE2-controlled immunoediting of TAMS in tumors overexpressing Cox-2.

LB14

Quantitative Proteomic Analyses of THP-1 Organelles Reveal Inflammasome-Associated Relocation of Mitochondria-Associated Membranes during Early Salmonella Infection

Yuan Fang, Leonard J Foster
University of British Columbia, Center for High-throughput Biology, Vancouver, BC

Salmonella enterica is an intracellular bacteria pathogen that acts by injecting effector proteins into host cells to induce pyroptosis via NLRC4 inflammasome activation[1]. Mitochondria associated membranes (MAM) are important contact sites between mitochondria and ER; this unique structure has recently been reported to modulate inter-organelle lipid transport and selectively facilitate Ca^{2+} uptake from ER lumen to mitochondria[2]. Previous immunofluorescence experiments showed that NLRP3 inflammasome co-localized with Calreticulin (one MAM marker) at the perinuclear region, suggesting a correlation between MAM and inflammasome activation[3]. In the context of early *Salmonella* infection of THP-1 cells, we used dis-continuous sucrose gradient in combination with Stable Isotope Labeling by Amino acids in Cell culture (SILAC) to profile organelle proteomes. With this unbiased approach, we generated protein profiles for >1200 mitochondria and ER proteins. With the stimulation of *Salmonella* infection, a subgroup of mitochondria and ER proteins are significantly shifted to a heavier fraction (5% heavier in sucrose gradient). In this

subgroup there are known MAM markers[2]: Calnexin, Calreticulin, Inositol 1, 4, 5-trisphosphate receptor (IP_3R), Erlin2, BiP/grp78, which suggests subcellular relocation of MAM proteins during *Salmonella* infection. Interestingly, Apoptpsis-associate Speck-like protein containing a CARD (ASC), the adaptor protein for inflammasomes, was significantly recruited to this membrane fraction (>8 fold), suggesting that inflammasome complexes may be located at MAM membranes during *Salmonella* infection. SILAC immunoprecipitation experiments showed that ASC can interact directly with MAM proteins (VDAC and grp75), further confirming the above speculation. Surprisingly, the MAM fraction we characterized is heavier than the major mitochondria fraction, instead of a lighter fraction in the published MAM isolation protocol[4]. This may be linked to the perinuclear location of the inflammasome upon activation. In conclusion, quantitative protein profiling across density gradients allows us to accurately characterize the proteome of MAM membranes. To our knowledge, this data set would be the first report of MAM proteome after bacterial infection and the first proteomic evidence of the physical association between inflammasome and MAM.

Reference:

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LB15

Acute-Phase Serum Amyloid A Causes Th1 Differentiation in Murine CD4 T Cells

Rong L. He, Asona J. Lui

Chicago State University, Department of Biological Sciences, Chicago, IL

Serum amyloid A (SAA) is an acute-phase protein, released predominantly from the liver during large scale inflammation. It is also secreted at local sites of inflammation by other cell types like macrophages, endothelial and epithelial cells. High SAA levels, up to 1000 times normal, are associated with autoimmune conditions like Rheumatoid Arthritis (RA). SAA mRNA is found at very high levels in the arthritic synovium, along with a high concentration of certain subsets of T lymphocytes, notably helper T cell type 1 (Th1). The marker for Th1 cells is production of the cytokine interferon gamma (IFNgamma). The purpose of this study is to determine if SAA stimulation of murine T cells causes Th1 differentiation. CD4+ T-cells isolated from the spleens of wild type mice have been stimulated in vitro with SAA protein. IFNgamma production is measured and compared to that of the known inducer of Th1 cells, interleukin-12 (IL-12). Indeed, SAA stimulation induces IFNgamma production from CD4+ T-cells and can cause the auto-secretion of IFNgamma, similar to IL-12. Our data suggests that SAA and IL-12 signal through separate receptors, as SAA and IL-12 can co-stimulate T cells and result in much further increased levels of IFNgamma secretion. This combination is even able to cause measurable IFNgamma secretion without TCR ligation by anti-CD3 antibody and at earlier time points than IL-12 or SAA alone. Th1 differentiation is investigated further by detection of the IFNgamma and IL-12 receptors with flow cytometry. These results suggest that SAA and Th1 cells are found together in arthritic joints because SAA induces this T-cell subset's production, and is perhaps a missing link in our understanding of RA. (Supported by NIH EARDA Pilot Project Grant (2G11HD039879-10))