

**Ohio Center for Innovative
Immunosuppressive Therapeutics**

Research Forum



December 15-16, 2011
Park Inn Hotel
Toledo, Ohio

Program

Thursday, December 15

1:00 p.m. Introduction – Akira Takashima (UT) – Location: Ballroom #1

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- 1:50 p.m. **David Soler (CWRU)**
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- 2:10 p.m. **Bryan Doreian (CWRU)**
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Characterization of Macrophage Response to Ultraviolet Radiation in the Development of Human Malignant Melanoma

Petukhova, Tatyana A; McCormick, Thomas S; Gerstenblith, Meg R; Bordeaux, Jeremy S; Kim, Julian; Honda, Kord S; Cooper, Kevin D.

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Epidemiological studies have shown that solar ultraviolet radiation (UVR) is a major risk factor for developing melanoma. The mechanism of UVR-initiated melanomagenesis is complex and has been explored in a new murine model that permits for fluorescence-aided melanocyte imaging and isolation following *in vivo* UV irradiation. In the mouse, production of interferon gamma (IFN- γ) by macrophages recruited to neonatal skin post UVB irradiation enhanced melanoma growth, and blockade of IFN- γ abolished this effect. The phenomenon of macrophage recruitment to human dermis and epidermis in response to UVR has been described, but IFN- γ production and melanocyte activation has not been previously investigated. Thus, the proposed pilot study aims to translate the experimental findings from the murine model to human subjects. In order to investigate whether IFN- γ -produced by macrophages is anti-apoptotic to human melanocytes, we plan to quantify these factors in human subjects in a two-part project.

In a case-control study of 15 healthy volunteers and 15 patients with a personal history of melanoma, we plan to compare their immunologic skin response to UVR. Biopsy specimens of sunburned human skin will be microscopically analyzed with immunofluorescence and laser capture microdissection for the presence of macrophage IFN- γ production and melanocyte activation. This part of the study aims to test the assumption that the inflammatory cytokine response of high-risk patients for melanoma development will bear a similar protumorigenic signature to the melanoma mouse model. Additionally, a case-series study of 30 primary malignant melanoma tissue samples will be analyzed with previously established laboratory techniques for macrophage infiltration, co-localized IFN- γ production, and quantitative RT-PCR for IFN- γ RNA.

The long-term benefit of these studies will be to provide quantitative data about the effects UVR on pro-tumorigenic effects of IFN- γ , and therefore to potentially build a rationale for future studies in the prevention, detection, or treatment of human malignant melanoma.

***In vivo* Response of Sunscreen-protected Skin to Ultraviolet Light; a Proteomics-based Investigation**

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Background: Ultraviolet radiation (UVR) exposure on human skin causes cellular and molecular changes that lead to immunosuppression, DNA and tissue damage. Regular use of sunscreens is one of the methods commonly employed to prevent these UV-induced events.

Objective: The aim of our study was to use a proteomics-based analysis to determine whether application of a sunprotective agent on human skin *in vivo* can alter protein expression changes that occur after ultraviolet irradiation.

Methods: Twelve healthy participants were enrolled in the study. Their ages and Fitzpatrick skin type ranged from 19 to 53 and I-IV respectively. The minimal erythema dose (MED) for each person was calculated according to COLIPA recommendations. Four 2.5cm² areas of unexposed skin were selected and treated as follows: 1) normal control, 2) 1 MED-irradiated, 3) sunscreen control, and 4) sunscreen plus 1 MED. A commercially available product with a sunprotection factor (SPF) of 25 was used for skin sites 3 and 4. Simulated solar radiation (SSR) was used to deliver 1 MED to sites 2 and 4. A minimally invasive suction blister technique was used to harvest the epidermal tissue from each section before processing it for protein extraction. Epidermal cell proteome was separated using a gradient SDS PAGE-based approach followed by a label free quantitation of differentially expressed proteins using chromatographic peak volume compilations from the LC-MS/MS data.

Results: A total of 1930 proteins were successfully quantitated based on 8378 peptides. Using a 2-way factorial statistical analysis, a total of 365 proteins were found to be differentially regulated and having an interaction effect due to the sunscreen application on Control vs MED-treated patient samples. Our data confirms the significant roles of Stat3, PyCard, XAF1, HIST2H2BF, CLTC, KRT2, EPPKI and MYH9 as some of the proteins that are most regulated by the sunscreen protective effect. A dominant theme of the sunscreen effect is the downregulation of several acute-response signaling pathways. Protein-protein interaction analysis of the protein expression data using the Ingenuity Pathway Analysis (IPA) software reveals the suppression of Caspase and nF kappa B pathways suggesting an elevated antiapoptotic and anti-inflammatory cellular response due to the protective sunscreen effect following the acute UV insult.

Conclusion: Gel-based fractionation approach was very useful in mining the epidermal tissue proteome in spite of the overwhelming keratin amounts. State of the art LC-MS/MS methodology has been successfully implemented to perform differential proteomics in this study. Our findings provide mechanistic input to interpret some protective mechanisms of sunscreen application that reverses the known harmful effects of UV radiation. Further efforts are on to obtain mechanistic granularity in relation to the protein-protein interactions.

9-Cis Retinoic Acid is Successful in Curing Recalcitrant Cheiropompholyx in Human Skin

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Pompholyx is a common chronic dermatitis of the palms and soles that may lead to a significant impairment of occupation and quality of life. This skin condition is characterized by the onset of small blisters in the hands and feet which follow recurrent cycles of healing and eruptions. We present the case of a recalcitrant type of cheiropompholyx affecting exclusively the hands in a 33 year old male patient. Four years after its initial onset, pompholyx blisters went into remission after treatment with the pan-retinoid agonist 9-cis Retinoic Acid -a retinoic acid stereoisomer commonly known as Alitretinoin. During a 12 weeks-long treatment with a daily oral dose of 30 mg, the patient's laboratory values remained steady (Cholesterol 147, Trygcerides 56mg/dL, LDL 100mg/dL) and he suffered no side-effects from the treatment. The authors' findings suggest that Alitretinoin is an effective drug in treating chronic forms of Pompholyx that are resistant to other conventional topical therapies.

The Combination of Statins and PPAR γ Ligands Generates Hyper-Inflammatory Macrophages Mediating Tissue Destruction

Doreian BW, Rosenjack J, Lu KQ

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Polypharmacy is common in the elderly and amongst patients with chronic diseases, increasing the risk of adverse side effects due to unpredictable drug-drug interactions. In chronic metabolic diseases such as diabetes, treatment regimens often include glitazones for glycemic control and statins for prevention of cardiovascular disease. Both classes of drugs independently have anti-inflammatory actions, which may be beneficial for the treatment of the chronic underlying inflammation associated with diabetes. Here we report in this work that co-treatment with mevastatin and rosiglitazone generates a paradoxical hyper-inflammatory response in macrophages resulting in high levels of iNOS, TNF- α , and IL-1 β expression *in vitro* and *in vivo*. This induction was dependent upon both PPAR γ signaling via SOCS3 to inhibit STAT3 and upregulation of TNF- α signaling in a feed forward mechanism. Systemic co-administration of statin and rosiglitazone in mice results in *de novo* tissue destruction and delayed wound healing (21 days vs. 14 days in control). Treatment with an iNOS inhibitor restored normal wound healing. In summary, the presence of two medications with known anti-inflammatory properties resulted in paradoxical increased inflammation and tissue destruction.

Hyper-inflammatory Macrophages Promote Phagolysosome Formation and *M. tuberculosis* Clearance

Rosenjack J, Doreian, BW, Lu KQ

Department of Dermatology, Case Western Reserve University, Cleveland, OH, USA

Macrophages utilize phagocytosis as the first step for the elimination of microorganisms; however, phagolysosome fusion must occur for subsequent destruction of pathogens. The pathogen *Mycobacterium tuberculosis*, the main causative agent of tuberculosis, is able to evade degradation and clearance in macrophages by preventing phagolysosome formation. Additionally, infection of macrophages with *M. tuberculosis* downregulates their ability to generate pro-inflammatory factors, further restricting clearance of the pathogen. Our lab has generated a macrophage that expresses hyper-elevated levels of pro-inflammatory factors iNOS and TNF α , via a combination treatment with statin and rosiglitazone. As infection with *M. tuberculosis* overrides pro-inflammatory response of macrophages, we hypothesized that our hyper-inflammatory macrophage would reduce the ability of *M. tuberculosis* to resist phagolysosome formation. Mouse and human macrophages were infected with GFP-labeled *M. bovis* BCG and then received the combination treatment of mevastatin and rosiglitazone. Confocal microscopy demonstrated dramatic increase in colocalization of lysosomes with *M. bovis* BCG in mouse macrophages treated with both mevastatin and rosiglitazone compared to control (n=1). Macrophage aggregates resembling early granuloma formation were only present in the mevastatin and rosiglitazone treatment, signifying a strong physiological response of macrophages toward bacterial invasion and subsequent clearance. Similar increases in lysosome/ BCG colocalization and aggregation occurred in human macrophages (n=3). Human macrophages treated with mevastatin and rosiglitazone exhibited 66.10% colocalization compared to 31.67% in untreated macrophages (n=1). To determine if processing is mediated by iNOS, a specific iNOS inhibitor was given to BCG-infected macrophages treated with mevastatin and rosiglitazone. Treatment with iNOS inhibitor resulted in a 3-fold decrease in lysosome/BCG colocalization and little aggregation was observed. These findings suggest that our hyper-inflammatory macrophage is able to compensate for the downregulation of pro-inflammatory factors upon *M. tuberculosis* infection, thereby decreasing the ability of *M. tuberculosis* to override phagolysosome formation.

Vitamin D Regulates Inflammation in Monocyte/Macrophage

L Au, B Doreian, J Rosenjack, J Stuckert, K Lu

Case Western Reserve University, Department of Chemical Engineering, Dermatology Department

The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}_3$; calcitriol) plays a role as an important immune response regulator during inflammation. Recently, macrophages were demonstrated to be able to convert the oral form of vitamin D (25(OH)D) to calcitriol to help balance and regulate inflammation. In previous studies, we demonstrated that generation of hyper-inflammatory macrophages expressing high levels of tumor necrosis factor- α (TNF- α) and inducible nitric oxide synthase (iNOS) levels resulted in tissue destruction and delayed wound healing. Based on the immunosuppressive effects of vitamin D, we hypothesized that treatment of hyper-inflammatory macrophages with vitamin D would suppress hyper-inflammatory response and prevents tissue destruction. We report here that treatment of hyper-inflammatory macrophages with vitamin D prevented upregulation of pro-inflammatory factors *in vitro* (TNF- α and iNOS). Treatment of hyper-inflammatory murine macrophages prior to or after stimulation suppressed iNOS and TNF- α expression. Similarly, we observed the treatment of human monocyte-derived macrophages (MDMs) with vitamin D suppressed TNF- α expression. To test the immunosuppressive effects of vitamin D *in vivo*, hyper-inflammatory mice (that display macrophage-mediated tissue destruction and delayed wound healing) were treated systemically with calcitriol or 25(OH)D. Mice treated with vitamin D were protected from hyper-inflammatory mediated effects. Furthermore, treatment of VDR-/- mice with vitamin D did not suppress the hyper-inflammatory response suggesting that the immunosuppressive actions of calcitriol and 25(OH)D were via the vitamin D receptor and not an off target effect. Taken together, metabolism of vitamin D by macrophages helps regulate inflammation suppressing pro-inflammatory cytokine production, and may have a therapeutic benefit in conditions of chronic or exacerbated inflammation.

Hyper-inflammatory Macrophages Exhibit Robust Melanoma Cytotoxicity In Vitro and Resist the Anti-inflammatory Effects of TGF- β

J Stuckert, L Au, J Rosenjack, B Doreian, K Lu

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Various populations of macrophages may exist in the melanoma tumor micro-environment including: tumor associated macrophages (TAMs) that promote angiogenesis and tumor growth, classically activated pro-inflammatory tumoricidal macrophages and immune-regulatory Foxp3+ macrophages that are critical during the early phases of melanoma tumorigenesis. Both melanoma cells and TAMs secrete transforming growth factor beta (TGF- β) that promotes the conversion of macrophages to the immune regulatory phenotype. Our laboratory has generated highly-polarized hyper-inflammatory macrophages that display remarkable cytotoxicity against murine B16 melanoma *in vitro*. The observed B16 cytotoxicity suggests that macrophages with a hyper-inflammatory phenotype can resist conversion to a TGF- β -induced tumor-promoting phenotype. Murine B16 melanoma cells were co-cultured for forty-eight hours with hyper-inflammatory macrophages at a 4:1 effector to target ratio. Most remarkable was the significant reduction in B16 cells despite being allowed to form a well-established mono-layer for twenty four hours prior to the addition of hyper-inflammatory macrophages. There was no noticeable reduction in the B16 melanoma cells when co-cultured with wild-type macrophages. Our data show that TGF- β reduces but does not abolish the hyper-expression of pro-inflammatory mediators of hyper-inflammatory macrophages under a number of scenarios including: *in vitro* TGF- β treatment of murine and human hyper-inflammatory macrophages and *in vivo* TGF- β treatment of murine hyper-inflammatory macrophages. Given these observations, the induction of hyper-inflammatory macrophages will be beneficial against the development of melanoma because of their robust cytotoxic effects and resistance to the effects of TGF- β . We envision that our research may provide the foundation to improve the care of melanoma patients by pharmacologically inducing macrophages into a tumoricidal phenotype or preventing the expression of a tumor promoting phenotype.

Regulation and Versatility of Integrative Conjugating Elements in *Legionella pneumophila*

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L. pneumophila is an intracellular pathogen of lung macrophages and the agent of Legionnaire's disease. Many unique strains of *L. pneumophila* can survive in hospital water systems and cooling towers and are responsible for several pneumonia outbreaks around the world. *L. pneumophila* isolates exhibit significant genetic diversity, with only 70% of genes conserved in all six sequenced strains. Specifically, a family of type IV secretion systems likely contributes to the versatility of *L. pneumophila*. In addition to the canonical Dot/Icm system, three putative type IV secretion systems are encoded on genomic islands with features typical of mobile genomic elements that transfer between bacteria. Bioinformatic analysis revealed each accessory T4SS contains structural elements similar to that of integrating conjugative elements (ICE), a type of transposon that can excise autonomously from the chromosome and induce its own conjugative transfer. A PCR-based assay established that each element can exist in both episomal and chromosomal forms, confirming the elements' mobility. A qPCR assay analyzing template DNA prepared from cultures at different growth stages suggested that the mobility of two ICEs is regulated in response to metabolic cues. Ongoing work will assess conjugation potential and phenotypic traits conferred by these islands. We postulate that mobility and horizontal transmission of ICE elements contributes to diversity, versatility, and pathogenicity of *L. pneumophila*. A better understanding of ICE mobility can inform strategies to prevent the spread among bacterial pathogens of advantageous traits, such as antibiotic resistance.

Delineation of *Borrelia burgdorferi* Colonization and Dissemination Characteristics Within Murine Skin via Intravital Microscopy

J.P. Lavik, V. Shukla, A.L. Nestor-Kalinowski, R.M. Wooten

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Lyme disease is caused by *Borrelia burgdorferi* (Bb), a tick-transmitted spirochete, which remains at the skin inoculation site for ~48h before disseminating and initiating disease. Possessing many surface agonists, Bb predictably elicits efficient clearance by phagocytes *in vitro*. However, efficient immunoevasion occurs *in vivo* with an ID₅₀ ≤50 organisms. Because current *in vitro* models appear inadequate for delineating natural Bb pathogenesis, we have developed a confocal microscopy-based approach allowing *in vivo* assessment of host-pathogen interactions critical to disease development. Here, we visualize fluorescent Bb within the skin of anesthetized transgenic mice and collect 4-D data depicting spirochete and immune cell behavior. By 4h post-inoculation, most spirochetes at the entry site are immobile and fragmented. However, surviving spirochetes preferentially disseminate through dermal tissues at rates 40- to 300-times faster than local immune cells. These host cells appear to infiltrate the infection site in a manner characteristic of their cell type and studies to delineate the kinetics of this phenomenon are described. Our findings suggest rapid Bb motility is critical for immunoevasion and dissemination. We anticipate that expansion of our imaging model will allow true delineation of host-Bb interactions vital for Lyme disease progression, which may subsequently be targeted for curative therapies.

Delineating Murine Macrophage Receptors and Signaling Pathways Involved in *Borrelia burgdorferi*-Elicitation of IL-10

N. Zhang, R. M. Wooten.

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Lyme disease, caused by the spirochetal bacterium *Borrelia burgdorferi*, is the most common vector-borne disease in the United States and Europe. *B. burgdorferi* is an extracellular bacterium transmitted into the skin by an infected-*Ixodes* tick bite. Despite eliciting a strong, predominantly TLR2-mediated, inflammatory/immune response, *B. burgdorferi* persists within host tissues for extensive periods of time post-infection and periodically re-emerges to cause disease. One of the important reasons that *B. burgdorferi* evades host immune clearance is that it rapidly elicits high levels of IL-10, an anti-inflammatory cytokine, which suppresses the host innate immune response critical for efficient *B. burgdorferi* clearance. So far, the IL-10^{-/-} mouse line is the only murine model that shows significantly enhanced clearance of *B. burgdorferi*. However, the mechanisms of how *B. burgdorferi* elicits this dysregulated IL-10 response are largely unknown. Macrophages reside in skin tissues where *B. burgdorferi* is initially transmitted into the host, and they secrete rapid and high levels of IL-10 upon *B. burgdorferi* stimulation. The goal of this *in vitro* study is to elucidate 1) which surface receptor; 2) which intracellular signaling pathways; and 3) which transcription factors are involved in this Bb-elicited IL-10 response by macrophages.

To address this, primary macrophages were expanded from the bone marrow of wild-type and TLR2^{-/-} mice and used for all *in vitro* analyses. The inhibitors LY294002, SB203580 and BAY11-7082 were used to block the PI3K, p38 MAPK, and NF-κB signaling pathways, respectively. Inhibition was determined by Western blotting and fluorescence microscopy. Transcript and protein levels of cytokines and chemokines were assessed by qRT-PCR and ELISA, respectively.

Upon *B. burgdorferi* stimulation, IL-10 levels were significantly decreased in TLR2^{-/-} macrophages compared to wild-type. Activation of PI3K and p38 MAPK signaling pathways were substantially decreased in TLR2^{-/-} macrophages compared to wild-type. *B. burgdorferi*-induced IL-10 was suppressed in the presence of inhibitors against PI3K and p38 MAPK without affecting the levels of most proinflammatory mediators (IL-6, IL-12, MCP-1 and KC), and with varied effects on nitric oxide production; however, TNF-α levels were suppressed by both inhibitors. Inhibition of NF-κB resulted in the downregulation of IL-10 and all other pro-inflammatory mediators assessed, including TNF-α, IL-6, IL-12 and nitric oxide.

The detrimental IL-10 induction by macrophages in response to *B. burgdorferi* is mediated by surface receptor TLR2, and can be suppressed by blocking activations of PI3K, p38 MAPK and NF-κB signaling pathways. However, most pro-inflammatory mediators believed important for controlling *B. burgdorferi* infection were not affected by blocking PI3K and p38 MAPK. Thus, these pathways may provide targets for IL-10-blocking curative therapies for Lyme disease.

Exogenous Lipopolysaccharide Stimulation Promotes Enhanced Macrophage Clearance of *Burkholderia pseudomallei*

M. P. Bechill, R. M. Wooten

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Burkholderia pseudomallei (Bp), the etiological agent of melioidosis, is responsible for roughly 40% of septicemic mortality within endemic regions. The LD₅₀ via inhalation is ≤ 10 organisms, making it a putative bioweapon for which no vaccine is available. Bp is adept at growing and persisting within macrophages, though little is known regarding how it evades clearance. We hypothesize that Bp lipopolysaccharide (LPS) inadequately activates macrophages, thus allowing evasion of intracellular clearance. Our *in vitro* data indicates that macrophage pre-stimulation with a prototypic LPS and/or IFN γ can elicit enhanced and synergistic intracellular clearance. To better understand these protective mechanisms, a dose response comparison of LPS from different bacterial species indicated that *Burkholderia* LPS induces significantly less mitochondrial activity, but similar direct toxic effects on macrophages as prototypic LPS species. Pre-stimulation with prototypic LPS species had no effect on bacterial uptake by macrophages, but did significantly enhance subsequent intracellular clearance of Bp. ELISA analyses indicated that prototypic LPS elicits a substantial upregulation of certain soluble mediators associated with protective responses to melioidosis, such as TNF α , IL-12, and nitric oxide. However, supernatant transfer studies were unable to confer any protective effects to macrophages subsequently infected with Bp. Our current studies are continuing to delineate the immune mechanisms associated with activation via protective” LPS-species versus the relatively non-protective Bp LPS.

The Role of Serum Opsonization in Clearance of *Burkholderia pseudomallei* by Macrophages

Minal Mulye, R. M. Wooten

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The encapsulated Gram-negative bacterium *Burkholderia pseudomallei* (*Bp*) causes melioidosis, a major cause of severe sepsis in endemic regions, with mortality rates $\geq 40\%$. Due to its high infectivity through the aerosol route and lack of effective therapies, *Bp* has been given select agent status. Our goal is to identify mechanisms by which *Bp* evades intracellular clearance by murine macrophages through comparative *in vitro* studies with *Burkholderia thailandensis* (*Bt*), a closely related but relatively avirulent acapsular species. Our preliminary studies suggest that macrophages are inherently unable to clear either *Bp* or *Bt*. Hence we hypothesize that *Bp* is better able to resist complement deposition on its surface, resulting in inadequate macrophage activation and clearance compared to *Bt*. Our data indicates that significantly greater levels of C3 are deposited on the surface of *Bt* compared to *Bp*, but these levels do not promote complement-mediated direct killing in either strain. C3 opsonization does lead to enhanced uptake of both strains, but does not enhance their intracellular clearance by macrophages. Uptake of serum opsonized bacteria does not produce a synergistic effect with interferon- γ or FcR-mediated activation to enhance clearance compared to non-opsonized bacteria. Interestingly, macrophage uptake of unopsonized *Bt* produced a significant enhancement of multiple proinflammatory mediators compared to unopsonized *Bp*, and opsonization of both bacteria caused a substantial enhancement of these mediators compared to unopsonized *Bp*. Overall, these data suggest that complement opsonization can promote clearance of *Burkholderia* species by enhancing the production of inflammatory mediators by macrophages, thus recruiting appropriate immune cells to the site of infection and activating them to clear persisting bacteria.

Serum Opsonization Promotes the Efficient Uptake and Killing of *Burkholderia pseudomallei* and *Burkholderia thailandensis* by Human Neutrophils

M.E. Woodman, R.G. Worth, R.M. Wooten

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Burkholderia pseudomallei is the causative agent of melioidosis and is a major mediator of sepsis in its endemic areas. Because of the low ID₅₀ via aerosols and resistance to multiple antibiotics, it is considered a Category B bioterrorism agent by the CDC. *B. pseudomallei* is an encapsulated bacterium that can infect, multiply, and persist within a variety of host cell types. *Burkholderia thailandensis* is closely-related to *B. pseudomallei*, but lacks a polysaccharide capsule and is relatively avirulent in infection models. *In vivo* studies suggest that macrophages and neutrophils are important for controlling *B. pseudomallei* infections, however few details are known regarding how neutrophils respond to these bacteria. Our goal is to describe the capacity of human neutrophils to control highly virulent *B. pseudomallei* compared to the relatively avirulent *B. thailandensis* using *in vitro* analyses. *B. thailandensis* was more readily internalized than *B. pseudomallei*, but both displayed similar rates of persistence within neutrophils, indicating they possess similar inherent abilities to escape neutrophil clearance. Serum opsonization studies showed that both were resistant to direct killing by complement, although *B. thailandensis* acquired significantly more C3 on its surface than *B. pseudomallei*. Both *Burkholderia* spp. demonstrated significantly enhanced uptake and killing by neutrophils after serum opsonization. Serum-opsonized *Burkholderia* induced a significantly increased respiratory burst by neutrophils compared to unopsonized bacteria. Killing of serum-opsonized *B. pseudomallei* and *B. thailandensis* was prevented by inhibiting NADPH oxidation, indicating the importance of reactive oxygen species in this process. In summary, while complement opsonization is essential for efficient uptake and killing of both strains, the absolute levels of surface C3 does not correlate with increased killing of *B. pseudomallei* and *B. thailandensis*. These findings suggest that neutrophil interactions with serum opsonized bacteria may represent a key component for the effective clearance of *B. pseudomallei* infection.

Identification of a Novel Role for Properdin in Activation of Complement on Platelets

Gurpanna Saggu, Claudio Cortes, Heather N. Emch, Galia Ramirez, Randall G. Worth, Viviana P. Ferreira

University of Toledo, College of Medicine, Department Medical Microbiology & Immunology

Elevated numbers of activated platelets are found in the circulation of patients with atherosclerosis, coronary disease, and hypercholesterolemia. Stimulated platelets can activate the complement system on their surface. While complement system activation is essential for effective innate and adaptive immune responses, it can also lead to vascular inflammation, thrombosis, and thrombocytopenia, especially in patients with defective regulation of complement on their cells. The molecular mechanisms leading to the activation of the alternative pathway of complement on stimulated platelets remains elusive. Recently we, and others, have shown that properdin, a positive regulator of the alternative pathway of complement, can directly recognize certain surfaces, including necrotic cells and *Chlamydia pneumoniae*, and promote alternative pathway complement activation. Here, our data shows that the physiological forms of purified human properdin bind directly to activated platelets and promote alternative pathway complement activation, as measured by C3b and C9 deposition on their surface by flow cytometry. Moreover, our results indicate that properdin bound to activated platelets increases the recruitment of C3(H₂O) to the surface by 1.5-fold versus platelets alone. The C3(H₂O) recruited by properdin can subsequently form a novel cell-bound C3 convertase [C3(H₂O),Bb] on the surface of activated platelets when exposed to factors B and D, *in vitro*. In contrast, no convertase is formed on the activated platelets when properdin is absent. Finally, we determine that properdin released by PMA-stimulated neutrophils (a major source of circulating properdin) binds to activated platelets. Altogether, our data suggest a novel mechanism for alternative pathway activation on stimulated platelets that may contribute to the localization of inflammation at sites of vascular injury and thrombosis.

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Photodynamic Therapy with Pc 4 Induces Cell Death of *Trichophyton rubrum*

Sara Lin, Mauricio A. Retuerto, Pranab K. Mukherjee, Mahmoud A. Ghannoum, Kevin D. Cooper, Minh Lam and Elma D. Baron

Onychomycosis is the most prevalent nail disease in adults and *Trichophyton rubrum* is the most common pathogen. Furthermore, *T. rubrum* is responsible for the most common causes of dermatophytosis. Treatment is limited since topical antifungals are only partially effective and systemic agents, such as terbinafine, have serious adverse effects including liver damage. Photodynamic therapy (PDT) is a non-invasive treatment that uses a photosensitizing drug and light, which, in the presence of oxygen in the tissue, results in the destruction of pathologic cells or microbes. We have previously demonstrated that PDT using the silicon phthalocyanine Pc 4 can induce apoptotic cell death in *C. albicans in vitro*. In this study, we demonstrate the *in vitro* toxicity of Pc 4-PDT in *T. rubrum*. Confocal image analysis confirmed that Pc 4 permeates the cell wall and localizes to cytosolic organelles. Increased fungal killing was observed with increasing doses of Pc 4 from 0 to 2 μM followed by light at 1.0 J/cm². This was confirmed using a clonogenic assay. Similarly, the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide) assay showed that Pc 4-PDT impaired fungal metabolic activity in a Pc 4 dose-dependent manner. Of interest is that these results were obtained in both terbinafine-sensitive (23103) and terbinafine-resistant (MRL666) strains, indicating the potential of Pc 4-PDT as an effective agent against *T. rubrum*. Given that Pc 4-PDT has already demonstrated an excellent safety profile in Phase I clinical trials for skin cancer and psoriasis, we aim to initiate a pilot clinical trial to determine the efficacy of Pc 4-PDT *in vivo*.

Longitudinal Microbiome Shifts in Delayed Wound Healing and the Effect on Innate Immunity

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Microbial infections are recognized as one of the many destructive processes that delay wound healing. Conventional diagnostic cultures of wounds are largely biased toward microbes that are able to grow rapidly in standard culture media and are presumed to be significant. Lack of a reliable measure for wound infections leads to non-targeted use of antimicrobials, promoting development of resistant microbial strains and/or killing potentially beneficial commensal bacteria. Therefore, further understanding of the relationship between microbes and delayed healing is critical for development of more targeted and evidence-based treatment strategies. Our recent clinical study comparing baseline microbiomes between patients with wounds that did or did not heal at six months showed that wounds of patients with severely delayed healing had striking *increases in bacterial diversity. As these wounds often follow a non-linear path to healing—with phases of wound expansion, stalled healing, and resolution—further study is needed to understand the evolution of the microbiome as the wound progresses through different phases of repair.* We hypothesize that the increase in bacterial populations in stalled wounds is related to ineffective host innate immune responses enabling formation of micro-niches that allow growth of diverse microbiomes. We propose to determine prospectively, in a cohort of patients with chronic venous insufficiency wounds, whether the “signature” wound microbiome changes with the transition through different phases of wound healing. Findings from these studies will contribute to understanding of the microbiome changes associated with delayed repair. This will set the stage for further manipulation of the interaction between the immune response and microbial communities, including *in vitro* evaluation of macrophages following stimulation with microbial antigens collected from distinct healing phases and *in vivo* microbial transfer from healing to non-healing wounds to determine the effect on inflammatory responses and repair. Such studies have potential to lead to sophisticated chronic wound diagnostics and therapeutics.

Phenotypic Analysis of B Cell Responses in Pneumococcal Polysaccharide Vaccinated Individuals

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S. pneumoniae is a human pathogen that can cause severe disease and mortality in immunodeficient populations such as children <2 years of age, the elderly, and HIV+ patients. The primary antigens for *S. pneumoniae* are polysaccharides expressed on their surface which results in a T cell independent response. For adults, a 23-valent pneumococcal polysaccharide vaccine, Pneumovax®, is available which can induce a protective host antibody response. However, the population of B cells that responds to this polysaccharide-based vaccination remains unclear and controversial. We are using flow cytometry to identify the phenotype of polysaccharide 23F (PS-23F) specific B cells that respond to pneumococcal polysaccharide vaccination (PPV). Blood samples taken from healthy donors immunized on day 1 with PPV were analyzed 1 week post vaccination to identify the phenotypes of their circulating peripheral blood B-lymphocytes. We have directly labeled PS-23F with 5-DTAF (FITC) to identify polysaccharide specific responding B cells. We previously described the predominance of the CD27+ IgM+ phenotype in the polysaccharide responding CD19+ B cell population. We now show that the majority of the PPS specific CD27+ IgM+ B cells also express CD43 and CD5 cell surface markers. This phenotype is associated with B-1 cells which are classically known to produce natural T cell independent IgM+ antibodies and comprise part of our innate immune response. A more thorough understanding of the B-lymphocyte response to PPV is needed to develop more effective vaccine strategies for susceptible populations and to provide protection against a broader range of PS expressing *S. pneumoniae* pathogens.

Analysis of Human Polyreactive Pneumococcal Antibodies

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Streptococcus pneumoniae is a human bacterial pathogen which colonizes the nasopharynx and is a major cause of pneumonia, meningitis and acute otitis media. The main virulence factor of *S. pneumoniae* is the capsular polysaccharide. Antibodies against PPS provide protection against disease. Natural or nonspecific antibodies are believed to provide a first line of defense against pneumococcal invasion. These antibodies are part of the early phase of the adaptive immune reaction and protect against infection and/or decrease the bacterial load if infection is already present. It is thought that human polyreactive antibodies are selected into the marginal zone B cell compartment, as demonstrated in immunoglobulin transgenic mice.

Nonspecific human pneumococcal polysaccharide (PPS) IgG antibodies have not been extensively studied. We analyzed polyreactive antibodies that bind both PPS14 and PPS23F after absorption with CWPS and PPS22F. These antibodies were isolated from single PPS specific B-cells allowing for the analysis of human immunoglobulins with natively paired variable regions. Although isolated individually, these antibodies demonstrated similar characteristics. Each antibody possessed a variable light (VL) chain with a CDR3 made up of nine amino acids, with one exception, and few negatively charged amino acids. While these antibodies are polyreactive and structurally alike, kinetic analysis revealed unique KD values.

To investigate the contribution of the constant region of these isotypes and their effect on antibody avidity to PPS, these nonspecific variable regions were expressed as IgG1 or IgG2 and subjected to kinetic analysis. The IgG1 antibodies uniformly had a stronger avidity to PPS14 and PPS23F when compared to IgG2. To further document the importance of the constant region in antibody avidity and fine specificity, analysis of antibody F(ab)² fragment binding to PPS14 and PPS23F resulted in similar KD values. These studies suggest that polyreactive antibodies are determined by VH and VL CDR3 amino acid composition while fine specificity of these antibodies are modulated by antibody isotype.

Time to Appropriate Antibiotic Administration and Outcomes in Infants with Viral Bronchiolitis and Secondary Bacterial Pneumonia

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We have previously shown that longer time to correct antibiotic administration is independently associated with adverse outcomes in critically ill children with bacterial community acquired pneumonia (CAP). The impact of antibiotic timing on outcomes for children with viral bronchiolitis and secondary bacterial CAP is unknown. We hypothesized that in children with viral bronchiolitis and secondary bacterial pneumonia, a delay in the time from endotracheal intubation to administration of appropriate parenteral antibiotic will be associated with longer durations of mechanical ventilation, intensive care unit (ICU) stay and hospital stay. We reviewed medical records of children \leq 12 months of age admitted to the pediatric ICU at Nationwide Children's Hospital between January 2007 and December 2010 with an ICD-9 diagnosis of bronchiolitis, pneumonia or respiratory failure. Children with positive viral studies who were treated with invasive mechanical ventilation were included. Patients with tracheostomy or cystic fibrosis were excluded. Secondary bacterial pneumonia was defined as moderate or heavy growth of a pathogen on lower airway culture with many/moderate PMNs on Gram stain, infiltrate on chest X-ray, and fever or abnormal WBC count. Time to appropriate antibiotic was defined as time from intubation to the administration of the first parenteral antibiotic to which the organism(s) was susceptible. Thirty five patients were identified. Viral etiologies included respiratory syncytial virus (N=32), parainfluenza virus (N=2), and human metapneumovirus (N=1). The most frequent bacteria cultured included *M. catarrhalis* (N=17), *H. influenzae* (N=13), and *S. pneumoniae* (N=11). Median time from intubation to correct parenteral antibiotic was 1.6 (IQR: 0.2 –6.5) hrs. Longer time from intubation to correct parenteral antibiotic was associated with longer durations of mechanical ventilation ($p=0.02$) and ICU stay ($p=0.04$), with a trend toward longer hospitalization ($p=0.06$). In our cohort of critically ill children with viral bronchiolitis and secondary bacterial pneumonia, longer time to correct antibiotic was associated with adverse outcomes. These data provide evidence that secondary bacterial pneumonias in children with viral bronchiolitis contribute to outcomes and are deserving of prompt antibiotic treatment.

In Vivo Imaging of Behavioral Responses of Dendritic Epidermal T Cells to Mechanical Stress

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The epidermal compartment of mouse skin contains two dendritic shaped leukocyte populations: a) Langerhans cells (LC), which are antigen presenting cells of the dendritic cell family, and b) dendritic epidermal T cells (DETC), which are epithelial tissue-resident gamma/delta T cells. We previously reported motile activities of LC by recording time-lapse images of EGFP⁺ LC in the IA beta-EGFP knock-in mice under confocal microscopy. They exhibited a unique motion termed dSEARCH characterized by repetitive extension and retraction of dendrites, and inflammatory stimuli (e.g., tape stripping and hapten painting) augmented their dSEARCH motion. The purpose of this study was to visualize motile behaviors of DETC. We found the CX₃CR1-EGFP knock-in mice to contain large numbers (773 ± 104 cells/mm², n = 3) of EGFP⁺ epidermal cells with elongated dendrites – they uniformly expressed Thy-1 antigen and gamma/delta T cell receptor, but not MHC class II molecule, verifying their identity as DETC. We recorded time-lapse videos of EGFP⁺ DETC in the ear skin of anesthetized CX₃CR1-EGFP knock-in mice. In the steady state, DETC exhibited modest dSEARCH movement at the levels comparable to those observed with LC. Within 8 h after tape stripping, a majority of DETC began to withdraw their dendrites, while the remaining cells showed significantly ($P < 0.0001$) exacerbated dSEARCH motion. This observation was confirmed by measuring complexity of the cell shape: the shape factor values increased from 0.12 ± 0.07 before tape stripping (n = 100) to 0.37 ± 0.13 at 8 h ($P < 0.01$, n = 121) and 0.66 ± 0.14 at 24 h ($P < 0.001$, n = 107). A significant ($29.7 \pm 0.2\%$) reduction in DETC numbers was also observed at 24 h after tape stripping. In marked contrast, LC exhibited amplified dSEARCH motion, but not dendrite withdrawal or reduced cell numbers by 24 h after the same treatment. This is the first report unveiling in vivo motile behaviors of DETC, and our results imply that LC and DETC respond differently to a given mechanical stress.

An Anti-TCR β mAb Induces Long-term Allograft Survival as well as Prevents and Reverses the Onset of Type I Diabetes

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T-cells play a central role in cell mediated immune responses to foreign and self antigens that lead to organ transplant rejection as well as autoimmune diseases. Optimal T-cell activation and expansion requires antigen recognition via cognate T-cell receptors (TCRs) along with co-stimulatory and cytokine signals. Modulation of the TCR engagement with specific antigen-MHC complexes may regulate T-cell responses against auto- and allo-antigens. In the current study, we have investigated the immune regulatory effects of TCR engagement with its specific mAb with and without its cognate antigens. Herein, we found that in vivo administration of an anti-mouse TCR β mAb (H57-597) resulted in a preferential reduction of antigen-reactive T-cells with enrichment of CD4⁺FoxP3⁺ Treg cells (~30% among CD4⁺ cells in lymph nodes). In transplantation models, transient H57-597 mAb treatment produced long-term cardiac allograft survivals (>100 days; n=11) and significantly prolonged survivals of skin allografts in naïve recipients (p<0.005; n=6) as well as heart allografts in skin-sensitized recipients (p<0.005; n=4). While Treg cells were involved in maintaining donor-specific long-term graft survivals, immunity was retained against third party allografts. Strikingly, a single injection of H57-597 mAb completely inhibited the rapid development of type I diabetes (T1D) in RIP-OVAhi mice while a transient H57-597 mAb treatment at 8 weeks of age prevented the development of T1D in normoglycemic NOD mice (90% prevention; n=10). Moreover, a brief H57-597 mAb therapy after the onset of T1D induced remission in 6 out of 8 NOD mice for over 8 weeks. In contrast to an anti-CD3 mAb, administration of H57-597 mAb induced limited T-cell activation and proliferation signals correlating with lower levels of inflammatory cytokine production. Thus, transient modulation of the TCR β chain by H57-597 mAb exhibits potent and safe therapeutic effects to control auto- and allo-immune responses.

Therapeutic Alteration of the T cell Immune Synapse Promotes Selective Deletion of Antigen Responding T Cells While Preserving Regulatory T Cells

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The clinical success of organ transplantation can be attributed in large part to the development of effective immunosuppressive therapies. However, long-term immunosuppression causes severe side effects and fails to prevent the active inflammatory process responsible for acute and especially chronic allograft rejection. T cells play a major role in the rejection of allografts, which occurs after T cells become activated by sequential and timely engagement of several functional molecules that form an immune synapse with alloantigen presenting cells. In this study, the T cell immune synapse was targeted using monoclonal antibodies (mAbs) directed to the TCR beta chain (a central component of the immune synapse) and the adhesion molecule LFA1 (a peripheral component of the immune synapse) in order to induce selective deletion of antigen responding T cells. Transient therapy combining anti-TCR mAb with anti-LFA1 mAb resulted in significantly prolonged survival of Balb/c (H-2^d) skin allografts in C57BL/6 (H-2^b) recipients (mean survival time of 49 ± 10 days; $p < 0.01$) when compared to treatment with anti-TCR mAb alone (25 ± 5 days), anti-LFA1 alone (19 ± 3 days), and untreated controls (10 ± 1 days). In order to monitor T cell responses in the presence of these mAb treatments in vivo, OT-II cells (expressing a TCR specific for the OVA peptide) were CFSE labeled and adoptively transferred into C57BL/6 mice. The mice were then injected with the OVA peptide to stimulate the OT-II cells, and lymphoid tissues were harvested 72 hours later for analysis. The anti-TCR mAb in combination with anti-LFA1 mAb completely abolished the proliferation of OVA peptide-stimulated OT-II cells adoptively transferred into wild type mice. In contrast, anti-TCR mAb or anti-LFA1 mAb treatment alone resulted in residual OT-II cell proliferation upon OVA peptide stimulation. While total numbers of CD4 T cells were reduced by about 50% in the anti-TCR mAb plus anti-LFA1 mAb treated mice, the OVA-responding OT-II T cell population was reduced by greater than 90% when compared to the cell populations of untreated mice. A similar effect was observed in the mice treated with anti-TCR mAb alone but not in the mice treated with anti-LFA1 mAb alone. Three days after administration of the combination therapy in these mice, the fraction of CD4⁺CD25⁺Foxp3⁺ Treg cells was significantly enhanced to 18 ± 1 % of the CD4⁺ T cell population when compared to 11 ± 1 % of the CD4⁺ T cell population in untreated controls. This enrichment of Treg cells within the CD4⁺ T cell population was not observed in the mice treated with either of the mAbs alone. In vitro cultures of CFSE-labeled OT-II splenocytes stimulated with OVA peptide confirmed the nearly complete abolition of proliferation in the anti-TCR mAb plus anti-LFA1 mAb treated cells. Further work is underway to understand how these different mAbs influence the molecular signaling pathways of the T cell immune synapse. Together, these results suggest that manipulation of the T cell immune synapse produces simultaneous deletion of antigen-specific T cell clones while preserving Treg cells, thus moving closer toward an optimal therapy for induction of transplantation tolerance.

Prolonged Ischemia Increases the Infiltration of Activated and Effector-Memory T Lymphocytes in Cardiac Allografts

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Cardiac transplantation remains the sole treatment choice for several pathophysiologic heart conditions including end stage heart failure. During organ transplantation, ischemia-reperfusion injury (IRI) is an inescapable pathological challenge which may significantly detrimentally impact graft function and survival. While the molecular mechanisms resulting from tissue ischemia and reperfusion are increasingly being investigated and delineated, the cellular elements mediating IRI early on remain poorly understood. The presence of pre-existing alloreactive memory T cells in the transplant recipient is now widely recognized as a major barrier to tolerance induction and maintenance and these cells have been documented to infiltrate the graft within 24 hours of transplant. While these pre-existing alloreactive CD8⁺CD62L^{low} memory T cells are generally thought to exhibit heterologous immunity, whereby exposure to previous immune stimulants enhances their ability to cross-react with unrelated pathogens or alloantigens, the exact mechanisms promoting infiltration of these cells into the graft remain poorly defined. We have observed that early CD8⁺CD62L^{low} memory T cell infiltration into heterotopic cardiac allografts at 48 hours post-transplant is significantly influenced by the duration of ischemia imposed on the graft. The factors induced by prolonged ischemia that would be expected to mediate this early infiltration and graft tissue damage are under investigation. Since these early infiltrating memory T cells play a role in early tissue inflammation and organ dysfunction, these studies will provide novel insights into mechanisms underlying the poor outcomes associated with cadaver donor grafts.

Blocking Porcine Sialoadhesin Improves Extracorporeal Porcine Liver Xenoperfusion

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Background: Patients in fulminant hepatic failure (FHF) currently do not have a temporary means of support while awaiting liver transplantation. A potential therapeutic approach for patients in FHF is the use of extracorporeal perfusion with porcine livers as a form of “liver dialysis”. During a 72 hour extracorporeal perfusion of porcine livers with human blood, porcine Kupffer cells (KC) bind to and phagocytose human red blood cells (hRBC) causing the hematocrit to decrease to 2.5% of the original value. Our laboratory has identified sialoadhesin (Sn) on the surface of porcine KC as the lectin responsible for binding N-acetylneuraminic acid (Neu5Ac) on the surface of the hRBC. In order for extracorporeal porcine liver perfusion to be used as a therapy for patients in liver failure, all forms of anti-human rejection must be resolved. We evaluated whether targeting Sn prevents the recognition and subsequent destruction of hRBCs seen during extracorporeal porcine liver xenoperfusion.

Methods: Six wild type pig livers were perfused with isolated hRBCs for 72 hours. In one group, three livers were treated with an anti-Sn antibody. As a control group, three livers were treated with an antibody of the same isotype but with no known specificity for Sn.

Results: We have shown that the addition of an anti-Sn antibody to an extracorporeal porcine liver xenoperfusion model significantly reduces the loss of hRBC over a 72 hour period ($p < 0.01$). Sustained liver function was demonstrated by continued bile production and other markers.

Conclusions: This study suggests that the destruction of human erythrocytes in an extracorporeal porcine liver xenoperfusion model is, in part, mediated by Sn expressed on the surface of porcine KC. Furthermore, this data illustrates that the addition of anti-Sn antibody to the circulation of a pig-to-human xenoperfusion may inhibit the destruction of human erythrocytes by porcine Kupffer cells.