1st Annual Pulmonary Research Day

Abstracts

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Eric P. Newman Education Center

Washington University
School of Medicine

Basic Category

Abstract B1

Structural Determinants of Innate Inflammatory Signaling by TREM-2

Daniel L. Kober[1,2,5], Jennifer xM. Alexander-Brett[2,5], Michael J. Holtzman[2,3,5], and Tom J. Brett[2,3,4,5]
From the Molecular Microbiology and Microbial Pathogenesis Program[1], Department of Internal Medicine[2], Cell Biology and Physiology[3], Biochemistry and Molecular Biophysics[4], and Drug Discovery Program in Pulmonary and Critical Care Medicine[5]
Washington University School of Medicine, St. Louis, Missouri 63110

Introduction: TREM-2 is an innate immune receptor centrally involved in tuning inflammatory signals, and thus has been linked to several inflammatory diseases including COPD and neurodegenerative diseases. Recently, distinct genetic variations in TREM-2 have been linked to a number of neurodegenerative diseases. One set of mutations is causative for Nasu-Hakola disease (NHD), while another group of mutations strongly increase risk for Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). We hypothesized that investigating how these mutations impact TREM-2 structure and function would reveal mechanistic insight into how TREM-2 contributes to diseases.
Methods: To understand how distinct point mutations within the Ig domain of TREM-2 contribute to different diseases, we performed structural, biophysical, and functional studies of WT and mutant proteins.
Results: First, we determined the crystal structure of the TREM-2 Ig domain to 3.1 Å resolution. Mapping the location of neurodegenerative disease mutations onto the structure revealed a clear segregation. Residues linked to NHD were buried while residues linked to AD were surface-exposed, leading to the hypothesis that the first set of mutations likely destabilize the protein and negatively affect surface expression, while the second set affect ligand binding. Protein expression, solution studies, and thermal and chemical stability analyses confirmed that the buried mutations destabilize the protein and increase aggregation, while the surface mutations largely do not affect protein stability. Circular dichroism (CD) analysis of surface mutants confirmed these proteins are folded, and also revealed subtle spectral changes in the main AD mutant, R47H. Functionally, surface mutations with strong AD risk were shown to decrease TREM-2 binding to cell lines proposed to express a TREM-2-ligand (TREM-2-L). Interestingly, a separate surface mutation, T96K, produced increased binding despite slightly diminished thermal and chemical stability. Further analysis revealed that all of these mutations occur within a large conserved basic surface patch on the TREM-2 Ig domain, and extending this patch with basic mutations led to increased binding to TREM-2-L.
Conclusions: Our functional and structural analysis indicate not only that mutations associated with neurodegenerative disease segregate to create two distinct loss-of-function mechanisms, but indeed reveal a novel functional surface on TREM-2 that is not present in other members of the TREM family of receptors. These findings indicate that different molecular approaches for treatments will be needed depending on the mutation and also highlight a unique functional surface which greatly extends our knowledge of TREM-2 biology.

Abstract B2

Ion channel regulation and mucus production: Secreted CLCA1 modulates TMEM16A to activate Ca^{2+}-dependent chloride currents in human cells

Zeynep Yurtsever[1,2,4,6] , Monica Sala-Rabanal[3,4], Michael J. Holtzman[2,3,6], Colin G. Nichols[3,4], Tom J. Brett[2,3,4,5,6]

[1]Biochemistry Program, [2]Department of Internal Medicine, [3]Department of Cell Biology and Physiology, [4]Center for the Investigation of Membrane Excitability Diseases, [5]Biochemistry and Molecular Biophysics, [6]Drug Discovery Program in Pulmonary and Critical Care Medicine
Washington University School of Medicine, St. Louis, Missouri, 63110

Introduction: The protein CLCA1 plays a central role in airway biology in health and disease; as a potent modulator of calcium-activated chloride channels (CaCCs) it contributes to proper mucus function; but when overexpressed, it is a mediator of mucus cell metaplasia (MCM) in chronic airway diseases. Although CLCA1 has long been linked to asthma and COPD, and considered as a drug target for the treatment of these diseases, progress in this area has been limited due to the lack of mechanistic insight into CLCA1 function.
Methods: To understand how CLCA1 activates chloride currents, we performed biochemical studies of CLCA1 and used whole-cell patch clamp method as a functional readout of CaCC activity.
Results: We demonstrate in an epithelial cell model that CLCA proteins contain a consensus cleavage site that is recognized by a unique zincin metalloprotease domain located within the N-terminus of CLCA itself, and that this self-cleavage is required to produce an active form of CLCA1 that can activate CaCCs. We also show that secreted CLCA1 activates calcium-dependent chloride currents in human cells in a paracrine fashion, and endogenous TMEM16A/Anoctamin1 conducts the currents. Exposure to exogenous CLCA1 increases cell surface levels of TMEM16A and cellular binding experiments indicate CLCA1 engages TMEM16A on the surface of these cells. Altogether, our data suggest that CLCA1 stabilizes TMEM16A on the cell surface, thus increasing surface expression, which results in increased calcium-dependent chloride currents.
Conclusions: Our results identify the first Cl^- channel target of the CLCA family of proteins, establish CLCA1 as the first secreted direct modifier of TMEM16A activity, and solve a 20-year old mystery as to how CLCA proteins modulate Cl- currents. These findings suggest cooperative roles for CLCA1 and TMEM16A in the physiology and pathology of asthma, COPD, and cystic fibrosis.

Abstract B3

The Role of Autophagy in the Secretion of Mucins in Asthma

Allen Cole Burks[1], Yael Alevy[1], Thaddeus S. Stappenbeck[2],
Michael J. Holtzman[1], and Steven L. Brody[1]
Departments of Medicine[1] and Pathology and Immunology[2],
Washington University School of Medicine, Saint Louis, Missouri

The Th2 cytokine IL-13 stimulates airway goblet cell metaplasia and secretion of the inflammatory mucin MUC5AC in asthma and other airway diseases. Autophagy is a multifunction, multiprotein pathway of membrane formation, protein engulfment, and vesicle fusion. Our previous studies identified that, in airway epithelial cells, IL-13-activated secretion of MUC5AC requires autophagy. The components of autophagy are well described, but the regulatory factors required by epithelial cells for autophagy-mediated mucin secretion are not defined. Given the pathologic role of mucus hypersecretion in asthma, efforts to understand the mechanism of mucin secretion, offer potential novel therapeutic avenues.

We propose three specific aims to address the mechanism of autophagy-mediated mucin secretion in normal and asthmatic airway epithelium: (1) Identify downstream regulatory targets of autophagy in non-diseased airway epithelial cells, (2) Determine the effect of IL-13 independent autophagy activation on mucin secretion, and (3) Assess the role of polymorphisms in the autophagy gene, *ATG5,* to regulate IL-13-mediated mucin secretion in asthmatic airway epithelial cells.

Studies will be carried out in primary culture human tracheobronchial epithelial cells (hTEC). For the first aim, we will deplete hTECs of the autophagy factor gene *ATG5* using lentivirus for shRNA transduction. Autophagy deficient and control cells will be differentiated with IL-13 stimulation to generate goblet cells. After an IL13 washout period, the cells will be re-exposed to IL-13, then analyzed using transcriptional microarrays to identify differentially expressed genes. Lead candidates and pathways will be validated in hTECs and their roles in autophagy-mediated secretion assessed by targeted gene silencing. Second, we will uses novel small molecules and other known secretagogues to activate autophagy in hTECs independent of IL-13 to determine the degree to which Th2 cytokines and specific components of autophagy pathways are required for MUC5AC secretion. MUC5AC secretion and autophagy flux will be assayed from hTEC prepared using the same conditions as in Aim 1. Third, using airway epithelial cells from a well-phenotyped cohort of asthmatic subjects, we will evaluate the role of previously described ATG5 polymorphisms on autophagy activation and MUC5AC secretion. Primary culture airway epithelial cells from subjects with asthma will be differentiated with and without IL13, and then assayed for MUC5AC secretion and autophagy flux. Completion of these studies will identify regulatory pathways of IL-13 activated MUC5AC secretion and determine the status of autophagy activation in asthmatic derived airway epithelial cells, providing a potential mechanism for the enhanced MUC5AC secretion in asthma.

Abstract B4

Respiratory Virus Antagonism of Host BST-2 Antiviral Function

Nicole Fazio[1,2], Alvin Su[2], Eugene Agapov[2,3], Rose Tidwell[2,3], Michael J. Holtzman[2,3,4], and Tom J. Brett[2,3,4,5]
From the Program in Computational and Molecular Biophysics[1], and Departments of Internal Medicine[2], Drug Discovery Program in Pulmonary and Critical Care Medicine[3], Cell Biology and Physiology[4], and Biochemistry and Molecular Biophysics. [5]Washington University School of Medicine, St. Louis, Missouri 63110

Introduction: BST-2 is an interferon-inducible host anti-viral protein that blocks viral replication by inhibiting the budding of newly formed viruses from infected cells. It does this by inserting one membrane anchor into the virus and one into the host cell, which are connected by a 180 Å coiled-coil ectodomain. Given this general mechanism, BST-2 inhibits most enveloped viruses. However, most viruses have evolved countermeasures to antagonize BST-2. We hypothesize that by understanding mechanistically how viral countermeasures antagonize BST-2, strategies could be designed to inhibit the viral antagonists and thus capitalize on BST-2 function as a broad-spectrum antiviral against respiratory viruses (IAV and RSV).
Methods: We previously determined the crystal structure and biophysically characterized the BST-2 ectodomain. We used immunohistochemical (IHC) staining to investigate if BST-2 was upregulated in airway epithelium in response to respiratory virus infection (IAV and SeV). To determine which viral proteins downregulate BST-2 surface expression, we utilized flow cytometry (FC) on cells transfected with viral proteins.
Results: Our structural and biophysical analysis of the BST-2 ectodomain revealed it to be a conformationally dynamic 180 Å long coiled-coil ideally designed to be a molecular tether. We next investigated whether and where BST-2 was expressed in lung following IAV and SeV infection. We found that BST-2 mRNA increased in whole lung extracts, reflecting a response to virus-induced interferon. IHC staining revealed that BST-2 expression was upregulated in airway epithelium following virus infection, and that this expression localized to ciliated cells. Intriguingly, BST-2 expression was not detected in infected cells, suggesting the IAV and SeV antagonize BST-2 by triggering degradation of the protein. To investigate this, we transfected HeLa cells (which constitutively express BST-2) with candidate viral antagonist proteins (from IAV, RSV, and SeV) and analyzed for decrease in BST-2 surface expression using FC. These studies are undergoing and latest results will be presented.
Conclusions: BST-2 is expressed in airway epithelia in response to respiratory viral infection, however, it appears to be targeted for destruction by viral-encoded antagonists. Identifying which viral proteins target BST-2 and elucidating their mechanism of action will lead to the development of inhibitors that block viral antagonist activity, allowing us to exploit BST-2 antiviral function as a broad-spectrum treatment.

Abstract B5

Imaging Bacterial Infection Induced Inflammation using ^{68}Ga-Transferrin

Ruqi Wang, Nilantha Bandara, Suzanne Lapi, Delphine Chen
Department of Radiology

Introduction: Postoperative bacterial infections greatly contribute to the morbidity and mortality. However, current methods lack sensitivity and/or specificity for detecting infections. Positron emitting tomography (PET)/CT is a highly sensitive and quantitative imaging approach that could be used for diagnosing infections. Transferrin can be labeled with ^{68}Ga for bacterial imaging because holo-transferrin is one of the major iron sources for invading bacteria. In our study, we tested whether PET imaging with ^{68}Ga-transferrin could detect bacterial infections specifically in a mouse model.

Methods: Apo-transferrin was labeled with ^{68}Ga eluted from a ^{68}Ge/^{68}Ga generator in acetate buffer. The pseudomonas aeruginosa strain (PA01) was cultured in standard lysogeny broth (LB). Neutrophil and monocytes were isolated from human peripheral blood and activated in vitro using phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), respectively. In vitro tracer cell uptake by PA01, resting and activated neutrophil and macrophages was assessed by incubating the tracer with cells at 37°C for one hour and quantified by %injected dose (%ID) per 10^7 cells. C57BL/6J mice were inoculated with PA01, LPS or PBS control intratracheally and imaged by micro-PET (Inveon PET/CT or Focus 220 PET scanners, Siemens/CTI) at 6 hour or 24 hour post inoculation. The tracer uptakes in the lungs were quantified by %ID/ml and assessed across different groups. Mouse bronchoalveolar lavage (BAL) and lungs were harvested for the validation of bacteria counts by colony forming unit (CFU) assay and inflammation respond by IHC and flow cytometry using neutrophil and macrophage specific markers.

Results: PA01 ^{68}Ga-transferrin uptake in vitro was 0.14±0.02 %ID per 10^7 cells compared to activated neutrophil or macrophage uptake of 1.2±0.5% and 2.6±0.5 %ID per 10^7 cells, respectively. MicroPET imaging demonstrated 5.7±0.8 and 7.2±0.9 %ID/ml at 6 and 24 hour post-infection, respectively, which correlates positively with inflammation (an average of 0.97×10^6 versus 2.75×10^6 neutrophils in BAL) but negatively with bacterial counts. In addition, bacteria infected mice and LPS installed mice that were imaged at the same time point post infection and developed comparable inflammation respond showed similar uptake indicating the insignificance of bacterial uptake of the tracer.

Conclusion: The above results suggested that the uptake of ^{68}Ga-transferrin is likely targeting the neutrophil recruitment rather than bacterial proliferation in vivo. This tracer will most likely be more useful as a neutrophil marker in bacterial infection induced inflammation.

Abstract B6

Influenza A Virus Causes Chronic Inflammatory Lung Disease Linked to Acute but not Chronic Viral Clearance via MX1

Shamus P Keeler[1], Eugene Agapov[1], Rose Tidwell[1], Michael E. Hinojosa[1],Anand C. Patel[1,2], Slobodan Paessler[3], Jeffrey J. Atkinson[1], and Michael J. Holtzman[1,3]

[1]Pulmonary and Critical Care Medicine, Department of Medicine, [2]Department of Pediatrics, and [3]Department of Cell Biology, Washington University School of Medicine, St. Louis, MO, and [3]Galveston National Laboratory, Department of Pathology, and Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX

Clinical and experimental observations suggest that the development of chronic lower respiratory disease is linked to respiratory viral infection but there is no experimental model that establishes the long-term aspect of this relationship using a potent human pathogen. Here we use influenza A virus (IAV) to develop a model for chronic lung disease that progresses long after initial infection. We show that IAV (A/WS/33 strain) triggers chronic lung disease that is dependent on the severity of infection and viral level during the acute illness and is manifested by long-term airway mucus production and hyperreactivity along with persistently transcribed IAV in wild-type mice (C56BL/6 strain). The chronic mucus and IAV are localized to focal areas of residual inflammation within the lung. These inflammatory foci and airway hyperreactivity persist for at least 26 weeks after viral inoculation, whereas IAV is no longer detectable by this time. In mice that were reconstituted for deficient Mx1 function and given an increased IAV inoculum to match the acute illness manifested in wild-type mice, we found marked attenuation of chronic disease that was associated with lower lung levels of IAV at 12 days but similar levels at 49 days post-inoculation. Infection with a separate IAV strain (CA/2009) and species (ferrets) also resulted in long-term inflammatory lung disease. The results establish the capacity of a respiratory virus that causes acute illness in humans to also trigger chronic lower respiratory disease. The disease process features acute severity and chronic airway inflammation, hyperreactivity, and mucus production that are linked to acute viral level and clearance and therefore appears applicable to humans with chronic lower respiratory diseases such as asthma and COPD.

Abstract B7

Type I Interferon Signaling Mediates Recovery from Influenza A Infection

Ashley L. Steed, Lulu Sun, David J. Morales, Deborah J. Lenschow, and Thaddeus S. Stappenbeck
Department of Pediatrics, Division of Critical Care Medicine

BACKGROUND: While type I Interferon (IFN) signaling has largely antiviral properties across diverse viruses, the role of type I Interferon signaling during influenza A infection remains controversial. There exist multiple conflicting reports of whether IFN signaling is protective or harmful in the context of influenza infection. Mounting evidence has implicated a role for the immune system, not only in pathogen detection and clearance, but also in tissue repair. Consistent with this, we have previously demonstrated a link between type I IFN signaling and epithelial proliferation outside of the lung.

HYPOTHESIS and METHODS: We hypothesized that enhancement of endogenous type I IFN signaling would promote epithelial tissue repair and improve outcomes following influenza A infection. To define mechanistic determinants of IFN signaling following influenza A infection, we utilized a mouse model of H1N1 influenza A pneumonia and investigated outcomes in mice deficient in Immunity-related GTPase family protein M1 (IRGM1), an endogenous negative regulator of IFN signaling. These animals display enhanced type I IFN activity in the serum and lungs, thereby providing a novel gain of function model of IFN signaling.

RESULTS: Infection of IRGM1-/- animals with a dose of H1N1 that is fatal to 50% of wildtype animals showed 100% protection ($p=0.01$) and no outward signs of morbidity in IRGM1-/- mice, revealing a striking resistance to influenza A infection. We further demonstrated that this protection is largely mediated by type I IFN signaling. Measurement of viral plaque forming units showed that wildtype and IRGM-/- mice have equivalent lung viral loads during acute infection, suggesting that mechanistically IRGM1 deficiency does not enhance viral clearance. Instead, histological analysis of lung specimens after infection revealed that IRGM1-/- animals displayed enhanced epithelial repair. Fourteen days postinfection, IRGM1-/- animals had an expanded progenitor pool and more than two-fold increase in lung epithelial proliferation compared to controls ($p=<0.001$). Lastly, through analysis of macrophage depleted and conditional knockout animals, we implicated macrophages as critical mediators of type I IFN signaling and protection from influenza infection.

CONCLUSIONS: These findings demonstrate that upregulation of endogenous type I IFN signaling is sufficient to protect from influenza A infection through enhanced epithelial repair and implicate IRGM1 as a novel therapeutic target. Further work will focus on identifying IFN stimulated growth factors necessary for epithelial regeneration.

Abstract B8

Acute activation of Toll-like receptor 3 translates to long-term postviral disease.

X. Wang, C. Park, L. Benoit, A.C. Patel, E. Agapov, M. Colonna, A. Shaw, D.E. Byers, X. Jin, M.J. Holtzman
Division of Pulmonary and Critical Care, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA

RATIONALE: Chronic obstructive lung disease is one of the leading causes of death in the US, with substantial human and economic costs and current therapies are limited to symptomatic treatment. To identify the molecular mechanisms to allow for more directed therapies, we developed a high-fidelity mouse model of chronic lung inflammation using the natural rodent pathogen Sendai virus (SeV). While nucleic acid-sensing pattern recognition receptors are important for innate immune responses to viral pathogens, and acute inflammation, there have been few studies investigating their role in the context of chronic disease. Here we show that Toll-like receptor 3 (TLR3) signaling mediates both the acute and persistent inflammatory phenotype in a mouse model of chronic obstructive lung disease. Mice deficient in TLR3 have reduced monocytic infiltration in the lung following SeV infection. Monocyte recruitment, while unnecessary for viral clearance, appears to be critical for the development of long-term pathology. Our results suggest that TLR3 in the respiratory epithelium and endothelium plays an important role in mediating the acute response to viral infection, while TLR3 in the myeloid population mediates a chronic one, and that both are necessary for the development of long-term disease.

METHODS: Six to twelve week old wildtype C57/B6 and TLR3 KO mice were infected intranasally with Sendai virus. Animals were sacrificed on day 5 post-infection to assess acute lung pathology and count lung immune cell populations by flow cytometry. For assessment of chronic disease, animals were sacrificed on day 49 and lungs were isolated for either whole lung mRNA analysis or histopathology.

RESULTS: Acutely after viral infection, TLR3 deficient mice have decreased induction of chemokines critical for monocyte chemotaxis, CCL2 and CC7. This correlates with decreased numbers of monocytes in the lung following viral infection. Furthermore, we have found that TLR3 is both expressed and induced by virus on airway epithelial cells and pulmonary endothelial cells in vivo. We have found that deficiency of TLR3 results in impaired production of CCL2/7 from these cells, resulting in decreased monocyte recruitment and fewer numbers of differentiated myeloid cells later. TLR3 deficient mice also have reduced mucus production, airway pathology, and airway inflammatory cell infiltration at day 49 post-infection, and our studies using bone-marrow chimeras suggests that TLR3 expressed in macrophages and dendritic cells is necessary to drive the onset of chronic pathology.

CONCLUSIONS: These results indicate that TLR3 plays an important role in mediating the acute immune response to viral infection, and is necessary for the development of chronic airway disease after the infection as been resolved. Targeting TLR3 and its effector functions may be effective in blocking the development of virus-induced chronic obstructive lung disease.

Abstract B9

Mycobacterial Mediators of Inducible Bronchial Associated Lymphoid Tissue (iBALT)

Oliver A. Prince[1], Suhas Gondi[1], Hongmei Jiao[1], Deepak Kaushal[2], Shabaana A. Khader[1]
[1]Department of Molecular Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri [2]Division of Bacteriology, Tulane National Primate Research Center, Covington, Louisiana; Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB) in humans. *Mtb* infection is initiated within the alveolar spaces of the distal airway, where it encounters macrophages. Primary infection of the alveolar macrophage is responsible for invasion deeper into host tissues. A hallmark of TB infection is the formation of an immune cell aggregate called the granuloma, which was thought to wall of the infection from the surrounding uninfected tissue and mediate control. This view has changed with the observation of distinct non-protective and protective granuloma (iBALT) formation. We have recently shown that host neutrophils drive non-protective granuloma formation and mediate cell injury, necrosis and *Mtb* spread during active TB (Gopal et al. 2014). Protective granuloma is an organized lymphoid organ, whose initiation is mediated by interleukin-17 (IL-17) and structure is characterized by the presence of $CXCR5^+$ T-helper cells and $CXCL13^+$ antigen presenting cells for control of *Mtb* (Slight et al. 2013, Gopal et al. 2014 & Monin et al 2015). *Mtb* determinants that are responsible for non-protective granuloma formation during active TB are unknown. It is known that *Mtb* expresses an array of lipid moieties that have been reported to manipulate the host response in various animal models. We hypothesize that specific *Mtb* gene products, particularly those associated with murein metabolism, are principal components in the development of non-protective granuloma during TB. The Khader Lab is currently using a transposon derived *Mtb* library in order to assess *Mtb* determinants involved in granuloma formation during aerosol infection of animal models. Our readouts include both histological and fluorescent microscopy in order to grade granuloma formation relative to our positive control (wild-type, HN878). We are also actively employing cell fractions from wild-type *Mtb* in both *in vitro* and *in vivo* models with subsequent analysis through histological, immunohistochemical, flow cytometric and cytokine panels. Taken together our approach will identify *Mtb* factors associated with iBALT formation and offer fundamental insights into the mechanism, maintenance and/or avoidance of host control during active TB.

Abstract B10

Interleukin-17A negatively regulates the formation of necrotic lesions in pulmonary tuberculosis

Racquel Domingo-Gonzalez[1], Kristin Griffiths[1], June Treerat[1], Javier Rangel-Moreno[2], Joaquin Zuñiga[3], Shabaana A. Khader[1]

[1]Department of Molecular Microbiology, Washington University School of Medicine

[2] Division of Allergy/Immunology and Rheumatology, University of Rochester School of Medicine and Dentistry

[3]Department of Immunology, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas

Mycobacterium tuberculosis (*Mtb*), the causative agent of pulmonary tuberculosis (PTB), is estimated to infect a third of the global population. Despite use of anti-tuberculosis drugs, *Mtb* infection remains the second leading cause of death due to a single infectious agent, highlighting the need for a better understanding of host responses to clinically-relevant *Mtb* strains. W-Beijing strains were identified in 50% of East Asia TB clinical cases and represent 13% of global *Mtb* isolates. Human genome-wide association studies implicate a correlation between the cytokine Interleukin (IL)-17A expression and incidence of PTB. Here we explore the role of IL-17A in the *Mtb*-susceptible mouse strain C3HeB/FeJ, which form necrotic cavitary granulomas, thus modeling human PTB. Our study reveals that IL-17A is required for protection in response to infection with the hypervirulent W-Beijing strain HN878, but not the lab-adapted strain H37Rv. IL-17A inhibition increased lung bacterial burden and led to the development of non-protective, necrotic granulomas. The effects of IL-17A is independent of direct effects on macrophage killing, however, and blocking studies show an increase in myeloid-derived suppressor cells and a decrease in lymphocyte recruitment. These findings implicate a regulatory role for IL-17A in promoting effective immune responses to HN878 in naturally susceptible mice.

Abstract B11

Telomerase Function and Regulation in Idiopathic Pulmonary Fibrosis

Anna Ballard and Luis Batista
Department of Internal Medicine, Division of Hematology

Tissue homeostasis is closely tied to the health of adult stem cells. Recent evidence indicates a decisive role of telomerase in the long-term maintenance of these stem cell populations. As the telomeres that cap chromosome ends shorten with successive cellular divisions, the telomerase ribonucleoprotein synthesizes and adds telomeric repeats to chromosomes, thus preventing the activation of DNA damage responses that occur when telomeres become critically short. In humans, telomerase activity is limited to stem and progenitor cell populations, thereby contributing to the unlimited self-renewal of these cell types. Mutations in telomerase have been associated with systematic tissue defects and linked to a myriad of clinical conditions that manifest primarily in tissues holding high turnover rates (ex. hematopoietic system). Current models of such syndromes suggest that continued division of stem cells lacking functional telomerase leads to exacerbated erosion of telomeres that subsequently activates DNA damage responses, thereby exhausting stem cell populations and extinguishing their ability to regenerate and respond to injury over time. Relatively quiescent tissues, such as the lung however, depend less on stem cell function for maintaining homeostasis. This suggests that additional factors may be involved in the progression of pathologic conditions of the lung in a telomerase-mutant background. Mutations in telomerase have been found in up to 15% of patients living with idiopathic pulmonary fibrosis (IPF), a disease involving fibrotic lesions in the airspace that severely compromise respiratory function. We hypothesize that such defects in telomerase decisively contribute to the onset and progression of IPF through disrupting the function of alveolar epithelial type II (ATII) cells. To test this, we are engineering isogenic human stem cells harboring IPF-linked mutations in telomerase, surfactant protein C, and mucin 5B promoter, and aim to use these cells to generate mature ATII cells suitable for functional characterization *in vitro*. We have successfully generated endoderm in culture from wild type embryonic stem cells, and are able to analyze telomerase expression and activity at this developmental stage. From here, we are comparing the efficiency of endoderm generation between isogenic cell lines and will further differentiate these tissues into alveolar epithelium to assess the consequences of chosen mutations on the function of ATII cells by assaying for surfactant protein release and wound healing capabilities. We anticipate functional differences between ATII cells from mutated lines will shed insight on the impact of telomere dysfunction on lung tissue remodeling and open new avenues of research into IPF.

Abstract B12

Fibroblast-specific FGF signaling in bleomycin-induced pulmonary fibrosis

Robert D. Guzy[1], David M. Ornitz[2]
Department of Internal Medicine, Division of Pulmonary and Critical Care[1] and Department of Developmental Biology[2], Washington University School of Medicine, St. Louis, MO, USA

Rationale: Idiopathic Pulmonary Fibrosis (IPF) is characterized by progressive pulmonary scarring, decline in lung function, and often results in death within three to five years after diagnosis. Recently approved treatments for IPF target Fibroblast Growth Factor (FGF) signaling, however the mechanism through which FGFs contribute to pulmonary fibrosis remains unclear. *In vitro*, FGF signaling has been shown to be required for TGF-b induced fibroblast proliferation and collagen production, but this has not been shown *in vivo*. We therefore hypothesized that FGF signaling in lung fibroblasts is required for the generation of pulmonary fibrosis in mice treated with bleomycin.
Methods: Mice with tamoxifen-inducible Cre recombinase driven by the promoter for procollagen Ia2 (*Col1a2-CreER*) were crossed with the *ROSA26-mTmG* reporter. *Col1a2-CreER* mice were also crossed with floxed alleles for FGF receptors 1, 2, and 3 to generate *Fgfr1/2/3* fibroblast-specific conditional knockouts. Mice were treated with tamoxifen at p21 for two weeks followed by a 4-week washout period. At 9 weeks of age, mice were exposed to intratracheal bleomycin (1 mg/kg), and lungs were subsequently harvested, inflation-fixed in 4% PFA, and embedded in OCT for frozen sections or paraffin for H&E staining. In a parallel experiment, primary lung fibroblasts were isolated from dissociated lungs via flow cytometry.
Results: *Col1a2-CreER* targets peribronchial smooth muscle and interstitial fibroblasts under baseline conditions. Peribronchial smooth muscle cells targeted with *Col1a2-CreER* are Periosin+ and aSMA+, while interstitial fibroblasts targeted with *Col1a2-CreER* are PDGFRa+. The lineage of cells targeted by *Col1a2-CreER* expand after bleomycin treatment, and are concentrated in areas of fibrosis. Fibroblast-specific *Fgfr1/2/3* conditional knockout mice have efficient deletion of FGFRs 1-3 in response to tamoxifen. After treatment with bleomycin, $Fgfr1/2/3^{Col1a2\text{-}CreER}$ mice have improved survival, decreased pulmonary fibrosis, and decreased collagen expression.
Conclusions: *Col1a2-CreER* targets peribronchial smooth muscle and interstitial fibroblasts in the lung which give rise to fibrotic tissue in response to bleomycin, making them useful for the study of fibroblast-specific genes and pathways involved in the pathogenesis of pulmonary fibrosis. Furthermore, intact FGFR signaling in fibroblasts appears to be required for pulmonary fibrosis in response to bleomycin, implicating fibroblast-specific FGF signaling in the pathogenesis of pulmonary fibrosis.

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Abstract B13

Laser capture microdissection as a method to investigate the FGF9-Wnt induced transcriptional landscape during embryonic lung mesenchyme development

Drew Hagan[1], Yongjun Yin[1], David Ornitz[1]
[1]Department of Developmental Biology, Washington University School of Medicine, Saint Louis, MO USA

Introduction: Secreted Fibroblast Growth Factors (FGFs) are a class of signaling molecules that act directly on cells to regulate responses such as mitogenesis, differentiation, migration, and cell survival. Removal of developmental FGF9 expression (*Fgf9*$^{-/-}$) results in severe lung underdevelopment beginning at E12.5, recapitulating pulmonary hypoplasia phenotypes seen in human neonatal disorders. Beginning at embryonic day (E) 10.5, FGF9 localizes to the endodermal epithelium of the developing primary bronchi as well as the mesothelium that lines the thoracic pleura, both signaling to mesenchymal FGF receptors (FGFRs) 1 & 2. FGF9 is involved in the initiation of a complex network downstream of receptor binding, inducing Wnt/β-catenin signaling in the mesenchyme. β-catenin stabilization is further necessary to maintain the expression of FGFR1/2 and the capacity of the mesenchyme to respond to FGF9. The downstream components of the FGF9-Wnt/β-catenin signaling network in embryonic lung mesenchyme are poorly characterized. Laser capture microdissection (LCM) on frozen, unfixed samples is amenable to precise isolation of specific tissue subpopulations based on histology alone. In this study LCM was performed on *Fgf9* $^{+/-}$embryos at E12.5 to determine feasibility in preparation for performing massive parallel sequencing to define the transcriptional landscape downstream of the FGF9-Wnt/β-catenin signaling network.

Methods: E12.5 lungs were dissected in ice-cold PBS and briefly cryoprotected in 30% sucrose. Lungs were immediately embedded and frozen in OCT for sectioning (8 uM). Sections were affixed to PEN-membrane glass slides and stained with hematoxylin and eosin (H&E). Mesenchymal and epithelial tissues were distinguished based on histology under bright-field microscopy and isolated by LCM. Total RNA was extracted, purified, cDNA generated, and RT-qPCR performed on markers for lung epithelium and mesenchyme to assay sample purity.

Results: Fresh frozen lungs stained with H&E allowed for clear discrimination between epithelial and mesenchymal tissue (Fig. 1). RNA was of sufficient quantity and quality for RT-qPCR. Epithelial samples were depleted of mesenchymal markers Dermo1 (>50x), Wnt2a (>12x), and Lef1 (>5x). Mesenchymal samples were similarly depleted of epithelial markers CDH1 (>9x) and SHH (>20x).

Conclusion: H&E staining of fresh frozen embryonic lungs allows for suitable discrimination of mesenchymal and epithelial tissues. H&E staining in combination with LCM allows for RNA isolation of sufficient quality, quantity, and purity for downstream applications. Future work will be directed at using this method followed by massive-parallel sequencing to reveal the induced transcriptional landscape of the FGF9-Wnt/β-catenin signaling network through comparison between *Fgf9* $^{+/-}$ and *Fgf9*$^{-/-}$ animals.

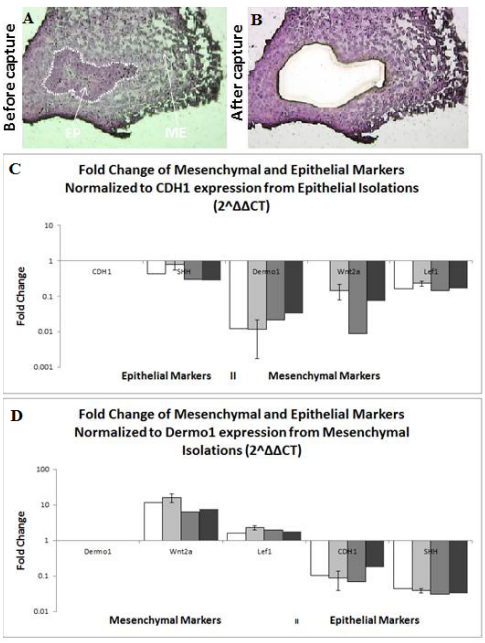


Fig. 1 LCM allows for isolation of pure epithelial and mesenchymal populations based on histology Photomicrographs of hematoxylin and eosin stained E12.5 lungs are shown before (**a**) and after (**b**) laser capture microdissection of epithelial tissue (LCM). Note the clear differentiation between the developing epithelial ducts (EP) and the mesenchyme (ME). Relative quantification of tissue specific markers from epithelial (**c**) or mesenchymal dissections (**d**). Fold change of markers normalized to pan-epithelial marker CDH1 (**c**) or pan-mesenchymal marker Dermo1 (**d**) from respective isolations. Collected RNA from all samples was of sufficient quantity and quality for robust reverse transcription and polymerase chain reaction amplification. Epithelial markers include CDH1 and SHH. Mesenchymal markers include Dermo1, Wnt2a, and Lef1. Error bars rep

Abstract B14

Important role for the transcription factor Zbtb7a in development of immune responses to lung associated self-antigens and Obliterative Airway Disease following MHC class I cross-linking by its antibodies

Deepak K Nayak[1], Monal Sharma[1], Muthukumar Gunasekaran[1], Zhongping Xu[1], Andrew Gelman[1], and T. Mohanakumar[1,2]
Departments of Surgery[1], Pathology and Immunology[2]

Introduction: Incidences of chronic rejection, bronchiolitis obliterans syndrome (BOS), remain a major challenge to long-term success of lung transplantation. *De novo* development of allo- and auto-immunity has been proposed as mechanisms that lead to development of fibroproliferative diseases including BOS. This often results in concomitant increase in the titer of donor specific antibodies to mismatched donor HLA and self-antigens (Kα1 tubulin (Kα1T) and Collagen V (ColV)) and augmentation of CD4 T cell responses.
Methods: Earlier, we identified Zinc finger and BTB domain containing 7A (Zbtb7a) as one of the early signature genes that are up-regulated following administration of antibodies to MHC class I. Zbtb7a is a master regulator of T- and B-cell neogenesis. In this study, using RNA interference (siRNA) based lentiviral gene-delivery, we selectively knocked down Zbtb7a expression in the pulmonary microenvironment and evaluated its effect on the development of antibodies and CD4 T-cell responses to Kα1T and ColV using a murine model of anti-MHC I induced obliterative airway disease (OAD). Expression profile of downstream genes following siRNA knockdown and anti-MHC I administration was examined by quantitative PCR.
Results: Expression of Zbtb7a mRNA and protein were significantly lower in mice receiving Zbtb7a targeted siRNA-lentivirus compared to that of scrambled siRNA-lentivirus. Following a 30-day intrabronchial anti-MHC class I administration, mice receiving Zbtb7a siRNA registered significant ($p<0.001$) reduction in serum anti-Kα1T and anti-ColV titers compared to mice receiving scrambled-siRNA. In addition, there were significant decreases in the Kα1T and ColV specific IL-17 and IFN-γ secreting T-cells in Zbtb7a knockdown mice. Further, Zbtb7a knockdown influenced the lung leukocyte profile where Zbtb7a-siRNA mice had significantly lower lung tropic T- and B-cells than scrambled control mice receiving anti-MHC I administration. Knockdown of Zbtb7a was found concurrent with over expression of transcription factor Runx3, a known repressor of Th17 differentiation.
Conclusion: Overall, Zbtb7a knockdown rendered protection from MHC class I antibody induced OAD. Mechanistic analysis revealed concomitant decreases in both humoral and cellular immunity to lung-associated self-antigens as a direct effect of Zbtb7a deficiency. We believe that Zbtb7a has a critical role in the pathogenesis of anti-MHC induced OAD by targeting both Th17 and B cell activation and trafficking pathways important in lower airways.

Abstract B15

BATF plays a crucial role in induction of autoimmunity and development of anti-MHC mediated Obliterative Airway Disease

Zhongping Xu[1], Muthukumar Gunasekaran[1], Nicholas Benshoff[1], Andrew Gelman[1], T. Mohanakumar[1, 2*]
Departments of Surgery[1], and Pathology and Immunology[2]

Introduction: Intrabronchial administration of antibodies (Abs) against MHC results in the induction of Th 17 mediated immune responses against lung associated self-antigens (SAgs), K-α 1 tubulin (Kα1T) and collagen V (ColV), leading to development of Obliterative Airway Disease (OAD) which has many pathological features seen in Bronchiolitis Obliterans Syndrome following human lung transplantation (LTx). Since BATF (B cell, activating transcription factor) is an upstream signaling mediator that controls development of Th17 responses and induction of autoimmunity, we proposed that BATF may play a critical role in induction of OAD.
Methods: Anti-$H2K^b$ was administered intrabronchially into Batf knockout (KO) mice of C57BL/6 background and wild type (WT) C57BL/6 mice.
Results: Histopathological analysis of the lungs harvested on days 30 and 45 following administration of Abs to Batf KO mice demonstrated significantly decreased cellular infiltration, epithelial metaplasia and fibrosis and no obstruction of small airways. In addition, these animals failed to develop Abs to SAgs, K-α1T and ColV. Further, significant reduction in the frequency of SAgs specific IL17 T cells in Batf KO animals was also seen compared to WT. There was also significantly decreased production of IL-6, IL-23, IL-17, IL-1β, FGF-6 and CXCL12 in Batf KO mice following Abs administration. Batf deficiency also resulted in decreased JAK2, STAT3, and RORγT expression following anti-MHC administration.
Conclusion: Therefore, we conclude that BATF play a critical role in induction of immune responses to SAgs and immunopathogenesis of OAD following administration of anti-MHC. Therefore, targeting BATF signaling pathways should be considered as a potential strategy for preventing the development of chronic rejection following human LTx.

Abstract B16

Post-Transcriptional Control of Immunologic Susceptibility to Lung Cancer

Saeed Arefanian, Stephanie H. Chang, Ryuji Higashikubo, Andrew E. Gelman, Daniel Kreisel, Alexander S. Krupnick
Department of Surgery, Division of Cardiothoracic Surgery, Washington University in St. Louis

Introduction: Lung cancer susceptibility or resistance can be influenced by inherited differences in function of natural killer cells (NK). Our objective is to investigate the immunologic mechanisms that affect NK function.
Methods: Genome-wide expression analysis was performed on NK cells from lung cancer resistant (B6 and C3H) and susceptible (A/J and 129) mice. Western blot and Flow-cytometric analysis were performed to study genetic data and NK cells phenotype, respectively. NK function was examined *ex vivo* by plate-bound antibody stimulation and lysis of the Lewis lung carcinoma (LLC) cell line using ^{51}Cr release. *In vivo* tumor immune response was evaluated using normal and NK-depleted mice via LLC injection into the flank with serial tumor diameter measurement.
Results: Genome-wide expression analysis demonstrated that higher levels of Sly1, an adaptor protein, correlate with lung cancer resistance. In human, NK cells with high Sly1 levels produced more TNF-α when stimulated by the lung cancer cell line A549 (correlation coefficient = 0.85, $p<0.0005$). Sly1$^-$ NK cells had lower levels of activating receptors NKG2D and Nkp46 as well as perforin, granzyme, and signaling adaptor molecules such as JAK, STAT and AKT compared to wild type NK cells (data not shown). LLC tumors grew more rapidly in Sly1$^-$ mice in an NK-dependent fashion (Figure 1A) and Sly1$^-$ NK cells demonstrated lower levels of LLC lysis in vitro (Figure 1B). NK cell activation with interleukin-2 (IL-2) abrogated all differences in surface receptor expression and tumor lytic capacity (Figure 1C). Assuming contributory role for stability or function of a regulatory or transcription factor, we investigated Sly1 co-immunoprecipitation and identified binding partners by mass spectrometry. Only ribosomal proteins co-precipitated with Sly1. Western blot analysis on wild-type NK cell lysates after sucrose density gradient fractionation confirmed that Sly1 localized solely to the ribosomal fraction.
Interestingly despite ribosomal location, gene expression analysis demonstrated lower levels of mRNA for activating receptors such as NKG2D and NK1.1, adhesion molecules (VLA4) and cytotoxic mediators (IFN-γ), suggesting that Sly1 potentially plays a role in mRNA stability or ribosomal binding, providing critical post-transcriptional control of NK cell function and lung cancer susceptibility.
Conclusion: Ribosomal disorders have been postulated to cause severe disorders such as fragile-X syndrome or Diamond-Blackfin anemia. We now demonstrate for the first time that a ribosome-associated protein plays a role in lung cancer immunosurveillance and resistance or susceptibility to it. We also demonstrate IL-2 treatment can reverse Sly1-mediated immunosuppression, opening a therapeutic approach.

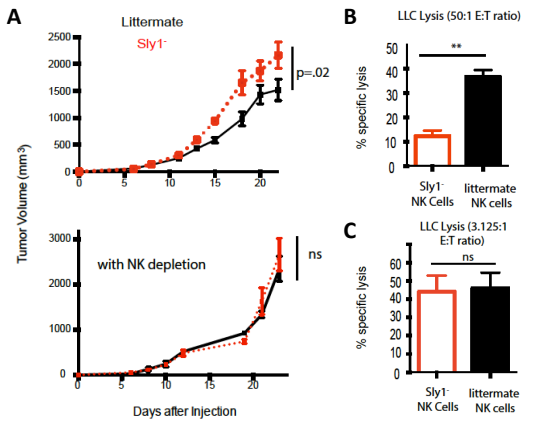


Figure 1: A) LLC tumor growth in Sly1- and wild type mice in the presence (top) or absence (bottom) of NK cells B) LLC lysis

Abstract B17

Impact of Minoxidil on the Anatomy and Physiology of the Pulmonary Vascular Circulation in a Mouse Model of Elastin Haploinsufficiency

Michael Ricafort, Russ Knutson, Li Ye, Joshua Danback, Beth Kozel
Department of Pediatrics, Washington University in St. Louis School of Medicine

Elastin is a key component to extracellular matrix, composing greater than 50% of the weight of large arteries. Elastin production and fiber assembly is complete in the early post-natal period. Williams Syndrome results from a submicroscopic deletion on chromosome 7, resulting in the loss of one copy of the elastin gene and elastin haplosinsufficiency. The result is a constellation of, among others, cardiovascular abnormalities, including pulmonary stenosis. Previous research has shown that elastin insufficient mice have significantly elevated pulmonary arterial pressures, RV hypertrophy, PA wall thickness, increased number of elastic lamellae, and reduced compliance compared to wild type mice (Eln +/+). Minoxidil is a potent, vasodilator that acts by opening ATP-dependent potassium channels. It has previously been shown to decrease PAP and PVR in humans, and increase elastin content in large arteries of rats. More recent research has shown that rats treated with minoxidil show increases in mRNA encoding components of elastin fibers, as well as increases in elastin content in large systemic arteries.

We will characterize the effects of treatment with minoxidil on pulmonary hemodynamics and structure in Eln +/- mice from weaning to three months. Groups of Eln (+/+) and Eln (+/-) mice will be randomized to treatment with minoxidil in feed water versus placebo in the following conditions: treatment from weaning to a total duration of 3 months, treatment from weaning to a total duration of 3 months, after which they will be off treatment for 1 month, and treatment from 7 days of age to a total duration of 3 months. At the end of treatment, their pulmonary arterial pressures will be measured, and they will be sacrificed. Their left pulmonary arteries will be harvested for measurement of arterial compliance. The main pulmonary artery will be collected for measurement of desmosine, an elastin specific protein. The right ventricular and total body weights will be measured to assess RV hypertrophy. In addition, vascular segments will be assessed by expression for differences in matrix gene expression.

Clinical Category

Abstract C1

Multiplexed Direct Genomic Selection to Identify Genetic Variation in *FOXF1* among Infants with Alveolar Capillary Dysplasia with Misalignment of the Pulmonary Veins (ACD/MPV)

Whitney Bour-Eldridge, M.D.[1], Daniel J. Wegner, M.S.[1], Ping Yang, B.S. [1], Hilary Heins, B.S.[1], Partha Sen, Ph.D. [2], Aaron Hamvas, M.D. [2], F.S. Cole, M.D.[1], Jennifer Wambach, M.D.[1]

[1] Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, United States 63110

[2] Department of Neonatology, Ann and Robert H. Lurie Children's Hospital of Chicago, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

Background: In infants with ACD/MPV diagnosed at autopsy, discovery of genomic disruption of forkhead box protein F1 gene (*FOXF1*) expression may be limited by degraded DNA from formalin-fixed paraffin embedded (FFPE) samples, targeting of exonic regions, and omission of regulatory, distant intragenic or intergenic copy number variants (CNVs). Using bacterial artificial chromosomes (BACs), Multiplexed Direct Genomic Selection (MDiGS) permits capture of large genomic regions for sequencing and assessment of CNVs regardless of DNA degradation.

Objective: To use MDiGS to identify exonic, intronic, and CNVs within and surrounding *FOXF1* among infants with ACD/MPV.

Methods: Using MDiGS with 4 biotinylated BACs to select for the *FOXF1* locus including 400 kb upstream and 200 kb downstream, we performed pooled next generation sequencing in two MiSeq runs on DNA from 21 infants with ACD/MPV (7=blood, 14=FFPE) after sonication and library construction with unique sequence indexes for each sample. We aligned sequencing data to the human genome (hg19) with NovoAlign, identified SNPs with SamTools, and discovered CNVs with in-house software. We used ANNOVAR to predict variant functionality and validated predicted functional variants with Sanger sequencing. We also evaluated quality (reads per targeted region), coverage, cost per kb sequenced per individual, and time to complete sequencing.

Results: We successfully sequenced 18 0f 20 high quality samples (infants and family) and 11 of 12 FFPE samples with greater than 10x coverage for over 90% of the regions for all 4 BACs. We identified a point mutation (c.T937C.p.Y313H) in FOXF1 predicted to be deleterious by ANNOVAR (4*/6 programs) and two large upstream deletions (>100 kb) in 2 infants with ACD/MPV. We detected two previously identified genomic variants and 1 previously identified deletion. Sample preparation and sequencing were completed in one month at a cost of $0.13 per kB per individual.

Conclusions: MDiGS with BAC capture is an efficient and inexpensive method to detect point mutations and CNVs in *FOXF1* for infants with ACD/MPV. MDiGS can be used to sequence distant genomic regions in fragmented DNA obtained from FFPE samples.

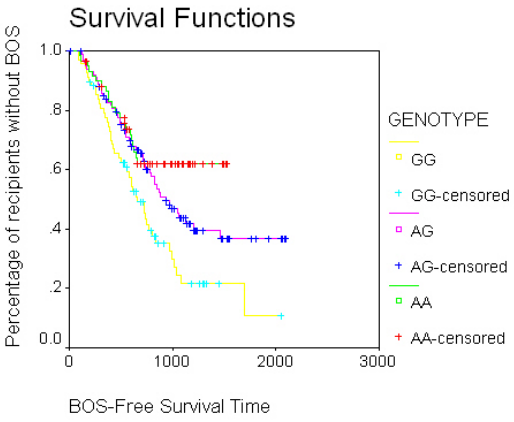
Funding Sources: National Institutes of Health (K08 HL105891 (JAW), K12 HL089968 (FSC), R01 HL065174 (FSC, AH), American Lung Association (JAW), the Saigh Foundation (FSC, AH).

Abstract C2

Lung Transplant Recipients with rs2241880 GG Genotype of Autophagy Gene ATG16L1 are at Risk of Developing Accelerated Chronic Rejection

Catherine Chen MD[1], Howard Huang MD[2], and Andrew Gelman, PhD[3]
[1]Division of Pulmonary and Critical Care, Barnes-Jewish Hospital
[2]Annette C. and Harold C. Simmons Transplant Institute, Baylor University Medical Center at Dallas
[3]Department of Pathology and Immunology, Washington University in St. Louis

Introduction: Autophagy is a catabolic process that allows cells to recycle cytoplasmic components and is implicated in regulating alloimmune responses by dendritic cells. Multiple studies have confirmed the association of the autophagy gene ATG16L1 (T300A) "risk" G allele with Crohn's disease but its role in regulating the survival of solid organ grafts remains unknown. Here we assess chronic rejection—bronchiolitis obliterans syndrome (BOS)—development risk in lung transplant recipients at rs2241880.
Methods: A retrospective chart review of 239 lung transplant recipients who underwent lung transplantation between January 1, 2009 and October 31, 2013, was performed. Demographic data of the donor and recipient were extracted, as well as first post-transplant Gram-positive (*S. aureus* or other) and Gram-negative infection (*P. aeruginosa*, other Gram-negative infection, or combined), first post-transplant Aspergillus infection (*A. fumigatus*, *A. niger*, *A. flavus*, other Aspergillus infections, or combined), first post-transplant cytomegalovirus (CMV) infection (blood, airway, or both), and first post-transplant respiratory viral infection (influenza, respiratory syncytial virus, adenovirus, other viral infections, or combined), as demonstrated by bronchial wash or bronchoalveolar lavage (BAL) sampling. Saliva samples were also collected to determine ATG16L genotype.
Results: 239 patients who underwent lung transplantation submitted saliva samples for genotyping. ATG16L genotype was able to be determined in 229 participants. 60 participants were AA, 101 AG, and 68 GG. Univariate analysis of ATG16L genotype demonstrated statistically significant difference in BOS-free survival between AA (wild type) and GG genotypes (p=0.0016). This result remained statistically significant when multivariate analysis was performed including Gram-positive bacteria, Gram-negative bacteria, CMV, Aspergillus, and CARV infections as covariates (p=0.008).
Conclusions: ATG16L GG genotype is independently associated with shorter BOS-free time in both univariate and multivariate analysis.



Abstract C3

The lung allograft virome

Brian C. Keller[1,2], Guoyan Zhao[2], Lindsay Droit[2], Scott A. Handley[2], Chandni Desai[2], Derek E. Byers[1], Herbert W. Virgin[2]
[1]Division of Pulmonary & Critical Care Medicine, Department of Medicine,
[2]Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, Missouri, USA

Introduction: Lung transplantation remains the definitive treatment for end-stage lung diseases. Relative to outcomes in other solid organ transplants, patient survival after lung transplantation is significantly lower due to the development of bronchiolitis obliterans syndrome (BOS). The exact etiology of BOS is not known, but viral infection has been linked to its development. Previous studies used _ENREF_13_ENREF_38culture-dependent and targeted PCR/microarray methods to analyze specific viruses; however these methods fail to detect the vast majority of viruses. In this study we sought to utilize high-throughput, next-generation sequencing (NGS) to characterize the virome (i.e. all viral genomes in a specific ecological niche) in the lung allograft and to determine whether components of the lung virome could predict clinical outcome, defined as time to BOS development.
Methods Bronchoalveolar lavage (BAL) samples were collected from patients during routine bronchoscopy after lung transplantation and separated into cellular and supernatant fractions by low speed centrifugation before storage at -80°C. Samples were thawed, ultra-filtered, and treated with lysozyme/DNase to minimize host genomic contamination. Total RNA or DNA was extracted from BAL supernatant and reverse-transcribed and PCR-amplified (RNA only) before library construction and NGS on the Illumina HiSeq 2500 platform. Sequence analysis was performed using a custom bioinformatics pipeline designed to focus analysis on non-host reads.
Results: Twenty-eight BAL samples, collected 6 months after bilateral lung transplantation, were processed and sequenced using NGS resulting in more than 15 million sequencing reads per sample. After removal of low quality sequences and contaminating host reads and clustering to remove PCR duplicates, 480,000 reads per sample remained. Comparison of remaining reads to a custom virus database revealed that the majority of viral reads were assigned as bacteriophage. Eukaryotic virus sequences were rare and were dominated by cytomegalovirus and anelloviruses. Read assignments were then tested for correlations with clinical metadata.
Conclusions: Compared to other body sites, the lung virome is much sparser. Bacteriophage predominate in the lung virome, which is not unexpected given previous studies characterization of the lung bacterial microbiome. Eukaryotic viruses are rare in the lung allograft and are represented primarily by members of the Herpesviridae and Anelloviridae.

Abstract C4

A Pilot Study of the IL-33 to MUC5AC Axis in Bronchoalveolar Lavage (BAL) Samples from Patients with Severe Respiratory Failure

Bradford C. Bemiss, Yael G. Alevy, Jennifer Tucker, Jennifer Alexander-Brett, Roger Yusen, Michael J. Holtzman, Derek E. Byers
Drug Discovery Program, Pulmonary and Critical Care Medicine, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA

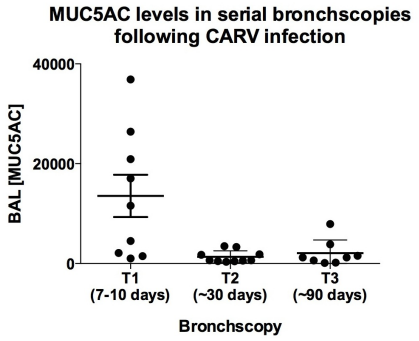
Background: Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death and a major cause of hospitalizations in the U.S each year. Respiratory viral infections contribute to at least one-half of all COPD exacerbations. In a mouse model of virus-induced lung disease, we discovered that IL-33 expression drives IL-13 production and subsequent mucous cell metaplasia characterized by upregulation of Clca and Muc5AC. In humans, we also found an increase of IL-33, IL-13, CLCA1, and MUC5AC in airway cores from patients with very severe (GOLD 4) COPD. In this pilot study, we examined the IL-33 to MUC5AC axis in BAL samples from patients intubated with severe respiratory failure with and without respiratory virus infections.
Methods: All intubated patients admitted to the MICU at Barnes-Jewish Hospital between March 2014 through November 2014 were considered for this study. Consent was obtained for bronchoscopy and collection of BAL. BAL were treated with protease inhibitors and stored at -20C. BAL levels of IL-33, CLCA, and MUC5AC were examined by ELISA.
Results: A total of 305 patients were screened and 10 were included. Reasons for exclusion were clinician deferral, malignancy, intubation >72 hours, and <40 years of age. Four patients had COPD and six did not. Of the patients with COPD, 50% (n=2) had viral cause of exacerbation. Three of the six patients without COPD had cultures positive for bacteria. No significant differences in the levels of IL-33, CLCA, or MUC5AC were found in COPD versus non-COPD patients. However, a trend toward higher levels of CLCA1 was found in the earlier stages of infection in all patients, and levels of CLCA1 and MUC5AC were increased in BAL samples from patients who smoked.
Conclusion: This pilot study lays the groundwork for future studies to examine the IL-33 to MUC5AC axis in airway samples from patients with respiratory failure due to viral infections.

Abstract C5

Levels of MUC5AC in bronchoalveolar lavage (BAL) fluid as a biomarker of viral respiratory tract infections in lung transplant recipients

Bradford C. Bemiss, Yael G. Alevy, Jennifer Tucker, Cassandra Mikols, Michael J. Walter, Murali Chakinala, Chad Witt, Roger Yusen, Elbert Trulock, Ramsey Hachem, Michael J. Holtzman, and Derek E. Byers
Drug Discovery Program, Pulmonary and Critical Care Medicine, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA

Introduction: Community acquired respiratory viral (CARV) infections contribute to the development of acute and chronic allograft injury following lung transplantation. One challenge has been to discern those patients with pathologic lower respiratory tract infections (LRTIs) from those with self-limited URTIs. In this pilot study, we examined the utility of an assay of airway mucus production, specifically MUC5AC generation, to identify LRTIs in serial bronchoscopic lung samples following transplantation.
Methods: Single-center, prospective cohort pilot study of lung transplant recipients who developed a URTI in the outpatient setting during the period of April 2006 and May 2008. Nasal lavage samples were collected and examined by virus-specific PCR (EraGen® PLx virus panel). Bronchoalveolar lavage specimens were collected at 7-10, 30, and 90 days following PCR-proven URTI onset and stored at -80°C with protease inhibitors. At the time of study, BAL supernatant was thawed and examined by viral multiplex PCR (GenMark® Respiratory Virus Panel) and standardized ELISA for MUC5AC as well as CLCA1 and IL-33. Spirometry was monitored for 6 months after the URTI. All subjects consented to this study under a protocol approved by the institutional review board.
Results: Of the 28 consented recipients, 3 died prior to completion of the study. Six subjects had PCR-confirmed viral URTIs a total of 10 times and underwent bronchoscopy collections. PCR assay of BAL samples showed respiratory syncytial virus (RSV, n=5), coronavirus NL63 (CV, n=2), influenza A H3N2 virus (IAV, n=2), and human rhinovirus (RV, n=1). Of these, 4 of the corresponding day 7-10 BAL specimens showed RSV (n=3) and IAV (n=1). MUC5AC levels were significantly increased in samples that were positive for RSV, IAV and RV, but not for CV, during the first week following URTI, compared to day 30 and day 90 samples. Total cell and neutrophil levels in BAL samples were also increased at day 7-10 compared to day 30 and day 90 but were not significantly associated with the presence of virus. The BAL sample levels of CLCA1 or IL-33 were not associated with viral detection. Only 1 of the 6 subjects that had a CARV LRTI developed a significant drop in FEV1 during the following 6 months.
Conclusions: The levels of MUC5AC in BAL fluid are increased within the first week following PCR-confirmed viral URTI in lung transplant recipients. Therefore, excess production of MUC5AC may be a biomarker of more severe CARV infections that involve the lower airway.
Funded by NIH



Abstract C6

Effect of preemptive antibody directed treatment of lung transplant recipients with donor specific antibodies for the clearance of antibodies to HLA and lung associated self-antigens: Impact for the development of chronic rejection.

Fangyu Zhou[1], Nancy Steward[1], Daniel Kreisel[1], T. Mohanakumar[1,2], Ramsey R. Hachem[3]
Department of Surgery[1], Pathology & Immunology[2], and Department of Medicine[3]

Introduction: *De novo* development of antibodies against mismatched donor HLA (DSA) after lung transplantation is associated with development of chronic rejection, bronchiolitis obliterans syndrome (BOS). It has been demonstrated that pre-emptive antibody directed treatment with intravenous immunoglobulin (IVIG) and rituximab or IVIG alone following *de novo* development of DSA can result in clearance of DSA and is associated with increased freedom from BOS. However, persistence of antibodies to lung associated self-antigens, K-α1 tubulin and Collagen V, increases the risk for development of BOS. The goal of this study is to determine the effect of preemptive treatment in the clearance of antibodies to lung associated self-antigens and its impact on BOS development.

Methods: Lung transplant recipients with *de novo* developed DSA defined by Luminex single antigen assay were subjected to antibody directed treatment. Pre- and post-transplant serum samples were analyzed for antibodies to self-antigens (Collagen I, Collagen V and K-α1 tubulin) using an ELISA developed in the laboratory. Results of DSA screening and antibodies to self-antigens were documented at each follow-up time points. Clinical information was reviewed at the end of follow-up.

Results: During the study, 28 lung transplant recipients developed DSA and all received IVIG and rituximab or IVIG alone. 21 (75%) patients cleared their DSA, however 7 of which (33%) had recurrence of DSA. 25 patients developed antibodies to self-antigens, 23 (92%) got resolved after treatment and 13 of which (57%) recurred. Seventeen patients who developed antibodies to self-antigens following the development of DSA demonstrated significantly more incidence of BOS. Furthermore, those who cleared antibodies to self-antigens only transiently had higher risk for development of BOS.

Conclusion: Pre-emptive treatment of lung transplant recipients with IVIG and rituximab or IVIG alone is effective in removing not only DSA but also antibodies to lung associated self-antigens. However, recurrence of antibodies to self-antigens is more frequent and is a risk factor for the development of BOS. Development of antibodies to self-antigens following *de novo* development of DSA is also strongly associated with poor long term outcome in lung transplant recipients.

Abstract C7

THE IMPACT OF PRE-TRANSPLANT ALLOSENSITIZATION ON OUTCOMES AFTER LUNG TRANSPLANTATION

James P. Bosanquet, MD[1], Chad A. Witt, MD[1], Bradford C. Bemiss, MD[1], Derek E. Byers, MD[1],, Roger D. Yusen, MD, MPH[1], G. Alexander Patterson, MD[2], Daniel Kreisel, MD, PhD[2], Thalachallour Mohanakumar, PhD[3], Elbert P. Trulock, MD[1], Ramsey R. Hachem, MD[1]
Washington University School of Medicine, [1]Division of Pulmonary & Critical Care Medicine, [2]Division of Cardiothoracic Surgery, [3]Department of Surgery

Background: Allosensitization can be a significant barrier to transplantation for some patients. Furthermore, early studies suggested that pre-transplant allosensitization is associated with worse outcomes after lung transplantation. However, human leukocyte antigen (HLA) antibody testing has evolved significantly over the past 20 year and current assays are highly sensitive and specific.
Methods: We examined the impact of pre-transplant allosensitization on post-transplant outcomes in the era of solid-phase multiplex HLA antibody detection assays in this retrospective single-center study of 304 adults transplanted at our center between 1/1/2006 and 12/31/2012. We accepted donor organs for allosensitized patients if a virtual crossmatch was compatible with all previously identified antibodies.
Results: In univariate and multivariate Cox proportional hazards models, pre-transplant allosensitization, the calculated panel reactive antibody (CPRA), and the number of pre-transplant HLA antibodies were not associated with the development of acute cellular rejection, lymphocytic bronchiolitis, donor-specific HLA antibodies, chronic lung allograft dysfunction, or graft failure.
Conclusions: We conclude that pre-transplant allosensitization does not adversely affect outcomes after lung transplantation when the reactive HLA are avoided in a potential donor.

Abstract C8

Effect of delayed chest closure on chest wall infections following lung transplantation

Aguilar PR[1], Krupnick AS[2], Hachem R[1]
Washington University in St. Louis, School of Medicine,
Departments of Medicine[1] and Cardiothoracic Surgery[2]

Introduction: Throughout cardiothoracic surgery, delayed chest closure is an accepted mechanism for addressing early post-operative right ventricular dysfunction and perioperative bleeding. In lung transplantation, this technique has also been widely accepted as an approach aimed at preventing propagation of allograft injury. Delayed chest closure has been associated with significant decreases in the incidence of severe PGD in a recent matched cohort analysis[1]. This approach, however, has the potential to increase the exposure of an intentionally sterile environment to non-sterile conditions in patients undergoing aggressive pharmacologic suppression of immune function.

Methods: We retrospectively reviewed 291 lung transplantation procedures performed at Barnes-Jewish Hospital between January 2010 and July 31, 2014 to assess the relationship between delayed chest closure and development of chest wall infections. Chest wall infections were defined as episodes of positive wound or pleural fluid cultures up to 6 weeks after lung transplantation.

Results: Out of 291 lung transplant reipients, 83 patients (28.5%) experienced delayed chest closure. 31 total patients (10.6%) had a culture-demonstrated chest wall infection during the 6 weeks following transplantation. Among patients with delayed chest closure, the incidence of chest wall infection was 16 out of 83 (19%) compared with 15 out of 193 (7.7%) among patients whose chests were closed at the time of initial transplantation. This difference was statistically significant ($p = 0.004$) and was associated with a hazard ratio of 3 for the incidence of chest wall infection when direct chest closure was compared to initial chest closure. Using binary logistic regression, we assessed for a relationship between chest wall infection and age, gender, body mass index or underlying lung disease. None of these factors were statistically related to the incidence of chest wall infection

Conclusions: While delayed chest closure has recently been associated with improvements in graft function among recipients of lung transplantation, this is associated with the cost of an increased frequency of post-transplant chest wall infections. Further work is needed to delineate mechanisms to improve infectious outcomes of these complex patients.

Reference:

1.) Shigemura N, Orhan Y, Bhama JK, et al. Delayed chest closure after lung transplantation: techniques, outcomes and strategies. J Heart Lung Transplant 2014;33:741-748.

Abstract C9

Smoking Cessation in Patients with Established Airway Disease: Initial Characterization of Cellular and Biomarker Sputum

G. Sajol[1], M. Glass[2], C.N. Burnham[2], M. Castro[1]
[1]Washington University St Louis - St Louis, MO/US, [2]Invion Ltd - Wilmington, DE/US

Rationale: Nadolol, a beta blocker with inverse agonist and biased ligand activity, is being studied as a teatment to aid smoking cessation in patients with established airway inflammation due to its demonstrated effect on airway healing.

Methods: The study population (n =104) includes patients with established chronic bronchitis as well as patients with chronic "smoker's cough", all of whom have repeatedly failed to quit, randomized 1:1 with placebo, who in a professional cessation effort combined with standard of care are titrated to maximum tolerated dose (Placebo v 10- 100mg nadolol) prior to last cigarette. Subjects enrolled to date (n=79), present with median age 48yo.(range 20 - 70), median FEV1 83% predicted (range 26-134%), median pack years' smoking 33.3 (range 4.8 -89), and multiple previous failures to quit. Samples were prepared at sites and were read at a single laboratory (Washington U, St. Louis).

Results: Median sputum diferentials were comparable regardless of COPD status (N/Y): 0% eosinophils; 7v 11% epithelial cells; 24 v 20% neutrophils; 19 v 12% macrophages; 0% lymphocytes, uncorrected for squamous cells.

Median sputum biomarker levels at baseline were comparable regardless of COPD status (N/Y): IL-6 31.25 v 31.25pgmL; IL-8 1915 v 2386pg/mL and MUC1 740 v 495microU/mL. Biomarkers in process include IL-13, MMP9, MUC5AC, MUC5B, p38 and ERK1/2 (assay of activity of beta arrestin pathway). Newly recruited subjects will have sputum analyzed at baseline and upon reaching maxmum tolerated dose of blnded treatment: placebo v 10 to 100mg/day nadolol. All subjects will have sputum analyzed at weeks 8 and 12 of blinded treatment. Nadolol will be compared to placebo as a treatment to assist smoking cessation, and for impact on cellular and supernatant markers of airway iflammation and healing.

Conclusion: The present clinical tral has appropriately combined smokers with and without established airway obstruction to test improvement in quit rates and markers of airway inflammation and healing.

Abstract C10

Expression of T-helper 2 inflammation in the upper and lower airway of children with severe asthma

Andrea M Coverstone, MD[1]; Leonard Bacharier, MD[1]; Anne Fitzpatrick, PhD, RN, CPNP[2]; Mario Castro, MD, MPH[3]

[1]Department of Pediatrics, Division of Allergy, Immunology and Pulmonary Medicine, Washington University in St Louis. [2]Department of Pediatrics, Division of Pulmonology, Allergy/Immunology, Cystic Fibrosis and Sleep, Emory University. [3]Department of Medicine, Division of Pulmonary and Critical Care Medicine, Washington University in St Louis

Introduction: Asthma, a disease of airway inflammation and reversible airway obstruction, is a leading cause of morbidity in children. Woodruff, et al. found that the epithelial genes POSTN, CLCA1, and SerpinB2 were significantly overexpressed in endobronchial biopsies from adults with asthma compared to healthy controls, and their expression correlated with expression of T-helper 2 (Th2) cytokines IL-13 and IL-5. Few studies to date have examined Th2 inflammation in children with severe asthma.

Methods: This is a prospective cohort of children ages 6-17 years with severe and mild-to-moderate asthma enrolled in the NHLBI Severe Asthma Research Program (SARP). Subjects were characterized with demographics and lung function at baseline and following an injection with systemic corticosteroids. Biological samples including nasal epithelial brushings, peripheral blood mononuclear cells (PBMCs), and sputum were obtained at each visit. 5 subjects with severe asthma had bronchoscopy with BAL and endobronchial biopsies and brushings. RNA was isolated from samples using a column-based extraction kit. Quantitative PCR was performed to evaluate expression levels of the epithelial genes POSTN, CLCA1, and SerpinB2 and cytokines IL5, IL4, and IL13.

Results: We enrolled a total of 25 children (16 severe asthma, 9 mild-to-moderate asthma). To date, gene expression has been performed on 7 pre-steroid and 5 post-steroid sputum samples, and 23 pre-steroid and 17 post-steroid nasal brush samples. Preliminary findings reveal varying degrees of expression of the epithelial and cytokine genes. The mean expression for the 3 epithelial genes in sputum was decreased following administration of corticosteroids (n=5, dCT 9.13 vs 13.22 p=0.04). Cytokine expression was unchanged. 6 of 7 sputum samples showed CLCA1 expression prior to steroids, while 0 of 5 samples showed CLCA1 gene expression after steroids. There is a positive correlation between epithelial gene expression in sputum and in the nose ($r^2 = 0.39$). Additional sputums and 5 endobronchial brushes will be analyzed for gene expression. PBMCs are available on 15 subjects pre- and post-steroid.

Discussion: Both the upper and lower airway of patients with asthma show variable expression of genes associated with Th2-driven inflammation. The gene expression from nasal brushes and sputum may serve as non-invasive biological markers of disease in pediatric severe asthma. Systemic corticosteroids appear to decrease expression of these genes in sputum. Determining how expression of these Th2 genes relates to clinical factors such as lung function or asthma control may help to determine who might benefit from more targeted asthma therapeutics, such as anti-IL5 or anti-IL13 monoclonal antibodies.

References:

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3. Peters MC et al. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. *JACI* 2014 ;133:388-94.

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Abstract C11

Longer-term follow-up of Human Metapneumovirus bronchiolitis and the risk for future wheezing and asthma

A Coverstone, MD[1], D. Burgdorf, RN[2], K. Sumino, MD, MPH[2], L Bacharier, MD[1] and M. Castro, MD, MPH[2]
[1]Department of Pediatrics, Division of Allergy, Immunology and Pulmonary Medicine, Washington University in St Louis. [2]Department of Medicine, Division of Pulmonary/Critical Care, Washington University in St Louis

Introduction: Human metapneumovirus (hMPV) produces a clinical syndrome comparable to RSV bronchiolitis; the latter has been linked to the development of persistent wheezing and asthma later in life. We previously reported an increased risk of recurrent wheezing and asthma one to three years following hospitalization or ER visit with hMPV bronchiolitis.
Methods: We performed a prospective cohort study of children ≤5 years of age without history of prior wheeze visiting the ER or hospitalized with hMPV bronchiolitis and an age-matched healthy control group undergoing elective surgery at the same medical center. The primary outcomes of wheezing episodes and physician-diagnosed asthma were obtained via follow-up visits.
Results: We enrolled 68 children and obtained follow-up data on 30 children with hMPV bronchiolitis and 27 healthy controls. Children were followed for a median duration of 34.9 and 31.8 months, respectively (combined range 1.6-79.9; p-value=0.61). We increased our median follow-up time by 18 months and maximum duration of follow-up by 40 months since our last description of this cohort. A greater proportion of hMPV+ children experienced recurrent wheezing episodes than healthy controls (63% vs 40%, p=0.006). hMPV+ children also experienced more total wheezing episodes in follow-up than healthy controls (3.7±3.9 vs 1.3±2.4, p=0.003). hMPV bronchiolitis was associated with a significantly increased likelihood for future episodes of wheezing (HR 3.47, 95%CI 1.58-7.63, p=0.002) compared to controls. There was no significant difference in the proportion of children who developed asthma between hMPV+ children and healthy controls (26% vs 13%, p=0.10). Both groups had similar characteristics at baseline; however, hMPV+ children had lower peripheral blood eosinophil percentage (0.89±1.17 vs 2.14±1.58, p=0.003) and more frequent exposure to maternal smoking (35.1% vs 13.3%, p=0.041) compared to healthy controls. To address these possible confounders, we used a Cox proportional hazard model and found that hMPV bronchiolitis was associated with significantly greater likelihood for asthma (HR 13.10, 95%CI 11.27-134.78, p=0.03) when adjusted for %blood eosinophil at time of enrollment. There was no difference in gender, race, gestational age, %blood eosinophils, IgE, eczema, history of breastfeeding, maternal smoking, smoke exposure, daycare status, or maternal history of asthma between those who developed asthma or wheezing compared to those who did not (p>0.05).
Conclusion: Following severe hMPV bronchiolitis, affected children maintain an increased risk for future wheezing episodes and asthma. Additional studies to determine the risk factors and pathogenesis of hMPV in the development of asthma are needed.

Abstract C12

Longitudinal assessments of lung function and bronchial hyperresponsiveness following severe RSV bronchiolitis

Maleewan Kitcharoensakkul, Mario Castro, Toni Schweiger, Brad Wilson, Kenneth Schechtman, Leonard Bacharier
Division of Allergy/Immunology and Pulmonary Medicine, Department of Pediatrics, Washington University at St. Louis School of Medicine

Rationale: It is well known that severe RSV bronchiolitis in infancy significantly increases risk of recurrent wheezing and asthma in later life. However, little is known about the long-term impact of severe RSV bronchiolitis on lung functions and bronchial hyperresponsiveness (BHR) of these children.
Methods: Of 206 children prospectively enrolled into the RSV Bronchiolitis in Early Life-1 (RBEL-1) cohort, 143 (69.4%) had ≥1 standard spirometry performed at mean ages of 5.4 (N=112), 6.6 (N=128), 7.4 (N=36), 8.4 (N=18), 10.8 (N=39), 12.6 (N=39) and 14.7 years (N=13). Standard deviation scores (z-scores) of forced expiratory volume in 1 sec (FEV_1), forced vital capacity (FVC) and FEV_1/FVC % predicted were calculated using published normative data. BHR was measured by methacholine challenge test (MCT) on 127 and 79 children at mean ages of 6.6 and 11.7 years, respectively. Abnormal BHR is defined as a provocative concentration of methacholine required to decrease FEV_1 by 20% (PC20) of ≤8 mg/ml. The comparison of PC20 was analyzed by using Wilcoxon sign-rank test and a mixed model, repeated measures analysis of variance was used for longitudinal changes on z-scores of spirometry values.
Results: Of 143 children, 81 (56.6%) were male and 74 (51.7%) were Caucasian. The age at most recent spirometry was 10.0 ± 2.9 (5.0 -16.3) years. Significant longitudinal declines in z-scores of FEV_1 and FVC were observed over the follow-up period ($p<0.004$), and did not differ by race, gender, or MD-diagnosed asthma. FEV_1/FVC % predicted z-scores declined over time, but only in children with MD-diagnosed asthma, $p < 0.0001$. There was an increase in geometric mean PC20 from 0.51 ± 0.07 mg/ml at 6 years to 1.66 ± 0.36 mg/ml at 12 years, with the prevalence of abnormal BHR decreasing from 96% at 6 years to 78% at 12 years, $p=0.0003$. There is a positive, but weak correlation (0.31, $p=0.007$) between PC20 adjusted for FEV_1 at 6 and 12 years.
Conclusion: Following severe RSV bronchiolitis, there is a significant decline in pre-bronchodilator lung function among these children across ages to puberty. Although abnormal BHR was observed in most patients at age 6, there is a decrease in the degree of their BHRs at age 12 and a small proportion normalized. The findings suggest long-term, and potentially progressive, impact of severe RSV bronchiolitis on lung function in early adolescence despite lower degrees of BHR. Ongoing follow-up will help clarify the long-term pulmonary sequelae of severe RSV bronchiolitis.

Abstract C13

Biological Variability of Dendritic cells and Regulatory T cells in Peripheral Blood of Normal Adults

[1]Kitcharoensakkul M, [1]Bacharier LB, [2]Burgdorf, D, [2]Yin-Declue HU, Boomer JS, [2]Wilson B, [2]Schechtman K, [2]Castro M

1 Division of Allergy/Immunology and Pulmonary Medicine, Department of Pediatrics, Washington University at St. Louis School of Medicine, 2 Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Washington University at St. Louis School of Medicine.

Rationale: Studies evaluating circulating dendritic cells (DCs) and natural and induced regulatory T cells (nTregs, iTregs) are often cross sectional and are difficult to interpret in diseased states without understanding the biologic variability of these cell populations in humans.

Methods: We investigated the day-to-day variability of DCs, nTregs (FoxP3+CD25+CD4+) and iTregs (Granzyme B –GZB, Th1/2 cytokines following CD3/46 activation *in vitro* for 3 days) from peripheral blood mononuclear cells (PBMC) collected on 3 consecutive days in 10 healthy adults. The intraclass correlation coefficients (ICCs) were used to evaluate intraindividual variability.

Results: In these 10 adults (6 nonatopic) the %PBMC of plasmacytoid DC, myeloid (mDC1 and mDC2) were 0.27±0.12, 0.22±0.1, and 0.02±0.02, with ICCs 0.91, 0.90, and 0.17 respectively. nTregs were 3.27±1.27% of CD4+ cells, with ICC 0.86. For iTregs, following CD3/46 stimulation, the average GZB expression was 35.3±17.7% CD4+ cells with ICC 0.77. The ICCs for IL-10, TNF-α, IFN-γ, IL-4, and IL-5 production by iTregs were 0.49, 0.63, 0.68, 0.74, and 0.82 respectively. The reliability was lower for IL-4 after adjusting for atopy (0.57). For other cytokines, there were no significant changes of the ICCs (<0.1) after adjusting for age, gender and atopy. The greatest variability for iTregs was found for the control condition (PBS with IL-2) - ICC 0-0.42.

Conclusions: There is little day-to-day biologic variability in quantification of nTregs, pDC and mDC1 in PBMC by flow cytometry in normal adults. However there is substantial variability in measuring mDC2 and iTreg production of IL-10 arguing that an average of several measurements should be taken to better approximate the true values.

Abstract C14

Effects of Vitamin D3 Therapy on Subjects with Asthma Complicated by Sinonasal Disease

Junfang Jiao, Tonya S. King, Matthew McKenzie, Leonard B. Bacharier, Anne E. Dixon, Chris Codispoti, Ryan Dunn, Nicole Grossman, Nijra Lugogo, Sima Ramratnum, Russ Traister, Michael Wechsler, Mario Castro for the NHLBI AsthmaNet

Rationale: Chronic sinonasal disease and asthma frequently coexist and share many common pathophysiological characteristics. The AsthmaNet Vitamin D in Asthma (VIDA) study previously demonstrated that Vitamin D_3 supplementation in adults with persistent asthma and Vitamin D insufficiency did not improve treatment failures. However, Vitamin D_3 may have different effects in those who have chronic sinonasal disease. We sought to investigate the effects of Vitamin D_3 supplementation on sinonasal disease and asthma control in these subjects.

Methods: Adult patients with symptomatic asthma and Vitamin D insufficiency were randomized to receive placebo (n=207) or high dose of oral vitamin D_3 (4,000 IU/day, n=201) in addition to inhaled corticosteroid (ICS) for 28 weeks. After 12 weeks on study drug, participants then underwent tapering of the ICS if they met criteria for asthma control. A five-item sinonasal questionnaire (SNQ) was used to indicate the presence of sinonasal disease (SNQ score $\geq$1.0).

Results: Baseline SNQ scores were 1.08±SD and 1.18±SD in placebo and Vitamin D_3 groups, respectively (p=0.13). Baseline characteristics were similar except for more asthma symptoms, higher lung function, higher vitamin D level and less blacks in those with sinonasal disease compared to without. Overall, there was no significant difference in treatment failures, lung function or airway hyperresponsiveness in those receiving vitamin D_3 with sinonasal disease compared to placebo. However, in those with sinonasal disease there were more exacerbations in those who received vitamin D (RR 2.9, 95%CI 1.1-7.7, p=0.03) compared to those without. This effect was even greater in blacks with sinonasal disease receiving Vitamin D_3 (RR 4.7, 95% CI 1.0-20.8). Similar effects were seen with a higher cutpoint for sinonasal disease (SNQ $\geq$1.5).

Conclusions: In patients with Vitamin D insufficiency and persistent asthma complicated by sinonasal disease, Vitamin D_3 supplementation is not associated with a better clinical outcome compared with placebo. In fact, Vitamin D3 may lead to more asthma exacerbations, especially in blacks with sinonasal disease.

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Abstract C15

Electronic cigarettes: the nicotyrine hypothesis

Aaron Abramovitz, MD[1], Amy McQueen, PhD[1], Raul E. Martinez, MS[2], Brent J. Williams, PhD[2], Walton Sumner, MD[1]

[1] School of Medicine, Washington University in St. Louis, St. Louis, MO. [2] School of Engineering & Applied Science, Washington University in St. Louis, St. Louis, MO

Background: There are conflicting reports about the efficacy of electronic cigarettes (e-cigs) as nicotine delivery devices and as smoking cessation products. The differences in these observations may be explained by varying nicotyrine content in the studied electronic cigarette liquid (e-liquid). Nicotyrine blocks nicotine metabolism by the cytochrome P450 2A family of enzymes (CYP2A) in airways and liver. These enzymes convert nicotine to cotinine and cotinine to 3-hydroxycotinine. In humans, nicotine is metabolized primarily by hepatic CYP2A6.

Hypothesis: Nicotyrine forms by the gradual oxidation of nicotine in e-liquids exposed to air. E-cigs aerosolize nicotyrine along with nicotine: Users who inhale nicotine and nicotyrine together achieve measurable serum nicotine levels because nicotyrine blocks nicotine metabolism by CYP2A13 in airways. Users who consume nicotyrine by any route impair hepatic CYP2A6. With CYP2A6 impaired, nicotine clearance is delayed and nicotine withdrawal symptoms are attenuated. Small, relatively infrequent nicotine doses can then sustain satisfying nicotine levels. E-cigs filled with aged e-liquids are much more effective replacements for cigarettes than are e-cigs filled with new nicotine solutions because only aged e-liquids contain pharmacologically active amounts of nicotyrine.

Supporting observations: The e-cig market is growing rapidly. Established vapers absorb nicotine from their own e-cigs. Smokers who successfully switch to e-cigs (vapers) change patterns of nicotine use dramatically, as if they were wearing an effective nicotine patch. In spite of the compelling anecdotal evidence that e-cigs work to deliver nicotine for the growing number of experienced vapers, smokers trying e-cigs to help them quit smoking may not absorb sufficient levels of nicotine from new e-cigs. Vapers may consider recently purchased e-liquids and disposable e-cigs to be unsatisfying. Additionally, e-cigs have been no more effective than conventional nicotine replacement in some formal smoking cessation studies. Vapers report the benefits of aging their e-liquids by exposure to air over time. Mouse models suggest that CYP2A inhibition by nicotyrine or methoxsalen slows nicotine metabolism and lowers the doses of nicotine that are rewarding or aversive.

Implications: Behavioral and pharmacokinetic e-cig studies should be interpreted with attention to likely levels of nicotyrine delivery: future e-cig studies should routinely measure nicotyrine content, assess CP2A6 activity, confirm nicotine delivery, and use either very new or aged e-liquids. Appropriate doses of nicotyrine added to conventional nicotine replacement therapies could result in products that more effectively replace cigarettes and e-cigs for most nicotine users. In the meantime, e-cigs delivering variable amounts of nicotyrine are probably much safer than conventional cigarettes. Impaired CYP2A enzyme function may be an inherent, but usually small, risk of e-cig use.

Abstract C16

Fluid Balance in the ICU: Interventions to Minimize Fluids in Patients with Septic Shock

Catherine Chen MD, Marin H. Kollef MD
Division of Pulmonary and Critical Care, Barnes-Jewish Hospital

Introduction: Since Rivers demonstrated that early goal-directed therapy improved survival in septic shock in 2001 fluid resuscitation has become a mainstay of treatment of septic shock. However, there is evidence that excessively positive fluid balance is associated with worse outcomes in acute lung injury and acute renal failure. We hypothesize that a protocol of daily fluid status assessment can decrease intravenous fluids administered and cumulative fluid balance in patients with septic shock determined to be not volume responsive.
Methods: Patients with septic shock in the medical ICUs who continued to require vasopressors at least 12 hours after adequate initial fluid resuscitation were approached; pregnant patients, minors, and those with pre-existing end-stage renal disease or whose goals of care were consistent with comfort measures only were excluded. Once consented, participants were stratified based on presence or absence of acute respiratory distress syndrome (ARDS) and randomized to usual care or daily fluid assessment groups. Daily administered IV crystalloid, colloid, and blood volumes, and daily urine output and fluid removal during renal replacement therapy were recorded.
Results: At interim analysis, 82 participants had been enrolled. Participants in the usual care group received 3919 mL (2747 – 9941 mL) of study fluid by day 3 and 7653 mL (4229 – 13,033 mL) by day 5; intervention group received 4286 mL (3139 – 6129 mL) by day 3 ($p = 0.54$) and 6204 (4927 – 8455 mL) by day 5 ($p = 0.28$). Cumulative fluid balance in the control group by day 3 was +3039 mL (655 – 8990 mL) and +2851 mL (-1125 – 9148 mL) by day 5; fluid balance in the intervention group was +1809 mL (48 – 4972 mL) by day 3 ($p = 0.28$) and +1852 mL (-2236 – 4664 mL) by day 5 ($p = 0.39$).
There was no statistically significant difference between the groups in maximal norepinephrine dose during enrollment, use of vasopressin during enrollment, or mean arterial pressure. There was no statistically significant difference in rate of renal replacement therapy between groups.
Conclusions: With a protocol of daily fluid status assessment, there may be a trend toward decreased IV crystalloid fluid administration between days 3 and 5 in patients with refractory septic shock. There may also be a trend toward decreased net fluid balance at days 3 and 5 in patients who undergo daily fluid status assessment. Importantly, there does not appear to be any difference in adverse outcomes

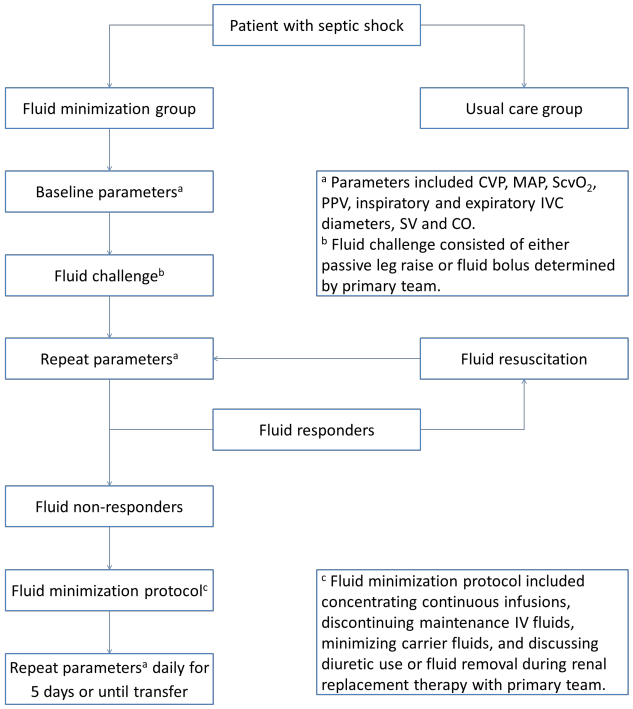

Figure 1. Research protocol.

Abstract C17

Training, Utilization and Impact of Ultrasound in the ICU

Adam Anderson, Warren Isakow
Department of Pulmonary and Critical Care Medicine, Department of Medicine, Washington University School of Medicine, St. Louis MO

The majority of admissions to a medical intensive care unit are associated with respiratory failure or shock; however, the underlying etiology is frequently difficult to define. Bedside ultrasonography has been shown to be an invaluable tool in the emergency department and critical care medicine. We hypothesize that a universal bedside ultrasound screening protocol can be implemented with accurate image capture and interpretation resulting in a change in medical management. To improve critical care ultrasound training and ensure fellows' competence, a curriculum has been created that includes readings, didactics, and an ultrasound conference. Supervision for image acquisition and interpretation at the bedside is an ongoing process. As part of each unique admission for a patient with respiratory failure or shock, the fellow is expected to perform a point of care bedside ultrasound focusing on limited echocardiography, lungs, abdomen and a vascular screen. If the leading diagnosis is hemorrhagic shock, an ultrasound is recommended, though not required. All images are saved locally for image review. A worksheet, that becomes part of the medical record, is completed at the time of the examination documenting the differential diagnosis both before and after the ultrasound, image interpretation and limitations, as well as any changes in the diagnostic or therapeutic plan. Using chart review, the ultrasound worksheet can be compared to standard of care diagnostics including radiographs, formal echocardiography and vascular duplex scans. To assess appropriate image interpretation, all examinations will be reviewed by an intensivist certified in critical care ultrasound. Examples of image acquisition, techniques and analysis are reviewed in the weekly educational ultrasound conference. Upon completion, we will be able to evaluate our ultrasound curriculum, the competency of image acquisition and appropriate application compared to formal studies and attending physician interpretation. Clinically, we will be able to assess the impact of point of care ultrasound in the diagnostic or therapeutic plan in this patient population.

Abstract C18

Risk factors for loss of sliding lung in a Medical Intensive Care Population with Acute Respiratory Failure.

Mallow, C., Isakow, W.
Department of Medical Education, Department of Pulmonary and Critical Care

Purpose: Point of Care Bedside Ultrasound is now widely utilized as a rapid technique to evaluate patients with a range of acute pulmonary emergencies, including acute pneumothorax. We attempted to identify patient related risk factors for absent lung slide in Medical ICU patients with respiratory failure who did not have pneumothoraces, to help define appropriate use of this technology in the future.
Methods: Data was collected on 158 patients admitted to the MICU with Acute Respiratory Failure who were undergoing routine admission lung ultrasound. The lung ultrasound exam consisted of 3 views of each hemithorax. A Sonosite M-Turbo Ultrasound machine was used with a p21x transducer (5.1 MHz) set in abdominal preset mode. 4 second clips at each location on the hemithorax were stored for review. Demographic and patient characteristics were recorded to identify risk factors for loss of lung slide in the absence of a pneumothorax, which was confirmed by Chest X-Ray in all patients and by CT when available for review.
Results: There were a total of 3 right sided pneumothoraces in the 158 patients,, all were detected by bedside lung ultrasound with loss of lung slide. There were 13 additional patients that demonstrated loss of lung slide on the right. Statistically significant risk factors by multivariate analysis for loss of slide in the absence of pneumothorax were, hypercarbic vs hypoxemic respiratory failure ($p<0.05$), low BMI ($p<0.001$) and history of prior chest tube insertion ($p=0.03$ lateral, $p=0.06$ apical). On evaluation of the left hemithorax, 7 patients failed to demonstrate lung slide, all at the apical evaluation site. Multivariate analysis confirmed low BMI ($p=0.001$) and male sex ($p=0.017$) as risk factors as well as a trend in patients with a history of COPD/asthma ($p=0.077$). Risk factors which were not significant bilaterally included the presence of ARDS, ILD, prior thoracic surgery and history of lung transplantation.
Conclusions: Hypercarbic respiratory failure, male sex, prior chest tube insertion and low BMI were the predominant risk factors that were clearly associated with the loss of lung slide in the absence of pneumothorax.
Clinical Implications: Our study identifies some of the potential risk factors associated with loss of lung slide in the absence of pneumothorax. Interestingly, prior thoracic surgery and a prior history of lung transplantation were not risk factors for loss of lung slide in this study. Prior chest tube insertion likely causes a pleurodesis, causing loss of lung slide by ultrasound. The higher likelihood of absent lung slide in hypercarbic patients is likely related to underlying structural lung disease, mainly advanced emphysema. Lung slide was hardest to detect in the apical position in our study and became easier to detect in the lung bases, an important finding when decisions are made to place a chest tube using only ultrasound findings.

Abstract C19

Title: Using the *Early Warning System* to Identify and Establish Goals of Care/Advanced Directives in a High Risk Patient Population

David Picker MD MBA, Maria Dans MD, Kevin Heard BS, Thomas Bailey MD, and Marin H. Kollef MD.
Division of Pulmonary and Critical Care, Washington University School of Medicine

Background: The scope and nature of caring for patients in hospitals and intensive care units has drastically changed over time. Initially a care setting to restore patients to their previous, functional state of health, inpatient care has unfortunately become a part of the death process. Nearly forty percent of our population dies in hospitals; with over twenty percent spending time in the intensive care units (1). The choice and decision regarding end of life care remains a personal one, and one in which medical providers must deliver to the best of their capabilities. If dying in an intensive care setting is congruent with the patient's goals, then we must facilitate the process in the most supportive way possible. However, data would suggest that many patients would rather prefer end of life care *outside* of hospitals (2). Identifying and breaking down barriers to the reasons this currently does not occur seems an essential component of providing autonomy to patients. The *Early Warning System* provides an excellent opportunity to provide an intervention at a critical point in the patient's hospital course. This system uses a real time algorithm to alert healthcare providers when their patients are at risk for clinical deterioration. These individuals are at 5 times higher risk for transfer to the ICU, and almost 9 times higher risk of death. Using this alert allows for a timely discussion about goals of care/advanced directives.

Methods: We will use a randomized control trial to help determine whether an intervention at time of *Early Warning System* generation will have an effect on patient's goals of care/advanced directive. Eligible patients will be medical floor patients at Barnes-Jewish Hospital in St. Louis, and be able to discuss end of life issues. The intervention group, in which a formal discussion will take place, will be compared to a population of individuals who generate an alert and receive existing standard of care.

Results: Our primary outcome we will be examining the difference in advance directives on file at time of discharge. Secondary outcomes will study rates of ICU transfer between groups. This study will hopefully enroll approximately 300 individuals per group.

Conclusions: Data collection is currently ongoing. Conclusions deferred pending evaluation of data.

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Abstract C20

Improving Acute Care Teams by Educating Residents Through Case Based Lectures and Simulation Center Training

Abhaya P. Trivedi[1], Christopher Mallow[1], Marin Kollef[1]
[1]Washington University, St. Louis, MO

RATIONALE: Rapid response teams (RRT) or acute care teams (ACT) were established in an effort to prevent cardiopulmonary arrest and improve outcomes of acutely decompensating patients. These teams are advocated by regulatory agencies and have been implemented in most hospitals. The ACT at our hospital is led by a medicine resident and is comprised of an intensive care nurse, a respiratory therapist, and other medicine house staff members. Formal training for the medicine house staff was started in 2012 through a dedicated simulation center session. However, this only occurred once during their intern or second year. Because one session was thought to be inadequate, additional sessions were added this year with the goal of showing that resident comfort level and knowledge improve with further training.
METHODS: All residents in the internal medicine residency program attended a case based lecture that was interactive in nature and discussed several ACT scenarios. A survey was distributed prior to the educational session, labeled pre-test or P0. The survey assessed comfort level and incorporated 10 knowledge based questions. After the lecture, the residents completed a survey, labeled post-test #1 (P1). The interns will participate in simulation center training throughout the year and then complete a survey, labeled post-test #2 (P2). P0 and P1 were compared to measure the benefit of the case based lecture. Once the simulation center training is completed, all three surveys will be compared to determine the benefit of additional training.
RESULTS: Residents agreed in P0 and P1that the knowledge obtained from simulation center training is more applicable than lectures. From P0 to P1 residents transitioned from feeling neutral to agreeing that case based lectures are more applicable than didactic lectures. From P0 to P1 residents' knowledge-based average score improved from 6.89 to 8.68. This improvement was statistically significant with a p value of 0.0001.
CONCLUSION: The addition of one teaching session improved resident education in management of ACT scenarios as evidenced by the increase in knowledge based scores. House staff agreed that interactive teaching through cased based lectures and simulation center training is more applicable than didactic lectures. This relatively simple educational tool of a case based lecture can be utilized not only for ACT training, but also for other aspects of resident education. Simulation center training will be completed throughout the year to determine if there is an added benefit to resident comfort level and knowledge in managing acutely decompensating patients.

FUNDING SOURCE: None

Other Submitted Abstracts

Abstract 01

A multicenter study of 2013 – 2014 Influenza-associated morbidity and mortality at large U.S. Extracorporeal Membrane Oxygenation referral centers

Raquel R. Bartz[1], John W. Hollingsworth[1,2], Patrick Aguilar[3], Katherine Klein[7], Hillary Babcock[3], Daryl J. Kor[4], John K Bohman[4], Dan Schuller[5], Britton Bloughs[5], Alicia Swink[5], Karsten Bartels[6], Lisa Foley[6], Michelle Barron[6], Ashu Babuk[6], Mani A. Daneshmand[1], Cameron Wolfe[1], and Pauline K. Park[7]. [1]Duke University School of Medicine, Durham NC; [2]Ohio State University, Columbus, OH; [3]Washington University School of Medicine, St. Louis, MO; [4]Mayo School of Medicine, Rochester, MN; [5]Baylor Health System, Dallas, TX; [6]University of Colorado School of Medicine, Denver, CO; [7]University of Michigan School of Medicine, Ann Arbor MI

Rationale: Multiple countries have reported on ICU and extracorporeal membrane (ECMO) outcomes of influenza positive patients. However, there are few reports of influenza-related hospitalization outcomes and utilization of ECMO despite the expanding use of this technology for severe hypoxemic respiratory failure from the United States.

Methods: All influenza positive patients admitted to six large ECMO referral centers were retrospectively evaluated and described. Data was reported in medians and interquartile range (IQR) 25-75% for skewed data and mean and standard deviation (SD) for normally distributed data.

Results: From 2013 – 2014 influenza season, 650 subjects were hospitalized at six large ECMO referral centers and diagnosed as being influenza positive by rapid flu test, PCR test, or culture. The predominant type of influenza was H1N1pdm2009 (N = 462, 71%) or un-subtyped A (N = 119, 18%). The mean age was 47.5 (SD 18.7) years old. Patient demographics included Caucasian (53%), African American (33%), Hispanic (6%), and Asian (3%). Mean body mass index was 29 (SD 9.5). Of patients admitted to the hospital, vaccination status was known in 545; 48% were unvaccinated, 35% were vaccinated and 16% were unknown, of patients admitted to the ICU vaccination status was known for 148; 42% unvaccinated, 28% vaccinated, and 29% unknown. Of 650 patients, insurance status was known in 498 with 87% insured. Of hospitalized patients, 209 (32%) were admitted to the intensive care unit (ICU). Mechanical ventilation was used in 16%, vasopressors in 12%, and renal replacement therapy in 4% of patients. ECMO was utilized in 34 or 5% of overall hospitalized patients. Median ICU length of stay (LOS) in days was 9 (IQR 3-20). Median overall hospital LOS was 5 (IQR 3-12), 17 (IQR 7-30) for those admitted to the ICU, and 39 (IQR 22-47) for those placed on ECMO. A total of 51 (7.5%) patients died. In hospital death occurred in 46 (22%) of those admitted to the ICU and 12 (35%) of those placed on ECMO.

Conclusions: During the 2013-2014 US influenza season, morbidity and mortality related to influenza was significant in a relatively young patient population and ECMO is increasingly utilized to treat severe hypoxemic respiratory failure in this patient population.

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Abstract 02

Cricothyroidotomy Training During Pulmonary and Critical Care Fellowship

Bradford C. Bemiss[1], Bradley D. Freeman[2], Warren Isakow[1], Brian Nussenbaum[3], Swarup S. Varaday[4]
[1]Pulmonary and Critical Care Medicine, Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA. [2]Acute and Critical Care Surgery, Department of Surgery, Washington University School of Medicine, Saint Louis, MO, USA. [3]Department of Otolaryngology, Washington University School of Medicine, Saint Louis, MO, USA. [4]Department of Anesthesiology, Washington University School of Medicine, Saint Louis, MO, USA

Background: Pulmonary/Critical Care (P/CC) fellows are frequently called upon to manage critically ill patients' airways in the intensive care units. During the course of taking care of critically ill patients, intubations are consistently more fraught with complication than in stable patients intubated in the OR. Around 10% of the intubations performed in the intensive care unit (ICU) are difficult intubations. One of the dangers of multiple attempts at tracheal intubation is a distortion of the anatomy and swelling of the larynx. Among a large cohort, the most common root cause related to difficult airways was a lack of education and training. It is unclear how many P/CC training programs routinely teach cricothyroidotomy to their trainees. Use of a percutaneous cricothyroidotomy kit (PCK) has been extensively studied as a non-surgical method for cricothyroidotomy. After a three-minute video demonstration participants were able to perform a cricothyroidotomy with a PCK in <30s. Confidence is increased after more than one attempt at the procedure on a mannequin or cadaver. At our hospital, a PCK is available in all airway boxes in the ICUs. We hypothesized that after implementing an instructional program using a model airway, P/CC fellows will have increased knowledge and confidence when caring for patient with difficult airways.
Methods: In order to assess P/CC fellows at Barnes Jewish Hospital they were asked to assess their confidence in performing cricothyroidotomy a pre- and post-training survey. In addition, all P/CC fellowship programs in the country were asked to respond to a survey to determine the prevalence and method of training in cricothyroidotomy throughout the country.
Results: Fifteen fellows responded to the pre-training survey. Fellows were reasonably experienced at endotracheal intubation, with the majority having performed at least 25 (n=12). Fellows were generally familiar with rescue therapies in difficult intubations with use of a bougie (n=14), fiber optic intubation (n=10), and video laryngoscopy (n=15). Only two fellows had seen a cricothyroidotomy, previously. Seven of fifteen fellows were uncomfortable with difficult intubations and 13 of 15 were unfamiliar with the steps of a cricothyroidotomy.
In the institutional survey, the large majority of programs offered some training in difficult airways (92%) and cricothroidotomy (77%). Hands-on training in cricothyroidotomy is still ongoing at this time.
Conclusion: While our current data set is incomplete, the training of P/CC fellows at our institution in difficult airway management and cricothryoidotomy is inadequate and should be remedied by further hands-on training.

Abstract 03

Reslizumab Treatment for Moderate to Severe Asthma With Elevated Blood Eosinophil Levels

Castro M, Zangrilli J, Wechsler ME, Bateman ED, Brusselle GG, Bardin P, Murphy K, Maspero J, O'Brien C, Korn S

Rationale: Elevated blood eosinophil levels are a risk factor for future asthma exacerbations. Reslizumab is a humanized monoclonal antibody against interleukin-5 that disrupts the maturation, growth and chemotaxis of eosinophils.

Methods: We performed two duplicate, randomized, double-blind, placebo-controlled, parallel-group trials of patients with moderate/severe asthma and elevated blood eosinophil levels despite medium to high doses of inhaled glucocorticoids, who had had one or more exacerbations in the prior year. Patients received intravenous reslizumab (3.0 mg/kg) or placebo every four weeks for one year. The primary endpoint was the frequency of clinical asthma exacerbations. Lung function, quality of life, asthma control and safety were assessed.

Results: Patients were randomized to reslizumab (n=477) or placebo (n=475). Baseline characteristics across studies were similar. In both studies, patients receiving reslizumab achieved reductions in clinical asthma exacerbations (study 1 RR 0.50[95%CI 0.37, 0.67], study 2 RR 0.41 [95%CI 0.28, 0.59], both $p<0.0001$) versus placebo. Lung function improved by the first assessment (Week 4) and was maintained to one year in both studies (change in FEV_1 0.145 L, p=0.0004 and 0.123 L, p=0.0016). Significant improvements were observed in Asthma Quality of Life Questionnaire (0.378, p=0.0001 and 0.268, p=0.0071) and Asthma Control Questionnaire scores (-0.326, p=0.0003 and -0.356, $p<0.0001$). Common adverse events on reslizumab were similar to placebo.

Conclusions: Reslizumab significantly reduced the annual rate of clinical asthma exacerbations and resulted in a sustained improvement in secondary measures of asthma control compared with placebo in patients with moderate to severe asthma with an elevated blood eosinophil count.

(Funded by Teva Pharmaceuticals: Study 3082, NCT01287039 and Study 3083, NCT01285323 in clinicaltrials.gov)

Abstract 04

Survival Following Extracorporeal Membrane Oxygenation as a Bridge to Lung Transplantation: An International Review

Catherine Chen MD, Derek E. Byers MD, PhD
Division of Pulmonary and Critical Care, Washington University of St. Louis

Introduction: Several reports have examined extracorporeal membrane oxygenation (ECMO) as a bridge to lung transplantation in patients with severe respiratory failure, but these studies are small and the overall benefits of this treatment are unclear. Here we compiled results from 10 case series to better predict the survival at one year following ECMO bridge and lung transplantation in this high-risk population.
Methods: In a comprehensive review, we identified 10 case series from single-center and multi-center studies from Europe and North America that were published between 2011 and 2013 to examine the success of lung transplantation for patients initiated on ECMO prior to transplantation and the survival at one year following lung transplantation. Data were examined using a random effect model for meta-analysis and the results were summarized as the overall survival rate with 95% confidence intervals.
Results: A total of 251 patients were included in this analysis. Of these, 199 survived to lung transplantation, and 153 survived to one-year following lung transplantation. Based on a random effects model, the rate of survival to transplantation following the initiation of ECMO was 0.77 (0.66-0.85, 95%CI). Of the patients who were transplanted, the rate of survival at one year was 0.77 (0.67-0.81, 95%CI). Overall, the survival rate for all patients who were started on ECMO with the intent to transplant was 0.63 (0.50-0.74 CI) at one year following transplantation.
Conclusion: Survival for patients with severe respiratory failure who require ECMO while awaiting lung transplantation is lower than the general rate of survival for all patients following lung transplantation. These results provide a benchmark for future studies to improve the outcomes for patients with the worst forms of respiratory failure who need lung transplantation.

Abstract 05

Height Accuracy in Critically Ill Patients

Catherine Chen MD[1], Mollie Gowan PharmD[2], Jennifer Bushwitz PharmD[2], Donna Prentice ACNS-BC, CCRN[3], Marin H. Kollef MD[4]

[1]Division of Pulmonary and Critical Care Medicine, Barnes-Jewish Hospital. [2]Department of Pharmacy, Barnes-Jewish Hospital. [3]Department of Nursing, Barnes-Jewish Hospital. [4]Division of Pulmonary and Critical Care Medicine, Washington University in St. Louis

Introduction: In the ICU, height is used to calculate ideal body weight, body surface area, and creatinine clearance, which in turn affect tidal volume and drug dosing. Currently, height recorded in the electronic medical record is patient-reported or estimated by medical staff. Therefore, it is critical to establish the accuracy of the heights recorded in the medical record and to determine if differences exist between standing and supine height. The purpose of this study was two-fold: to determine the accuracy of heights recorded in patient charts, which are frequently self-reported or staff-estimated, and to describe the difference between standing and supine heights.

Methods: To assess height accuracy, patients admitted to the medical ICU were screened to determine source of recorded height. Those with staff-estimated height were approached for consent; those with bilateral lower extremity contractures or amputations were excluded. Height was measured with patients supine on a maximally inflated mattress using straight edges placed at the crown of the head and sole of the foot with a tape measure stretched between. Recorded and measured heights, gender, age, and serum creatinine were recorded. To compare standing and supine heights, healthy volunteers were recruited. Standing and supine heights were measured by investigators using the method described above. Self-reported, standing, and supine heights were recorded.

Results: Approximately one-quarter of ICU patients had heights that were staff-estimated. In those ICU patients, measured height differed from recorded height by a median of -4.4 cm (range -17.8 to +3.8 cm), with recorded height preferentially greater than measured height ($p=0.02$). In healthy volunteers, measured standing height differed from self-reported height by a median of -2.1 cm (range -5.1 to +0.4 cm), with self-reported height preferentially greater than measured height ($p=0.48$). Significant inter-rater variability was noted while measuring supine heights, due to mattress inflation/deflation cycling.

Conclusions: Heights documented in the medical record in ICU patients is routinely over-estimated or over-stated. Measuring supine height is difficult due to mattress inflation/deflation cycling. Development of a protocol and tool to accurately measure heights of critically ill patients is necessary for appropriate drug dosing and ventilator management.

Abstract 06

Height Accuracy in Critically Ill Patients

Catherine Chen MD[1], Mollie Gowan PharmD[2], Jennifer Bushwitz PharmD[2], Donna Prentice ACNS-BC, CCRN[3], Marin H. Kollef MD[4]

[1]Division of Pulmonary and Critical Care Medicine, Barnes-Jewish Hospital. [2]Department of Pharmacy, Barnes-Jewish Hospital. [3]Department of Nursing, Barnes-Jewish Hospital. [4]Division of Pulmonary and Critical Care Medicine, Washington University in St. Louis

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Abstract 07

Presence of complement factor C3-related proteins within human lung epithelial cells

Hrishikesh S Kulkarni, MD[1], Kathy Liszewski, PhD[2], Michelle Elvington[2], PhD, John Atkinson, MD[2]
[1] Division of Pulmonary and Critical Care Medicine, Department of Medicine, Washington University of Saint Louis, St Louis, MO. [2] Division of Pulmonary and Critical Care Medicine, Department of Medicine, Washington University of Saint Louis, St Louis, MO

Introduction: The complement system forms a first line of defense against pathogens, principally via the cleavage of complement factor 3 (C3). However, dysregulated activation of the system can lead to intrinsic tissue damage in the host. C3a - an activated component of the system - is tonically generated within cell lines of lymphoid ($CD4^+$ T cells and B cells) origin and is required for CD4 T cell survival. However, its presence and role in lung epithelial cell function has not been defined.
Methods: BEAS-2B, a transformed human epithelial cell line, was used (CRL-9609; ATCC, VA). Western Blots were performed using cell lysates to detect the α-chain of C3 (rabbit-anti-human C3a antibody) and intact C3 (goat-anti-human C3 antibody). Flow cytometry was done using intact as well as fixed and permeabilized cells (F/P) to detect intact C3 both extracellularly and intracellularly in unstimulated BEAS-2B cells using FITC-goat-anti-human C3 antibody.
Results: An intracellular C3-like protein was identified in a dose-dependent manner in unstimulated BEAS-2B cells in both reduced and non-reducing conditions. Intracellular C3 was identified in F/P cells using FACS, whereas there was minimal C3 identified extracellularly in non-F/P cells.
Conclusions: These preliminary data shows the presence of intracellular C3-related proteins that are constitutively present in unstimulated human lung epithelial cell lines.
Implications: These findings form the basis of our current work on identifying its mechanism of production, intracellular location and the process by which it becomes activated, and to apply the knowledge to a disease model of obstructive airway disease.

Abstract 08

MDCT Characteristics of Pre-Specified Asthmatic Cluster Phenotypes in the Severe Asthma Research Program

A. Sheshadri[1], J. Jiao[2], L. Carlstrom[1], J.P. Sieren[3], M. Escher[3], G. Sajol[1], H. Yin-Declue[1], J. Tarsi[1], R. Schutz[1], B. Wilson[1], H. Li[4], K.B. Schechtman[1], W.C. Moore[4], E.R. Bleecker[4], E.A. Hoffman[3], M. Castro[1]
[1]Washington University at St. Louis School of Medicine - St. Louis, MO/US, [2]Washington University School of Medicine - St. Louis, MO/US, [3]University of Iowa Carver College of Medicine - Iowa City, IA/US, [4]Wake Forest University School of Medicine - Winston-Salem, NC/US
Severe Asthma Research Program

Rationale: Cluster analyses in severe asthma reveal distinct clinical phenotypes that differ in key areas such as health care utilization and medication usage. The use of multi-detector row CT (MDCT) can enrich traditional cluster analyses with geographic assessments of airway morphometry and lung densitometry. Here, we describe the MDCT characteristics of previously defined clinical clusters (Moore WC, et al., AJRCCM 2009) of subjects in the Severe Asthma Research Program (SARP).

Methods: We performed unsupervised hierarchical cluster analyses on 1264 subjects from SARP using 34 pre-defined clinical and physiologic variables. Clusters 1 and 2 had early-onset asthma with normal lung function, Cluster 3 consisted of older obese women with moderate lung function impairment, and Clusters 4 and 5 had severe airflow obstruction and significant health care utilization. Of these subjects, 216 had chest MDCT (supine, maximal bronchodilation) performed at total lung capacity (TLC) and functional residual capacity (FRC, n=173) which were available for analysis. We analyzed MDCT scans with Pulmonary Workstation 2 (VIDA Diagnostics, Iowa City) and measured the following at TLC at generations 3-6: wall area (WA), wall thickness (WT), wall area percent (WA%), wall thickness percent (WT%), lumen area (LA), eccentricity, branch angle (BA), center-line length (CLL). We measured lung density using air trapping (%AT, % lung<856 HU at FRC), and emphysema-like lung (%ELL, % lung<950 HU at TLC). Analysis of mean differences among clusters was done using either the Kruskal-Wallis test or analysis of variance. Analysis of proportions was done using the chi-square test.

Results: MDCT characteristics of the cohort (gen. 3) are summarized in **Table 1.** The following morphometric measures differed among the pre-specified clusters (ANOVA $p<0.05$): WA (gen. 3), WT (gen 4,5), WT% (gen. 3-5), WA% (gen. 3-6), eccentricity (gen. 3,5), LA (gen. 3,4), and LA/CLL (gen. 4) but not BA. With lung density, %AT differed significantly among clusters ($p<0.001$) but not %ELL ($p=0.0501$). In pre-specified analyses comparing clusters 4 and 5 with clusters 1 and 2, clusters 4 and 5 had significantly higher WT% (gen. 3-6), WA% (gen. 3-6), WA (gen 4), eccentricity (gen 3,5), %AT, %ELL, and smaller LA (gen. 3-5), and LA/CLL (gen. 4).

Conclusions: Quantitative MDCT can enrich traditional cluster analyses in identifying distinct phenotypes of patients with asthma. This additional characterization has potential implications in guiding therapeutic approaches and determining responses to specific therapies.

Table 1: MDCT Characteristics of Pre-Specified Cluster Phenotypes in the Severe Asthma Research Program

Measures	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	p-value
WT%	0.33±0.05	0.35±0.05	0.34±0.06	0.38±0.07	0.39±0.07	<0.0001
WA%	0.61±0.03	0.63±0.03	0.62±0.03	0.65±0.04	0.65±0.03	<0.0001
Eccentricity	1.32±0.12	1.35±0.10	1.35±0.09	1.35±0.09	1.41±0.13	0.02
WT (mm)	3.07±0.36	3.20±0.40	3.22±0.50	3.23±0.39	3.25±0.34	0.35
WA (mm^2)	28.5±5.0	29.1±5.4	31.8±8.3	28.1±6.0	26.8±5.4	0.04
BA (degrees)	149.8±5.1	149.4±4.3	148.4±4.9	149.3±3.8	150.3±4.7	0.65
LA (mm^2)	18.9±4.7	18.2±4.6	20.1±6.9	16.2±5.1	15.1±4.9	0.003
LA/CLL (mm)	1.95±0.82	1.98±0.59	2.20±1.06	1.87±0.86	1.73±0.72	0.16
ELL (%)	2.3±2.8	3.1±4.2	2.6±3.7	3.9±4.0	5.0±6.0	0.0501
AT (%)	7.2±13.8	8.2±8.0	7.6±6.9	19.1±18.9	21.4±16.8	<0.0001