6th Annual Research Symposium

Thursday, April 13th, 2023

Vanderbilt Student Life Center

VANDERBILT UNIVERSITY MEDICAL CENTER

Welcome to the 6th Annual VI4 Research Symposium

presented by the

Vanderbilt Institute for Infection, Immunology and Inflammation

and co-hosts:

Vanderbilt Center for Immunobiology
Division of Rheumatology
Division of Pediatric Infectious Diseases
Center for Structural Biology
Vanderbilt Microbiome Innovation Center
Division of Infectious Diseases
Vanderbilt Tuberculosis Center

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and VI4's programs and centers:

Center for Personalized Microbiology and microVU
Program in Computational Microbiology and Immunology
Program in Imaging of Infection and Immunity
Vanderbilt Microbiome Innovation Center

Agenda

8:30 - 9:00 am	Registration
9:00 - 9:05 am	Welcome : Eric Skaar, PhD, MPH
9:05 - 9:40 am	**Susan Buchanan, PhD** "Structural Insight into Outer Membrane Protein Folding in Bacteria and Mitochondria"
9:40 - 10:15 am	**Jason Weinstein, PhD** "Ode to joy: CD9 expressing T follicular helper (Tfh) cells orchestrate $CD11c^{+}Tbet^{+}$ B cells in infection and autoimmunity"
10:15 - 11:30 am	Poster Session #1
11:30 - 12:30 pm	Lunch
12:30 - 1:05 pm	**Eduardo Amaral, PhD** "BACH1 regulates host resistance in *M. tuberculosis* infection by modulating glutathione metabolism"
1:05 - 1:40 pm	**Elizabeth Johnson, PhD** "Dietary lipids as modulators of gut microbiome function"
1:40 - 2:55 pm	Poster Session #2
2:55 - 3:25 pm	Trainee Presentations
3:25 - 4:00 pm	**Audrey Odom John, MD, PhD** "Parasites, puppies, and...pus: targeted antimalarial and antibacterial prodrugs"
4:00 - 4:35 pm	**David Raulet, PhD** "Regulation and immunotherapy responses of NK cells in antitumor immune responses "
4:35 - 4:45 pm	Closing Remarks
4:45 - 6:00 pm	Reception

Guest Speakers

Eduardo Amaral, PhD

Post Doctoral Researcher

National Institute of Allergy

and Infectious Diseases

Dr. Amaral earned a bachelor's degree in biology from North Fluminense State University in Rio de Janeiro/Brazil and both Master's and Ph.D. degrees in immunology conferred by the Biomedical Science Institute at the São Paulo University in São Paulo/Brazil. His doctoral and post-doctoral training have collectively focused on using cell biological and genetic approaches to investigate the mechanism of immunopathogenesis in tuberculosis induced by hypervirulent mycobacterial strains. Specifically, during his post-doctoral in Dr. Sher's lab, he made an important contribution to the TB field describing a major role of ferroptosis in *Mycobacterium tuberculosis* (Mtb)-induced macrophage necrosis, pointing out Gpx4 as a critical enzyme to regulate this cell death process and host resistance to the pathogen. Currently, Dr. Amaral is studying the molecular mechanism by which Mtb modulates host oxidative stress responses and screening potential candidates to be used as target for host-directed therapy.

Guest Speakers

Susan Buchanan, PhD

Senior Investigator

National Institute for Diabetes &

Digestive & Kidney Diseases

Dr. Buchanan is Chief of the Section on Structural Biology of Membrane Proteins in the National Institute for Diabetes & Digestive & Kidney Diseases, at the National Institutes of Health. She received her Ph.D. from the Johann-Wolfgang-Goethe Universität in Frankfurt, Germany in 1990. She completed postdoctoral fellowships at the MRC Laboratory of Molecular Biology, Cambridge, UK, and at UT Southwestern Medical Center, Dallas, before returning to the UK to establish a research group at Birkbeck College, London in 1998. She joined the NIDDK as a tenure track investigator in 2001 and is currently a senior investigator and Chief of the Laboratory of Molecular Biology and Deputy Scientific Director, NIDDK.

Guest Speakers

Audrey Odom John, MD, PhD

Professor of Pediatrics

University of Pennsylvania

Dr. John is the Stanley Plotkin Endowed Chair and Chief of the Division of Pediatric Infectious Diseases at the Children's Hospital of Philadelphia. She is Professor of Pediatrics and of Microbiology in the Perelman School of Medicine at the University of Pennsylvania. Dr. John earned her M.D. and Ph.D. in biochemistry from Duke University School of Medicine. She completed her pediatrics residency and pediatric infectious diseases fellowship at the University of Washington through the special alternative pathway. Dr. John's research focuses on microbial metabolism, particularly in the malaria parasite *Plasmodium falciparum*. Her translational interests include development of new anti-infective agents to combat antimicrobial resistance, and development of non-invasive breath-based diagnostics for pediatric infections. Dr. John is an Investigator in the Pathogenesis of Infectious Diseases of the Burroughs Wellcome fund and has been recognized with several honors, including the Emerging Leader Award at Duke University School of Medicine, a Young Investigator Award of the American Chemical Society – Infectious Diseases, and the inaugural IDea Incubator Grand Prize winner of the Infectious Diseases Society of America. Her research has been funded by the NIH, US Department of Defense, and multiple foundations.

Guest Speakers

Elizabeth Johnson, PhD

Assistant Professor of

Molecular Nutrition

Cornell University

Dr. Johnson is an Assistant Professor of Molecular Nutrition at Cornell University. Her research program focuses on understanding how nutrients in infant diets affect gut microbiome function and the overall health of babies. She studied biology at Spelman College before receiving an NSF graduate research fellowship to pursue a PhD investigating cell cycle transcriptomics at Princeton University. Dr. Johnson went on to study lipid dependent host-microbe interactions during her postdoctoral training in the lab of Dr. Ruth Ley before joining the faculty at Cornell University in the Division of Nutritional Sciences. Dr. Johnson is a Pew Biomedical Scholar, a CIFAR Global Scholar in the Humans & the Microbiome Program and the recipient of an NIH NIGMS Early-Stage Investigator Outstanding Investigator Award.

Guest Speakers

David Raulet, PhD

Professor of Immunology
and Pathogenesis
University of California, Berkeley

Dr. Raulet is a tumor and viral immunologist who holds the Esther and Wendy Schekman Chair in Cancer Biology at UC Berkeley, and directs Berkeley's Immunotherapeutics and Vaccine Research Initiative. He made fundamental contributions to understanding the specificity and function of innate lymphocytes including natural killer (NK) cells, and of T cells. His efforts focus on mechanisms of tumor immunosurveillance and approaches for immunotherapy of cancer and mechanisms of viral recognition. His group elucidated roles for inhibitory and activating receptors on NK cells and T cells in tumor recognition, including NKG2D, its ligands and others. He discovered mechanisms that inhibit, desensitize or inactivate NK cells in the tumor environment, which represent potential targets for cancer immunotherapy. His lab probed how innate pathways such as the cGAS-STING pathway provoke antitumor immunity. He is developing protocols to mobilize NK cells for therapy of cancers that are resistant to T cell recognition. He previously made fundamental contributions to our understanding the specificity of the T cell antigen receptor, TCR genes, and T cell development in the thymus. He is a member of the National Academy of Sciences, a recipient of the William B. Coley Award for Distinguished Research in Tumor Immunology, a Fellow of the AAAS and a Distinguished Fellow of the American Association of Immunologists.

Guest Speakers

Jason Weinstein, PhD

Assistant Professor of Medicine

Rutgers University

Dr. Weinstein is an assistant professor and Chancellors' Scholar at Rutgers University. He has a long-held interest in immunology, particularly in understanding T-B collaboration in normal and autoimmune responses. His graduate work focused on understanding autoreactive B cell generation and function in ectopic lymphoid tissue found in various autoimmune diseases. Dr. Weinstein completed his postdoctoral training at Yale University, assessing the developmental requirements of T follicular helper (Tfh) cells and their regulation of germinal center B cells in both infection and autoimmunity. In addition to these cellular studies, he used genomic approaches to identify novel Tfh-cell specific enhancers from chronically inflamed tonsils. The Weinstein lab combines advanced approaches in cellular immunology and genomics to investigate the dynamic genetic regulation and function of pathogenic T and B cells from early to advanced autoimmunity in comparison to those following pathogen challenge.

Poster Abstracts

Session #1

10:15 am - 11:30 am

Click the names to read their abstract.

Taseer Ahmad
Lindsay Bass
Tomas Bermudez
Katy Bunn
Sophia Chou
Kara Eichelberger
Kyle Enriquez
Eden Faneuff
Emilie Fisher
Alejandra Flores
Brittney Gimza
Emily Green
Samantha Grimes
Clinton Holt
Matthew Jung
Dillon Kunkle
Mona Mashayekhi
Melanie McKell
Tess McNeely
Matthew Munneke
Bianca Nguyen
Jennifer Noto
Chiamaka Okoye
Hualiang Pi
Javier Ramirez-Ricardo
Seth Reasoner
Kennedy Reed
Mohammad Saleem
Kaitlyn Schaaf
Catherine Shelton
Jennifer Shuman
KayLee Steiner
Audrey Thomas
Mandy Truelock
Tegy Vadakkan
Matt Vukovich
Michelle Wiebe

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Poster Abstracts

Session #2

1:40 pm - 2:55 pm

Click the names to read their abstract.

Alexandra Abu-Shmais
Juan Barraza
Kaeli Bryant
Chelsea Campbell
Heather Caslin
Cara Charnogursky
Martin Douglass
Matthew Dungan
Sergio Escobar
Jeffrey Freiberg
Azuah Gonzalez
Erin Green
Hamilton Green
Owen Hale
Parker Jamieson
Nikhita Joshi
Jacob Kim
Shannon Kordus
Madelyn Landis
Grace Morales
Caitlin Murdoch
Kateryna Nabukhotna
Donald Okoye
Seth Reasoner
Pollyana Ribeiro Castro
Gabriel Rodriguez
Cherie Saffold
Nicolas Shealy
Clara Si
Alaina Skellett
Anna Smith
Rachael Smith
Ty Sornberger
Matt Stier
Kacie Traina
Sirena Tran
Jacqueline Van Ardenne
Paige Vega
Steven Wall

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Antioxidant Flavonoid Diosmetin is Cardio-Protective in a Rat model of Myocardial Infarction induced by Beta 1-Adrenergic Receptor Activation

Taseer Ahmad, Taous Khan, Annet Kirabo, Abdul Jabbar Shah

Myocardial infarction (MI), is a common and life-threatening manifestation of ischemic heart diseases. The most important risk factor for MI is hypertension. Flavonoids have been found to be efficacious in ischemic heart diseases by alleviating oxidative stress and beta-1 adrenergic activation but the mechanistic link is not clear. We hypothesized that antioxidant flavonoid diosmetin is cardio-protective in a rat model of MI induced by beta 1-adrenergic receptor activation. To test this hypothesis, we evaluated the cardioprotective potential of diosmetin on isoproterenol-induced myocardial infarction in rats by performing lead II electrocardiography (ECG), cardiac biomarkers including troponin I and creatinine kinase by using biolyzer 100, as well as histopathological analysis. We found that diosmetin (1 and 3 mg/kg) attenuated isoproterenol-induced elevation in T-wave and deep Q-wave on the ECG, as well as heart to body weight ratio (0.53 ± 0.01 vs. 0.34 ± 0.02, $p< 0.001$), and infarction size (55 ± 3.16 vs 13.33 ± 1.83 $p< 0.001$). In addition, pretreatment with diosmetin attenuated the isoproterenol-induced increase in serum troponin I (0.68 ± 0.012 vs 0.310 ± 0.0115, $p< 0.001$). These results demonstrate that flavonoid diosmetin may provide therapeutic benefit in myocardial infarction.

Clonally expanded B cells in anti-histidyl-tRNA synthetase syndrome patients exhibit an autoreactive-prone memory phenotype and bind Jo-1 autoantigen

Lindsay Bass, Alberto Cisneros 3rd, Dena Liu, Jennifer Young-Glazer, Leslie J. Crofford, Erin M. Wilfong, and Rachel H. Bonami

Anti-histidyl-tRNA synthetase syndrome (Jo-1 ARS) is a potentially life-threatening rheumatic disease diagnosed in part by the presence of autoantibodies against the anti-histidyl tRNA synthetase autoantigen (Jo-1). Jo-1 ARS can involve multiple tissues including muscle, lung, skin, and joints. Jo-1 binding B cells have been identified in the peripheral blood of patients with Jo-1 ARS, but the mechanisms that promote B cell recognition of Jo-1 autoantigen are unknown. Using single-cell transcriptomic, phenotypic, and immune repertoire profiling, we identified clonally expanded B cell populations unique to Jo-1 ARS patients compared to healthy controls. These clonally expanded B cells exhibited an autoreactive-prone memory (CD21lo CD27+) B cell phenotype and underwent limited class switching. We recombinantly expressed a subset of B cell receptors (BCRs) isolated from clonally expanded B cells unique to Jo-1 ARS patients and determined that 20% bound Jo-1 autoantigen. As a complementary approach, we used human hybridoma technology to capture Jo-1-binding B cells from Jo-1 ARS patients. Jo-1-binding BCRs identified through both methods ranged from germline to highly (25%) mutated, with some of these mutations being required for Jo-1 autoantigen recognition. Multiple Jo-1 binding B cells expressed the VH4-34 heavy chain gene, which has been associated with autoimmunity in several studies. These data suggest somatic hypermutation can strengthen Jo-1 binding but is not required for Jo-1 recognition in structurally varied germline BCRs. Tracking phenotypic and immune repertoire changes in Jo-1 binding B cells isolated from Jo-1 ARS patients could be used to evaluate therapeutic responses in future clinical trials.

Drug Resistance without a cost? Common and uncommon routes to fosfomycin resistance in Uropathogenic *Escherichia coli*

Tomás A. Bermudez, John R. Brannon, Neha Dudipala, Seth Reasoner, Michelle Wiebe, Mia Cecala, Omar Amir and Maria Hadjifrangiskou

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide. Uropathogenic *E. coli*, the primary cause of UTIs is becoming increasingly resistant to commonly used antibiotics, driving a relative increase in the use of the unique antibiotic fosfomycin. Fosfomycin irreversibly inhibits the bacterial enzyme MurA, halting peptidoglycan synthesis. Fosfomycin enters *E. coli* through two transporters, GlpT and UhpT, which transport glycerol-3-phosphate (G3P) and glucose-6-phosphate (G6P) respectively. Consequently, loss-of-function mutations in *uhpT*, or *glpT* genes lead to fosfomycin resistance and have been identified during in vitro clinical testing. However, given their role in importing glycolysis intermediates into the cell, mutations in the *uhpT* and *glpT* transporter genes are considered of high biological cost to bacteria. However, work from our lab and others demonstrated that glycolysis is dispensable during acute UTI and both G6P and G3P are scarce in urine. These observations prompted us to test the hypothesis that uhp mutations do not lead to a fitness cost during UTI. We report that indeed, loss of *uhp* gene function does not impair UPEC pathogenesis and report clinical isolates that lack Uhp altogether. Moreover, we identify a suite of novel genes – other than *glpT* or *uhpT*, the disruption of which leads to fosfomycin resistance.

Activated T cells secrete extracellular vesicles in the allergic airway that enhance eosinophil viability

Kaitlyn E. Bunn, Brenna G. Giese, Cherie E. Saffold, and Heather H. Pua

Extracellular vesicles (EVs) are secreted membrane particles that mediate intercellular communication by delivering cell-derived cargoes. We previously found that immune cells secrete EVs into the airways during allergic lung inflammation in mice. The goals of this study were to determine the contribution of T cells to allergic airway EVs, identify T cell EV protein cargoes, and assess the effects of T cell EVs on eosinophils, important mediators of allergic inflammation. To determine the contribution of T cells to airway EVs, allergic airway inflammation was induced in mice with T cell membrane labeling, and bronchoalveolar lavage fluid (BALF) was subjected to EV flow cytometry. We found that 2% of EVs in BALF of mice with induced allergic airway inflammation were of T cell origin. To identify T cell EV protein cargoes, EVs were purified from primary mouse T cell culture media by size exclusion chromatography (SEC), ultrafiltration, and density gradient flotation for mass spectrometry. We identified a total of 957 T cell EV proteins by mass spectrometry, including cell membrane proteins known to be involved in eosinophil survival, such as CD40L, CD47, and CD22. To assess effects of T cell EVs on eosinophils, bone marrow-derived eosinophils were treated with EVs purified from resting and activated T cell culture, and eosinophil viability was assessed. We found that EVs secreted by activated T cells, but not resting T cells, enhance eosinophil viability. This effect on viability is EV concentration-dependent and can be abrogated with surface protein shaving. These results provide evidence that T cells secrete EVs carrying protein cargoes into the allergic airway and that T cell EVs play a role in allergic airway pathology by improving eosinophil survival through a mechanism that involves EV surface protein(s). T cell EVs may be important and potentially targetable components of allergic and other immune-mediated reactions.

Single Cell Multiomic Approaches to Define the Immunopathology of Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis

Sophia Chou, Chelsea Campbell, Amy Palubinsky, Ramesh Ram, Yueran Li, Andrew Gibson, and Elizabeth Phillips

Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN) is a severe, cutaneous drug hypersensitivity reaction that results in roughly 60,000 immune-mediated emergencies annually in the United States with up to 50% mortality rate. SJS/TEN is largely mediated via HLA-class I drug-antigen presentation to CD8+ T cells resulting in clinical manifestations including development of blisters and sloughing of skin due to extensive keratinocyte death. At present, there is no established therapeutic option for the treatment of SJS/TEN. Additionally, the cellular and molecular mechanisms of SJS/TEN are not well defined due to low incidence of disease, difficulty obtaining patient samples, and a lack of animal models. As such, we have established a 10X single-cell (sc)-5'-TCR-RNA-CITE-seq platform in order to define sc-TCR, gene, and protein expression in an unbiased fashion on immune cells of interest at sites of SJS/TEN tissue damage. Herein, we highlight our single cell multiomic pipeline to investigate the immunopathogenesis of SJS/TEN at an individual patient level. Utilizing the unique pathogenesis of SJS/TEN, a single cell approach can be conducted on digested affected or sloughed skin and blister fluid, which is a natural single cell suspension. Antigen specific TCRαβ pair sequences within blister fluid can be identified via scTCR-seq, allowing for synthetic reconstruction and further testing within *in vitro* settings. Whole transcriptomic analysis including identification of cell types driving SJS/TEN pathogenesis can be identified though scRNA-seq while surface protein expression of involved cells is determined through scCITE-seq. Taken together, our multiomic pipeline fulfills a critical need in defining the cellular and molecular mechanisms of SJS/TEN and could ultimately lead to the implementation of targeted therapeutics for the treatment of SJS/TEN.

Exploring the intersection of metabolism and virulence in cross-kingdom interactions

Kara R. Eichelberger, Brian M. Peters, James E. Cassat

The bacterium *Staphylococcus aureus* and the opportunistic fungus *Candida albicans* are medically important co-infecting pathogens due to frequent co-isolation from polymicrobial infections. *C. albicans* enhances *S. aureus* virulence in part through increased activation of the *S. aureus* accessory gene regulator, or Agr, system. The overarching goal of this study is to determine mechanisms by which *C. albicans* enhances *S. aureus* Agr activation and increases virulence during co-infection. *C. albicans* grows as budding yeast or filamentous hyphae, and the ability to switch between these two morphologies is important for virulence and for interactions with *S. aureus*, which primarily associate with *C. albicans* hyphae. We hypothesized that *C. albicans* enhances *S. aureus* Agr activation via a morphogenesis-dependent mechanism. Using *C. albicans* morphogenesis mutants and an *S. aureus* Agr reporter strain, we determined that *C. albicans* hyphal growth was not required for enhanced *S. aureus* Agr activation during co-culture. However, we identified that the *C. albicans* master transcriptional regulator Efg1 was necessary for enhanced *S. aureus* Agr activation and Agr-mediated cytotoxicity following co-culture. Because Efg1 regulates morphogenesis and glycolytic gene expression in *C. albicans*, we tested the role of glucose in mediating enhanced *S. aureus* Agr activation during co-culture. We found that co-culture in media containing excess glucose enhanced Agr activation, while co-culture in media containing low glucose does not. Additionally, *C. albicans* hgt4Δ/Δ, which is defective for glucose uptake, fails to enhance *S. aureus* Agr activation. Interestingly, *C. albicans* co-culture also enhanced the cytotoxicity of an *S. aureus* Agr mutant towards human macrophages, but not murine macrophages. *C. albicans*-mediated enhancement of *S. aureus* Δagr cytotoxicity towards human macrophages also required Efg1. Collectively, these data implicate a critical role for the nutritional environment in shaping virulence outcomes of cross-kingdom interactions between *S. aureus* and *C. albicans.*

Temporal modelling of the biofilm lifecycle as a statistical and experimental framework for examining the time-dependence of biofilm community dynamics

Kyle T. Enriquez, Eric P. Skaar

Consensus models of biofilm formation show biofilm formation is a dynamic process where microbes form communities that attach, adhere, accumulate, disaggregate, and detach from surfaces and one another. Biofilms have been implicated in infection pathogenesis and associated with rising rates of antimicrobial resistance. These findings suggest biofilm and biomass-associated bacterial communities are key populations for study of infectious diseases and improving clinical outcomes. There is currently a gap in knowledge surrounding the behavior of these communities as a function of time. This lack of granularity makes the study of bacterial strategies for community development difficult to translate from *in vitro* study. To address this gap, we employ a well-characterized crystal violet biomass accrual and planktonic cell density assay to address biofilm measurement as a function of time. This assay is coupled to a statistical framework which allows quantitative comparisons of distinct biofilm communities and growth conditions. These measurements across time, species, and media conditions in a 96-well format are reactive to environmental changes and are adaptable to media fluctuations. These measurements can reliably be condensed into response features of area under the curve, linear models, and cubic spline models that describe the time-dependent behavior of adherent biomass and planktonic cell density. Using this strategy, this assay was applied to *Staphylococcus aureus* and *Pseudomonas aeruginosa* in conditions of metal starvation in both nutrient rich and poor media to demonstrate rigor and translational potential. Consistent with the literature, significant differences were seen in metal deplete conditions, including a significant increase in adhered biomass in the absence of calcium and a loss of biomass in setting of zinc depletion across assays for *S. aureus*. Taken together, these results suggest that a time-dependent analysis of biofilm formation may provide novel information regarding the impact of exogenous factors on biofilm formation *in vitro*.

Myeloid PTEN inhibits innate host defense during methicillin-resistant *Staphylococcus aureus*-induced sepsis

Eden Faneuff, Cedrick Shili, Min Joo Kim, Ana Salina, Leticia Penteado, Amondrea Blackman, and C. Henrique Serezani

Sepsis is defined as life-threatening organ dysfunction and damage caused by an aberrant host immune response to systemic infection or injury. Due to the abundant production of different classes of inflammatory mediators, investigating the role of endogenous proteins that influence multiple signaling pathways might provide important clues in understanding sepsis pathogenesis. Phosphatase and tensin homolog (PTEN) is a protein/lipid phosphatase, tumor suppressor, and a negative regulator of the PI3K/AKT/mTORC pathway. PTEN inhibits FcR-mediated phagocytosis, bacterial killing, and the expression and actions of the Toll-IL1R (TIR) adaptor MyD88. Whether PTEN influences the outcome of Methicillin-resistant *Staphylococcus aureus* (MRSA) infection remains to be determined. Given the pleiotropic effects of this phosphatase on both antimicrobial response and TLR activation, we hypothesize that PTEN is a key signaling intermediate that perturbs cellular function and increases organ damage in sepsis. Our data showed that MRSA infection increased PTEN expression in the murine kidney and PTEN deletion in myeloid cells ($PTEN^{\Delta myel}$) decreases MRSA loads and kidney abscess area compared to $PTEN^{fl/fl}$ mice. $PTEN^{\Delta myel}$ leads to increased neutrophil but not monocyte/macrophage migration to the kidney. Furthermore, infected $PTEN^{\Delta myel}$ show reduced IL-1β and TNF-α in the kidney. *In vitro* data demonstrate that $PTEN^{-/-}$ macrophages have increased phagocytosis and reactive oxygen species production. These data indicate that during MRSA-induced sepsis, PTEN expression in myeloid cells increases the inflammatory response while decreasing antimicrobial effector functions in the kidney.

Improving anti-tumor CD8+ T cell function through manipulating glutamine metabolism

Emilie Fisher, Channing Chi, Rachael Smith, Matthew Madden, Ayaka Sugiura, Jeffrey Rathmell

Immunotherapies that bolster the anti-tumor effects of cytotoxic CD8+ T lymphocytes (CTLs) have improved outcomes for many, yet most patients fail to achieve complete and durable responses. Investigations into strategies to further enhance the cancer killing capacity of these cells are therefore crucial in the effort to improve immunotherapies. One avenue for manipulation of CTLs is through alterations to cellular metabolic programs. T cells radically alter their metabolism upon activation, and prior studies have shown pan-inhibition of glutamine metabolism (glutaminolysis) using the inhibitor 6-Diazo-5-oxo-l-norleucine (DON) enhances the function of CTLs in the tumor microenvironment. However, DON's toxicity combined with this drug's lack of specificity demonstrate a need for a more directed glutaminolysis targeting approach. I utilized a targeted glutaminolysis CRISPR library to investigate the role of individual enzymes targeted by DON. To interrogate the importance of these enzymes specifically in anti-tumor CTLs, I developed a model in which CRISPR-library edited antigen-specific CTLs were adoptively transferred into tumor-bearing mice. After 7 days of *in vivo* selection, next generation sequencing was performed on isolated CTLs to evaluate the survival advantage or disadvantage of these gene knockouts. While multiple DON targets resulted in *decreased* CTL fitness, deletion of the gene encoding for glutamine synthetase (GS) – the enzyme responsible for catalyzing the conversion of glutamate to glutamine – conferred a fitness advantage to CTLs. Upon inhibition of GS, cells may increase cellular levels of glutamate, and preliminary data suggests this results in a dose-dependent decrease in glycolysis and concurrent increase in mitochondrial respiration capacity. Lastly, *in vitro* data demonstrate increased inflammatory cytokine production of CTLs upon CRISPR-mediated genetic knockout or pharmacologic inhibition of GS.

Identities of Packaged Reovirus Defective Viral Genomes Are Independent of the RNA Polymerase Source

Alejandra Flores, Julia R. Diller, Monique S. Porter, Kristen M. Ogden

During viral replication, defective viral genomes (DVGs), nonfunctioning genetic material, can be generated due to modifications such as mutations, deletions, and structural changes. Previous studies indicate that mammalian orthoreovirus (reovirus), which has a segmented double-stranded RNA genome, accumulates DVG segments featuring internal deletions during serial passage. Two strains of reovirus, T1L and T3D^{I}, exhibited distinct DVG profiles, where T1L packaged similar DVG segments in replicate lineages, while packaged T3D^{I} DVGs differed in number and size. Since sequence-directed recombination at distinct sites has been documented, we hypothesized that the distinct T1L and T3D^{I} polymerase complexes produce dissimilarity in the DVG pattern. Using reverse genetics, we exchanged the polymerase and its cofactor, which are encoded by segments L1 and M1, respectively, between T1L and T3D^{I}. T3D^{I}-T1L1M1 formed larger plaques than T3D^{I}, while T1L-T3L1M1 formed smaller plaques than T1L. Each virus had similar replication kinetics to the parent that matched its L1 and M1 segments. To compare packaged DVGs, we completed ten serial passages in triplicate lineages for T1L-T3L1M1 and T3D^{I}-T1L1M1. Viral titers, which were calculated by plaque assay, were maintained within a similar range but exhibited some variability across passages and lineages. Using RT-PCR, we detected a consistent DVG segment profile across lineages for each virus for multiple segments. These findings suggest that the polymerase and its cofactor are not likely responsible for differences in T1L and T3D^{I} DVG pattern. We sequenced DVGs from segments L1 and S4 and found that they were identical across lineages, suggesting a selective process during recombination, packaging, or another step in the replication cycle. Collectively, these findings indicate that although the polymerase and its cofactor control aspects of replication, they do not specify packaged reovirus DVG identity. Nevertheless, DVG synthesis or packaging may be an orchestrated event.

Investigating antibiotic failure in *Staphylococcus aureus* osteomyelitis

Brittney Gimza, Kara Eichelberger, Casey Butrico, Chris Good, Thomas Spoonmore, Jeffery Spraggins, Isaac Thomsen, and Jim Cassat

Osteomyelitis is a devastating bone infection most commonly caused by *Staphylococcus aureus* that remains difficult to treat even with effective antibiotics. In addition to genome-encoded antibiotic resistance, *S. aureus* has multiple mechanisms that contribute to antibiotic recalcitrance *in vivo*, including: formation of biofilms, metabolic changes that render antibiotics less effective, colonization of antibiotic-protected host niches, and growth within sequestered inflammatory tissue lesions known as abscesses. To improve the outcome of osteomyelitis treatment, a better understanding of the factors that lead to antibiotic failure is needed. One mechanism known to contribute to both biofilm and abscess formation, and may also contribute to antibiotic failure, is the interaction of *S. aureus* with the mammalian coagulation system. *S. aureus* expresses multiple virulence factors that usurp components of the clotting cascade to build a "shield" around the organism in host tissues. We hypothesize that staphylococcal virulence factors that interact with components of the vertebrate clotting cascade to promote shielding of *S. aureus* during the early stages of infection contribute to antibiotic failure during osteomyelitis. To test this, we use our laboratory's established murine osteomyelitis model that recapitulates antibiotic treatment failure in osteomyelitis. Our data reveal that vancomycin treatment is completely ineffective when delayed 24 hours post-infection, yet the infecting organisms remain susceptible to vancomycin *ex vivo*. We are leveraging this model to characterize the role of *S. aureus* coagulases and fibrin(ogen)-binding proteins in protecting bacteria from antibiotic treatment. Additional bacterial mechanisms contributing to antibiotic failure during osteomyelitis are being identified using a transposon sequencing approach. Finally, using a newly developed antibiotic-responsive fluorescent reporter we seek to visualize the interaction of antibiotics and *S. aureus in vitro* and *in vivo*. The generation of new tools to visualize and mechanistically dissect the interaction of *S. aureus* with antibiotics will offer insight into mechanisms of treatment failure.

Deleted in malignant brain tumors 1 (DMBT1) glycoprotein is lost in early colonic dysplasia

Emily H. Green, Hannah M. Lunnemann, Megan E. Rutherford, John A. Shupe, Robert J. Coffey, D. Borden Lacy, Nicholas O. Markham

Colorectal cancer (CRC) is the third most common cancer in the United States and is responsible for more than 50,000 deaths annually. Deleted in Malignant Brain Tumors 1 (DMBT1) is a secreted glycoprotein highly expressed at epithelial barrier sites in the human gastrointestinal tract. Published data show DMBT1 is upregulated in gastric metaplasia and gastric adenocarcinoma, but the expression and mechanistic role of DMBT1 in CRC precancerous lesions is unknown. Recent research suggests DMBT1 slows epithelial proliferation and may inhibit the development of CRC. Our preliminary data show the *DMBT1* gene is downregulated in distal colonic dysplasia. We hypothesize DMBT1 is a tumor suppressor specifically acting in early CRC dysplasia. Here, we use spatial transcriptomics to reveal down-regulated *DMBT1* expression in dysplastic foci compared with normal colonocytes (2.41 Log-fold change, adjusted p-value < 0.01). In mouse colon tissue, we use immunofluorescence to characterize DMBT1 protein expression. The DMBT1 staining is predominantly in mid-crypt colonocytes with cytoplasmic localization and enhanced staining near the apical border. It is not heavily expressed in $MUC2^{+}$ goblet cells. The proximal colon contains more DMBT1 staining than the distal colon, particularly in areas of epithelial injury. We further show DMBT1 expression in 3 different mouse models of CRC: azoxymethane/dextran sodium sulfate, *C. difficile*-associated tumorigenesis in Apc^{min}, and $Lrig1^{CreER/+};Apc^{fl/+}$ mice. In 100% of the dysplastic foci (n = 57 foci from 11 mice), the immunofluorescent staining of DMBT1 protein is reduced in precise correlation with up-regulated β-catenin. We are currently evaluating the expression of DMBT1 in human colitis-associated cancer samples, including tissue microarrays containing early dysplasia. Future work will focus on mechanistic experiments to determine how loss of DMBT1 might be advantageous for tumor development in human CRC. Potentially, the loss of DMBT1 staining may aid with clinically distinguishing dysplasia from reactive changes in inflammatory bowel disease.

A mutation in the coronavirus nsp13-helicase impairs enzymatic activity and confers partial remdesivir resistance

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Coronaviruses (CoVs) encode nonstructural proteins 1-16 (nsps 1-16) which form replicase complexes that mediate viral RNA synthesis. Remdesivir (RDV) is an adenosine nucleoside analog antiviral that inhibits CoV RNA synthesis. RDV resistance mutations have been reported only in the nsp12 RNA-dependent RNA polymerase (nsp12-RdRp). We here show that a substitution mutation in the nsp13-helicase (nsp13-HEL A335V) of the betacoronavirus murine hepatitis virus (MHV) confers partial RDV resistance independently, and additively when expressed with known RdRp RDV resistance mutations. The MHV A335V substitution also results in a fitness cost in competition with WT MHV and does not have cross-resistance to the cytidine nucleoside analog parent compound of molnupiravir. Biochemical analysis of the SARS-CoV-2 helicase encoding the homologous substitution (A336V) demonstrates that the mutant protein retains the ability to associate with other replication proteins but has impaired helicase unwinding and ATPase activity. Together, these data highlight a novel determinant of nsp13-HEL enzymatic activity, define a new genetic pathway for RDV resistance, and demonstrate the importance of surveillance for and testing of helicase mutations that arise in SARS-CoV-2 genomes.

Epitope-focused Influenza Vaccine Against Conserved Peptides

Clinton Holt, Nada Abbadi, Matthew Vukovich, Maria del Pilar Quintana, Jarrod Mousa, Ian Setliff, Ivelin Georgiev

Influenza is a disease that infects approximately 20 million individuals in the U.S. each year resulting in around 400,000 hospitalizations. One challenge in preventing these cases is the high genetic diversity of influenza strains such that immunizations typically do not provide high levels of protection against every circulating strain or against zoonotic strains. We address this limitation with an epitope-focusing approach toward two highly conserved peptides in influenza hemagglutinin (HA). First, peptides on HA were chosen based on sequence conservation, conformation in the native structure, and their neutralization-sensitivity. They were then conjugated to the immunogenic carrier protein Keyhole Limpet Hemocyanin and either administered by themselves to mice or as a prime followed by immunizations with HA. Vaccines were then assessed through viral challenge studies, serum binding assays, serum neutralization assays, and the characterization of monoclonal antibodies using LIBRA-seq. Immunization with peptide alone or as a prime prior to immunization with hemagglutinin resulted in protection from viral challenge. Peptide immunizations alone resulted in the elicitation of heterosubtypic neutralization. LIBRA-seq was applied to characterize the antigen-specificity of tens of thousands of mouse B cells. We present a simple strategy for developing conserved epitope-focused vaccines using a two-stage immunization approach. This strategy resulted in several metrics of protection when applied to developing an influenza vaccine. Further, we present a new technique for analyzing murine vaccine results, applying LIBRA-seq to generate antigen-specificity maps of the B cells in the spleen from mice in various immunization groups.

Apolipoprotein N-acyltransferase is essential for colonization of the stomach by *Helicobacter pylori*

Matthew S. Jung, Holly M. Scott Algood and Mark S. McClain

Bacterial lipoproteins play important roles in adhesion, invasion, colonization, host defense evasion, and antibiotic resistance. These proteins are post-translationally modified by the addition of two or three acyl chains to their amino terminal ends. The acyl chains help to anchor the proteins to the bacterial membrane. Acylation is catalyzed by three enzymes (Lgt, LspA, and Lnt). We previously showed in *Helicobacter pylori*, a Gram-negative bacterium that can lead to peptic ulcers and gastric cancer, that *lgt* and *lspA* are essential for bacterial growth whereas *lnt* was not essential. The deletion of the apolipoprotein N-acyltransferase gene (*lnt*) prevents the addition of the third fatty acid chain on the lipoprotein amino terminus, resulting in diacylated lipoproteins. The number of acyl chains on a lipoprotein is known to influence the balance between proinflammatory and immunosuppressive signaling via mammalian Tlr2 receptors. In this study, we deleted the *lnt* gene in *H. pylori* WT strain *J166* and characterized the mutant in vivo and in vitro. Both *C57BL/6* and *Tlr2-/-* mice were infected with either *H. pylori J166*, the isogenic *lnt* mutant, or a complemented mutant strain. At 2 and 4 weeks, the *lnt* mutant failed to colonize either mouse strain, while *J166* and the complemented mutant bacteria successfully colonized the mice. Murine bone marrow derived macrophages (BMDM) were stimulated with either live bacterial strains (*H. pylori J166*, the *lnt* mutant, or complemented mutant strain), or lipoprotein extracts of WT or *lnt* mutant *H. pylori* strains. Gene expression was measured through qRT-PCR and displayed an increased proinflammatory response to diacylated lipoproteins compared to triacylated lipoproteins. This work demonstrates that triacylation of lipoproteins modulates the innate immune response and is essential for the ability of *Helicobacter pylori* to colonize the stomach.

An AT3 family acyltransferase participates in *Acinetobacter baumannii* nutrient metal acquisition and virulence

Dillon E. Kunkle, Matt J. Munneke, & Eric P. Skaar

Acinetobacter baumannii is a multidrug-resistant nosocomial bacterial pathogen that causes a range of diseases including respiratory and wound infections. *A. baumannii* is the leading cause of hospital-acquired pneumonia and has been identified as a major pathogen coinfecting COVID19 patients. The WHO has categorized *A. baumannii* as the most critical bacterial pathogen for the development of new therapeutics. Nutrient transition metals are essential to all life forms, including pathogenic bacteria. Vertebrates exploit this requirement by sequestering metals from invading pathogens in a process known as nutritional immunity. We have shown that the struggle for nutrient metals at the host-pathogen interface is a critical determinate of *A. baumannii* infection outcome. However, the mechanisms that *A. baumannii* employs to respond to, and overcome, nutritional immunity remain poorly understood. We have identified a gene, *A1S_3410*, which encodes for a membrane bound AT3 family acyltransferase that is induced during nutrient metal limitation. Our findings indicate that A1S_3410 targets cytoplasmic proteins for acyl-transfer, in contrast to AT3 acetyltransferase homologues which target extracellular carbohydrates. Loss of *A1S_3410* results in a reduced capacity to survive in metal-limiting environments, diminished metal acquisition, and attenuated virulence in a mouse model of pneumonia. Collectively, these results suggest a previously unappreciated role of post-translational protein modification in the maintenance of bacterial metal homeostasis.

The Effect of Sodium-Glucose Cotransporter-2 Inhibition on Systemic and Adipose Tissue Inflammation in Individuals with Obesity and Pre-Diabetes

Mona Mashayekhi, Celestine N. Wanjalla, Eric Olson, Christian M. Warren, Heather L. Caslin, Samuel S. Bailin, Curtis L. Gabriel, Charles R. Flynn, Alyssa H. Hasty, Nancy J. Brown, J. Matthew Luther, Spyros A. Kalams, John R. Koethe

The adipose immune environment is dysregulated in obesity and contributes to systemic inflammation and cardiometabolic diseases. Sodium-glucose cotransporter-2 (SGLT2) inhibitors reduce glucose and weight, and decrease cardiovascular events through unknown mechanisms. In this pilot, we tested the hypothesis that SGLT2 inhibitors reduce adipose inflammation in humans as a mechanism for cardiovascular benefit. Six individuals with obesity and pre-diabetes were treated with the SGLT2 inhibitor empagliflozin for 12 weeks. Blood and subcutaneous adipose were collected at baseline, 2- and 12-weeks. We compared these findings to results from individuals after hypocaloric diet-induced weight loss (N=5). Within-individual change from baseline was tested using Wilcoxon signed-rank, and between-individual difference using Mann-Whitney U. Baseline characteristics for the empagliflozin-treated group were age 51.5±16.5 years, weight 107.1±21.9kg, BMI 39.6±7.8kg/m2, and fasting glucose 100.8±9.3mg/dL. Baseline characteristics for the diet-treated group were comparable. Average weight loss was -0.3±2.5kg after empagliflozin and -7.1±5.6kg after diet at 12 weeks (P=0.02). Empagliflozin increased anti-inflammatory M2-like CD206+CD163+ macrophages in adipose from baseline to 2 weeks, with a diminished effect at 12 weeks (2 weeks: +5.0±0.5%, P=0.04; 12 weeks: +1.7±3.0%, P=0.43). Empagliflozin also caused a directional, but not statistically significant, decrease in pro-inflammatory T helper (Th)-1 cells in adipose and blood from baseline to 12 weeks (adipose: -1.6±1.8%, P=0.11; blood: -2.6±3.7% of CD3+ cells, P=0.14). By comparison, despite significantly greater weight loss, hypocaloric diet did not decrease Th1 cells in adipose or blood. SGLT2 inhibitors have proven paradigm-shifting in reducing cardiovascular disease, yet we do not understand the mechanisms involved in this beneficial response. These preliminary data support the hypothesis that SGLT2 inhibition increases anti-inflammatory adipose macrophages and may reduce pro-inflammatory T cells independently of weight loss. We next plan to define the effects of SGLT2 inhibitors on adipose inflammation and assess the association with measures of cardiovascular function in a randomized trial.

Discovery of Novel Therapeutic Targets against *Acinetobacter baumannii* by Understanding Nutritional Immunity at the Macrophage-Pathogen Interface

Melanie C. McKell, Kelsey Voss, Channing Chi, Jeff Rathmell, & Eric P. Skaar

Acinetobacter baumannii is a Gram-negative respiratory pathogen with increasing antibiotic resistance. Indeed, the World Health Organization has listed *A. baumannii* as the highest priority pathogen for which new therapeutics are urgently needed. Metals are required by nearly all living organisms for almost all biological processes; because of this, both host and pathogen have developed numerous mechanisms to sequester and use metals during infection. This struggle for nutrient metals provides a unique interface for studying host-pathogen interactions. Myeloid cells, including neutrophils and macrophages, are among the first line of defense against *A. baumannii*. Despite this, there is limited understanding of how these cells handle infection with *A. baumannii*. To better understand this gap in knowledge, I have developed an approach to identify macrophage metal handling genes that are necessary for combating *A. baumannii* infections *in vivo*. This approach analyzes *in vivo* myeloid metal genes necessary for lung infection with *A. baumannii* by combining a targeted CRISPR library with a bone marrow chimera model in a Cas9 expressing mouse. Following infection, mutated myeloid cells are isolated from the lungs to analyze genes that are beneficial to macrophage survival during infection. Preliminary data from this screen shows several genes that have not previously been associated with macrophage infections, providing exciting avenues for future discovery.

Investigating *Clostridioidies difficile* genes important during zinc limitation in the presence of the intestinal microbiota

Tess P. McNeely, Matthew J. Munneke, Eric P. Skaar

Clostridioides difficile is an intestinal bacterial pathogen that causes severe diarrhea, pseudomembranous colitis, toxic megacolon, and even death. This bacterium currently places significant financial strain on the healthcare system, and the incidence of *C. difficile* infection (CDI) is on the rise. CDI occurs during a state of intestinal microbiota dysbiosis since the microbiota regulates the immune response to CDI and compete with the *C. difficile* for nutrients, but specific mechanisms of competition have not been fully defined. Zinc is a vital nutrient for *C. difficile* growth, and bioavailability of zinc is linked to disease severity. The immune system exploits the necessity for zinc by producing the metal-chelating protein calprotectin (CP). CP binds zinc from the intestinal environment to starve *C. difficile* and other bacterial pathogens. We hypothesize that representative members of the microbiota compete with *C. difficile* for zinc. Considering the host-induced zinc limitation and gut dysbiosis as a CDI determinant, we hypothesized that there are specific genes within the *C. difficile* genome that are important to maintain zinc homeostasis in the competitive host environment. To test this hypothesis, we used the next-generation sequencing technique, Transposon Sequencing (Tn-Seq) by competing a *C. difficile* transposon mutant library with the gut bacteria *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* in zinc deplete and replete conditions. We discovered that a mutation in *feoB1*, a putative ferrous iron transporter, was selected against during zinc limitation, validating our selective conditions. Our future work is focused on generating a mutant library, enabling us to have a comprehensive analysis of the genes required to overcome zinc starvation in the presence of commensals. Taken together, Tn-Seq can be used to determine genes important for colonization and infection. This improved understanding of mechanisms for colonization and infection in *C. difficile* could lead to new generation therapeutics that specifically inhibit these mechanisms.

Clostridioides difficile scavenges an unconventional nucleobase during colonization of the gut

Matthew J. Munneke, M. Wade Calcutt, Valérie de Crécy-Lagard, & Eric P. Skaar

Clostridioides difficile is the leading cause of nosocomial infectious diarrhea, and infection typically proceeds following perturbation of the gut microbiota. During infection, *C. difficile* faces competition for nutrients from both the microbiota and the host immune system. Amongst the nutrients depleted following gut perturbation are conventional nucleobases, and nucleobase metabolism is critical for pathogenesis. We hypothesize that *C. difficile* possesses unique metabolic mechanisms to salvage nucleobases during infection. We found that *C. difficile* can utilize 4-thiouracil (4-TU) as a uracil source, an unconventional nucleobase in the vertebrate gut. The metabolism of 4-TU is mediated by proteins containing domain of unknown function 523 (DUF523). *C. difficile* encodes for two DUF523 paralogs, one of which is required for growth in the presence of 4-TU and which we have named, TudS. Additionally, *Escherichia coli* lacks a DUF523 homolog and is unable to grow in the presence of 4-TU. We found that heterologous expression of *C. difficile tudS* or addition of exogenous uracil is sufficient to restore growth of *E. coli* in the presence of 4-TU. Due to the structural similarity between 4-TU and uracil, we hypothesized that 4-TU is toxic because of misincorporation into RNA. Indeed, we discovered that 4-TU is incorporated into RNA in the absence of TudS and exogenous uracil. To identify additional components involved in 4-TU metabolism, we conducted a genetic screen in the presence of 4-TU. We discovered that mutations in uracil phosphoribosyltransferase, a component of the uracil salvage pathway, are sufficient to overcome 4-TU-mediated growth inhibition and prevent 4-TU incorporation into RNA. These data suggest that 4-TU hijacks the uracil salvage pathway for incorporation into RNA, and TudS converts 4-TU to uracil in the first step of 4-TU salvage in *C. difficile*. We hypothesize that this metabolic mechanism gives *C. difficile* an advantage in the competitive gut environment.

CCR10 promotes blood pressure elevations and renal damage in a mouse model of hypertension

Bianca A. Nguyen, Ziche Chen, Daniel Fehrenbach, Xue Zhong, Na Xiong, Meena Madhur, Matthew R. Alexander

Hypertension is the leading risk factor of morbidity and mortality. Emerging evidence suggests inflammation plays a key role in hypertension and related end-organ damage. Regulatory T cells (Tregs) function primarily by limiting inflammation. However, Tregs also have tissue-specific functions and play pathogenic roles in some chronic diseases by limiting angiogenesis. As such, the role of Tregs in chronic hypertension remains unclear. We recently identified $CCR10^+$Tregs as an immune population selectively decreased in hypertensive patients. CCR10 is a chemokine receptor important for recruitment of immune cells to the skin and $CCR10^+$Tregs exhibit a gene expression profile consistent with decreased angiogenic potential. Although skin has not been well studied in hypertension, prior reports in humans demonstrate skin microvascular rarefaction as a putative mechanism by which blood pressure (BP) is increased. Thus, we hypothesized that $CCR10^+$Tregs promote hypertension through skin infiltration leading to enhanced microvascular rarefaction. Herein, we demonstrate mice with Angiotensin II(AngII)-induced hypertension have increased abundance of $CCR10^+$ Tregs in the skin with a corresponding decrease in circulation compared to normotensive controls. In addition, AngII infusion increased skin protein levels of CCL27, a ligand for CCR10. Importantly, we demonstrate that CCR10-deficient mice are more resistant to AngII-induced systolic BP elevations ($p=0.004$) and albuminuria ($p=0.02$) compared to wild type mice. CCR10-deficient mice also exhibit higher levels of skin $CD31^+$ endothelial cells, consistent with reduced cutaneous microvascular rarefaction. VEGFa levels, a key mediator of angiogenesis, are also increased in the skin of CCR10-deficient compared to control mice. Finally, using PrediXcan, we demonstrate that higher genetically predicted levels of CCR10 are associated with increased risk of hypertension in humans. Taken together, our data suggest CCR10 promotes AngII-induced BP elevations and renal damage in mice at least in part by promoting Treg recruitment to the skin leading to microvascular rarefaction.

Targeting hypoxia-inducible factor-1 alpha suppresses *Helicobacter pylori*-induced gastric injury via attenuation of both microbial virulence and proinflammatory host responses

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Helicobacter pylori-induced inflammation is the strongest known risk factor for gastric adenocarcinoma. Hypoxia-inducible factor-1 alpha (HIF-1α) is a key transcriptional regulator of immunity and carcinogenesis. To examine the role of this mediator within the context of *H. pylori*-induced injury, we first demonstrated that HIF-1α levels were significantly increased in parallel with the severity of gastric lesions in humans. In interventional studies targeting HIF-1α, *H. pylori*-infected mice were treated with or without dimethyloxalylglycine (DMOG), a prolyl hydroxylase inhibitor that stabilizes HIF-1α. *H. pylori* significantly increased proinflammatory chemokines/cytokines and inflammation in vehicle-treated mice; however, this was significantly attenuated in DMOG-treated mice. DMOG treatment also significantly decreased function of the *H. pylori* type IV secretion system (T4SS) *in vivo* and significantly reduced T4SS-mediated NF-κB activation and IL-8 induction *in vitro*. These results suggest that prolyl hydroxylase inhibition protects against *H. pylori*-mediated pathologic responses, and is mediated, in part, via attenuation of *H. pylori* virulence and suppression of host proinflammatory responses.

Protein-protein interactions required for secretion of *Helicobacter pylori* CagA, a bacterial oncoprotein

Chiamaka D. Okoye, Kaeli N. Bryant, W. Hayes McDonald, Mark S. McClain, and Timothy L. Cover

Helicobacter pylori strains that produce CagA and the Cag Type IV Secretion System (T4SS) are associated with an increased risk of stomach cancer. The Cag T4SS delivers CagA, a bacterial oncoprotein, into gastric epithelial cells. The molecular architecture of the Cag T4SS and mechanisms of CagA recruitment and secretion are not fully understood. Our previous studies show that CagF, a putative chaperone for CagA, can be used as a bait for isolating CagA and the Cag T4SS outer membrane core complex. The goals of the current study are to elucidate the protein-protein interactions required for CagA recruitment and secretion. We immunopurified epitope-tagged-CagF from *H. pylori* under multiple conditions, including pre-treatment of intact *H. pylori* with water-soluble and membrane-permeable crosslinkers, varying crosslinker concentrations and varying solubilization conditions. Samples were analyzed by SDS-PAGE, silver staining, Western blotting, and mass spectrometry. Following treatment of *H. pylori* with DSS (a membrane-permeable crosslinker), immunoblot analysis revealed immunoreactive bands with increased molecular mass, consistent with successful crosslinking. Mass spectrometry analyses of immunoprecipitated HA-CagF preparations indicated that both CagA and CagF were isolated after crosslinker treatment, but core complex proteins were no longer isolated under these conditions. We propose that multiple pools of CagA and CagF exist in *H. pylori* and that the type of crosslinker conditions influences how they interact with each other and with the Cag T4SS core complex. These results open up new directions for mapping specific sites of protein-protein interactions between CagF, CagA, and Cag T4SS core complex components.

Clostridioides difficile ferrosome organelles resist nutritional immunity

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Iron is indispensable for almost all forms of life but toxic at elevated levels. To survive within their hosts, bacterial pathogens have evolved iron uptake, storage, and detoxification strategies to maintain iron homeostasis. However, these iron homeostatic systems are largely undefined in the human pathogen *Clostridioides difficile*. *C. difficile* is a Gram-positive, spore-forming anaerobe and the leading cause of nosocomial and antibiotic-associated infections in the United States. Here, we report that *C. difficile* undergoes an intracellular iron biomineralization process and stores iron in membrane-bound ferrosome organelles containing non-crystalline iron phosphate biominerals. We found that a membrane protein (FezA) and a P_{1B6}-ATPase transporter (FezB), repressed by both iron and the ferric uptake regulator Fur, are required for ferrosome formation and play an important role in iron homeostasis during transition from iron deficiency to excess. Additionally, ferrosomes are often localized adjacent to cellular membrane invaginations as revealed through the visualization of lamellas prepared using focused ion beam scanning electron microscopy followed by cryo-electron tomography analysis. Furthermore, using two mouse models of *C. difficile* infection (CDI), we demonstrated that the ferrosome system is activated in the inflamed gut to combat calprotectin-mediated iron sequestration and is important for bacterial colonization and survival during CDI. The discovery of ferrosome nanoparticles in pathogenic bacteria has the potential to reshape our understanding of host-pathogen interactions during infection, redefine the concept of trace element storage in anaerobes, unveil important insight into how gut microbes cope with changes in elemental levels within the host, and provide a prototype for production of metal nanoparticles and drug delivery vesicles, opening countless new avenues of research.

Elucidating the role of Activated Leukocyte Cell Adhesion Molecule (ALCAM) in the biogenesis and function of tumor extracellular vesicles

Javier Ramirez-Ricardo, Ariana von Lersner, Bong Hwan Sung, Dolores Di Vizio, Andries Zijlstra Alissa M. Weaver and Heather H. Pua

Activated Leukocyte Cell Adhesion Molecule (ALCAM) is a widely expressed transmembrane adhesion protein that regulates cell motility and metastasis, especially in bladder cancer. Given the important roles of extracellular vesicles (EVs) in tumorigenesis, we asked whether ALCAM might also impact EV biogenesis or function. To study the effects of ALCAM on cancer cells, we generated ALCAM-deficient HT1080 fibrosarcoma and UMUC3 bladder tumor cell lines using CRISPR-Cas9. EVs were isolated from control and ALCAM knockout cell culture supernatants by serial ultracentrifugation to generate 10,000g and 100,000g pellets. EVs were analyzed by Nanoparticle tracking (ZetaView), western blots, and single EV flow cytometry with dimensional reduction analysis after staining with di-8-ANEPPS. Although no differences were observed in EV counts between HT1080 WT and ALCAM deficient cells, we observed an increase in EV counts by nanoparticle tracking in both the 10,000g and 100,000g pellets of UMUC3 ALCAM-deficient bladder tumor cells compared with controls. By western blot, we observed reduced amounts of CD9 and Syntenin, with relatively preserved levels of Alix and TSG101 in the lysates of 100,000g pellets of UMUC3 ALCAM-deficient cells. Flow cytometry identified differences in EV subpopulations between control and ALCAM-deficient cell lines. This work identifies changes in EV number and protein cargos in a bladder tumor cell line in the absence of ALCAM expression. This finding suggests that ALCAM may regulate EV biogenesis and/or cargo loading in cancer cells.

Defining the Infant Male Urobiome and Moving Towards Mechanisms in Urobiome Research

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It is now established that the urinary bladder harbors a community of microbes termed the urobiome, a niche which remains understudied. In this study, we present the urobiome of healthy infant males from samples collected by transurethral catheterization. Using a combination of extended culture and amplicon sequencing, we identify several common bacterial genera that can be further investigated for their effects on urinary health across the lifespan. Many genera were shared between all samples suggesting a consistent urobiome composition among this cohort. Likewise, early life exposures including mode of birth (vaginal vs. Caesarean section) and prior antibiotic exposure did not alter urobiome composition. In addition, we report the isolation of culturable bacteria from the bladders of these infant males, including *Actinotignum schaalii*, a bacterial species that has been associated with urinary tract infection in older male adults. Herein, we isolate and sequence 9 distinct strains of *A. schaalii* enhancing the genomic knowledge surrounding this species and opening avenues for delineating the microbiology of this urobiome constituent. Furthermore, we present a framework for using the combination of culture-dependent and sequencing methodologies for uncovering mechanisms in the urobiome.

High Fat Diet-Induced Colonocyte Mitochondrial Dysfunction Links Inflammatory Monocyte Influx and Colonic Crypt Hyperplasia

Kennedy K. Reed, Mariana X. Byndloss

A Western-style, high-fat (HF) diet rich in saturated fat causes dysbiosis in the intestinal microbiota and low-grade inflammation in the intestines. Dysbiosis, an imbalance of healthy and unhealthy microbes within the microbial community, is known to cause non-communicable diseases such as obesity and type 2 diabetes, mainly in individuals exposed to a HF diet. However, the mechanisms linking a HF diet, intestinal inflammation, and gut dysbiosis are largely unknown. Our preliminary data from in vitro research and mouse models of diet-induced obesity demonstrated that high saturated fat content in the HF diet induces mitochondrial dysfunction in colonocytes. Thus, we hypothesize that colonocyte mitochondrial dysfunction due to saturated fatty acids trigger the CCR2-dependent recruitment of inflammatory monocytes into the colonic mucosa, promoting colonic crypt hyperplasia and intestinal dysbiosis. Interestingly, human colonic epithelial cells (Caco-2 cells) exposed to increasing concentrations of the saturated fatty acid palmitate showed increased production of the monocyte chemoattractant CCL2. For our in vivo studies, we exposed male mice to a HF (60% fat) or control low-fat (LF, 10% fat) diet for 11 weeks. In mice, we observed a HF diet-induced CCL-2 expression in colonocytes, which was accompanied by an increased influx of inflammatory monocytes to the intestinal mucosa, and colonic crypt hyperplasia. Additionally, consumption of a HF diet caused changes in gut microbiota composition, including intestinal expansion of Enterobacteriaceae, a marker of intestinal dysbiosis. The HF diet-induced colonic crypt hyperplasia and intestinal dysbiosis was abrogated in CCR2-/- mice, demonstrating the contribution of CCL-2-dependent recruitment of inflammatory monocytes to intestinal disease associated with diet-induced obesity. We are currently performing in vitro and in vivo studies using the antioxidants MitoQ and NAC to determine the contribution of HF diet-induced mitochondrial dysfunction to inflammatory monocyte recruitment and microbiota dysbiosis associated with our phenotype. Overall, we conclude that a HF diet, due to its high content of saturated fatty acids, causes colonocyte mitochondrial malfunction, leading to the CCR2-dependent recruitment of inflammatory monocytes into the colonic mucosa and, ultimately, colonic crypt hyperplasia and microbiota dysbiosis.

Dendritic cell-specific SMAD3, downstream of JAK2, contributes to inflammation and salt-sensitivity of blood pressure in humans and mice

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Background: High salt consumption is associated with increased cardiovascular risk and higher morbidity and mortality in salt-sensitive hypertensives than in salt-resistant normotensives. Salt sensitivity of blood pressure (SSBP) is an independent predictor of death due to cardiovascular disease. Although the role of SMAD3 has been extensively studied in kidney fibrosis during renal artery stenosis and other cardiovascular disorders, the role of this pathway in immune cells contributing to SSBP is yet to be defined.

Hypothesis: We hypothesized that antigen-presenting specific SMAD3, downstream of JAK2, mediates IsoLG-adducts formation, IL-6 production, T cell activation, and inflammation and contributes to SSBP.

Method. We enrolled two cohorts of participants. We isolated monocytes from cohort one, treated them with normal or high salt, and performed RNA-seq analysis. We used an inpatient salt load and salt depletion protocol to phenotype for salt-sensitive and salt-resistant participants in cohort 2 and performed CITE-Seq analysis.

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In additional experiments, we generated dendritic cell-specific JAK2 knockout mice (DC^{JAK2KO}) and performed molecularly and flow cytometric immune phenotyping along with both noninvasive tail-cuff and state-of-the-art radio telemetry blood pressure and heart rate (HR) monitoring in the L-NAME/high salt model of salt sensitivity. We used immunohistochemistry and Fluorescent *In Situ* Hybridization for spatial and differential infiltration and expression of JAK2, SMAD3, ENAC-γ, and IL-6 in dendritic cells as well as fibrosis and infiltrating macrophages in the kidney.

Results: Both bulk and single-cell transcriptomic analyses of human myeloid antigen-presenting cells revealed that high salt treatment in vitro and in vivo upregulates genes of the JAK-STAT-SMAD pathway and downregulates downstream regulators, including the suppressor of cytokine signaling (SOCS) genes. DC^{JAK2KO} mice exhibit attenuated salt-sensitive hypertension (*SBP, 121.6 vs. 138.5, SEM±3.3, n=6*) and reduced heart rate compared to the wildtype littermates during L-NAME/high salt regimen. This was associated with reduced phosphorylation/activation of SMAD3 in total leukocytes (*982.6 vs 434.7, SEM±107.3*), dendritic cells (63.6 vs 18.8, SEM±10.73), and monocytes (*17.5 vs 106.2, SEM±34.1*). Inflammatory markers, IsoLG-protein adducts (*7.1 vs 25.9 SEM±3.6*), IL-6 (*6.8 vs 26 SEM±5.9*), and TGF-β1 (*38 vs 91.8 SEM±26.4*) in DC were significantly attenuated. Similarly, these markers were downregulated in total leukocytes and monocytes. The $CD8a^{+}$ Central memory T (T_{CM}) and effector memory T (T_{EM}) cells exhibit lowered IL-17A (*14.6 vs 26.3, SEM±5.1; 5.7 vs 38.5, SEM±13.96*) and IFN-γ (*26.3 vs 1.104.7 SEM±59.01; 11.6 vs 52.3 SEM±15.6*) expression. DC^{JAK2KO} mice also showed attenuated infiltration of total leukocytes, DCs, monocytes, and lymphocytes in the kidney.

Conclusion: These results indicate that dendritic cell-specific SMAD3 downstream of JAK2 plays an essential role in SSBP.

MMP inhibition during Influenza A virus infection limits shedding of the alveolar epithelial glycocalyx and reduces lung injury independent of inflammation

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Background: The epithelial glycocalyx, a layer of heparan sulfate-rich glycosaminoglycans, protects the alveolar epithelium during homeostasis. We found that the alveolar epithelial glycocalyx is shed in patients with acute respiratory distress syndrome (ARDS) and in mice with influenza A (IAV). The specific mechanisms of glycocalyx cleavage during viral infection are not well understood. We previously showed that the sheddase matrix metalloprotease-7 (MMP-7) has increased expression in the lung during ARDS and murine IAV pneumonia. Sheddases like MMP-7 are membrane-bound enzymes that cleave extracellular portions of proteins and glycoproteins. Therefore, we hypothesized that MMP-7 or other sheddases cause shedding of the alveolar epithelial glycocalyx during IAV infection. **Methods**: We used a mouse model of IAV pneumonia to quantify alveolar epithelial glycocalyx shedding in the presence or absence of the pan-MMP inhibitor Ilomastat (ILO). C57BL/6 mice were infected intranasally with IAV A/PR/8/34 (H1N1) at 30,000 PFU/mouse. Ilomastat (100mg/kg, Selleckchem) or vehicle control was given daily by intraperitoneal injection. On day 6, bronchoalveolar lavage (BAL) and lung tissue were collected. Glycocalyx shedding into BAL was quantified by ELISA of Syndecan-1 (a glycocalyx component), inflammation by BAL differential cell counts, permeability by BCA of total BAL protein, and IAV viral load by RT-qPCR of genome segment 8 in whole lung homogenate.

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Results: In alignment with our hypothesis, MMP inhibition by ILO reduced glycocalyx shedding compared to control (median ILO: 1.41 +/-0.88ng/mL Syndecan-1, control: 2.51 +/- 1.97ng/mL, p=0.018). Early in infection, mice treated with ILO had significantly less weight loss than control (12% vs. 16% respectively, $p<0.05$), although this effect had resolved by day 6. We examined the effect of ILO on lung injury and found that ILO-treated animals had reduced lung permeability (ILO: 1680 +/-254µg/mL protein, control: 2459 +/-190µg/mL, p=0.498). To test if attenuation of lung injury was driven by a differential immune response, we examined BAL immune infiltration and found that ILO did not reduce inflammation (ILO: $4.0x10^5$ +/- $1.9x10^5$ cells/mL vs. control: $4.5x10^5$ +/- $1.3x10^4$ cells/mL, p=0.611). ILO did reduce viral load in the lungs 2-fold (p= 0.015). **Conclusions**: Together, these data indicate that inhibiting MMPs with ILO during IAV infection reduces alveolar epithelial glycocalyx shedding and protects alveolar-capillary barrier integrity. This occurs independent of changes in inflammation. This suggests that MMP sheddase activity may drive IAV-induced lung injury through shedding of the alveolar epithelial glycocalyx. Future studies will examine whether MMP-7 is the primary sheddase responsible for glycocalyx shedding during IAV.

An early-life microbiota metabolite protects against obesity by regulating intestinal lipid metabolism

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The mechanisms by which the early-life microbiota protects against environmental factors that promote childhood obesity remain largely unknown. Using a mouse model in which young mice are simultaneously exposed to antibiotics and a high-fat diet, we show that *Lactobacillus* species, predominant members of the small intestine microbiota, regulate intestinal epithelial cells (IECs) to limit diet-induced obesity during early-life. A predominant *Lactobacillus* species, *L. murinus* (depleted during exposure to antibiotics and a high fat (HF) diet), protects against excess weight gain and adiposity during consumption of a HF diet in colonized gnotobiotic mice. We determined that *L. murinus* activates peroxisome proliferator activated receptor γ (PPAR-γ), a regulator of intestinal lipid metabolism, in small intestine IECs and thereby reduces adiposity. To investigate how *L. murinus* regulates PPAR-γ in IECs, we performed untargeted metabolomics in the small intestine and determined that a *Lactobacillus*-derived metabolite, phenyllactic acid (PLA), was depleted in mice exposed to antibiotics and a high-fat diet. Significantly, we then showed that PLA protected against metabolic dysfunction caused by early-life exposure to antibiotics and a high-fat diet by increasing the abundance of PPAR-γ in small intestine IECs. Therefore, PLA is a microbiota-derived metabolite that activates protective pathways in the small intestine epithelium to regulate intestinal lipid metabolism and prevent obesity during early life.

Remodeling of the gastric environment in *Helicobacter pylori*-induced atrophic gastritis

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Helicobacter pylori colonization of the human stomach is a strong risk factor for gastric cancer. In this study, we used a Mongolian gerbil model to investigate molecular alterations in *H. pylori*-induced atrophic gastritis, a premalignant condition characterized by inflammation and loss of specialized cell types in the gastric corpus. Histologic evaluation revealed varying levels of parietal and chief cell loss in *H. pylori*-infected animals, and transcriptional profiling using NanoString methodology revealed loss of molecular markers for these cells in animals with atrophic gastritis compared to infected animals with non-atrophic gastritis or uninfected animals. We then used imaging mass spectrometry to assess the spatial distribution and relative abundance of gastric proteins. We detected 13 corpus-specific peptides in uninfected animals; 9 of these were reduced in abundance and 4 exhibited diffuse delocalization in infected tissues with atrophic gastritis. None of these alterations were detected in animals infected with a Δ*cagT H. pylori* mutant (which is unable to secrete the oncoprotein CagA). LC-MS/MS analyses of corpus and antral tissues revealed about 300 corpus-specific proteins in uninfected stomachs. Of these, 74 exhibited decreased abundance in tissues with atrophic gastritis, including subunits of the potassium-transporting ATPase (parietal cell-specific enzyme) and proteins involved in mitochondrial energy generation and membrane transport. Additionally, 45 were delocalized in tissues with atrophic gastritis, including proteins related to mitochondrial function or cellular signal transduction. Collectively, these results reveal that loss of parietal and chief cells in *H. pylori*-induced atrophic gastritis is accompanied by extensive loss and redistribution of corpus-specific proteins.

Mitochondrial Trans-2-Enoyl Coenzyme A Reductase (Mecr) Regulates CD4$^+$ T Cell Function

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Many inflammatory diseases and cancer are driven by dysregulation of T helper cells (Th cells). We have previously shown that manipulation of metabolic pathways can affect Th cell differentiation and disease development. In this study, we used a custom CRISPR library to investigate the role of lipid metabolism in survival and proliferation of Th cells. In pooled genetic screens using in vivo models of allergic airway disease and inflammatory bowel disease, CD4$^+$ T cells were transduced with this lipid metabolism library and adoptively transferred into *Rag1*$^{-/-}$ recipients. After disease was apparent, the relative abundance of each guide RNA was determined in infiltrating CD4$^+$ T cells in diseased tissues to identify immunometabolic regulators of CD4$^+$ T cell recruitment and persistence in inflammation. Mitochondrial trans-2-enoyl-coenzyme A reductase (*Mecr*) was a significantly depleted gene, demonstrating a role for this gene in T cell-mediated lung inflammation. Although recessive mutations in humans cause an inborn error of metabolism, there are currently no published data on the function of Mecr in immune cells. Therefore, we used CRISPR/Cas9 to test the role of Mecr in T cell function and metabolism. *Mecr*-knockout in vitro caused no significant changes in T cell cytokine production or transcription factor expression. When tested side-by-side with control and *Mecr*-knockout Th17 cell in a model of transfer inflammatory bowel disease model, *Mecr*-knockout cells were depleted and had lower Tbet expression compared to a non-targeting control in the spleens, mesenteric lymph nodes, and lamina propria. Preliminary data also show that *Mecr*-knockout in CD4$^+$ T cells have a reduced oxidative consumption rate and increased extracellular acidification rate. Together, these results show that Mecr plays an important role in T cell immunometabolism and will be further explored metabolically and in the context of T cell-mediated inflammation.

Structural and Immunological Analysis of FlgG, a *Clostridioides difficile* Surface Antigen

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Clostridioides difficile infection (CDI) is a leading cause of nosocomial and antibiotic-associated infections in the United States, contributing to approximately 500,000 infections, 29,000 deaths, and $4.8 billion in healthcare costs each year. Recent vaccine strategies by Pfizer and Sanofi Pasteur targeting the etiologic agents of CDI, the TcdA and TcdB toxins, were unsuccessful at garnering durable mucosal immunity and preventing colonization. This may be due to a heavy focus on stimulating a systemic IgG response, rather than the prevention of *C. difficile* colonization at the site of infection. A recent *in silico* vaccinology study identified 16 surface proteins on *C. difficile* as putative non-toxin immunogens that may prevent bacterial colonization. The 16 putative non-toxin antigens were codon-optimized from the virulent *C. difficile* R20291 background, expressed, and purified. Four of the proteins were screened for IgG binding partners in CDI patient sera by ELISA. Of the four proteins tested, flagellar basal body protein FlgG elicited a significant IgG binding response in some of the patient sera samples. Electron microscopy was used to characterize the structure of FlgG alone and in-complex with other *C. difficile* flagellar proteins. Vaccination with FlgG and subsequent *C. difficile* challenge experiments are currently underway to test FlgG antigenicity and protective capabilities. Immunophenotypes from sera, spleens, gut-associated lymphoid tissues, and colonic/cecal lamina propria will be comprehensively analyzed by fluorescence-activated cell sorting. Histopathology scoring of the colon and ceca of vaccinated and challenged mice will be used to evaluate immunization safety and visualize alterations of epithelial disease markers upon challenge. If vaccination and challenge are successful, LIBRA-Seq. (linking B cell receptor to antigen specificity through sequencing) will be performed to identify monoclonal antibodies specific to FlgG. Discovery and validation of a clinically-relevant surface antigen on *C. difficile* capable of protecting against lethal challenge and colonization will inform subsequent vaccination strategies against the public health threat of CDI.

Beyond cellular vacuolation: *Helicobacter pylori* VacA toxin induces oxidative stress and impairs mitochondrial respiration

Mandy D. Truelock, Nora J. Foegeding, Mark S. McClain, Timothy L. Cover

Helicobacter pylori are gram-negative bacteria that colonize the stomach in over 50% of the world's population. Infection with *H. pylori* is associated with an increased risk of developing peptic ulcer disease and gastric cancer. *H. pylori* strains producing active forms of a secreted toxin (VacA) are associated with increased risk of peptic ulcer disease and gastric cancer compared to strains producing hypoactive forms. Previous studies show that VacA induces oxidative stress. In this study, we seek to further define the consequences of VacA-induced oxidative stress and how it leads to metabolic impairment in gastric cells. To investigate VacA's ability to induce oxidative stress-related changes, we conducted untargeted metabolomic analyses of VacA-treated AGS gastric epithelial cells compared to buffer-treated control cells. Several hundred metabolites were differentially abundant in VacA-treated cells compared to control cells, and pathway analysis showed there were significant alterations in numerous pathways, including taurine and hypotaurine metabolism, glutathione metabolism, and cysteine and methionine metabolism, which are correlative with oxidative stress. Additionally, we observed that VacA-treated cells exhibited significant increases in 8-hydroxyguanosine, a marker of RNA damage caused by oxidative stress, as well decreases in taurine levels, which is protective against oxidative stress. Several metabolites functioning as antioxidants were also depleted in response to VacA intoxication. Taurine deficiency is linked to decreased fatty oxidation, which is consistent with the acylcarnitine decrease we detected in cells intoxicated with VacA, potentially leading to impaired mitochondrial respiration. To study mitochondrial respiration in VacA-treated cells, we used Seahorse methodology and observed that VacA decreased the cells' maximum rate of respiration and their spare respiratory capacity, which is a measure of the cells' ability to respond to increased energy demands. We propose a model in which VacA's ability to induce oxidative stress in gastric epithelial cells leads to metabolic dysregulation and impaired mitochondrial respiration.

Vanderbilt University Cell Imaging and Shared Resource (CISR)

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The Vanderbilt Cell Imaging Shared Resource (CISR) is an institutional, fee-for-service, advanced microscopy resource. The CISR provides researchers with access to state-of-the-art imaging equipment and expert technical support for sophisticated microscopy and analysis of tissue and cellular anatomy and physiology. As of 2023, the CISR independently manages 17 advanced optical microscopes, 1 transmission electron microscope and 2 scanning electron microscopes. These instruments and the array of advanced capabilities offered by them are available to support any investigator with an appointment at Vanderbilt University or Vanderbilt University Medical Center. The CISR supports over 200 labs per year (over 400 individual users) for 12,000+ microscope hours per year and 100+ publications associated with these microscope hours. The CISR has been in existence for over two decades and grown with time in number of personnel, instruments, users, technology offered, and funding sources. In 2007, the CISR incorporated the EM core and in 2016, added the new Nikon Center of Excellence (COE). The CISR continuously strives to add new microscope technologies to support our wide variety of biomedical researchers. In the past 3 years, we have added lightsheet microscopy for imaging larger samples (up to 1 cm) as well as Focused-Ion-Beam Scanning Electron Microscopy for volume EM. To promote these new imaging technologies, we also support sample preparation necessary for these microscopes including tissue clearing for lightsheet and new EM preparation techniques such as freeze substitution. For VI4 members, CISR staff offer microscope and image analysis training on both commercial and open source software as well as support for optimizing imaging, image analysis, and sample preparation protocols.

Rational Design of a Multivalent HIV-1 Vaccine

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The HIV-1 virus is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) and continues to be a significant public health threat. The highly mutable HIV-1 envelope glycoprotein (Env) is the sole target of neutralizing antibodies and is the focus of vaccination attempts. Early vaccine attempts consisted of Env immunogens from arbitrarily selected HIV-1 strains and resulted in the elicitation of strain-specific neutralizing antibodies. Recent work identified factors that are essential for eliciting broad protection. These factors include number and choice of Env strains to represent global Env diversity, broadly neutralizing antibody epitope availability, and glycan shield coverage. Our lab developed an algorithm that simultaneously incorporates the three factors listed above to select a set of Env strains to be used in immunizations. Multiple sets of Env strains selected by the algorithm were expressed and validated in terms of antigenicity and structure. Guinea pigs were immunized with the Env strain sets and post-vaccination serum was tested for binding, neutralization, and effector functions. All immunogens elicited robust antibody responses that can bind to and neutralize tier 2 heterologous HIV-1 pseudoviruses. Additionally, vaccine elicited antibodies displayed antibody-dependent cellular phagocytosis activity against tested Env strains. This work will evaluate an algorithm-based selection of HIV-1 Env strains in a cocktail immunization strategy. Elicitation of broad antibody protection will justify future studies on this algorithm aimed at further expanding neutralization breadth.

Contribution of acid resistance mechanisms to UPEC pathogenesis

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Acid stress is a substantial challenge to bacterial life. Acidic conditions can damage the bacterial cell envelope and can disturb vital physiological processes, such as enzymatic activity, protein folding, membrane- and DNA maintenance. Consequently, bacteria are equipped to withstand acidic conditions. *Escherichia coli* occupies numerous environmental and host niches with varying pH. Six acid resistance (AR) mechanisms, termed AR1-AR6, one of which I have identified, operate in *E. coli*. Uropathogenic *E. coli* (UPEC), which is responsible for >75% of urinary tract infections (UTIs) persist for years in the host, colonizing the gut and the vagina (pH 3.8 - 5) asymptomatically for long periods of time, while causing acute and chronic infection in the bladder. No studies elucidated which AR mechanisms are critical for UPEC infection or asymptomatic colonization. To bridge this gap in the field, we created a collection of AR gene deletions and evaluated them in a murine model of UTI and measured the transcript levels of each AR during UPEC's interaction with urothelial cells. We show that AR4 is upregulated during intracellular infection and that deletion of a combination of both AR4 and AR5 resulted in reduced ability of UTI89 to adhere to and become internalized by bladder epithelial cells. During acute UTI, mutants lacking AR6, or a combination of AR2 and AR4 had a significant defect colonizing the bladder. These data suggest that AR6 and a combination of AR2 and AR4 are critical for the initial stages of infection. Ongoing studies are tracking the contribution of each AR mechanism for long-term colonization of asymptomatic niches. Our ultimate goal is to develop strategies to block these mechanisms as a method to enhance displacement of UPEC by commensal bacteria.

Toward a Human Antibody-Antigen Atlas

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The B cell compartment of the adaptive immune system plays a critical role in multiple disease settings, including in the generation of antigen-specific antibodies against invading pathogens. Despite antibody discovery research efforts, there is limited data regarding the fundamental rules governing antibody-antigen recognition. Recently our laboratory has developed a technology termed Linking B cell Receptor to Antigen Specificity by Sequencing (LIBRA-seq) that enables the rapid identification of antigen-specific B cells by turning B cell receptor (BCR):antigen interactions into sequence-able events. Leveraging this novel technique, we isolated strain specific and cross-reactive antibodies against several viral pathogens, simultaneously revealing patterns in antibody chain usage, variable gene usage, and somatic hypermutation frequency. Furthermore, the presence of both public and private clonotypes was observed. Inclusion of a diverse antigenic library, representing multiple strains across 5 distinct viral families, has enabled analysis of the BCR molecular features underlying antigen reactivity with greater breadth than achieved in previous studies. Moreover, continued generation of large-scale antibody sequence: antigen specificity datasets will enable the application of machine learning algorithms that may facilitate the development of predictive methods, such as for computational antigen specificity prediction directly from antibody sequence.

Identification of mucosal barrier alterations that impact attenuated *Clostridioides difficile* infection of Cystic Fibrosis intestinal epithelia

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Clostridioides difficile is a bacterium that can cause gastrointestinal disease. Most *C. difficile* infections (CDI) occur in hospitals after patients receive antibiotics. Thus, CDI has been shown to progress when *C. difficile* is transmitted to individuals with disturbed microbiota (dysbiosis), where it colonizes and produces toxins driving disease. CDI are prevalent hospital-acquired infections in the US, causing 29,000 deaths per year. People with Cystic Fibrosis (CF), a genetic disease that thickens mucosal secretions throughout the body, including the gastrointestinal tract, have increased rates of *C. difficile* colonization, but decreased rates of CDI, compared to individuals without CF. Yet, people with CF are recurring hospital visitors and frequently receive antibiotic therapy. We hypothesize receptor-ligand binding interactions between *C. difficile* and the intestinal mucosa drive CDI. To test this hypothesis, we are applying *C. difficile* to human-derived colonoids as an infection model to characterize the impact of mucosal barrier in CDI disease progression using a combination of transcriptomics and quantitative microscopy. We have collected fluorescent images of *C. difficile*-injected colonoids and shown that the fitness of *C. difficile* is impacted by mucin in co-culture. This work will leverage CF-mediated CDI attenuation in the identification *C. difficile* and host factors that result in CDI, while reshaping our understanding of human disease across different genetic backgrounds.

Assembly of the *Helicobacter pylori* Cag Type IV Secretion System Outer Membrane Core Complex

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Helicobacter pylori colonization of the stomach is the strongest known risk factor for gastric cancer. The *H. pylori cag* pathogenicity island encodes the Cag type IV secretion system (T4SS), which has a key role in pathogenesis. We previously analyzed the Cag T4SS outer membrane core complex (OMCC) by cryo-EM and showed that it contains five protein components (Cag3, M, T, X, and Y). In this study, we sought to define how individual components contribute to OMCC formation and detect interactions between the OMCC and other T4SS subassemblies. We used immunoprecipitation (IP) methods to isolate the OMCC from a strain containing an intact Cag T4SS and a mutant strain lacking an OMCC component (*ΔcagT*). Complexes were visualized by cryo-EM and the proteins present in the purification were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cryo-EM analysis of the *ΔcagT* OMCC showed that it contained a well-defined periplasmic ring with 17-fold symmetry and a poorly-organized outer membrane cap. This contrasts with the wild-type OMCC, which has a 17-fold-symmetric periplasmic ring and a 14-fold-symmetric outer membrane cap. To identify additional components of the Cag T4SS, we utilized crosslinkers to stabilize the wild-type T4SS during IP. LC-MS/MS analysis revealed two putative components of the T4SS inner membrane complex (CagE and CagV) isolated upon addition of DSP or DTTSP. These data indicate that CagT has a key role in assembly of the outer membrane cap but is not required for assembly of the periplasmic ring. We propose that the OMCC interacts with the T4SS inner membrane complex through CagE and CagV.

Multiomic and Functional Approaches to Define Risk and Immunopathogenesis of Allopurinol Associated Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis

Chelsea N. Campbell, Sophia R. Chou, Amy M. Palubinsky, & Elizabeth J. Phillips

Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN) is a rare T-cell mediated, HLA class I restricted hypersensitivity reaction that is typically drug induced. SJS/TEN is characterized by extensive keratinocyte death resulting in blistering and sloughing of skin and other mucous membranes including ocular and urogenital involvement. SJS/TEN is thought to be a single disease across a spectrum of severity defined by percentage of body surface area detachment. There is currently no established evidence-based treatment for SJS/TEN other than cessation of causal drug and implementation of supportive care in a critical care setting. One of the most common causes of SJS/TEN is allopurinol (ALP), a xanthine oxidase inhibitor frequently prescribed for the treatment of gout. HLA-B*58:01 is strongly associated with the development of ALP-SJS/TEN; however, the cellular and molecular mechanisms involved in pathogenesis are not well defined. We have established a single cell multiomic pipeline paired with functional approaches in patients with confirmed HLA-B*58:01 restricted ALP-SJS/TEN to identify additional risk factors associated with disease pathogenesis. We have found ALP-SJS/TEN blister fluid to be heavily infiltrated by CD8+ T cells with distinct TCRαβ sequences that are not shared between patients. To validate HLA restriction and TCR reactivity following drug stimulation, TCRαβ sequences were used to create patient specific cell lines for *in vitro* functional analysis. Data from this analysis suggests that in some cases of ALP-SJS/TEN, the active metabolite of ALP, oxypurinol (OXP), is able to directly interact with either the clonally expanded TCR or HLA-B*58:01. We could not, however, confirm this for other HLA-B*58:01 restricted cases of ALP-SJS/TEN, suggesting that other factors, including specific peptides at sites of tissue damage or other HLA binding pocket modifiers are involved in disease pathogenesis. Taken together, these data will inform the cellular and molecular mechanisms that are necessary and sufficient in the immunopathogenesis of ALP-SJS/TEN.

Weight cycling may induce lipid handling in mast cells

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Weight cycling worsens diabetes risk in both humans and mice. We recently found that weight loss and weight cycling induce many immunological changes in the adipose tissue of mice by single cell RNA-sequencing. We also found a substantial increase in adipose mast cells with weight cycling, but not weight loss. The purpose of this study was to confirm and characterize the changes in mast cells with weight cycling. However, using flow cytometry (cKit+FcER1+) and Toluidine blue staining, we found that adipose mast cells are greatest in the weight loss group, in contrast to our prior study using single cell RNA-sequencing. Upon further analysis, we found two unique mast cell populations in our single cell sequencing dataset: one that we consider traditional mast cells and one with lower expression of mast cell surface and protease genes and elevated expression of lipid handling and antigen presentation genes. The second fraction appeared similar to lipid-associated macrophages and accounted for most of the mast cells in the weight cycled group. By flow cytometry, we confirmed the %CD9+, MHCII (I-A/ I-E)+, and CD74+ mast cells were highest in weight cycled mice. We also found two mast cell populations in human subcutaneous adipose tissue that appear similar to the murine populations in our dataset, with one population significantly correlating with weight variability. Together, these data suggest that weight cycling may induce a population of lipid-associated mast cells. We next aim to induce lipid handling in bone marrow derived mast cells by repeated exposure to adipose conditioned media in order to understand the mechanisms which induce lipid handling.

Prevalence of nasopharyngeal carriage of macrolide resistance-associated erm genes among healthy children and adults in a peri-urban community in Peru

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Background: Erythromycin ribosome methylase (*erm*) genes, which confer macrolide resistance, are commonly detected in healthcare settings. Yet, their prevalence among healthy individuals in the community is unknown. Here we provide an initial assessment of *erm* nasopharyngeal carriage in healthy children and adults.

Methods: Nasopharyngeal swabs were systematically obtained at enrollment and weekly thereafter from children and adults enrolled in a household-based prospective cohort study in Lima, Peru. Samples were sequenced using the Illumina Respiratory Pathogen/ID AMR Panel to detect common respiratory bacteria and antimicrobial resistance genes. We defined 'any *erm* gene' (*erm*) as the detection of at least one of the specific *erm* gene classes. We compared the prevalence of erm carriage at enrollment among age groups (ages 0-4, 5-17, 18-44, and 45+ years) using the Fisher's exact test.

Results: 114 individuals were included in this analysis; 74% were female and median age was 24.2 years (IQR 4.6, 41.8). An *erm* gene was detected in 51 (44.7%) of individuals, most commonly *ermC* (15.8%) and *ermB* (7%). The prevalence of *erm* gene detection was high and similar among age groups: [0-4 years (19/33, 57.6%), 5-17 years (12/20, 60.0%), 18-44 years (11/34, 32.4%) and 45+ years (9/27, 33.3%) (p=0.056)].

Conclusions: These preliminary results indicate that *erm* genes were commonly detected in healthy community-dwelling children and young adults in Lima, Peru. Future analysis will assess changes in *erm* carriage over time, transmission among household members, and its clinical relevance.

Clostridioides difficile increases undecaprenyl pyrophosphate recycling and drug efflux in response to iron starvation

Martin V. Douglass, and Eric P. Skaar

Clostridioides difficile infection (CDI) is the leading nosocomial intestinal infection in the United States and an urgent threat to public health. CDI onset begins with *C. difficile* outcompeting both the host microbiota and the innate immune response for limited nutrients. A critical factor in the host immune response to CDI is the innate immune protein calprotectin (CP) that chelates essential nutrient metals from the pathogen through a process termed nutritional immunity. CP is essential for the host to combat CDI, yet how *C. difficile* overcomes CP to acquire nutrients is not well understood. To uncover how *C. difficile* responds to nutritional immunity, we evaluated the transcriptional changes that *C. difficile* undergoes when challenged with CP. We identified a putative two-component system (TCS), *2822* and *2823*, to be transcriptionally increased in the presence of CP and iron chelators. Mutants lacking this TCS exhibit a growth defect in iron limiting conditions. Furthermore, we found 2822/2823 regulates three genes immediately downstream: *2821, 2820,* and *2819*. Based on bioinformatic predictions, 2820 and 2819 encode an ATP driven efflux pump, and 2821 encodes an undecaprenyl pyrophosphatase. Further experiments revealed that 2822/2823 is activated by the antibiotic bacitracin, and mutants lacking the TCS are extremely sensitive to the cell surface targeting molecules bacitracin and vancomycin, the latter of which is clinically relevant. Our results support a model in which *C. difficile* overcomes nutritional immunity by coordinating an increase in undecaprenyl pyrophosphate recycling and drug efflux to defend against external threats such as cell envelope targeting antimicrobials.

Paired Immunoglobulin-like Receptor B in Macrophages Regulates the Pro-resolving Response to Efferocytosis

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Maintenance of tissue homeostasis in the human body requires the efficient removal of millions of dead cells each day. Efferocytosis, the macrophage phagocytic process of apoptotic cell (AC) clearance, enhances inflammation resolution by promoting anti-inflammatory macrophage metabolism and cytokine production. Failures of efferocytosis promote the development of chronic inflammatory diseases, including atherosclerosis. Recently, immunomodulatory receptors, a class of receptors known to broadly regulate macrophage inflammatory responses, have been implicated in efferocytosis. Paired immunoglobulin-like receptor B (PirB) is an understudied immunomodulatory receptor previously shown to inhibit inflammatory cytokine production in macrophages. We hypothesized that PirB is a novel promoter of efferocytosis-mediated inflammation resolution. We examined atherosclerotic lesions from the aortic root of *Ldlr*$^{-/-}$ mice after 8 or 16 weeks of Western diet feeding and observed decreased levels of PirB in lesional macrophages in mice with larger necrotic cores, dysfunctional efferocytosis, and more advanced atherosclerosis. To test if PirB regulates efferocytosis, we generated bone marrow-derived macrophages (BMDMs) from Control and *Pirb*$^{-/-}$ mice, co-cultured them with labeled ACs, and then assessed efferocytosis using flow cytometry. While Pirb-/- BMDMs bound and internalized ACs, they failed to undergo lysosomal acidification, suggesting ACs are not being digested. Additionally, an unbiased query of validated protein:protein interactions identified phagolysosomal maturation machinery as binding partners of PirB, suggesting loss of PirB impedes AC digestion by dysregulation of phagolysosomal maturation. As expected, control BMDMs fed ACs upregulated the pro-resolving metabolic pathway, oxidative phosphorylation, and cytokine IL-10. Conversely, *Pirb*$^{-/-}$ BMDMs upregulated pro-inflammatory cytokines and maintained elevated glycolysis upon AC feeding. Our results suggest that PirB is a unique regulator of inflammation resolution by regulating AC digestion and metabolic rewiring of macrophages post-efferocytosis.

Role of TonB-dependent transporters in *Helicobacter pylori* colonization of the stomach

Sergio Escobar, John T. Loh, Jennifer H. Shuman, Timothy L. Cover

Helicobacter pylori s contain more than 50 genes predicted to encode outer membrane proteins, six of which are predicted to be TonB-dependent transporters (TBDTs). TBDTs bind and transport various substrates, utilizing a complex of three inner membrane proteins (TonB-ExbB-ExbD) that transduce energy in the form of proton motive force to the outer membrane. Early studies assigned the names "*fecA*-like" and "*frpB*-like" to six *H. pylori* genes predicted to encode TBDTs, based on sequence relatedness to genes in other species; the encoded proteins were predicted to have roles in iron acquisition. The three *fecA*-like genes and three *frpB*-like genes are non-identical. Thus far, there has been relatively little study of the functions of these proteins, however, *frpB*-3 is reported to have a role in nickel acquisition. In this study, we tested the hypothesis that *H. pylori* TonB-dependent transporters contribute to colonization of the stomach. We generated mutant strains in which each of the *fecA*- and *frpB*-like genes were knocked-out individually. Next, we experimentally infected C57BL/6 mice with the wild-type or one of the mutant strains. At two weeks post-infection, mice were euthanized, their stomachs were extracted and homogenized, and the gastric homogenates were cultured to evaluate the bacterial burden of *H. pylori*. Notably, each of the mutant strains successfully colonized mice. In comparisons of mice infected with single mutant strains to mice infected with the wild-type strain, we did not detect significant differences in the proportion of mice colonized or in the density of *H. pylori* colonization. Therefore, we hypothesize that there is redundancy in the functions of individual *H. pylori* TBDTs.

Understanding the role of arginine metabolism in antibiotic tolerance during *Staphylococcus aureus* infections

Jeffrey A. Freiberg, Valeria M. Reyes Ruiz, and Eric P. Skaar

Staphylococcus aureus is a leading cause of a wide range of bacterial infections and is associated with significant morbidity and mortality. These poor outcomes are driven by the high rates of antibiotic treatment failure seen with many types of *S. aureus* infections. Antibiotic tolerance, which is defined as the ability of bacteria to survive in the face of antibiotics through phenotypic changes without the acquisition of antibiotic resistance, is the major driver of antibiotic treatment failure in *S. aureus* infections. To identify staphylococcal proteins responsible for antibiotic tolerance, mature bacterial communities were grown using a methicillin-resistant *S. aureus* (MRSA) strain representative of the predominant clinical isolate. These bacterial communities were exposed to multiple clinically relevant antibiotics along with a no antibiotic control and subsequently subjected to whole proteome analysis using LC-MS/MS. This analysis revealed that antibiotic exposure increases the abundance of proteins involved in the arginine deiminase pathway of arginine degradation and decreases the abundance of the proteins ArgG and ArgH, which are involved in arginine biosynthesis in *S. aureus*. In parallel, transposon sequencing experiments also identified mutants containing transposon insertions disrupting the expression of ArgG and ArgH as beneficial to the survival of *S. aureus* during antibiotic treatment. Further experiments identified an increase in antibiotic tolerance in mature *S. aureus* communities when arginine is depleted, suggesting that the depletion of arginine through regulation of bacterial metabolism can induce antibiotic tolerance in *S. aureus*. This research suggests manipulating arginine levels and metabolism during *S. aureus* infections may present an exciting new target to adjuvate anti-*Staphylococcus aureus* antibiotic therapy.

Trained immunity impairs inflammation resolution and efferocytosis

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Chronic inflammation is a major driver of atherosclerotic cardiovascular disease, and therapeutics that target inflammation reduce clinical cardiac events beyond levels seen with conventional strategies targeting cholesterol alone. Recent work has demonstrated that advanced atherosclerosis is also characterized by the failure of an active repair process termed 'inflammation resolution'. The resolution program, aimed at curtailing inflammation, includes efficient phagocytosis of apoptotic cells (efferocytosis). Recent findings suggest innate immune cells maintain 'memory' of prior exposure to inflammatory stimuli, a phenomenon known as 'trained immunity'. In response to inflammatory stimuli, macrophages undergo an epigenetic rewiring that primes them to mount an augmented response upon a second exposure. Atherogenic stimuli, including oxLDL, have recently been shown to be potent triggers of trained immunity, implicating trained immunity in atheroprogression. While trained immunity has been shown to promote proinflammatory pathways, we hypothesized that trained immunity in macrophages promotes inflammation by impairing efferocytosis.

We treated murine bone marrow progenitors with oxLDL for 24 hours, then washed and differentiated them into macrophages (BMDMs) for seven days. We co-cultured BMDMs with fluorescently-labeled apoptotic cells (ACs) and quantified their efferocytic capacity using flow cytometry. Trained BMDMs were able to ingest a first AC better than untrained BMDMs yet had an impaired ability to take up additional cells. Using an *in vivo* approach, we transplanted donor marrow from *Ldlr*$^{-/-}$ mice fed a Chow-Diet or a Western-Diet into naïve C57BL/6 recipient mice. After engraftment, we elicited peritoneal macrophages using zymosan and injected fluorescently-labeled ACs to the peritoneum to assess efferocytic capacity. C57BL/6 mice that received marrow from *Ldlr*$^{-/-}$ mice fed a Western-Diet had impaired efferocytosis compared to those receiving marrow from *Ldlr*$^{-/-}$ mice fed a Chow-Diet. These data suggest that the effects of oxLDL/Western-Diet training are durable effects that demonstrate heritability, suggesting a possible epigenetic mechanism.

The intestinal microbiota regulates nutritional immunity and resistance to *Acinetobacter baumannii* during respiratory infection

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A major risk factor for nosocomial infections is the administration of broad-spectrum antibiotic therapy. In the case of gastrointestinal infections, this frequently leads to disruption of the microbial community inhabiting the gastrointestinal tract, resulting in loss of colonization resistance and outgrowth of enteric pathogens. However, prior or inappropriate administration of broad-spectrum antibiotics has also been associated with development of nosocomial pneumonias, including respiratory infections with the emerging gram-negative bacterial pathogen *Acinetobacter baumannii*. Here, we establish a murine model of broad-spectrum antibiotic treatment prior to *A. baumannii* intranasal infection and observe enhanced mortality and higher bacterial loads in mice receiving prophylaxis. Control of infection is restored to antibiotic-treated mice following fecal microbiota transfer, suggesting that perturbations to the intestinal microbiota drive pneumonia susceptibility. Transcriptomic profiling via single-cell RNA-sequencing (scRNA-seq) identified a blunted signature of neutrophil activation in the infected lungs of mice receiving antibiotic prophylaxis, and neutrophil depletion ameliorated differences between antibiotic-treated and non-treated groups, indicating that components of the microbiota drive neutrophil activation and control of *A. baumannii* pneumonia. Neutrophils from antibiotic-treated animals exhibited decreased transcription of several factors contributing to the restriction of nutrient metals from invading pathogens, including *S100A9*, encoding calprotectin, and *Lcn2*, encoding Lipocalin-2. Moreover, antimicrobial prophylaxis of *S100A9*$^{-/-}$ and *Lcn2*$^{-/-}$ mice did not exacerbate susceptibility to *A. baumannii* pneumonia, suggesting that signals from the intestinal microbiota prime neutrophil-mediated nutritional immunity defenses in the lung. Future work will be targeted at dissecting the molecular mechanisms by which the gut microbiota influences neutrophil activation and effector functions in the lung.

Stochastic Lipoprotein Mutations that Impact Biofilm Morphology in uropathogenic *Escherichia coli*

Hamilton Green, Timothy. William, Gerald Van Horn, Maria Hadjifrangiskou, Jonathan Schmitz

Uropathogenic *Escherichia coli* (UPEC), the primary cause of urinary tract infections (UTIs), forms biofilms on catheter devices, on the urothelial surface and inside host cells. Two primary biofilm matrix components in UPEC are cellulose and curli, both of which take up the Congo Red (CR) dye when bacteria are spotted on CR-agar plates. In this work, we identified clinical UPEC isolates that form colony biofilms with prominent intra-strain heterogeneity in their ability to take up CR. We focused on a poorly characterized morphotype that forms a "*peppermint*" pattern, because isolates with this "peppermint" morphotype were more frequently isolated from patients with recurrent UTIs (rUTIs). Here, we uncover genetic mutations associated with the peppermint phenotype of one peppermint isolate deriving from rUTIs, VUTI148. Bacterial strains were grown overnight in Luria broth and 10ml of overnight culture was spotted on CR- agar plates. Colony biofilms were incubated for 11 days at room temperature and images were taken of biofilm development at 11 days. Red and white subpopulations from the colony biofilm were isolated and subjected to gDNA isolation and next generation sequencing on an Illumina platform. Obtained reads were compared between white and red samples. Genomic analyses of red subpopulations uncovered 4 independent mutations in the *nlpI* gene that encodes an outer membrane lipoprotein. A clean *ΔnlpI* mutant phenocopied the *nlpI* mutant subpopulations, displaying increased rugosity and higher CR uptake. Increased rugosity is not due to differences in cellular replication, because colony biofilms from Δ*nlpI* and the wild-type strain have similar bacterial titers. We postulate that loss of NlpI leads to changes in the extracellular matrix of UPEC, possibly through the regulation of curli and cellulose.

Insights into the ecology and evolution of *Lactobacillus crispatus*: spontaneous mutation rates and interactions with *E. coli*

Owen Hale, Michelle Yin, Megan Behringer

Lactobacillus crispatus is a prevalent bladder commensal bacterium that has been associated with urinary health and specifically a decreased risk of urinary tract infections (UTIs). Unique aspects of *L. crispatus* such as its aerotolerant anaerobic lifestyle and production of mutagens like hydrogen peroxide are likely to impact its mutation rate. Therefore, it is important to characterize the mutation rate and spectrum of this bacterium across the diverse conditions in which it is found. We followed over 40 independently derived *L. crispatus* lines for 1100 generations. We cultured each line anaerobically for 100 days, passaging them through 50 single-cell bottlenecks following a classic mutation accumulation approach. Using whole-genome sequencing, we were able to identify all single nucleotide variants as well as small insertions and deletions. We found that *L. crispatus* exhibits a uniquely high mutation rate characterized by a spectrum dominated by GC to AT transitions. This result is unexpected given the culture environment which would minimize oxidative stress. However, the production of DNA damaging metabolites such as hydrogen peroxide may explain the high mutation rate. By establishing the spontaneous mutation rate in a minimally selective environment, we provide a baseline for characterizing mutation rates in other environments such as the human body. In addition to characterizing the mutation rate of *L. crispatus*, we began to investigate the genetic determinants of its interactions with the most common UTI pathogen, *Escherichia coli*. Using RB-TnSeq, we demonstrated that *L. crispatus* directly kills *E. coli* in co-culture and found that disrupting genes related to cell wall and cell membrane homeostasis in *E. coli* can increase resistance to this killing effect. This study points to potential mechanisms by which *L. crispatus* protects the bladder from infections and how *E. coli* can develop resistance to inhibition by the bladder microbiota and cause UTIs.

High-Throughput Mapping of the Antibody Response to Multivalent HIV-1 Vaccination

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A challenge for HIV-1 vaccine design is eliciting effective HIV-1 antibodies that recognize a diverse range of viral variants. HVTN124 is a first-of-its-kind 5-valent prime-boost human vaccine clinical trial aiming to elicit cross-reactive neutralizing antibodies (NAbs) against HIV-1. HVTN124 utilized two different vaccination strategy groups. Group 1 received a DNA prime at day 0, followed by protein boost at day 168. Group 2 received both the DNA prime and protein boost at day 0. Both groups elicited polyclonal cross-reactive HIV-1 antibodies in serum; however, Group 1 displayed higher IgG titers than Group 2. To date, the monoclonal antibody response has not yet been characterized for either group. Monoclonal characterization is important for HIV-1 vaccine design as it informs features of antibodies that may help define their effectiveness. These include the ability of a vaccine to elicit antibodies that recognize diverse epitopes across inter- and intra-clade HIV-1 variants, or cross-react, and neutralize against virus. I propose to perform high-throughput antigen specificity mapping of B cell receptor (BCR) sequences from PBMCs isolated from vaccinated individuals using LIBRA-seq, which uses barcoded antigens to map BCR-antigen specificities. This study will be the first to look at the B cell responses to HIV-1 multivalent vaccination at such degree. Our overall goal is to use LIBRA-seq and antibody characterization assays to evaluate the antibody responses from the multivalent HVTN124 human vaccine clinical trial. Dissecting the response from this new strategy will improve upon what is currently known for HIV-1 vaccine design by providing novel insights into antibody responses to a multivalent vaccine candidate in humans, as a potential template for further vaccine engineering and optimization.

Assessing the Role of IL-17RA in the Stomach Epithelium During *H. pylori* Infection

Nikhita Joshi, Lee C. Brackman, Matthew S. Jung, Holly M. Scott Algood

Heliobacter pylori is a Gram-negative pathogen that colonizes the stomach, and its presence is attributed with severe disease outcomes such as gastric cancer. Interleukin-17 has been shown to play an important role in the inflammatory response during *H. pylori* colonization of the gastric mucosa. Interleukin-17 (IL-17) is the principal cytokine produced by T helper 17 cells and mediates protective innate immunity against pathogens by inducing the activation and mobilization of neutrophils (PMNs). IL-17 is also known to be expressed by other immune cells, including CD8+ T cells, natural killer T cells, and innate lymphoid cells. The IL-17 receptor is expressed in epithelial cells, fibroblasts, and hematopoietic cells. In this study, *Foxa3*^{cre}*Il17ra*$^{fl/fl}$ (IL-17RA$^{\Delta epi}$) and *Il17raf*$^{fl/fl}$ (control) mice are utilized to test the hypothesis that IL-17RA signaling in epithelial cells protects against hyperinflammation after *H. pylori* infection. The genotypes of the mice and the specificity of the Cre recombinase are being determined by DNA genotyping, real-time PCR (qRTPCR), and flow cytometry. As determined by examination of histological stains, IL-17RA$^{\Delta epi}$ often develop lymphoid follicles and increased inflammation by 3 months post infection, whereas control mice do not. Gene expression analysis of stomach tissues at this same time point indicate that both acute (i.e., PMNs) and chronic responses (i.e., lymphocytes, IL-17, IL-21 expression) are significantly increased in the IL-17RA$^{\Delta epi}$ mice compared to controls. These data suggest that a deficiency of IL-17RA in epithelial cells is sufficient to drive chronic inflammation and hyperactivation of the Th17/Tfh- B cell axis but is not required for recruitment of PMNs. Future studies will investigate which cell type (i.e., fibroblasts) might respond to IL-17 to recruit PMNs during *H. pylori* infection.

Loss of SLAM-associated protein (SAP) disrupts germinal center T-B lymphocyte interactions and reduces diabetes incidence in transgenic anti-insulin VH125^{SD}.NOD mice

Dudley H. McNitt, Jacob Kim, Chrys Hulbert, James W. Thomas, and Rachel H. Bonami

The autoimmune disease type 1 diabetes (T1D) results from the destruction of insulin-producing beta cells in the islets of Langerhans in the pancreas. High-affinity anti-islet autoantibodies predict T1D and suggest that germinal centers (GCs) are important early in T1D pathogenesis. Signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) is an intracellular signaling protein that is necessary for stable T-B interactions and cognate T follicular helper (Tfh)/GC B cell maturation in GCs. We hypothesized that SAP is required for islet autoantigen-reactive GC Tfh/GC B cells to form, and in turn for T1D to develop. To address this hypothesis, we used the VH125^{SD}.NOD BCR transgenic model, in which 1-2% of B cells bind insulin and compared their fates when SAP was/was not present (SAPko). *SAP*-sufficient VH125^{SD}.NOD mice rapidly developed T1D whereas *SAPko* VH125^{SD}.NOD mice had reduced T1D incidence. In the early stages of T1D onset, insulitis was reduced in *SAPko* mice, while the formation of organized tertiary lymphoid structures in the pancreas was low and did not differ across groups. Loss of *SAP* significantly reduced the number of GC B lymphocytes in the pancreas and pancreatic draining lymph nodes, while the number and subset of GC Tfhs were the same in *SAPko* as *SAP*+ mice. Our data suggest that stable T-B lymphocyte conjugates supported by SAP are not required for generating mature GC Tfhs but fail to support the development of anti-insulin GC B cells, which may be critical for the development of pro-inflammatory diabetogenic Tfhs.

Understanding the roles of tcdE and tcdL during toxin secretion in *Clostridioides difficile*

Shannon L. Kordus, Rubén Cano Rodríguez, Evan Krystofiak, Natalie Loveridge, Kevin Childress, and D. Borden Lacy

Understanding how large macromolecules are transported across a cell wall is a complex and poorly understood biological process. The nosocomial pathogen *Clostridioides difficile* produces two large toxins, TcdA (308 kDa) and TcdB (270 kDa). Although toxin function in host cells has been extensively studied, little is known about how these toxins are secreted from the bacterium. TcdA and TcdB are encoded on a pathogenicity locus (*PaLoc*) which also encodes the holin-like protein, TcdE, and the remnants of an endolysin, TcdL. While bacteriophages use holin/endolysin systems to trigger bacterial cell lysis and escape, multiple reports now suggest that TcdE is used for the secretion of the toxins by forming a channel within the cell membrane. While TcdL was only recently discovered, there are data to suggest that it can interact with TcdB to help facilitate toxin translocation. To further understand toxin secretion, we created strains with *tcdE, tcdL,* or *tcdE* and *tcdL* deleted. Our data indicate that both TcdE and TcdL are required for secretion. Furthermore, we found that TcdA and TcdB are released in a temporal manner where TcdA is secreted before TcdB. The research presented here will address the outstanding questions of how TcdE and TcdL interact with *C. difficile* toxins to create a pore and how the toxins can be released with mechanisms other than cell lysis.

Uncovering the Relationship Between Renal Cell Carcinoma, Immune Checkpoint Blockade, and CD8 T Cell Metabolism and Effector Function

Madelyn Landis, Rachel Hongo, Zaid Hatem, Katy Beckermann, Kim Rathmell, Jeff Rathmell

Renal cell carcinoma (RCC) is the 9th most common cancer type in the United States with 75k new diagnoses and 15k deaths each year. Immune checkpoint blockade therapies are the frontline treatments for RCC metastatic disease, and these therapies target immune checkpoints such as PD1 and CTLA4. Not every patient responds to immune checkpoint blockade therapy and elucidating the connection between patient response and anti-tumor T cell function continues to be a point of study. The tumor microenvironment (TME) of RCC is thought to be immunosuppressive and hinder the anti-tumor effector function of RCC tumor infiltrating lymphocytes (TILs). T cell metabolism has been shown to be critical to anti-tumor effector function and is found to be dysregulated in RCC TILs. To investigate the effects of immune checkpoint blockade on RCC associated T cell function, TILs from patient derived RCC samples are studied for T cell activation, expansion, metabolic function, and effector function by flow cytometry, mitochondrial stress tests, and tumor killing assays. *In vitro* co-stimulation of RCC T cells with aCD3 and aCD28 rescues cytotoxic T cell effector function with expression of IFNgamma and Granzyme B as well as causing expansion of CD8 T cells following *in vitro* treatment with cytokine IL-2. CD8 TIL treated with aPD1 show increased expansion, mitochondrial oxygen consumption rate (OCR), and extracellular acidification rate (ECAR) compared to CD3/CD28 alone. These results show the critical relationship between T cell metabolism with immune checkpoint blockade and cellular expansion in RCC.

Insights to Pathogenic Potential: Genomic Analysis of *Escherichia coli* from Asymptomatic and Symptomatic Patients

Grace Morales, Seth Reasoner, Benjamin Abelson, Maria Hadjifrangiskou, Jonathan Schmitz

Urinary tract infections (UTI) afflict over 50% of women over the course of their lifetime. While uropathogenic *Escherichia coli* (UPEC) causes >75% of reported UTIs, UPEC lacks a specific molecular signature. In the clinical setting this poses a significant dilemma when patients present with asymptomatic bacteriuria (ASB), in which significant numbers of *E. coli* is found in the urine of patients, but without the associated symptoms of UTI. Both ASB and UTI-causing *E. coli* carry similar types of virulence factors, and – to date – no genomic signature exists to tell these two *E. coli* types apart. In this study, we sequenced approximately 800 *E. coli* strains from the urine of symptomatic and asymptomatic patients. We utilized a custom bioinformatic pipeline to characterize strains outside of the traditional virulence factors, hypothesizing that a signature lies not in the carriage of distinct virulence genes, but rather in discrete polymorphisms that change tropism and pathogenic potential. Included in this analysis are applications of microbial Genome-Wide Association studies as well as machine learning methodology to detect potential loci associated with the pathotype in an unbiased manner. We've identified distinct patterns of SNPs within these strains that could lead to their pathogenic potential. This work more robustly defines true uropathogenic strains with the aim of identifying a molecular signature to differentiate strains with pathogenic potential.

Maintenance of organismal and cellular homeostasis by an ancient zinc metallochaperone

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Metals are essential micronutrients required for cellular function across all kingdoms of life. Zinc (Zn) serves many roles in human health and disease, functioning as a cofactor for both structural and enzymatic proteins for an estimated 10% of the proteome. Dysregulated Zn homeostasis is detrimental to cellular homeostasis and underlies diseases including cancer and neurodegeneration, yet the mechanisms by which Zn promotes these pathologies remain largely unexplored. Zn delivery to target proteins is critical as intracellular concentrations of free Zn are virtually non-existent. In conditions of Zn limitation, it is predicted that specialized proteins called metallochaperones are necessary for the transfer of metals to critical metalloprotein clients. We have identified the first vertebrate Zn metallochaperone, which we named Zn regulated GTPase metalloprotein activator 1 (ZNG1). We have demonstrated that vertebrate ZNG1 binds to and transfers Zn to metalloproteins to promote their enzymatic activity *in vitro*. To determine the *in vivo* role of ZNG1 proteins, we generated animal and cell culture models deficient in *Zng1*. Collectively these models revealed that ZNG1 activity is required for proteostasis and cellular respiration in conditions of Zn deprivation. Our findings suggest that this previously uncharacterized vertebrate ZNG1 enzyme family regulates intracellular Zn mobilization controlling fundamental cellular processes. (*continue next page*)

Further, we identified numerous candidate ZNG1 client Zn metalloproteins that are associated with cellular proliferation and brain function, including the Zn-finger transcription factor ZFHX3. Notably, ZFHX3 controls the expression of several secreted neuropeptides in the brain that have systemic roles in the intestine. Preliminary data demonstrates dysregulated expression of these ZFHX3 target neuropeptides, raising the possibility that ZNG1 regulates animal physiology via interactions with ZFHX3. Ultimately, this work will vertically advance our understanding of Zn homeostasis in animals and the contribution of Zn metabolism to the progression of human neurodegeneration.

Clostridioides Difficile Transferase Toxin Activates the NLRP3 Inflammasome

Kateryna Nabukhotna, John A. Shupe, & D. Borden Lacy

Clostridioides difficile is a Gram-positive, spore-forming anaerobe and the leading cause of hospital-associated diarrhea in the United States. Besides producing the main virulence factors, toxin A (TcdA) and toxin B (TcdB), some of the common epidemic *C. difficile* strains secrete *C. difficile* transferase toxin (CDT) suggesting that it increases the severity of *C. difficile* infection (CDI). To provide a framework to understand how CDT functions and contributes to *C. difficile* pathogenesis we investigated its role in host inflammasome enhancement. In contrast to published reports, our data from HEK293 TLR reporter cell lines and murine bone-marrow derived dendritic cells (BMDCs) indicate that CDT does not prime host inflammasomes via TLR2/6 and TLR4 pathways. Instead, CDT directly activates the inflammasome as assessed by the secretion of cleaved caspase-1 and cleaved IL-1β into the supernatants of BMDCs. Surprisingly, in bone marrow-derived macrophages (BMDMs), a cell binding and pore-forming component of the toxin, CDTb, alone activates the inflammasome and is dependent on K^+ efflux. The presence of the enzymatic component, CDTa, inhibits this activation. The activation was not observed in both BMDCs and BMDMs derived from NLRP3$^{-/-}$ mice suggesting the involvement of the NLRP3 inflammasome. To evaluate the physiological role of the NLRP3 inflammasome during CDI, wild-type and NLRP3$^{-/-}$ C57BL6 mice were infected with *tcdA*$^+$*tcdB*$^+$*cdtA*$^+$*cdtB*$^+$ and *tcdA*$^-$*tcdB*$^-$*cdtA*$^+$*cdtB*$^+$ strains of *C. difficile*. This study indicates that CDT might manipulate the host immune responses during CDI.

ANATOMY OF THE IMMUNE RESPONSE TO *FRANCISELLA TULARENSIS* LVS INFECTION IN THE LUNGS.

G. Donald Okoye, Amrendra Kumar, Holly M. Scott Algood, Sebastian Joyce

The respiratory mucosa is under constant immune surveillance because of its vulnerability to infectious diseases. Infection of the lungs with the live vaccine strain (LVS) derived from *Francisella tularensis* (Ft) subspecies holarctica models pulmonary tularaemia-like disease in mice. Current evidence suggest many immune cells and cytokines respond to Ft LVS infection, but how these immune system components are integrated to mount a protective response remains unclear. Hence, in a transcriptomics study at single cell resolution, we characterized the acute immune landscape in the lungs of C57BL/6 mice at day 0 and day 7 post intranasal inoculation with Ft LVS. Defining features of the immune response include a robust type 1 immune response characterized by accumulation of inflammatory neutrophils and the expansion of innate-like effector lymphocytes, primarily interferon-γ producing NKT1, NK, and effector $CD8^+$ T cells. Increased accumulation of MAIT17 over MAIT1 cells is another feature, which is in line with previous findings linking MAIT cells and IL-17 to LVS immune response. Surprisingly, MAIT17 cells either maintained or upregulated type 1 inflammatory markers. Additionally, a highly active MAIT cell subset has increased *Nr4a1* expression, which encodes Nur77, suggesting a T cell receptor-mediated activation. By contrast, day 7 NKT1 cells poorly upregulated *Nr4a1* expression but induced *Il18r1* expression suggesting, a cytokine-mediated sequelae. Consequently, we predict that unconventional T cells integrate innate cues to control tularaemia-like disease caused by murine Ft LVS infection.

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Detailed Profiling of the Intestinal Microbiome of Children with Cystic Fibrosis Treated with Elexacaftor-Tezacaftor-Ivacaftor

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The intestinal microbiome influences pulmonary outcomes in cystic fibrosis (CF), forming the gut-lung axis. Recently approved small molecule modulators restore function of the pathogenic mutated CFTR channel. The impact of CFTR modulators on the intestinal microbiome remains unclear. Therefore, we investigated the longitudinal intestinal microbiome changes of 39 children with cystic fibrosis treated with the CFTR modulator, elexacaftor-tezacaftor-ivacaftor (ELX/TEZ/IVA). We collected stool samples at 4 timepoints: 2 timepoints before ELX/TEZ/IVA treatment, and 2 after ELX/TEZ/IVA treatment. We used shotgun metagenomic sequencing to uncover the widespread effects of ELX/TEZ/IVA on the intestinal microbiome. We find that microbiome diversity is increased following ELX/TEZ/IVA coupled with a decrease in intestinal carriage of *Staphylococcus aureus*, the predominant respiratory pathogen in children with CF. We further detect reduced abundance of microbiome-encoded antibiotic resistance genes. In all, this study represents the first comprehensive analysis of the intestinal microbiome in children with CF receiving ELX/TEZ/IVA.

Role of Bacteroides in driving colonic hyperproliferation in response to dietary inulin in mice

Pollyana R. Castro, Nicolas G. Shealy, Renan O. Corrêa, Marco Aurélio R, Vinolo, & Mariana X. Byndloss

Dietary soluble fibers, such as inulin, are known for their ability to impact the gut bacterial community, and their consumption has usually been associated with health improvement in mice and humans. Still, in specific circumstances, inulin can promote inflammatory states. How interactions between dietary inulin and the gut microbiota determine a beneficial vs. a detrimental outcome during inulin supplementation is a critical but poorly understood question. Therefore, we proposed to investigate the influence of inulin diet on epithelium homeostasis and how it influences the composition and function of the colon microbiota. For this, mice were fed for 30 days with a diet containing 5% of the insoluble fiber cellulose or the same diet enriched with 10% of the soluble fiber inulin. Using a combination of histochemistry, host cell transcriptomics, 16S microbiome analysis, germ-free, and gnotobiotic mouse models, we analyzed the impact of inulin intake on the colonic epithelium and colon bacteria. A fiber-rich diet containing inulin altered the colon epithelium by increasing the proliferation of intestinal stem cells leading to deeper crypts and longer colons. These effects were dependent on the inulin-modified gut microbiota, as no modulations were observed in animals deprived of microbiota (GF model and antibiotics treatment). Moreover, inulin supplementation caused an expansion of commensal *Bacteroidales* spp. We also demonstrated that *B. ovatus* but not *B. thetaiotaomicron* could grow in inulin-enriched media. These data indicate that *B ovatus* and its metabolites may be involved in inulin-induced hyperproliferation in the colon of mice. Additional analyses will be carried out using gnotobiotic models, untargeted metabolomics and streamlined genetic manipulation of diverse *Bacteroides* to confirm this hypothesis.

Tumor cell secretome induces the expression of the immune checkpoint NKG2A on CD8⁺ T cells

Gabriel J. Rodriguez-Garcia, Henry A. Schares, Brian O. Bachmann, Young J. Kim, Michael J. Korrer, Jeffrey C. Rathmell

PD-1 blockade has been approved for head and neck squamous cell carcinoma (HNSCC) patients. However, only about 20% of HNSCC patients respond to PD-1 blockade. We hypothesize that other inhibitory receptors play a role as a resistance mechanism to PD-1 blockade. Our laboratory and others found that the NKG2A/HLA-E pathway is overexpressed in HNSCC patients. NKG2A is an inhibitory receptor expressed by NK cells and CD8⁺ T cells. How NKG2A expression is regulated on human T cells is not clear. Additionally, we previously identified a significant increase in NKG2A⁺ T cells in the tumors when compared to T cells in matching PBMC samples. Therefore, we hypothesize that the tumor microenvironment is inducing NKG2A's expression. We have identified that tumor conditioned media (TCM) of HNSCC cell lines induce the expression of NKG2A on activated CD8⁺ T cells isolated from PBMCs of healthy donors. In our efforts to identify what factor(s) in the TCM are inducing NKG2A, we boiled the TCM to denature proteins or used filters to exclude molecules larger than 3kDa. However, both boiled and filtered TCM show similar induction of NKG2A. In addition, we further divided the media through hydrophobic fractionation of the filtered TCM and showed NKG2A is induced by the most hydrophobic fraction. Altogether, these findings suggests that NKG2A is being induced by a heat stable small hydrophobic molecule. This is a novel mechanism for tumor immune evasion, in which tumor cells induce NKG2A expression through secretion of small molecules. Our findings can lead to targets for novel cancer therapies or biomarkers for NKG2A blockade response.

Differential Secretion of Extracellular Vesicle-Bound YRNAs in the Allergic Airway

Cherie Saffold, Carleigh Gray, Heather Pua

YRNAs are ~110 nucleotide stem loop noncoding RNAs that form YRNA fragments (YsRNAs) in response to apoptotic and inflammatory stimuli. Intracellularly, full length YRNAs regulate the cellular location and protein-protein interactions of Ro60, an RNA binding protein that coordinates the degradation of misfolded RNA. Recently, YRNAs and YsRNAs have been identified secreted from cells in extracellular vesicles (EVs), which are lipid-delineated particles containing cargo used for cell-cell communication. Published studies have shown that these EV-bound YRNAs can also be differentially secreted in response to inflammatory stimuli. To examine how EV-bound YRNAs function as a communication mechanism during allergic lung inflammation, we tested if murine YRNAs (RNY1 and RNY3) and their YsRNAs are selectively secreted in EVs within the bronchoalveolar lavage fluid (BALF) in a mouse model of asthma. We found that although YRNAs/YsRNAs in BALF are stable when treated with RNAse and Proteinase K, they degrade when treated with detergent and RNase suggesting that they are present in vesicles within this biofluid. In the allergic mouse lung, the RNY1 5' fragment increases in whole BALF. In vesicles purified from allergic BALF, both the RNY1 5' fragment and RNY3 increase, suggesting that YRNAs are differentially packaged into EVs upon allergic airway inflammation. These data contribute to the growing evidence that YRNAs are selectively secreted and are a potential mode of cell-cell communication in inflammatory states.

Diet-induced susceptibility to *S.* Tm-gastroenteritis in a genetically resistant background

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Successful enteric pathogens must overcome microbiota-mediated colonization resistance to expand and cause disease. The bacterial pathogen *Salmonella* Typhimurium (*S*. Tm) elicits gut inflammation to gain access to nutrients that enable *S*. Tm to outcompete the gut microbiota. However, little is known about how dietary changes affect the nutrient pool in the inflamed gut and the host's susceptibility to *S*. Tm infection. I have shown that CBA/J mice fed an L-amino acid (AA) rich diet harbor significantly more *S*. Tm in feces from day one post-infection compared to chow-fed mice. CBA/J mice are genetically resistant to *S*. Tm infection and normally develop *S*. Tm-induced gastroenteritis within 7-10 days post-infection (d.p.i.). However, CBA/J mice fed the AA diet 48hrs before infection developed marked gastroenteritis within 3-5 d.p.i. Ileal and cecal tissue from mice fed an AA diet showed edema, epithelial damage, and immune cell influx to the submucosa, all consistent with *S*. Tm-induced gastroenteritis. Interestingly, the *S*. Tm-induced ileitis phenotype observed only in AA-fed mice resembles features of the human disease, suggesting that this may be an important new animal model to understand human Salmonellosis. 16S rRNA sequencing, bulk RNAseq and untargeted metabolomics revealed significant changes in the gut microbiota composition, transcriptomic, and metabolic landscape of mice fed an AA diet. Specifically, AA diet-fed mice showed substantial depletion of *Lachnospiraceae* and expansion of *Akkermansiaceae* compared to whole protein-fed littermates. Additionally, cecal content from mice fed an AA diet demonstrates distinct amino acid profiles from that of chow-fed animals. (*continue next page*)

One distinction is a marked decreased abundance of tryptophan-derived metabolites, despite a significant enrichment in AA diet-fed mice. Tryptophan-derived metabolites (i.e., indoles) have been shown necessary for the activation of nuclear receptors expressed in the gut epithelia and implicated in controlling the inflammatory tone of the gut microenvironment. Taken together, this data suggests that diet-driven alterations in the metabolic landscape of the gut may alter the microbiota's ability to confer resistance to *S*. Tm, likely through increased inflammatory status. This project will aid in understanding enteric bacterial pathogens by demonstrating a mechanism by which environmental factors overcome genetic resistance to infection.

Host RANKL signaling impacts *Salmonella enterica* subspecies enterica serovar Typhimurium infection outcomes

Clara Si, Christopher Peek, Jenna Petronglo, Juan Barraza, Teresa Torres, Mariana Byndloss, & James Cassat

Although infection by the Gram-negative bacterial pathogen *Salmonella enterica* most commonly presents as infectious gastroenteritis, *Salmonella spp*. can breach the intestinal barrier to cause fatal disseminated disease. A hallmark of systemic salmonellosis is bacterial survival within myeloid-lineage phagocytes, including macrophages and monocytes, during hematogenous seeding of distant organs. Mononuclear phagocytes and their effector mechanisms, such as inflammasome mediated IL-1ß release, are crucial for host anti-bacterial responses. Monocytes are unique in that they can also differentiate into bone-resorbing osteoclasts upon stimulation with the canonical osteoclastogenic cytokine Receptor Activator of Nuclear factor KappaB-Ligand (RANKL). However, bone is not the only milieu where cells encounter RANKL, as RANKL signaling is also vital for antigen-sampling in the gut, secondary lymphoid organ development, and immune cell crosstalk, all of which shape critical host defenses against *Salmonella*. Elucidating how this key tissue homeostatic signaling axis intersects with infectious niches is important for understanding infection outcomes. Using a combination of *in vitro* and *in vivo* approaches, we found that RANKL treatment resulted in a ~50 to 100-fold increase in bacterial burdens during *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S*Tm) infection of murine and human monocytes. Live-cell imaging using *S*Tm fluorescent reporters also demonstrated increased fluorescence in RANKL-treated monocytes. RANKL treatment significantly decreased IL-1ß abundance during *S*Tm infection and exposure to purified *S*Tm inflammasome activators.
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Additionally, RANKL significantly reduced the expression of host genes associated with macrophage effector functions, such as the transcription factor *IRF8*. *In vivo*, anti-RANKL treatment altered *S*Tm dissemination and host responses to infection. Taken together, these data suggest that RANKL signaling disrupts key monocyte effector genes to render cells more susceptible to intracellular bacterial infection; we hypothesize that this is achieved through dysregulated vacuolar trafficking and inflammasome responses. Future work will focus on deriving a mechanism for IL-1ß ablation and extending *in vivo* findings.

Somatic hypermutation occurs among anti-insulin B lymphocytes at the earliest detectable stage of type 1 diabetes in at-risk participants but is dispensable for insulin autoantigen recognition

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Insulin autoantibodies are among the best predictive type 1 diabetes (T1D) biomarkers, yet the molecular basis for B lymphocyte recognition of insulin in humans has not been well-studied. To address this knowledge gap, we isolated n = 29 monoclonal anti-insulin hybridoma lines from the peripheral blood of n = 11 Type 1 Diabetes TrialNet Pathway to Prevention participants who were positive for ≥ 2 islet autoantibodies and thus at high risk for diabetes. These participants were categorized as Stage 1 or Stage 2 based on normal or impaired glucose tolerance test results at the time of blood draw, respectively. Anti-insulin BCRs isolated from these participants expressed diverse variable heavy chain (VH) gene segments, with some VH gene segments occurring in at least three independent lines and at a greater frequency than was present in the unselected BCR repertoire (n = 9218 BCRs). This suggests some VH may hold increased potential to generate insulin-binding BCRs. Only 17% (5/29) of anti-insulin BCRs showed any appreciable somatic hypermutation (>2% mutated), with only one exhibiting >10% mutation. Analysis of these BCRs with respect to participant disease stage showed that 2/5 of the mutated BCRs were isolated from Stage 1 participants, suggesting that mutated anti-insulin BCRs can arise during the earliest detectable stage of pre-symptomatic T1D. Polyreactive binding to other antigens is a common feature of autoreactive BCRs that have been characterized in other autoimmune diseases.
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We found that 60% of insulin-binding BCRs isolated from stage 1 T1D individuals were polyreactive for other antigens, which dropped to 40% in stage 2 T1D participants. BCRs that exhibited insulin-selective binding were also observed, even among germline (non-mutated) BCRs. These data suggest that BCR mutation (and by extension, affinity maturation) is not required for insulin autoantigen recognition in people at high risk for subsequent diabetes development. Future studies will be required to determine whether polyreactivity aids in initial immune tolerance breach and expansion of anti-insulin B lymphocytes early in T1D.

Developing a Genetic Toolkit for Paeniclostridium Sordellii

Anna Smith, Shannon Kordus

Paeniclostridium sordellii are a spore-forming, gram-positive anaerobe known to cause rare and opportunistic but fatal uterine infections globally. Many isolates of P. sordellii from uterine swabs of healthy human patients are non-pathogenic and ubiquitous within the host's microbiome, however pathogenic strains like the clinical isolate ATCC9714 produce a lethal cytotoxin (TcsL) and are usually only identified from patient bodies post-mortem. Currently, there is a growing body of research working to characterize P. sordellii. As such, it is useful to have the ability to create specific gene mutations within the pathogen for use in vitro and in vivo assays and as a control for comparison against wild-type genotypes. While some methods such as targetron insertion, clostron disruption, and site-directed mutagenisis have proven to be successful at generating P. sordellii mutants, there are limitations to the data acquired using these strains when so much of the sequence is abandoned in the genome, and can add to the list of unknown variables that may be affecting the outcome of assays where these mutants are used as controls. Therefore, it is our opinion that there is a need for a reliable system to create complete whole-gene knockdowns, however such a system has not to our knowledge been optimized for P. sordellii. We have chosen to focus our approach by utilizing a series of genetic tools and methods that have been extensively studied and established in other Clostridial species for many years, including inducible-expression systems, fluorescent reporter genes, and eventually, CRISPR Cas9 directed allelic exchange to generate clean knockouts, all by way of plasmid insertion through conjugation using either Escherichia coli or Bacillus subtilis as donor vectors. We have successfully demonstrated that P. sordellii is a willing recipient of our plasmids and are responsive to xylose-inducible and constitutively expressed mScarlet fluorescent protein expression and which can be observed through confocal microscopy.

Activating the cGAS/STING pathway suppresses regulatory T cell function and induces antiviral interferon secretion in a temperature-sensitive manner

Rachael C. Smith, Darren R. Heintzman, Kelsey C. Voss, Channing Chi, Jeff C. Rathmell, John T. Wilson

Vaccines are essential to mitigating the spread and risk of viral infections. However, there is an urgent need for new or improved vaccine adjuvants, as current FDA-approved adjuvants are generally ineffective at inducing T cell-mediated antiviral immune responses. Nucleic acid agonists have emerged as potential adjuvants that stimulate T cell immunity by activating nucleic acid sensors, such as the Stimulator of Interferon Genes (STING), in innate immune cells, which in turn produce antiviral Type I Interferons (IFN-I). Recently, it was identified that STING is also highly expressed in T cells and can be activated by viral infection and cell-stress (*e.g*. fever-range hyperthermia) induced mitochondrial DNA leakage. However, the role of STING in regulatory CD4+ T cells (Tregs) is not well-understood. Furthermore, although temperature increases are a hallmark of inflammatory immune responses, the effects of hyperthermia on STING activation in Tregs remain unknown. Here, we report the STING agonist thio-cGAMP induces a pro-inflammatory phenotype in murine Tregs, which normally exert anti-inflammatory, immunosuppressive functions, at physiological (37°C) and fever-range (39°C) temperatures. Using flow cytometry, we found thio-cGAMP treated Tregs displayed significantly decreased viability and expression of Treg markers CD25 and FoxP3, immunosuppressive cytokine TGFβ, and inhibitory immune checkpoint protein CTLA-4. Surprisingly, thio-cGAMP treated Tregs also secreted high levels of IFN-I, suggesting that STING can promote antiviral responses by Tregs.
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Additionally, hyperthermia consistently and significantly enhanced the pro-inflammatory phenotype when combined with thio-cGAMP. In summary, these data provide the first evidence that STING agonists can induce IFN-I production by Tregs, suppress their anti-inflammatory phenotype, and that these effects are potentiated by hyperthermia. Thus, this study motivates further investigation of regulatory T cell function in fever-range hyperthermia and suggests the potential for Treg-targeted STING agonists as a method to improve antiviral T cell responses.

A cross-reactive monoclonal antibody lineage targeting the neuraminidase active site following heterologous influenza exposure

Ty A Sornberger, Seth J Zost, Rachael M Wolters, Luke Myers, Elaine C Chen, Robert H Carnahan, & James E Crowe Jr

Influenza is a viral respiratory disease that affects more than 1 billion people annually. Current vaccines provide protective immunity by inducing immune responses to the major surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA). These proteins are responsible for functions, such as attachment and fusion of the virion and host cell, migration through mucus, and egress from the infected cell. Current FDA-approved influenza antivirals, such as oseltamivir, inhibit NA enzymatic activity; however, their efficacy is inconsistent across influenza subtypes. Monoclonal antibodies (mAbs) that target NA provide an alternative approach to preventing and treating influenza infection. Here, we describe an antibody lineage that targets the IAV NA glycoprotein and exhibits cross-reactive NA binding between subtypes. Using 10X sequencing and microscale expression of paired heavy and light chain sequences, antibody clonotypes were determined to be IAV NA-specific. Enzyme-linked immunosorbent assays (ELISAs) of recombinantly expressed mAbs to recombinant IAV NA antigen – derived from H3N2 (rN2) and H7N9 (rN9) – show that most antibodies of this lineage bind rN2 with some also binding rN9. The introduction of an N-linked glycosylation site at position 245 of H3N2 NA during the 2014-2015 influenza season resulted in a wide decrease in binding by mAbs of this lineage. Structural biology studies have shown that this glycan overlaps with the NA active site, suggesting that mAbs of this lineage likely target the NA active site. In summary, this study identifies an antibody lineage with potent binding to the neuraminidase active site that may be harnessed for antiviral therapies. The presence of heterosubtypic mAbs can also inform vaccine design by targeting epitopes conversed between influenza A virus subtypes.

Sepsis Alters the T Cell Immunometabolic Landscape in Critically Ill Patients

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Sepsis is a dysregulated host response to infection that precipitates systemic multiorgan injury. There are 1.1 million incident cases of sepsis and over 200,000 associated deaths annually in the United States alone. Despite this, no targeted therapies exist for the treatment of sepsis beyond antibiotics and procedural source control for the inciting infection and supportive care for the individual organ failures. Severe sepsis requiring admission to the intensive care unit (ICU) is associated with both aberrant hyperactive immunologic pathways and concurrent profound cell-mediated immunosuppression, the latter of which is associated with complications including secondary infections and ineffectual organ recovery that account for substantial morbidity and mortality. Herein, we aim to determine the immunometabolic abnormalities associated with the imbalance of regulatory and effector pro-inflammatory T cells in sepsis. Peripheral blood mononuclear cells were isolated from septic and non-septic critically ill patients admitted to the Medical and Surgical ICUs at Vanderbilt University Medical Center. CD4+ and CD8+ T cell subsets were enumerated by flow cytometry and evaluated for functional markers and metabolic dependencies via SCENITH, a single cell functional energetic assay. We identified that CD4+ T regulatory cells (Tregs) from septic patients have increased overall metabolic activity, less mitochondrial dependence, more glycolytic capacity, and an increased suppressive phenotype with higher PD-1 expression. Concurrently, we observed high levels of mitochondrial dependence in CD8+ effector T cells in all critically ill patients but uniquely identified reduced fatty acid and amino acid oxidative capacity in these cells in the context of sepsis.
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This correlated with reduced expression of the activation marker CD25 on CD8+ effector T cells in septic patients. Collectively, these data identify key immunometabolic alterations in regulatory and effector T cell subsets that may be both etiologic and pharmacologically targetable in reversing the pathologic suppression of cell-mediated immunity in life-threatening sepsis.

A potential RNA pyrophosphohydrolase impacts zinc homeostasis in *Acinetobacter baumannii*

Kacie A. Traina, Jeanette M. Critchlow, Erin R. Green, & Eric P. Skaar

Acinetobacter baumannii is a Gram-negative, opportunistic human pathogen and a leading cause of ventilator-associated pneumonia. Multi-drug resistant *A. baumannii* infections are becoming increasingly prevalent, reinforcing the need for new therapeutic development. To colonize and survive in the host, *A. baumannii* must acquire essential nutrient metals, including zinc (Zn). The vertebrate host actively sequesters these nutrient metals to defend against infection in a process called "nutritional immunity." *A. baumannii* employs specific mechanisms to combat Zn dysregulation. To better understand Zn stress in *A. baumannii*, a transposon-sequencing (Tn-seq) screen was performed to profile the fitness of a WT *A. baumannii* ATCC 17978 library in Zn deplete, replete, and excess environments. A mutant in A1S_0414 (*Δ0414*) was selected against in high Zn, suggesting A1S_0414 plays an important role in maintaining Zn homeostasis. A1S_0414 is a predicted RNA pyrophosphohydrolase (RppH), an important enzyme in bacterial mRNA degradation. While RppHs have been preliminary studied in other bacterial species, no RppH has been discovered yet in *A. baumannii*. The role of RppH in Zn homeostasis in any bacterial species also remains unknown. Through experiments with a marked deletion strain (*Δ0414*), we confirmed sensitivity to Zn toxic conditions, as seen in the Tn-seq. Complementation experiments of *Δ0414* mirrored WT growth curves, demonstrating that A1S_0414 is important in Zn toxic environments. In a vertebrate host, bacterial pathogens can encounter both Zn deplete and Zn toxic environments.

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To assess the role of A1S_0414 in pathogenicity, a murine pneumonia model was utilized to show that infections with a *Δ0414* strain resulted in a significant decrease in bacterial burden in the lungs as compared to WT *A. baumannii*. Ongoing work focuses on determining the role of A14_0414 in maintaining proper Zn regulation. Defining mechanisms by which *A. baumannii* maintains Zn homeostasis, possibly through A1S_0414, could identify new antimicrobial targets.

Role of the CagY (VirB10) Antenna Projection in *Helicobacter pylori* Cag Type IV Secretion System Activity

Sirena C. Tran, Mark S. McClain, Timothy L. Cover

Helicobacter pylori strains containing the cag pathogenicity island are more likely to cause gastric adenocarcinoma or peptic ulcer disease than strains that lack this genetic element. The *cag* PAI encodes an effector protein (CagA, designated as a bacterial oncoprotein) and a type IV secretion system (Cag T4SS). Within the Cag T4SS outer membrane core complex (OMCC), 14 CagY (VirB10) proteins assemble into a ring-like structure known as the antenna region, which is predicted to span the outer membrane. Similar VirB10 antenna regions are present in T4SS outer membrane complexes from multiple bacterial species. Individual CagY components of the antenna region contain two α-helices connected by an unstructured loop, and the entire antenna region sequence is highly conserved among *H. pylori* strains. In this study, we investigated the role of the CagY antenna region in OMCC assembly and Cag T4SS function. An *H. pylori* mutant strain with deletion of the entire CagY antenna projection (ΔAP) retained the capacity to produce CagY and assemble an outer membrane core complex, but this mutant lacked T4SS activity (CagA translocation and IL-8 induction in AGS gastric cells). In contrast, a mutant strain with Gly-Ser substitutions in the unstructured CagY AP loop retained Cag T4SS activity. Mutants containing CagY antenna loops with shortened lengths were defective in CagA translocation and exhibited reduced IL8-inducing activity compared to control strains. These data indicate that the CagY AP region is required for Cag T4SS activity, but not for the assembly of the Cag T4SS outer membrane core complex.

Uncovering Molecular Heterogeneity in *S. aureus* Abscesses with Multimodal Molecular Imaging

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Staphylococcus aureus (S. aureus) is the leading cause of death associated with bacterial infections and is most often associated with formation of soft tissue abscesses. These abscesses have a complex organization forming the interface between bacteria and the host immune response. Bacteria group together at the center of the abscess in staphylococcal abscess communities (SACs). SACs are surrounded by both viable and necrotic neutrophils. As the infection progresses, the abscess is encapsulated by fibrin deposits to limit further tissue damage. Characterization of the host-pathogen interactions in *S. aureus* abscesses is critical to the development of preventative measures and therapeutics, but conventional microscopic assays provide limited information about the molecular composition of the abscess. Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) is a sensitive, label-free, highly multiplexed technology for mapping biomolecules *in situ*. Advancements in spatial resolution for MALDI IMS have enabled the correlation of these molecular signatures to specific tissue structures and cell types allowing for more in-depth analysis of abscess pathology. Histological staining provides complementary information about cellular and morphological organization in and around the abscess, such as identification of viable and necrotic neutrophils and fibrosis. By combining stained microscopy with MALDI IMS, these data reveal trends in molecular distributions as they relate to changes in host cellular neighborhoods due to infection.
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Using this approach, we have identified a variety of phospholipid classes including both odd and even chain saturated cardiolipin and lysyl-phosphatidylglycerol species with distinct localizations in the SACs, as well as phosphatidylinositol and phosphatidylserine species which localize to regions containing neutrophils. These lipids play important roles in both host immune signaling pathways as well as in *S. aureus* defenses. Understanding the heterogeneity of lipid profiles between abscesses with different host cellular neighborhoods is essential to building a more detailed molecular model of *S. aureus* abscess progression.

Epithelial, stromal, and microbial remodeling in the *Tnf*$^{\Delta ARE}$ mouse model of Crohn's-like intestinal inflammation

Paige N. Vega, Alan J. Simmons, Jiawei Wang, Yanwen Xu, Yilin Yang, James Ro, Amrita Banerjee, Mariah H. Harned, Qi Liu, & Ken S. Lau

Crohn's disease is characterized by chronic inflammation of the gastrointestinal tract and can be modeled by TNF overexpression in *Tnf*$^{\Delta ARE/+}$ mice. We use droplet-based single-cell RNA-sequencing to profile epithelial and stromal cells in wildtype and *Tnf*$^{\Delta ARE/+}$ mice with mild to severe ileal and colonic inflammation. We found an emergence of innate immune cell types and transcriptomic changes in lymphocytes in both stages of inflammation in the ilea and colon of *Tnf*$^{\Delta ARE/+}$ mice. Surprisingly, we identified a loss of normal fibroblast populations and an emergence of pro-inflammatory fibroblasts in *Tnf*$^{\Delta ARE/+}$ mice at both stages of inflammation. Absorptive enterocytes, but not colonocytes, in *Tnf*$^{\Delta ARE/+}$ with severe inflammation displayed major transcriptomic changes when compared to normal or *Tnf*$^{\Delta ARE/+}$ mice with mild inflammation. Enterocyte transcriptomic changes reflected a switch from normal absorptive function toward an upregulation of inflammatory response genes. Shotgun DNA sequencing on intestinal lavages from the ileum and colon revealed increased abundance of the obligate intracellular bacterium *Chlamydia muridarum* in *Tnf*$^{\Delta ARE/+}$ mice compared to wildtype cagemates. While ileitis is spontaneous, colonic inflammation was dependent on the housing facility, where "clean" facility mice were free of colonic inflammation. We transferred and co-housed mice from the "clean" facility with those in the "dirty" facility and found *Tnf*$^{\Delta ARE/+}$ mice developed colitis, suggesting that TNF-induced colitis is microbiome-dependent.

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We provide a full characterization of cell types in inflamed ilea and colon from the *Tnf*$^{\Delta ARE/+}$ model of Crohn's-like disease, and moreover, identify cell types and microbes that are involved in chronic inflammation. Future studies aim to reveal the epithelial cell type that harbors *Chlamydia muridarum* and the role of Paneth cells and their antimicrobial products in persistent infection. Revealing mechanisms by which host cell-cell and host-microbe interactions drive intestinal inflammation is critical for understanding Crohn's disease and will lead to novel therapeutic targets.

Pediatric SARS-CoV-2 antibodies exhibit broad neutralization and belong to adult public clonotypes

Steven C. Wall, Naveenchandra Suryadevara, Changil Kim, Andrea R. Shiakolas, Clint M. Holt, Emma B. Irbe, Perry T. Wasdin, Yukthi P. Suresh, Elaine C. Chen, Seth J. Zost, Elizabeth Canfield, James E. Crowe Jr, Mary Ann Thompson-Arildsen, Daniel J. Sheward, Robert H. Carnahan, and Ivelin S. Georgiev

From the beginning of the COVID-19 pandemic, children have exhibited different susceptibility to SARS-CoV-2 infection, reinfection, and disease, compared to adults, with factors such as prior seasonal coronavirus exposure, innate immunity, and longevity of antigen-specific immune responses being implicated. Neutralizing antibodies are associated with protection against SARS-CoV-2 and are therefore an important variable to consider when evaluating differences in protection. Yet there is currently limited information about the types and specificities of neutralizing antibodies in children, with studies generally limited to assessment of polyclonal responses. Here we assess SARS-CoV-2-specific antibody repertoires in a pediatric cohort, using the LIBRA-seq single-cell sequencing technology. Our results show that neutralizing antibodies in children possess similar genetic features compared to antibodies identified in adults, with multiple pediatric antibodies belonging to previously established public antibody clonotypes in adults. Notably, pediatric-derived antibodies showed potent neutralization of currently circulating SARS-CoV-2 variants, including BA.4.6, BA.2.75.2, BQ.1.1, XBB, and XBB.1.5, that have cumulatively resulted in resistance to virtually all approved monoclonal antibody therapeutics. Our results show that children can rely on similar SARS-CoV-2 antibody neutralization mechanisms compared to adults, and are an underutilized source for the discovery of effective antibody therapeutics to counteract the ever-evolving pandemic.

A Message from the Director

Thank you for joining us for our first in-person VI4 Annual Symposium since 2019. This is a day full of exciting talks, learning, and community that highlights the outstanding research and training that happens within VI4. We are grateful for the support you have shown VI4 over the past four years when we held our symposium virtually, and we are glad to be back together again.

We invite you to check out our website to learn more about other upcoming events and opportunities to connect with the VI4 community. Thank you to all of our partners, who helped us put together a world-class symposium!

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