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THE UNIVERSITY OF
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1872

OCIIT Research Symposium

November 4 –5, 2010

Hilton Columbus / Polaris

8700 Lyra Drive, Columbus, Ohio 43240

Thursday, November 4th, 2010
Polaris Ballroom C

5:00 p.m. Opening Remarks - Akira Takashima, Kevin Cooper

Session I Dendritic Cells
Moderator Bryan Doreian

5:15 p.m. Visualization of in vivo behavioral responses of Langerhans cells to UVB irradiation Ran Lu, Akira Takashima. University of Toledo College of Medicine.

5:30 p.m. Discovery of a new dendritic cell subset, termed “gr-DC”, derived from a granulocyte precursor. Shuo Geng, Hironori Matsushima and Akira Takashima. University of Toledo College of Medicine.

5:45 p.m. Nerve-derived substance P (SP) and calcitonin gene related peptide (CGRP) sustain CD11c⁺ and CD4⁺ cutaneous cell infiltration and contribute to acanthosis in the KC-Tie2 mouse model of psoriasis Stephen M. Ostrowski, Abdelmadjid Belkadi, Candace M. Matheny, Doina Diaconu and Nicole L. Ward. Case Western Reserve University.

Session II Alopecia
Moderator Ran Lu

6:00 p.m. Gene expression profiling reveals similarities and distinct differences between lymphocytic and neutrophilic cicatricial alopecias. Taneeta Ganguly, Sreejith P. Panicker, Mary Consolo and Pratima Karnik. Case Western Reserve University.

6:15 p.m. Cross-talk between AHR and PPAR Gamma Signaling Pathways in Cicatricial Alopecia. Sreejith P. Panicker, Taneeta Ganguly, Mary Consolo and Pratima Karnik. Case Western Reserve University

--- New Faculty Speaker ---

6:30 p.m. Outer membrane vesicle immunization overcomes active immunosuppression by *Francisella tularensis*. Jason F. Huntley University of Toledo College of Medicine.

7:00 p.m. Dinner (*Polaris Ballroom D*)

9:00 p.m. Hospitality Suite Opens (*Destination TBA*)

Friday, November 5th, 2010
Polaris Ballroom C

7:30 - 8:45 a.m. Breakfast

9:00 a.m. Opening Remarks - Akira Takashima, Kevin Cooper

Session III Innate Immunity I
Moderator Sreejith Panicker

9:15 a.m. *In vivo* imaging of *Borrelia burgdorferi* and murine innate immune cells during early cutaneous infection. John-Paul Lavik, Vipul Shukla, R. Mark Wooten. University of Toledo College of Medicine.

9:30 a.m. *Borrelia burgdorferi* Elicits Dysregulated Production of IL-10 by Macrophages through a TLR2-dependent, yet Phagocytosis-independent Mechanism. Yutein Chung, Rudel Saunders, Joshua P. Waldman, and R. Mark Wooten. University of Toledo College of Medicine.

Session IV Immune Cell Signaling
Moderator John-Paul Lavik

9:45 a.m. Immune Regulatory Effects of Common gamma-Chain Cytokine Signal Deprivation on T-cell responses Mithun Khattar, Wenhao Chen and Stanislaw M Stepkowski. University of Toledo College of Medicine.

10:00 a.m. Put your SOCS on STAT: Cellular Mechanisms Regulating Hyper-inflammatory Macrophage Generation. Doreian BW, Rosenjack J, Cooper KD, Lu KQ. Case Western Reserve University.

10:15 a.m. Generation of a hyper iNOS-expressing macrophage lead to a severe delay in wound healing. Doreian BW, Rosenjack J, Cooper KD, Lu KQ. Case Western Reserve University.

10:30 - 11:00 am Break

Session V Skin Cancer

Moderator Mithun Khattar

- 11:00 a.m. Proteasome inhibition with bortezomib leads to a GATA3-dependent increase in CTLA-4, a mechanism that may provide insight to CTLA-4 regulation in CTCL.** Heather Gibson, Pierluigi Porcu and Henry Wong. The Ohio State University.
- 11:15 a.m. Radiation therapy in the management of unilesional primary cutaneous T-cell lymphomas.** Derek V. Chan, Savina Aneja, Kord Honda, Sean Carlson, Min Yao, Henry Koon, Jerald Katcher, and Kevin D. Cooper. Case Western Reserve University.

--- New Faculty Speaker ---

- 11:30 a.m. Pigmentation and skin cancer genome-wide association studies: a review and meta-analysis.** Gerstenblith MR, Shi J, and Landi MT. Case Western Reserve University.

12:00 noon – 1:00 pm

Lunch (*Polaris Ballroom F*)

Session VI**T cell therapeutics****Moderator John-Paul Lavik**

- 1:15 p.m. Differential *CTLA-4* expression in human CD4⁺ versus CD8⁺ T cells is associated with increased transcription factor NFAT1.** Heather M. Gibson, Barbara M. Aufiero, Adam J. Wilson, Mikehl S. Hafner, Qing-Sheng Mi* and Henry K. Wong. The Ohio State University.
- 1:30 p.m. Translational development of silicon phthalocyanine Pc 4 for photodynamic therapy: an update.** Baron ED, Malbasa C, Lam M, McCormick TS, Oleinick NL, Cooper KD. Case Western Reserve University.
- 1:45 p.m. Mechanistically-based optimization of UV radiation therapy in psoriasis.** Diana Carlson, Thomas S. McCormick, Kevin D. Cooper. Case Western Reserve University.
- 2:00 p.m. Caspase Activation following Turbo UVB radiation therapy for psoriasis.** Ning Lu, Diana Carlson, Thomas S. McCormick, Kevin D. Cooper. Case Western Reserve University.
- 2:15 p.m. Cytotoxic Effects of Photodynamic Therapy with the Silicon Phthalocyanine Pc 4 on *Candida albicans*.** Paul Jou, Minh Lam, Ali A. Lattif, Yoojin Lee, Christi Malbasa, Pranab K. Mukherjee, Nancy L. Oleinick, Mahmoud A. Ghannoum, Kevin D. Cooper and Elma D. Baron. Case Western Reserve University.
- 2:30 p.m. - 3:00 p.m. Break**

Session VII Pneumococcal Polysaccharide Immunity
Moderator Heather M. Gibson

- 3:00 p.m. Promiscuous Variable Light Chain Recombination with Pneumococcal Polysaccharide Specific Variable Heavy Chain**
Rebecca Thompson, Jason Mosakowski, Jieying Wang, Noor Khaskhely and M.A.J. Westerink. University of Toledo College of Medicine.
- 3:15 p.m. Human Immunoglobulin Variable Regions in Immune Response to *Pneumococcal Polysaccharide*.** Jieying Wang, Jason Mosakowski, Rebecca Thompson, Noor Khaskhely and M.A. Julie Westerink. University of Toledo College of Medicine.
- 3:30 p.m. Identification and single cell sorting of Pneumococcal Polysaccharide Specific B Cells by Flow Cytometry and analysis of variable gene repertoire.** Jason Mosakowski, Noor Khaskhely, Rebecca Thompson, Jieying Wang, and M.A. Julie Westerink. University of Toledo College of Medicine.

Session VIII Microbial Immunity
Moderator Derek Chan

- 3:45 p.m. The Role of Exogenous Immune Stimulants on Macrophage Clearance of *Burkholderia pseudomallei*.** Michael P. Bechill, Michael E. Woodman, William E. Grose, R. Mark Wooten. University of Toledo College of Medicine.
- 4:00 p.m. *Trypanosoma cruzi* Calreticulin, a Virulence Factor that Binds C1 on the Parasite Surface.** G. Ramírez, V. P. Ferreira and A. Ferreira. University of Toledo College of Medicine.
- 4:15 p.m. The Role of Serum Opsonization in Clearance of *Burkholderia pseudomallei* by Macrophages.** Minal Mulye, William E. Grose, R. M. Wooten. University of Toledo College of Medicine.
- 4:30 p.m. CARMA3 required for *S.aureus*-induced TNF- α production through NF- κ B activation in Raw264.7 cells.** Byung Cheol Lee, and Zhixing K. Pan * University of Toledo College of Medicine.
- 4:45 p.m. WRAP UP & AWARDS**

Abstracts

Thursday, November 4th

Visualization of in vivo behavioral responses of Langerhans cells to UVB irradiation

Ran Lu and Akira Takashima. Department of Microbiology and Immunology, University of Toledo College of Medicine.

UVB radiation reduces surface densities of Langerhans cells (LCs), although underlying mechanisms remain unknown. By combining two technologies of I-A beta-EGFP knock-in mice and intravital confocal imaging, we recently reported dynamic behaviors of EGFP+ LCs. The purpose of this study was to visualize the impact of UVB irradiation on dynamic movement of LCs in living animals. I-A beta-EGFP mice received local UVB radiation via four TL 20W/01RS lamps only on the left ears and then monitored under a confocal microscopy at different time points. By recording images of epidermal EGFP+ LCs in the same microscopic fields before and after UVB radiation (intermittent imaging), we measured the impact on LC influx versus LC efflux. UVB radiation (3,000 J/m²) elevated the 24 h LC efflux rate from $1.6 \pm 0.1\%$ (sham-exposed right ears) to $34.3 \pm 2.8\%$ ($p < 0.001$, $n = 3$) without altering the LC influx rate. FACS analyses showed no significant changes in the % of annexin V+ apoptotic LCs. Our results demonstrate for the first time that UVB radiation reduces LC densities by promoting LC emigration from the epidermis. In time-lapse imaging (in which images were recorded every 2 min for up to 60 min), EGFP+ LCs exhibited “dSEARCH” motion characterized by repetitive extension and retraction of dendrites and limited lateral migration of cell bodies. Single UVB radiation (3,000 J/m²) augmented both dSEARCH activity (3.4-fold, $p < 0.0001$, $n = 29$) and lateral migration (3.6-fold, $p < 0.0001$, $n = 352$) of EGFP+ LCs at 24 h post-radiation as compared to the cells in sham-exposed control sites. Importantly, these changes in motile behaviors were already detectable at 6 h post-radiation when LC densities remained unchanged. When the UVB-irradiated skin sites were monitored 60 days later, all the above parameters measured for EGFP+ LCs returned to the baseline levels observed in sham-exposed skin. Thus, we conclude that UVB radiation induces dramatic, but reversible, changes in motile behaviors of LCs. It is tempting to speculate that such changes may contribute to UVB-induced immunosuppression.

Discovery of a new dendritic cell subset, termed “gr-DC”, derived from a granulocyte precursor

Shuo Geng, Hironori Matsushima and Akira Takashima
Department of Medical Microbiology and Immunology, University of Toledo College of Medicine

Working with our newly generated transgenic mice expressing DsRed gene under the control of IL-1 β promoter, we observed that DsRed⁺ cells became detectable in 24 h in GM-CSF-supplemented bone marrow (BM) cultures. The DsRed⁺ cells were CD11b⁺/Ly6G⁺/CD11c⁻/MHC II⁻ and exhibited a characteristic morphology of neutrophil precursors, known as “band cells”. Surprisingly, a band cell population purified from WT C57BL/6 mice (CD45.2) began to exhibit features of dendritic cells (DCs) when cultured for 6 days with GM-CSF and BM feeder cells from B6-SJL mice (CD45.1); these features included expression of CD11b, CD11c, MHC II, and CD205, inclusion of oval-shaped nuclei, extension of long dendrites, and a potent ability to present OVA peptides to both OT-I CD8 T cells and OT-II CD4 T cells. Importantly, they retained surface expression of a granulocyte marker Ly6G (which is not detectable on any of the currently known DC subsets) and were, thus, termed “gr-DCs”. Moreover, we confirmed surface expression of Ly6G in small fractions of CD11b⁺/CD11c⁺/MHC II⁺ DCs isolated from the spleen (2.5%) and the peritoneal cavity (1%) of WT mice, indicating the in vivo presence of gr-DC populations. Markedly increased (>100-fold) numbers of gr-DCs were found in peritoneal exudates after i.p. injection of thioglycolate (TG). When band cells purified from C57BL/6 mice were i.v. transferred to B6-SJL mice, significant numbers of CD45.2⁺/CD11c⁺/MHC II⁺ gr-DCs were recovered from the TG-inflamed (but not untreated) peritoneal cavity. Affymetrix Genechip analyses further revealed a cluster of genes (170 in total including cathelicidin and CD62L) that are expressed by gr-DCs, but not by monocyte-derived DCs purified in parallel, and some of these findings were confirmed at protein levels. Thus, under inflammatory conditions, band cells can give rise to a novel DC subset while retaining some features of granulocytes, thereby perhaps participating in adaptive immune responses.

Nerve-derived substance P (SP) and calcitonin gene related peptide (CGRP) sustain CD11c⁺ and CD4⁺ cutaneous cell infiltration and contribute to acanthosis in the KC-Tie2 mouse model of psoriasis

Stephen M. Ostrowski^{1,2}, Abdelmadjid Belkadi¹, Candace M. Matheny², Doina Diaconu² and Nicole L. Ward^{1, 2, 3} Departments of Neurosciences¹, Dermatology², Case Western Reserve University, Cleveland, OH 44106, USA; and The Murdough Family Center for Psoriasis³, University Hospitals, Case Medical Center, Cleveland, OH 44106, USA.

Participation of the nervous system in psoriasis is supported by increases in nerve fibers and neuropeptides in psoriatic skin and by clinical case reports linking nerve injury to disease remission. The KC-Tie2 psoriasis mouse phenocopies human psoriasis and contains ~2.5-fold more PGP9.5⁺ cutaneous nerve fibers and a ~4-fold increase in sensory nerve-derived SP and CGRP. To investigate the mechanisms by which nerve injury leads to remission of psoriasis, we surgically denervated KC-Tie2 mouse skin and observed that beginning 1d following denervation, CD11c⁺ cell numbers decreased by 40% (p=0.011), followed by a 30% improvement in acanthosis at 7d (p=0.004) and a 30% decrease in CD4⁺ T cell numbers by 10d (p=0.03). Small molecule inhibition of SP signaling in innervated KC-Tie2 mouse skin resulted in similar decreases in cutaneous CD11c⁺ and CD4⁺ cell numbers (p<0.003) and restoration of SP signaling using receptor agonists in denervated KC-Tie2 skin prevented decreases in CD11c⁺ and CD4⁺ cells; no change in acanthosis was observed. In contrast, small molecule inhibition of CGRP signaling in innervated KC-Tie2 mouse skin resulted in similar decreases in cutaneous CD4⁺ cell numbers (p<0.003) and improved acanthosis (p=0.009) and restoration of CGRP signaling using receptor agonists in denervated KC-Tie2 skin prevented decreases in CD4⁺ cells and reversed denervated mediated improvements in acanthosis; CD11c⁺ cells remained unaffected. These data demonstrate that sensory nerve-derived peptides mediate psoriasiform dendritic cell and T cell infiltration and acanthosis and introduce targeting nerve-immunocyte/keratinocyte interactions as potential psoriasis therapeutic treatment strategies.

Gene Expression Profiling Reveals Similarities and Distinct Differences between Lymphocytic and Neutrophilic Cicatricial Alopecias

Taneeta Ganguly, Sreejith P Panicker, Mary Consolo and Pratima Karnik
Department of Dermatology, University Hospitals Case Medical Center, Case Western Reserve University, Cleveland, OH

We previously reported that PPAR γ -regulated pathways play an important role in the etiology of the lymphocytic cicatricial alopecia (CA), lichen planopilaris (LPP). Treatment of LPP patients with PPAR γ agonists showed a marked reduction of CA severity score and a dramatic decrease in inflammatory infiltrate. To understand the molecular pathogenesis of other lymphocytic (central centrifugal cicatricial alopecia (CCCA), frontal fibrosing alopecia (FFA) and neutrophilic (folliculitis decalvans (FD), tufted folliculitis (TF) CA, gene expression profiles of unaffected and affected scalp tissue from CA patients were compared with normal controls. Cholesterol biosynthesis was significantly decreased both in lymphocytic and neutrophilic CA. On the other hand, fatty acid metabolism was decreased in lymphocytic but not in neutrophilic CA. In addition, cytochrome P450 genes are upregulated in the unaffected tissue of all CA suggesting a role for xenobiotic metabolism in the pathogenesis of all CA. PXR is upregulated only frontal fibrosing alopecia (FFA) while CAR/RXR activation occurs only in central centrifugal cicatricial alopecia (CCCA). Thus, PXR and CAR may be used as biomarkers to distinguish between these two lymphocytic CA sub-types. In addition, we have identified distinct patterns of chemokine and cytokine genes in neutrophilic and lymphocytic CA. These novel studies allow the molecular classification of CA subtypes and provide new therapeutic targets for these challenging hair diseases.

Cross-talk between AHR and PPAR Gamma Signaling Pathways in Cicatricial Alopecia

Sreejith P Panicker, Taneeta Ganguly, Mary Consolo and Pratima Karnik
Department of Dermatology, University Hospitals Case Medical Center, Case Western Reserve University, Cleveland, OH

We previously showed that Peroxisome Proliferator Activated Receptor Gamma (PPARG) is crucial for healthy pilosebaceous units and loss of this function triggers the pathogenesis of lichen planopilaris (LPP), a cicatricial alopecia (CA). However, the mechanisms responsible for loss of PPARG signaling in CA are not understood. Microarray analysis of unaffected and affected scalp biopsies from patients with lymphocytic and neutrophilic CA revealed that the most significant biological pathways upregulated are Aryl Hydrocarbon Receptor (AHR) signaling and xenobiotic metabolism by cytochrome P450 (CYPs). The AHR is a ligand-activated transcription factor best known for mediating the toxicity of dioxin. The CYP1A1 gene, a direct target of AhR-mediated transcription, is significantly upregulated (~20 fold) in all paired unaffected and affected lymphocytic (LPP, frontal fibrosing alopecia, central centrifugal alopecia) and neutrophilic (tufted folliculitis) CA. Pathway analysis of CA microarray data revealed that AHR and PPARG signaling converge on biological processes as diverse as growth response, metabolism, inflammation and tissue remodeling by impinging on common cellular target genes. Treatment of hair follicle outer root sheath (ORS) cells with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) showed a > 5-fold decrease in PPARG gene expression and a significant increase in expression of CYP1A1 gene suggesting the activation of AHR. PPARG agonist (Pioglitazone - PIO) treatment of ORS cells caused a significant decrease in CYP1A1 and AHR gene expression. The inhibitory effects of PIO treatment on AHR are abrogated when GW9662, a PPARG-specific antagonist, is administered concomitantly with the agonist, thus demonstrating that PIO is acting through PPARG. These data confirm in vivo observations and suggest a negative feed-back regulation between PPARG and AHR. Our data suggest that cross-talk between AHR and PPARG occurs in all CA, but, sensitization of diverse signal transduction cascades may contribute to clinical differences in CA.

Outer membrane vesicle immunization overcomes active immunosuppression by *Francisella tularensis*.

Jason F. Huntley

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

Francisella tularensis is one of the most dangerous bacterial pathogens known, due to its ease of aerosolization, low infectious dose, and ability to cause rapid, fatal disease in humans. Previous studies have demonstrated that *F. tularensis* actively suppresses early proinflammatory responses following pulmonary infection—allowing the pathogen to replicate and spread systemically. Vaccine development efforts against *F. tularensis* have made limited progress in preventing infection and understanding protection. Indeed, a live attenuated vaccine strain of *F. tularensis* (LVS) has shown moderate efficacy against aerosolized *F. tularensis* exposure, but is not licensed for human use due to efficacy and safety concerns. In an effort to develop an acellular (subunit) vaccine against *F. tularensis*, we previously demonstrated that purified *F. tularensis* outer membrane vesicles (OMVs) protected mice from pulmonary challenge with virulent, Type A *F. tularensis*. In subsequent studies, we have shown that, when mixed with the human-relevant adjuvants immune stimulating complexes (ISCOMs) and CpG DNA, OMVs provided enhanced protection (>80%) against *F. tularensis* challenge. Because little is known about protective immune responses against Type A *F. tularensis* pulmonary infections, we have performed comprehensive immunological assessments of immunized and challenged mice to characterize the immune cells and molecules responsible for bacterial clearance and disease resolution. Through passive immunization, we have established that OMV-specific sera conferred equivalent levels of protection as OMV immunization, suggesting that antibodies play a significant role in protection. Additionally, we have examined cytokine expression profiles from non-immunized and OMV-immunized mice at various time points following *F. tularensis* challenge and have found that both Th1 and Th2 responses correlated with bacterial clearance. Finally, we have identified an OMV-induced CD4⁺ T cell subset that rapidly converts into IFN γ -producing effectors cells to promote sterilizing immunity. Taken together, these studies provide crucial information for the development of a safe, efficacious acellular human vaccine to prevent tularemia.

Abstracts

Friday, November 5th

***In vivo* imaging of *Borrelia burgdorferi* and murine innate immune cells during early cutaneous infection.**

John-Paul Lavik, Vipul Shukla, R. Mark Wooten.

Department of Medical Microbiology and Immunology, University of Toledo College of Medicine.

After tick transmission, the spirochetal bacterium, *Borrelia burgdorferi* (Bb), remains at the skin inoculation site for up to 48 hours before disseminating and initiating Lyme disease. Bb possess many surface agonists that should elicit potent inflammatory responses and clearance from skin. However, the ID₅₀ is <50 spirochetes indicating that efficient immunoevasion occurs. This discrepancy suggests that traditional *in vitro* models, which display efficient Bb clearance by phagocytes, are inadequate for accurately characterizing Bb pathogenesis. Thus, we have devised a confocal microscopy method for visualizing infectious fluorescent Bb within intact skin tissues of living mice and in real-time. For these studies, Bb are injected into ear skin of anesthetized mice and four-dimensional data regarding Bb motility during natural infection are collected. This data is complemented by quantitative PCR analyses tracking the temporal fall and rise of spirochete numbers *in vivo*. By 4 hours, the majority of Bb at the injection site are immobile, while Bb adjacent to the site retain motility. Two movement types are seen: 1) a back and forth shift that may aid Bb in finding an optimal migration path and 2) a directed run that navigates dermal structures. Analysis indicates an average Bb velocity of ≥ 100 micrometers per minute in skin, which is 10x to 100x faster than any immune cell velocity observed in our model. This suggests that Bb motility is a primary mechanism for host cell evasion and indicates that our imaging techniques will allow true delineation of host-Bb interactions critical for Lyme disease development.

***Borrelia burgdorferi* Elicits Dysregulated Production of IL-10 by Macrophages through a TLR2-dependent, yet Phagocytosis-independent Mechanism**

Yutein Chung, Rudel Saunders, Joshua P. Waldman, and R. Mark Wooten
Department of Medical Microbiology and Immunology, University of Toledo Health Science Campus, Toledo, OH, USA.

Borrelia burgdorferi (Bb) is a tick-borne spirochete that causes Lyme disease. Bb efficiently evade host defenses and persist in host tissue despite eliciting strong immune responses that involve Toll-like receptor 2 (TLR2)-mediated events. Virulent Bb are known to rapidly elicit substantial IL-10 production by skin-resident antigen presenting cells (APCs) such as macrophages (MØs), and blocking IL-10 significantly enhances Bb clearance and APC immune functions. We hypothesize that the mechanisms involved in the dysregulated IL-10 response are initiated rapidly and distinct from those involved in proinflammatory responses. To begin to address this, BMMs were pretreated with cytochalasin D (CytoD) to block Bb phagocytosis and/or antibodies that specifically neutralize TLR2 function, thus allowing delineation of early responses to Bb. Our results demonstrated that the production of IL-10 and IL-6 by MØs in response to Bb is independent of phagocytosis; whereas most pro-inflammatory mediators required phagocytosis. IL-10 produced in response to intact Bb was only partially reduced after TLR2-blockage, while IL-10 elicited by the Bb lipoprotein OspA was completely abrogated by TLR2-blockage. Our findings demonstrate that elicitation of IL-10 production from BMMs by Bb can occur through the interactions on the surface of BMMs via a TLR2-dependent fashion. In addition, Bb lipoproteins may play an important role in the elicitation of IL-10 production from BMMs. We are currently investigating the roles of down-stream signaling cascades such as PI3 kinase and MAP kinase pathways on the production of IL-10 by BMMs in response to Bb.

Immune Regulatory Effects of Common gamma-Chain Cytokine Signal Deprivation on T-cell responses

Mithun Khattar, Wenhao Chen and Stanislaw M Stepkowski. Department of Medical Microbiology and Immunology, University of Toledo College of Medicine

Optimal T-cell activation and expansion requires cytokine signals, namely the binding of common gamma-chain (γ_c) cytokines to their cognate receptors activate Janus tyrosine kinase (Jak) 3 pathway. Because of its obligatory role in T-cell activation and homeostasis as well as its predominant expression only in T cells that actively participate in immune responses, Jak3 has been considered as an attractive target for immunosuppressive therapy of T-cell mediated diseases. To promote Jak3-targeted therapy, it is critical to precisely understand its mechanistic role during early T-cell activation. In the present study, we aim to delineate the tolerogenic effects of γ_c cytokine/Jak3 signaling deprivation on different T-cell subsets during early phases of priming. We show highly reduced survival of CD8 T-cells under cytokine/Jak3 inhibiting conditions (by using neutralizing anti-IL-2 and IL-4 mAbs or Jak3 inhibitors) during activation, whereas CD4 T-cells were significantly more resistant. These results also correlated with highly reduced Stat5 phosphorylation, BCL-2 expression, as well as mitochondrial membrane potential in CD8 T-cells, relative to CD4's. The persistent Stat5 phosphorylation of Stat5 in CD4 T-cells under γ_c /Jak3 signal blockade suggested alternate pathways of cell survival that were independent of γ_c /Jak3 signals, as shown by lack of IL-2 production in these cells. In an in vivo SEB infection model, we have also shown that Jak3 inhibitor therapy was significantly effective in deleting the SEB reactive CD8+ T-cell whereas the CD4+ T-cells remained protected. Taken together, JAK3-based therapy has potential barriers such as the resistance of CD4 T cells which limits its effectiveness. We are continuing studies to investigate the differential regulation of apoptotic pathways in CD4 Vs CD8 T-cells during early events of activation and further design novel approaches to selectively and efficiently delete allo-antigen specific T-cells in order to promote transplantation tolerance.

Put your SOCS on STAT: Cellular Mechanisms Regulating Hyper-inflammatory Macrophage Generation

Doreian BW, Rosenjack J, Cooper KD, Lu KQ, Department of Dermatology, Case Western Reserve University

Recent evidence from our lab has demonstrated that treatment with a PPAR γ agonist in the absence of IL-6 drives macrophages to express super-elevated levels of iNOS and TNF- α , resulting in tissue damage. Here we show that these hyper-inflammatory macrophages can be generated in other organs; their presence was observed in both lung and peritoneal models of inflammation, indicating a possible role for mediating chronic inflammation and tissue damage in other organs. To investigate a potential intracellular signaling mechanism responsible for hyper-induction of iNOS under our treatment conditions, we examined transcription factors involved in the cooperative regulation of iNOS, specifically nuclear factor κ B (NF- κ B), and signal transducer and activator of transcription 3 (STAT3). STAT3 has been previously demonstrated to inhibit NF- κ B-mediated transactivation of iNOS, resulting in decreased iNOS expression. Additionally, PPAR γ ligands have been shown to increase SOCS3 expression as well as decrease STAT3 phosphorylation, both potential mechanisms to decrease nuclear translocation STAT3. Given this, we hypothesized that in our experimental condition, STAT3-mediated suppression of NF- κ B-transactivation of iNOS is diminished via increased SOCS3 inhibition of STAT3 activation. This then would result in unregulated, and thus elevated, iNOS expression by NF- κ B. We show that *ex vivo* rosiglitazone treatment of IL-6^{-/-} thioglycollate-elicited macrophages stimulated with LPS results in increased SOCS3 protein in the cytoplasm at 15 minutes and a subsequent decrease of STAT3 with a concomitant increase in NF- κ B p65 protein at 60 minutes in the nucleus. As hypothesized, we observed hyper-induction of iNOS expression as analyzed by qPCR under these conditions. Lastly, to directly assess the functional role of STAT3 in transrepression of NF- κ B and iNOS expression in our hyper-inflammatory cells, we treated IL-6^{-/-} bone marrow derived macrophages (BMDM) with a STAT3 decoy oligodeoxyribonucleotide. Transfection with the STAT3 decoy significantly increased iNOS expression following LPS stimulation, while mismatched decoys had no effect. In summary, our data demonstrate a mechanism of hyper-iNOS generation in macrophages mediated by increased SOCS3 expression, decreased nuclear STAT3 accumulation, and increased NF- κ B activation and activity.

Generation of a hyper iNOS-expressing macrophage leads to a severe delay in wound healing

Doreian BW, Rosenjack J, Cooper KD, Lu KQ

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

Macrophages (M Φ) are cellular responders to injury capable of being driven toward either a pro-inflammatory or an anti-inflammatory phenotype. Glitazones, a family of PPAR- γ agonists commonly used to treat type II diabetes, are known to drive macrophages towards an anti-inflammatory state. We hypothesized that a skin environment enriched with macrophages in the presence of a PPAR- γ agonist would generate anti-inflammatory responses that would promote anti-inflammatory responses, and would result in accelerated wound healing. IL-6 helps to regulate IL-10, a cytokine produced by anti-inflammatory macrophages. We developed an excisional wound healing model in IL-6 $^{-/-}$ mice treated with a PPAR- γ agonist under inflammatory conditions. Contrary to our prediction of accelerated healing, the result show exacerbated pro-inflammatory responses resulting in de novo local tissue destruction and subsequent delayed wound repair. IL-6 $^{-/-}$ mice under experimental conditions demonstrated: dramatic enlargement of wounds up to 136 \pm 4% of the initial size between 0-2 days; failure to initiate wound healing with sustained enlarged wounds for 7 days; delayed complete re-epithelialization compared to controls (23 vs. 12 days, n=15, p<.0001). We found a 24-fold increase in iNOS expression, an indicator of pro-inflammatory responses, by RT-PCR from wounded skin of IL-6 $^{-/-}$ mice compared to controls (n=3, p<0.03). Triple-color confocal microscopy demonstrated co-localization of intense iNOS staining with cells expressing both M Φ markers CD11b and F4/80 (n=3). Administration of a blocking antibody (anti-CD11b) targeting monocytes and M Φ resulted in decreased skin iNOS expression by RT-PCR (n=3, p<0.001), decreased numbers of iNOS $^{+}$ M Φ by immunostaining (n=3, p<0.001), and most importantly restored wound healing to 12 days (n=3, p<0.001). Finally, treatment with a specific iNOS inhibitor (1400W), prevented wound enlargement between days 0-2 and restored animals to a normal healing curve demonstrating the criticality of iNOS in tissue destruction (n=4, p<0.001). In summary, our data demonstrate a novel mechanism of delayed wound repair mediated by hyperinflammatory M Φ and suggest a role for hyperinflammatory M Φ in destructive inflammatory diseases.

Proteasome inhibition with bortezomib leads to a GATA3-dependent increase in CTLA-4, a mechanism that may provide insight to CTLA-4 regulation in CTCL.

Heather Gibson, Pierluigi Porcu and Henry Wong. Comprehensive Cancer Center, The Ohio State University.

The costimulatory molecule CTLA-4 functions as an immunomodulator generally associated with suppression of T cell proliferation. Though structurally similar to CD28, which is expressed constitutively on T cells, transcriptional regulation of CTLA-4 is highly regulated. CTLA-4 is expressed at elevated levels in cutaneous T cell lymphoma (CTCL), which may contribute to suppression of anti-tumor response as the disease progresses. The transcriptional regulator GATA3 is also over-expressed in CTCL, but its significance in CTLA-4 regulation has not been elucidated. Both transcript and protein levels of GATA3 are augmented by proteasome inhibition. We show by polyubiquitin immunoblot that the proteasome pathway is dysregulated in CTCL. Here we demonstrate a role for GATA3 in transcriptional regulation of CTLA-4 using the proteasome inhibitor bortezomib. Bortezomib treatment leads to a dose-dependent increase in both GATA3 and CTLA-4 expression in normal CD4 T cells at both the transcript and protein level. Flow cytometric analysis confirms the elevated CTLA-4 is properly trafficked to the cell surface. By cotransfection of a GATA3 expression vector with a CTLA-4 promoter luciferase construct into Jurkat T cells, we see that elevated GATA3 expression can enhance CTLA-4 promoter activity in a dose-dependent manner. In primary CD4 cells, we detect specific binding of GATA3 to the CTLA-4 proximal promoter in bortezomib-treated CD4 cells by ChIP assay. These results support a potential mechanism for increased CTLA-4 observed in CTCL T cells where GATA-3 is also elevated. Additionally, this work provides insight into potential effects on T cell function from proteasome inhibition with bortezomib. As GATA-3 supports differentiation of Th2 T cells, bortezomib may be useful in immune modulation in diseases associated with Th1 dominance, such as graft versus host disease (GVHD).

Radiation therapy in the management of unilesional primary cutaneous T-cell lymphomas

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ABSTRACT

Background: Unilesional presentations of mycosis fungoides (MF)-type cutaneous T-cell lymphoma (CTCL) may offer opportunities for definitive treatment with intent to cure, as opposed to more advanced disease involving multiple body areas, where remissions are not expected to be long term.

Objective: To ascertain the disease-free survival for patients with unilesional or clustered oligolesional cutaneous T-cell lymphoma treated with radiotherapy.

Methods: We reviewed the records of all patients brought before the Multidisciplinary Cutaneous Oncology Tumor Board at University Hospitals Case Medical Center from 1997 to 2010. A total of 10 patients diagnosed with unilesional or oligolesional cutaneous T-cell lymphoma were treated with radiation therapy.

Results: 100% of patients treated with radiation therapy achieved a complete response verified by clinical examination within 2 months after radiation therapy. Following radiation therapy, 30% of patients reported a relapse, of which two-thirds occurred within the previously irradiated area. Among patients who relapsed, the mean time to relapse was 42.3 months. We combined our data with a previous study and conducted a pooled data analysis which showed that the 1 and 5 year disease-free survivals after radiation therapy were 92.7% and 83.4% respectively.

Limitations: The study is limited by the small sample size and variable time of follow up.

Conclusion: Local radiation therapy appears to be an effective treatment for patients presenting with unilesional or localized cutaneous T-cell lymphoma.

Keywords: unilesional; CTCL; mycosis fungoides; radiation therapy

Pigmentation and skin cancer genome-wide association studies: a review and meta-analysis.

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We performed a meta-analysis of summary results from 11 genome-wide association studies (GWAS) of pigmentation, sun sensitivity, nevi, melanoma, basal cell carcinoma (BCC), and squamous cell carcinoma and five replication studies expanding upon GWAS findings. Loci associated with pigmentation, freckling, sun sensitivity, and skin cancer included MC1R (melanocortin-1 receptor), TYR (tyrosinase), ASIP (agouti signaling protein), TYRP1 (tyrosinase-related protein 1), OCA2 (oculocutaneous albinism type II), and SLC45A2 (solute carrier family 45, member 2). Loci associated with nevi and melanoma included MTAP (methylthioadenosine phosphorylase) and PLA2G6 (phospholipase A2, group VI). Loci associated with only pigmentation and/or sun sensitivity included TPCN2 (two-pore segment channel 2), KITLG (kit ligand), SLC24A4 (solute carrier family 24, member 4), HERC2 (hect domain and RCC1-like domain 2), IRF4 (interferon regulatory factor 4), and SLC24A5 (solute carrier family 24, member 5). Loci distinctly associated with BCC included PADI6 (peptidylarginine deiminase, type VI), RHOA (ras homolog gene family, member u), TERT-CLPTM1L (telomerase reverse transcriptase-CLPTM1-like protein), KLF14 (kruppel-like factor 14), CDKN2A/B (cyclin-dependent kinase inhibitor 2A/B), and KRT5 (keratin 5). These findings suggest that melanoma development may occur distinctly via nevi or pigmentation pathways and that BCC can develop via pigmentation or independent of pigmentation. Only three of five skin cancer GWAS, however, adjusted for pigmentation or sun sensitivity, so the effects on skin cancer cannot be fully separated from those on pigmentation. GWAS results must be interpreted cautiously since causal variants cannot be identified. MC1R exemplifies this since the functional 'red hair color' (RHC) MC1R alleles are not on the platforms used for these GWAS, and further genotyping showed that signals on chromosome 16 were due to RHC alleles.

Differential *CTLA-4* expression in human CD4⁺ versus CD8⁺ T cells is associated with increased transcription factor NFAT1

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CTLA-4 is a member of the costimulatory molecule family that functions as a negative regulator of T cell activation. Originally identified in murine CD8⁺ T cells, it has been found to be rapidly induced on human T cells. Furthermore, CTLA-4 is expressed on regulatory T cells (T_{reg}). Targeting the expression of CTLA-4 has clinical utility in the treatment of melanoma. Whether the expression of CTLA-4 is differentially regulated in human T cells is unclear. Here we analyzed CTLA-4 in normal human CD4⁺ and CD8⁺ T cell subsets and show for the first time that CTLA-4 is expressed significantly higher in the CD4⁺ T cells than in CD8⁺ T cells. CTLA-4 is higher at the protein and the transcriptional level in CD4⁺ T cells. The increased in CTLA-4 expression is from activation of the CTLA-4 promoter, which undergoes acetylated chromatin conformation at the proximal promoter and is dependent on NFAT1. Furthermore, we show that blocking CTLA-4 on CD4⁺ T cells in mixed-lymphocyte studies lead to greater proliferation in comparison to CD8. These findings demonstrate the differential regulation and function of CTLA-4 on CD4 and CD8 T cell subsets. This suggests that the CD4 T cells have potent regulatory roles via CTLA-4 and that modulating CTLA-4 function preferentially targets the CD4⁺ T cell subset.

Translational development of silicon phthalocyanine Pc 4 for photodynamic therapy: an update.

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Photodynamic therapy is a non-invasive light treatment modality that is currently used for neoplastic as well as inflammatory diseases. Initially, porphyrins and porphyrin-based drugs were used for PDT, but a number of adverse effects such as pain and prolonged photosensitivity were often encountered. Other more novel PDT agents are in various stages of investigation. An example is the silicon phthalocyanine Pc 4, which is in clinical trials for PDT of cutaneous pathologies. We have successfully completed a Phase 1 trial for cutaneous neoplasms in 43 subjects which demonstrated that Pc 4-PDT has an excellent safety profile. Because of Pc 4-PDT effects on T-cells observed in vitro, as well as the encouraging clinical response of patients with cutaneous T-cell lymphoma, we hypothesized that Pc 4-PDT is a treatment option for T-cell mediated diseases, including psoriasis. We are currently conducting a Phase 1 trial in psoriasis in which we have treated 12 subjects to date with Pc 4 from 0.001 to 0.1 mg/ml, followed by 675 nm red laser from 50-150J/cm². The treatment is well tolerated with no adverse effects. Partial clinical response has been observed in one subject after a single session of Pc 4-PDT. As this is a dose escalation trial, it may indicate that we have just begun to approach a minimally effective dose. It is also possible that repeat/multiple PDT sessions would yield better efficacy. Analysis of known Pc 4-PDT induced apoptotic events is being performed on skin biopsies obtained from the subjects.

Mechanistically-based optimization of UV radiation therapy in psoriasis

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Background: Psoriasis is a common skin disease (prevalence of 2%) responsible for significant morbidity and disability. Understanding the mechanisms of UV therapy, a mainstay of treatment, is very likely to lead to novel approaches, which utilize the same mechanisms, but without incurring the drawbacks of phototherapy. In order to reduce the dose of UVB delivered, our current focus is on pre-activation of T cells followed by lower UVB dose regimens. This may have direct bearing on developing a new, efficacious, and more tolerable treatment schedule for patients with psoriasis that has a firm and rational scientific basis.

Objective: Our hypothesis is that imiquimod, a Toll-like receptor agonist, or corticosteroid pretreatment can affect susceptibility of abnormal psoriatic lesional cells to UVB (excimer) laser-induced apoptosis.

Methods: This pilot study is a single-blind, vehicle-controlled comparison of imiquimod versus clobetasol propionate cream combined with excimer UVB phototherapy for psoriasis using both histological and clinical response endpoints. Following a 5 day course of treatment with topical imiquimod or clobetasol, excimer laser treatment was applied at either 3 or 47 hours after the last topical treatment. Epidermal thickness, apoptosis and mRNA gene expression were assessed via H&E, CD3 & caspase-3 IHC staining, and rtPCR respectively. Clinical plaque assessments including the lesional psoriasis area severity index (L-PASI), physician's static global assessment (PSGA) of target lesions, physician's dynamic global assessment (PDGA), and target lesion site reaction assessment were recorded.

Results: To date, we have enrolled and treated 5 patients in our imiquimod-treated cohort. Our preliminary results show that in patients treated with imiquimod vehicle control for 5 days followed by excimer laser treatment 3 hours later, the L-PASI score decreased by $33\% \pm 13\%$. In patients treated with imiquimod for 5 days followed by excimer laser treatment 3 hours later, the L-PASI score decreased by $29\% \pm 12\%$. In patients treated with imiquimod for 5 days followed by excimer laser treatment 47 hours later, the L-PASI score decreased by $15\% \pm 27\%$. In a single patient treated with steroid vehicle control for 5 days followed by excimer laser treatment 3 hours later, the L-PASI score decreased by 33%. In the same patient treated with steroid for 5 days followed by excimer laser treatment 3 hours later, the L-PASI score decreased by 43%. In the same patient treated with steroid for 5 days followed by excimer laser treatment 47 hours later, the L-PASI score decreased by 29%. Only one of five patients developed a transient increase in erythema in plaques pre-treated with imiquimod compared to the vehicle treated plaques.

Limitations: The study is limited by the small sample size.

Conclusion: Increased clinical efficacy of UVB in combination with imiquimod pretreatment has not been observed.

Caspase Activation following Turbo UVB radiation therapy for psoriasis

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Psoriasis is a chronic inflammatory skin disease that affects 4.5 million people in the US. Histologically, psoriatic lesions contain hyperproliferating keratinocytes, with infiltrating granulocytes and activated T cells. While the exact pathogenesis of psoriasis remains unknown, treatments targeted against T cells and APC-T cell interactions have shown promise clinically. However, these therapies pose the threat of activating latent infections or overly suppressing the immune system. Recently, single wavelength, 308nm UVB Phototherapy with excimer laser has emerged as an effective treatment against psoriasis. Its mechanism of action has been reported as immunosuppression in local tissue and induction of apoptosis, particularly of activated T cells. A drawback of UVB treatment is that the high doses required for therapeutic effect may predispose patients to skin damage and even cancer. We propose that pre-treating patients with topical Imiquimod, a TLR7/8 ligand and potent immune activator, can allow for lower doses of UVB treatment due to maximal T cell activation. Patients with moderate to severe psoriasis were recruited for the study and underwent a washout period of two weeks. They were then treated with either vehicle or a 5% topical imiquimod cream once daily for 5 days. Lesions were subsequently lasered and biopsies were taken at 1 hour and 48 hours post UVB treatment. Half of each biopsied lesion was fixed with formalin and embedded in paraffin while the other half stored frozen in OCT. Frozen samples were used for quantitative PCR to measure levels of Mx A, an imiquimod response gene. qPCR data revealed higher levels of Mx A in untreated psoriatic skin when compared to normal skin and an augmented signal following Imiquimod application. Paraffin samples were stained for CD3 and activated caspase 3 as a marker of apoptosis induction using immunofluorescence. Caspase 3 activity was not detectable at either 1 hour or 48 hour time points in patients with psoriasis. We therefore performed a time course on normal controls, giving 4 MEDs of UVB light with excimer laser and followed by biopsies 24 and 48 hours after UVB exposure. Using normal skin, IF stain demonstrated positive caspase 3 in keratinocytes as early as 24 hours with increased activity up to 48 hours. The absence of caspase 3 staining in psoriatic skin, even at 48 hours post UV, indicates that either caspase may not be a suitable marker for induction of apoptosis or a time point longer than 48 hours may be necessary to see its activity. Alternatively, pathologies found in psoriatic skin, such as keratinocyte hyperproliferation and dysregulated immune responses, may contribute to a delay or inhibition of caspase 3 activation.

Cytotoxic Effects of Photodynamic Therapy with the Silicon Phthalocyanine Pc 4 on *Candida albicans*

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ABSTRACT: The high prevalence of antifungal drug resistance necessitates the development of novel agents against infections from opportunistic fungal pathogens. *Candida albicans* is a ubiquitous fungus that continues to represent a major health burden, especially in immunocompromised patients. Manipulation and optimization of apoptosis, or programmed cell death, in fungal pathogens may provide a basis for future therapies. Photodynamic therapy (PDT), originally developed as a cancer treatment, has been demonstrated in mammalian cells to generate reactive oxygen species (ROS), such as singlet oxygen, through the interaction between tissue molecular oxygen, light, and a photosensitizing drug. The resulting ROS instantaneously oxidizes nearby molecules, including lipids and proteins, leading to apoptosis. Silicon phthalocyanine, Pc 4, is a second-generation photosensitizer activated by 675 nm light, and is currently in Phase 1 clinical trials for cutaneous pathologies. Using Pc 4, we performed a pre-clinical investigation aimed to determine the *in vitro* susceptibility of yeast-form *C. albicans* to PDT. Confocal image analysis confirmed that Pc 4 penetrates *C. albicans* cell wall and membrane, localizing to cytosolic organelle membranes, including mitochondria, within 15 minutes of incubation at 37°C. Photoirradiation of Pc 4-treated *C. albicans* resulted in exposure of phosphatidylserine residues on the outer surface of the plasma membrane as evidenced by positive Annexin-V-FITC staining as well as observable changes in nuclear morphology, both of which are characteristic of apoptosis in mammalian cells. Clonogenic assay indicates that incubation with 1µM Pc 4 followed by light exposure at 2.0 J/cm² reduced cell survival by ~90% (LD90). Metabolic assays using XTT and the FUN-1 fluorescence probe show an immediate drop in metabolic activity after Pc 4-PDT. These preliminary data suggest that Pc 4-PDT is cytotoxic against *C. albicans* likely due to the induction of apoptosis and could serve as an alternative therapy against candidiasis.

Promiscuous Variable Light Chain Recombination with Pneumococcal Polysaccharide Specific Variable Heavy Chain

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Streptococcus pneumoniae is a human bacterial pathogen which colonizes the nasopharynx and can result in pneumonia, meningitis and acute otitis media. The adult 23 valent pneumococcal polysaccharide (PPS) vaccine has an 80% protective efficacy in healthy young adults. However efficacy in the elderly is drastically reduced despite normal antibody levels. This phenomenon is thought to be linked to antibody structure.

Previous studies in both *Haemophilus influenzae* type b polysaccharide (Hib PS) and PPS have suggested that the use of a specific variable light chain may be essential in maintaining polysaccharide epitope specificity. Variable chains obtained from human antibodies specific for PPS4, PPS14 or PPS23F were cloned into mammalian expression vectors. The goal was to identify the variable light chain(s) required for binding PPS23F and to investigate the binding requirements of PPS23F. A 23F specific heavy chain was paired with light chains and transfected into human epithelial kidney cells. The secreted immunoglobulins were tested by ELISA for binding to pneumococcal polysaccharide 4, 14 and 23F. Independent of variable light chain specificity, all clones bound exclusively to PPS23F. We have thus demonstrated that pneumococcal polysaccharide variable heavy chain gene usage is promiscuous in in vitro recombination with many variable light chains in contrast to previous studies.

Human Immunoglobulin Variable Regions in Immune Response to *Pneumococcal Polysaccharide*

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Background: *Streptococcus pneumoniae* is a serious human bacterial pathogen causing invasive diseases and deaths worldwide. The current 23-valent pneumococcal polysaccharide vaccine (PPV) provides effective protection in healthy young adults. However, vaccine efficacy in the elderly is significantly reduced despite the fact that the antibody response is similar to that of young adults. This could be due to different gene usage of the antibody leading to lower avidity and functional activity.

Aim: The ultimate goal of this study is to determine the molecular structure of PPS-specific immunoglobulin (Ig) variable regions of heavy (V_H)- and light-chain (V_L) and further investigate the relationship of PPS-specific antibody structure and its function.

Methods: PPS-specific-single B cells from healthy young adults were isolated from peripheral blood using flow cytometry seven days after vaccination with the 23-valent PPV. B cells were then expanded in culture and V_H and V_L pairings of PPS-specific antibodies were cloned and sequenced.

Results & Conclusions: We identified B cells that specifically recognize PPS using optimized sorting technique by FACS and sequenced V_H and V_L of antibodies specific for PPS serotype 14 and 23F. We examined V_H/V_L gene usage and CDR3 domain of the variable region. We determined the dominant V_H/V_L gene families responsible for immune response to PPS14 and 23F. This novel approach allows us to determine naturally paired V_H/V_L gene usage in response to PPV suitable for large-scale population analysis.

Identification and single cell sorting of Pneumococcal Polysaccharide Specific B Cells by Flow Cytometry and analysis of variable gene repertoire

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The currently available pneumococcal polysaccharide (PPS) vaccine is highly effective in young adults but has lower efficacy in the elderly. Previous studies have shown that while the elderly immune response to the vaccine is diminished levels of post-vaccination PPS specific antibodies are similar to those of young adults. The mechanism behind this is not well understood. One possible explanation is a shift in variable heavy (V_H) and variable light (V_L) gene use by B cells in the young and elderly. Previous methods investigating PPS specific B cell V_H/V_L use in humans were limited by either not being high throughput enough for population studies, hybridomas, or not maintaining native V_H/V_L pairing, combinatorial libraries. We have conjugated PPS serotypes 14 and 23F to fluorochromes and biotinylated 23F enabling the identification and single cell sorting of PPS 14 and 23F specific B cells from young adult donors. Sorted cells were analyzed for V_H/V_L use, CDR3 length, and mutation rate. We have adopted a method previously used to identify B cells specific for a protein antigen and adapted it for polysaccharide specific B cells. This method is beneficial for the study of the immune response to polysaccharide antigens and for the generation of humanized monoclonal antibodies to these antigens.

**The Role of Exogenous Immune Stimulants on Macrophage Clearance of
*Burkholderia pseudomallei***

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Burkholderia pseudomallei (Bp) is a Gram-negative bacillus that causes melioidosis. Main routes of infection are through skin abrasions or inhalation. The inhalation lethal dose (LD₅₀) for Bp is ≤10 organisms, with a ≤90% mortality rate if untreated, and thus has potential as a bioweapon. No vaccine is available against Bp, thus it is important to identify immune evasion mechanisms. Bp infects many host cell types, but appears particularly adept at infecting and surviving within macrophages, making this central immune cell a prime model for study. Based on the current literature, we hypothesize that Bp lipopolysaccharide (LPS) inadequately activates macrophages, thus allowing evasion of intracellular clearance. Our current goal is to treat macrophages with different exogenous agonists to assess whether they can enhance intracellular clearance of Bp by macrophages and define the effective immune mechanisms. We also will compare these effects on clearance of *Burkholderia thailandensis* (Bt), a closely related but relatively avirulent acapsular strain that possess LPS that is similar in structure. Bp and Bt displayed a relatively high resistance to kanamycin, which is used to differentiate between killing of intracellular and extracellular bacteria, but a single optimal dose was ascertained, allowing comparison between the strains. Intracellular growth experiments showed that Bp and Bt display a 4 to 6 hour lag phase, followed by a rapid growth phase that leads to macrophage lysis within 12 hours, with no inherent differences between the strains. Intriguingly, stimulated macrophages appear to take-up/concentrate exogenous antibiotic to further enhance clearance of these strains, which may explain differences that exist for similar previously published studies. Macrophages treated with interferon gamma (IFN γ) and/or LPS demonstrated an enhanced and synergistic effect on clearance of both Bp and Bt. Future studies will compare the immune stimulatory properties of "protective" LPS-species versus the relatively non-protective Bp LPS, with the goal of identifying targets for curative therapies.

***Trypanosoma cruzi* Calreticulin, a Virulence Factor that Binds C1 on the Parasite Surface**

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Trypanosoma cruzi is the agent of Chagas' disease, an acute and chronic illness affecting 20 million people in Latin America, causing 50,000 deaths per year and considered the sixth most important neglected tropical disease worldwide. Trypomastigotes, the infective form of *T. cruzi*, are highly resistant to complement activity, while epimastigotes, the non infective form, are not. Several molecules expressed by trypomastigotes contribute to explain this resistance (CRP, T-DAF, trans-sialidases and specific lipases, among others). We and others have previously shown that *T. cruzi* calreticulin (TcCRT), an intra and extracellular, highly pleiotropic molecule, is translocated from the endoplasmic reticulum to the parasite surface. We have also determined that TcCRT S domain (TcS) binds to the collagenous tails of C1q, thus inactivating the classical complement pathway. In addition, TcCRT and C1q colocalize on the parasite surface, mainly in the area of flagellar emergence, which is the area that first contacts the host cell. We propose herein that the parasite uses this molecule to capture complement C1, in an infective strategy. This fact correlates with significant increases in TcCRT mRNA levels during early infection stages. Accordingly, mouse immunizations with TcCRT induced humoral responses that, after challenge, correlated with increased parasitemia. *In vitro*, whole anti-TcCRT Igs promote C1 deposits on trypomastigotes while, as expected, F(ab')₂ fragments, that lack the ability to bind C1q, decrease it. Likewise, pretreatment of the parasites with whole anti-TcCRT antibodies augmented parasitemia and mortality in mice. In contrast, pretreatment with anti-TcCRT F(ab')₂ fragments, was protective. Most important, while pretreatment of trypomastigotes with C1q increased infectivity in a macrophage murine cell line, as well as mice mortality and parasitemia, the F(ab')₂ fragments significantly interfered with the C1q-dependent infectivity. Therefore, the *T. cruzi* calreticulin/complement C1 interaction, besides inhibiting complement activation, is a virulence factor involved in the infectivity process of professional phagocytic cells, promoting parasite uptake at early stages of infection.

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

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Infection with the encapsulated Gram-negative bacterium *Burkholderia pseudomallei* (*Bp*) leads to melioidosis, a major cause of septic disease in Southeast Asia and Northern Australia. This disease has a 40% mortality rate even with antibiotic treatment. Its high infectivity through the aerosol route has led to its categorization as a potential bioweapon. *Bp* is resistant to many antibiotics and has no effective vaccine, thus there is great interest in developing protective therapies for at-risk individuals. As an intracellular pathogen capable of persisting within macrophages, we are interested in identifying mechanisms by which *Bp* evades macrophage clearance. To help identify virulence mechanisms, we currently perform comparative studies with *Burkholderia thailandensis* (*Bt*) which is a closely related but relatively avirulent acapsular species. We hypothesize that complement opsonization is critical for efficient intracellular clearance of *Burkholderia* species by macrophages. Our data indicates that *Bp* and *Bt* are equally resistant to complement-mediated direct killing compared to a serum-sensitive *E.coli* strain. Western blots and FACS analysis indicate that complement deposition is substantially greater on the surface of *Bt* compared to *Bp*, suggesting a role for *Bp* capsule in inhibiting surface opsonization. While complement opsonization appears to have no effect on bacterial uptake by macrophages, it did significantly enhance intracellular clearance of *Bt*, but not *Bp*. Future studies will further delineate the role of *Bp* capsule and specific complement receptors in complement-mediated immune evasion, with the goal of eventually identifying therapeutic targets to promote immune clearance.

CARMA3 required for *S.aureus*-induced TNF- α production through NF- κ B activation in Raw264.7 cells.

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CARMA3 (CARD and MAGUK domain-containing protein 3) is a scaffold molecule with not all biological functions known. It is well known that CARMA3 proteins play a critical role in mediating activation of the NF- κ B transcription factor in LPA induced GPCR signaling pathways. However, the molecular mechanism by which CARMA3 regulates activation of NF- κ B in bacterial induced TLR signaling pathways is still unknown. Here we show that CARMA3 physically associates with intracellular mediators and regulates TNF- α production through TLR induced NF- κ B in the mouse macrophage cell line Raw264.7 cells.

By generating CARMA3 knockout cells using the siRNA approach we show CARMA3 is required for TLR-induced NF- κ B activation in Raw264.7 cells. We found that CARMA3 deficiency significantly decreased TNF- α production through NF- κ B activation in LPS and *S. aureus* stimulated mouse macrophages. Also, CARMA3 forms a complex with Bcl10, Malt1, and TRAF6 in LPS and *S. aureus* stimulated Raw264.7 cells. Mechanically, *S. aureus* and LPS induced TLR2 and TLR4 signaling regulates TNF- α production through CARMA3-Bcl10-Malt1-TRAF6 complexes in Raw264.7 cells.

Taken together, we demonstrate that *S. aureus* and LPS-induced CARMA3 is required for TLR-induced NF- κ B activation in macrophages. Also, our results show a novel TLR-induced signaling pathway that leads to NF- κ B activation through a CARMA3-Bcl10-Malt1-TRAF6 complex in LPS and *S. aureus* stimulated-Raw264.7 cells. The molecular complex containing CARMA proteins, BCL10, Malt1, and TRAF6 is a key component in the new signal transduction pathways that regulate NF- κ B transcription factor in macrophages. Also, our results suggest molecular targets for designing therapeutic new agents for bacterial infectious diseases.