Center for Immunology

MEDICAL COLLEGE OF WISCONSIN

Thursday, March 12, 2020
HRC/Alumni Center

Scientific Retreat Schedule

8:00-8:45 a.m.	Reception/Continental Breakfast HRC H1210/H1230
8:45-9:00 a.m.	Introduction
9:00-10:00 a.m.	**Michael Farrar, PhD**, Professor and Virginia and David C. Utz Land Grant Chair in Fundamental Immunology, University of Minnesota *Entraining Immune Tolerance: When, Where, What and How*
10:00-10:15 a.m.	**Break**
10:15-10:55 a.m.	**Calvin Williams, MD, PhD** Professor and Chief of Pediatric Rheumatology, Departments of Pediatrics and Microbiology & Immunology, Medical College of Wisconsin; Chief Scientific Officer, Children's Research Institute *A Critical Role for Peripherally Induced Regulatory T cells in Mucosal Homeostasis*
10:55-11:10 a.m.	**Anthony E. Zamora, PhD** Assistant Professor Department of Medicine *Neoantigen-specific CD8+ T Cells from Pediatric Patients with ALL Exhibit Phenotypic and Functional Heterogeneity*
11:10-11:25 a.m.	Abstract # 21 – **Robyn Oldham** (Medin Lab) *T Cells Armed with Novel anti-CD30/anti-CD3 Bispecific Antibodies for Immunotherapy of CD30+ Malignancies*

11:25-11:40 a.m.	Abstract # 23 – **Wen Zhu** (Wang Lab) *Antibody Cloning Identifies Pathogenic and Non-Pathogenic Antibodies in Heparin-Induced Thrombocytopenia and Defines the Molecular Signatures That Differentiate the Two Groups*
11:45-12:55 p.m.	**Catered Lunch – HRC H1250**
12:55-1:35 p.m**.**	**Vera Tarakanova, PhD** Associate Professor Department of Microbiology & Immunology *Gammaherpesviruses and IRFs: Manipulating the Enemy*
1:35-1:50 p.m.	Abstract # 3 – **Yao Chen** (Cui Lab) *BATF Epigenetically Programs Progenitor to Cytolytic CD8 T cell Transition During Chronic Viral Infection*
1:50-2:05 p.m.	Abstract # 16 – **Christopher Jondle, PhD** (Tarakanova Lab) *Gammaherpesvirus Usurps Host IL-17 Signaling to Support Chronic Infection*
2:05-2:20 p.m.	**Break**
2:20-3:00 p.m.	**Antje Kroner-Milsch, MD, PhD** Assistant Professor Department of Neurosurgery *Inflammation and Hemorrhage After Spinal Cord Injury*
3:00-3:15 p.m.	Abstract # 18 – **Katy LaFond, MA** (Medicine) *NOS1 Provides a Molecular Mechanism from Which Neutrophils Respond to Inflammation*
3:15-3:30 p.m.	Abstract # 19 – **Mahmoud Abu Eid** (Dwinell Lab) *Inhibition of Oxidative Phosphorylation Modulates Anti-Tumor Immunity and Abrogates Melanoma*
3:30-5:00 p.m.	**Poster Presentations and Reception** Alumni Center

Abstract #: 1

CCL3 and its receptors contribute to secondary damage following spinal cord injury

Nicolas Pelisch, Antje Kroner

Department of Neurosurgery, Medical College of Wisconsin & Zablocki Veterans Affairs Medical Center, Milwaukee, WI, USA

Secondary damage after spinal cord injury (SCI) occurs due to a sequence of events after the primary injury, including but not limited to inflammation, contributing to increased lesion size and poor locomotor recovery. Thus, understanding secondary damage following SCI is critical to minimize tissue damage and improve neurological outcome. We have identified the expression of CCL3, a member of the CC chemokine family, and its receptors at different timepoints after contusive SCI in mice. Adult female 8-week old mice were subjected to a moderate contusion at thoracic level 11, and the expression levels of CCL3 and its receptors CCR1, CCR4 and CCR5 were characterized at the lesion site on the mRNA and protein level. CCL3 was upregulated after injury, with a peak at 6 hours and stayed upregulated for the duration of the experiment (28 days). Similarly, CCR1 and CCR5 expression was also increased at day 3 and 7-post injury, respectively. CCR4, in contrast, did not show any significant change. Next, we compared locomotor recovery in CCL3 knockout and wild type mice. The Basso Mouse Scale locomotor score (BMS) showed that the CCL3 knockout mice initially recovered better compared to the wild type group, but at later time points, the scores of the CCL3 knockout mice started to decline, while the wild type group showed a slight improvement. Histological analysis revealed a reduced tissue loss in CCL3 knockout mice. To further address the role of CCL3 in mediating secondary damage, we knocked-down CCL3 using a novel technology of antisense oligonucleotides. FANA antisense oligonucleotides (FANA ASO) can penetrate cells and tissues without the need of transfection. We used adult female C57BL/6J mice and intrathecally delivered CCL3 specific FANA ASO or a scrambled control immediately after contusion. Injection of CCL3 specific FANA ASOs significantly decreased the expression of CCL3 and its receptor CCR5 at day 3-post contusion. CCL3 knockdown led to a subtle functional improvement compared to control mice. In summary, we suggest that the proinflammatory chemokine CCL3 might be critically involved in the inflammatory response and contribute to secondary damage following contusive SCI *in vivo*, thereby providing a new potential target for SCI therapy.

Abstract #: 2

Single cell lineage mapping of a diverse virus-specific naïve CD4 T cell repertoire

Achia Khatun[1], Moujtaba Kasmani[1], Ryan Zander[2], Weiguo Cui[1,2*],

[1]Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, WI; [2]Versiti Blood Research Institute, Milwaukee, WI

Tracking how individual naïve T cells from a natural TCR repertoire clonally expand, differentiate, and make lineage choices in response to an infection has been challenging. Here, using single cell sequencing technology to identify clones by unique TCR sequences, we were able to trace the clonal expansion, differentiation trajectory and lineage commitment of virus-specific CD4 T cells individually, during an acute LCMV infection. Notably, we identified ~450 LCMV-specific CD4 T cell clones defined by uniquely paired TCR α and β chain sequences in each mouse at the peak of T cell expansion. Surprisingly, no clonal overlap has been observed even in two genetically identical mice. At the same time, single-cell transcriptomics of polyclonal antigen specific CD4 T cells revealed a higher degree of transcriptional heterogeneity towards two different Th1 (Type 1 helper T cell) and three different T_{FH} (Follicular helper T cell) subsets along with MPC (Memory Precursor Cell) and Treg (Regulatory T cell). Interestingly, Monocle 2 trajectory analysis also showed linear progressive differentiation from MPC to either Th1 or T_{FH} effector cell subsets at the population level. To further define the lineage choices at the single cell clonal level, clonal definition was restricted as clones having ≥2 cells with identical TCRs and their differentiation trajectory were analyzed using Monocle 2. Intriguingly, although most naïve CD4 T cells gave rise to multiple lineages at the clonal level, ~26% of naïve cells exhibited a preferred lineage choice towards either Th1 or T_{FH} cell subsets. In addition, many T cell clones don't necessarily follow a linear differentiation trajectory from MPC to effector cells, but rather progress into either Th1 or T_{FH} cell subsets. Mechanistically, we found that T_{FH} biased, but not Th1 biased lineage decision, was influenced by TCR structure, in particular the CDR3 motif of the TCR α chain. Collectively, our new findings from this work could provide insight and frameworks for vaccine designs that can generate more tailored cellular or humoral immunity against infection and cancers.

Abstract #: 3

BATF epigenetically programs progenitor to cytolytic CD8 T cell transition during chronic viral infection

Yao Chen[1,2], Weiguo Cui[1,2]

[1]Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, WI, USA; [2]Versiti Blood Research Institute, Milwaukee, WI, USA

During chronic viral infections and cancer, antigen-specific CD8 T cells gradually become functionally exhausted. Without the alteration of epigenetic landscape, immune checkpoint blockade can only transiently recover the function of exhausted T cells. Recent work from our lab and others have shown that there are at least three major subsets of virus-specific CD8 T cells during the late phase of chronic LCMV infection. These phenotypically and functionally heterogeneous T cells encompass $Ly108^{+}TCF1^{+}$ progenitor, CX_3CR1^{+} cytotoxic effector, and $Ly108^{-}$ CX_3CR1^{-} $PD\text{-}1^{hi}$ exhausted subsets. The progenitor subset, with "CD4 help", can further transition to CX_3CR1^{+} cytotoxic effector cells to overcome T cell exhaustion. Nevertheless, the detailed molecular mechanisms that underlie this developmental transition remain unknown. Our recently published work has shown that CD4 T cell-derived IL-21 upregulates BATF expression in CD8 T cells, which led us to hypothesize that BATF as pioneer chromatin modifier provides a permissive epigenetic landscape that allows this differentiation transition. Here, we first compared single cell RNA-sequencing data of virus-specific CD8 T cells from chronic and acute LCMV infection. Interestingly, we found that the progenitor and CX_3CR1^{+} subset in chronic infection shared similar transcriptional profiles with memory precursor and short-lived-effector T cell (SLEC) in acute infection, respectively. Consistently, computational analysis and genetic deletion model showed that T-bet (*Tbx21*) was required for the formation and function of the CX_3CR1^{+} subset during chronic LCMV infection. Furthermore, we demonstrated that three subsets of virus-specific CD8 T cells are epigenetically distinct, with the CX_3CR1^{+} subset being similar to SLEC from acute infection. Importantly, BATF binding motif was enriched in enhancers unique to the CX_3CR1^{+} and progenitor subsets but not in those unique to exhausted T cells. The binding of BATF to enhancers of multiple CX_3CR1^{+} subset signature genes, such as *Tbx21* and *Cx3cr1*, was confirmed by ChIP-seq. Lastly, BATF haplodeficiency caused profound reduction of the CX_3CR1^{+} subset while significantly increasing the proportion of $PD\text{-}1^{hi}$ subset. In summary, our data suggest that BATF is required to maintain a permissive chromatin structure that allows TFs, such as T-bet, to regulate the $Ly108^{+}$ progenitor to CX_3CR1^{+} cytotoxic effector transition. This study also suggests that redirecting progenitor cells towards cytotoxic differentiation may be a more feasible strategy to circumvent T cell exhaustion in treating chronic infections and cancer.

Abstract #: 4

CD8+ T cell Heterogeneity Revealed by ScRNA-Seq Analysis: Comparing Responders and Non-Responders to Immune Checkpoint Blockade Therapy

Alexandra Cohn[1], MSt, Achia Khatun[3], Moujtaba Kasmani[1,3], Ryan Zander, PhD[2] David Schauder, PhD[1,3], Weiguo Cui, PhD[2,3]

[1]Medical School, Medical College of Wisconsin; [2]Versiti Blood Research Institute; [3]Microbiology and Immunology, Medical College of Wisconsin

Recent scRNA-seq analysis from our lab and others has revealed heterogeneity in exhausted CD8+ T cells during chronic infection: CD8+ T cells in a progenitor-like population can differentiate into either effector cells or terminally exhausted cells, with the effector population being critically important for infection control. This differentiation process has been shown to be similar in cancer-findings relevant to the development of T cell-mediated tumor therapies such as immune checkpoint blockade therapy. It is not yet known why only some patients respond to these treatments. We hypothesized that the increased differentiation from progenitor CD8+ T cells into effector CD8+ T cells in response to immune checkpoint blockade therapy is a key prognostic factor for treatment outcome. In analyzing CD8+ T cell scRNA-seq data from melanoma patients (Sade- Feldman et al., 2018; GSE 120575), we found the ratio of effector to progenitor cells to be significantly higher in responders compared to non-responders, while frequency of each CD8+ population alone was not predictive of response. Differentiation from progenitor to effector T cells may be important in predicting outcomes in the TME, as has been shown similarly in chronic infection.

Abstract #: 5

The tale of two receptors: Determining the role of lipoprotein receptors, SR-BI and LDL-R, in gammaherpesvirus infection

Carlie Aurubin, Vera Tarakanova PhD

Department of Microbiology & Immunology, Medical College of Wisconsin

Gammaherpesviruses are ubiquitous pathogens that establish life-long infections in most adults and are associated with several cancers, including B cell lymphomas. Recent studies from our lab have demonstrated that endogenous lipid synthesis supports gammaherpesvirus replication and latency. However, the role of exogenous cholesterol exchange during infection had not been addressed until now. Exogenous cholesterol is carried in the serum by high density lipoproteins (HDL) and low density lipoproteins (LDL). These lipoproteins facilitate cellular cholesterol exchange by engaging the scavenger receptor B, type 1 (SR-BI) and low density lipoprotein receptor (LDL-R), respectively. We have found that SR-BI supports gammaherpesvirus replication at the level of early viral gene expression by attenuating the type I Interferon response in primary macrophages. Alternatively, we found that LDL-R modestly attenuates gammaherpesvirus replication during the early stages of infection through the attenuation of the endogenous cholesterol synthesis pathway, and does not appear to affect type I IFN signaling. These data suggest that while SR-BI exerts a pro-viral effect, LDL-R exerts an anti-viral effect in infection. Thus, revealing an exciting yet complex role for lipoprotein receptors during gammaherpesvirus infection.

Abstract #: 6

Bhlhe40 is regulates CD4 cell fate decision during viral infection

Christine Nguyen and Weiguo Cui

Medical College of Wisconsin & Versiti Blood Center of Wisconsin

CD4 T cells orchestrate the adaptive immune response by providing assistance to both CD8+ T cell and B cell responses against pathogens. In response to viral infection, naïve CD4+ T cells are activated into T helper 1 (Th1) and T follicular helper (T_{fh}) subsets which are regulated by their key transcriptional factors T-bet and BCL6, respectively. During LCMV infection, Th1 cells mediate the CD8 T cell pro-inflammatory response whereas T_{fh} cells assist in the formation of germinal centers and the maturation of B cells leading to production of neutralizing antibodies against the virus during late phase of infection. There is evidence that BCL6, the master transcriptional regulator of T_{fh} cells, functions as a transcriptional repressor by various mechanisms to inhibit Th1 effector genes, while simultaneously reinforcing the expression of T_{fh} signature genes. However, the transcriptional circuits underlying the fate bifurcation of Th1 and T_{fh} CD4+ T cell subsets are not fully understood. Previous work has shown the transcription factor Bhlhe40 represses multiple genes related to T cell differentiation and function. In Th1 cells, deletion of Bhlhe40 is shown to significantly increase IL-10, an anti-inflammatory cytokine, at the expense of IFN-γ production. This is supported by an increase in the transcription factor c-Maf, which is a key regulator of IL-10 production. Recent single cell RNA-seq and Bulk RNA-seq data from our lab indicate that Bhlhe40 is upregulated in the Th1 population and is decreased in the T_{fh} subset during LCMV infection. This leads us to propose that Bhlhe40 plays a role in transcriptionally regulating CD4+ T cell fate determination. We have tested the necessity of Bhlhe40 in CD4 T cells by using a mixed bone marrow chimera mice model in which Bhlhe40 is genetically deleted only in CD4 T cells and show that Bhlhe40 negatively regulates the frequency and function of T_{fh} cells by promoting Th1 differentiation during viral infection. This suggests that Bhlhe40 may be imperative in not only controlling the function of Th1 cells, but also in reinforcing their differentiation. Collectively, I hypothesize that Bhlhe40 regulates CD4 Th1/T_{fh} cell fate determination via a repressive mechanism by transcriptionally promoting Th1 differentiation while simultaneously suppressing Tfh differentiation. Identifying the molecular basis of CD4+ T cell fate determination will allow for novel antiviral therapies.

Abstract #: 7

Activating the antitumor immune response in pancreatic cancer

Vonderhaar E, Barnekow NS, McAllister D, Jing W, Johnson B PhD, and Dwinell MB PhD

MCW student, Department of Microbiology & Immunology, Medical College of Wisconsin

The median survival of all-comers with pancreatic ductal adenocarcinoma (PDA) is a mere 8 months. Its unique tumor microenvironment (TME), abundant in fibroblasts, extracellular matrix and tumor-promoting immune populations, drives therapeutic resistance and creates an immunologically tolerant space. Recently, activation of the innate immune system to augment adaptive antitumor immune responses has been employed in pre-clinical models with promising results. One such innate immune pathway involves the antiviral protein, Stimulator of Interferon Genes (STING). STING potently triggers inflammatory signaling cascades culminating in the expression of effector cytokines and chemokines. We aim to test the hypothesis that STING activation can initiate an effective antitumor response in PDA despite its highly immune-suppressed TME. Mice were implanted with syngeneic pancreatic tumor cells subcutaneously and treated with three intratumoral injections of murine STING agonist 5,6-Dimethylxanthenone-4-acetic acid (DMXAA). We have established that the subcutaneous tumors recapitulate the TME of the autochthonous tumors from which they were derived. DMXAA-treated mice had significantly increased survival and decreased tumor burden than controls. Additionally, STING pathway activation resulted in increased effector CD8+ T cells, decreased regulatory T cells and decreased the Foxp3:CD8 ratio within the tumor. A multiplex screen revealed that T cell-attractant chemokines, CXCL9 and CXCL10, were produced at higher levels in STING agonist-treated tumors. PDA cells, macrophages, dendritic cells and fibroblasts all contributed individually to the increased production of these chemokines. Interestingly, the frequency of tumor-reactive effector T cells in the spleen was higher in DMXAA-treated mice. To test the ability of local intratumoral STING agonist therapy to induce a global antitumor response, mice were co-implanted with tumors on each flank and received treatment in only the primary tumor. Contralateral tumors of DMXAA-treated mice were smaller and more inflamed than tumors of vehicle-control mice, reflecting responses within the primary tumor. Overall, activation of the STING pathway in PDA strongly reprograms the TME from immune-suppressed to immune-activated. A detailed mechanism by which STING activation results in an effective anti-PDA immune response remains to be determined. Ongoing and future studies will also utilize human and mouse bi-specific STING agonists.

Abstract #: 8

The Effects of Multiple Sclerosis and Ocrelizumab B Cell Depletion Therapy on Lymphocyte Populations

Cody Gurski, Bonnie Dittel, Ahmed Obeidat

Versiti Blood Research Institute, Milwaukee, Wi

Multiple sclerosis (MS) is a debilitating autoimmune disease of the central nervous system characterized by demyelination and inflammation. Traditionally considered to be a T cell mediated disease, recent studies have found that B cells may play a role in MS. This is supported further by the efficacy with which B cell depleting therapies attenuate disease in MS patients. Ocrelizumab is a humanized anti-CD20 antibody approved for both primary progressive and relapsing forms of MS. In this pilot study, we aimed to characterize the T and B cell populations in MS patients at timepoints before and after CD20 depletion with Ocrelizumab when compared to age and sex matched healthy controls. We also investigated which B cell subsets were refractory to depletion entirely, and those which reemerged early after infusion. We were unable to phenotype B cells after the initial infusion, as all ($CD19^+$) B cells were depleted and only a miniscule portion of class switched B cells repleted by the six month timepoint. We found that class switched ($CD19^+$ IgM^- IgD^-) B cells were also increased in MS patients when compared to healthy controls. Interestingly, several MS patients displayed a population of plasmablasts ($CD19^+$ $CD20^-$ IgD^- IgM^- $CD27^{HI}$ $CD38^{Hi}$) that were also depleted after infusion. We examined the ectonucleotidase, CD39, because it is a marker for T regulatory cell (Treg) suppressive activity to IL-17. In the Treg populations of MS patients and healthy controls, we found that $CD39^+$ Tregs are more prevalent in MS patients and that $CD39^+$ Treg numbers decrease after infusion. As we continue this pilot study, we hope to discover trends among other B and T cell subsets to further contribute to the understanding of how Ocrelizumab affects MS patients.

Abstract #: 9

Roles for biased agonist chemokine signaling in acute myelogenous leukemia

Halyko, M., D. McAlister, N. Barnekow, B.F.Volkman, and M.B. Dwinell

Department of Pediatrics, Department of Microbiology & Immunology, Department of Biochemistry, and the Center for Immunology, Medical College of Wisconsin

Acute myeloid leukemia (AML) is a hematological malignancy of adults and children with poor clinical outcomes. While the majority of pediatric patients respond to standard of care chemotherapy half will relapse with chemoresistant disease. The CXCL12-CXCR4 chemokine axis helps maintain AML cells in the chemoprotective bone marrow niche. Plerixafor is an FDA-approved CXCR4 antagonist currently being investigated in clinical trials for relapse/refractory AML. Our group and others have described that CXCL12 locked into a dimerized state (CXCL12-LD) is a biased agonist for CXCR4. We hypothesized that CXCL12-LD can mobilize CXCR4 expressing cells into the peripheral blood and may serve as a novel therapeutic for AML. As a first step, we injected tumor-bearing mice with either CXCL12-LD or Plerixafor and found increased mobilization of lymphocytes and monocytes into the peripheral blood. Similarly, we measured mobilization of CXCR4 expressing CD34+ hematopoietic progenitor cells at multiple timepoints following injection of CXCL12-LD. Having shown CXCL12-LD induced mobilization of CXCR4-expressing immune cells, the expression of CXCR4 by two human AML cell lines was confirmed using RT-PCR and flow cytometry. We next asked if CXCR4 expressed by AML could be activated by wild-type CXCL12, a balanced agonist that activates G protein and arrestin signaling, and CXCL12-LD biased agonist that activates G proteins. Treatment of AML cells with CXCL12-LD and wild type ligand stimulated calcium influx characteristic of CXCR4 activation. Moreover, AML cells undergo dose dependent chemotaxis in response to wild type CXCL12. In conclusion, our data shows that CXCR4 expressing progenitor cells and immune cells are mobilized by CXCL12-LD. CXCR4 is expressed AML cells, suggesting these malignant cells may be therapeutically targeted by the biased agonist.

Abstract #: 10

Mapping of the AML Cell Surface N-Glycoproteome and Identification of a Novel scFv for Immunotherapy

Charles Hay[1], Mary Faber[1], Robyn Oldham[1,2], Theodore Keppel[3,4], Amanda Buchberger Jones[3,4], Rebekah Gundry[3,4], Jeffrey Medin[1,2,3]

Departments of [1]Pediatrics, [3]Biochemistry, and [4]Center for Biomedical Mass Spectrometry Research, MCW, [2]Department of Medical Biophysics, University of Toronto

Acute myeloid leukemia (AML) is a cancer of the bone marrow wherein immature myeloid or blast cells function abnormally and do not mature into red blood cells, white blood cells, or platelets. With time, AML spreads to other tissues. AML is the 2nd most common pediatric leukemia. The pediatric survival rate has plateaued at 65%-75% and the overall survival rate of 27.4% stresses the need for alternative treatment strategies. A mAb therapy that targets AML cell surface proteins may have the potential to improve this low survival rate.

We used a mass spectrometry (MS)-based, discovery-driven cell surface capture (CSC) technique to map the *N*-glycoproteome of 4 AML cell lines. We identified 730 *N*-glycoproteins in total. A comparison of the resultant list against sources to eliminate proteins expressed on normal human cells and tissues yielded a final shortlist of 12 AML-specific proteins with therapeutic potential. The extracellular domain of one of these antigens, with an exogenously added polyhistidine affinity tag, was expressed in a mammalian cell culture system and purified. Assessment of the purified protein by SDS-PAGE revealed the protein to be larger in size than expected. This increased size was found to be due to post-translational glycosylation. The identified glycoprotein was used to pan a single-chain fragment variable (scFv) phage display library and subsequently tested for antigen binding specificity. This approach yielded one scFv clone whose sequence contained identifiable Ig framework regions and complementarity determining regions (CDRs) consistent with human variable light and heavy chains. The 18-mer G/S rich linker and 6xHis tag were also present as expected. The clone was determined by ELISA to be specific for the input AML antigen. This scFv will be used to create novel anti-AML immunotherapy reagents (such as bispecific antibodies and chimeric antigen receptors) to be tested *in vitro* and *in vivo* in a mouse model of AML.

Abstract #: 11

Role of hemopexin in secondary damage after spinal cord injury

Kishan Patel M2, Nicolas Pelisch MD/PhD, Antje Kroner MD/PhD, Department of Neurosurgery

Department of Neurosurgery, Medical College of Wisconsin

Introduction: Spinal cord injury (SCI) is a debilitating neurological condition with tremendous socioeconomic impact on affected individuals. Hemorrhage can be cytotoxic and pro-inflammatory through the release and degradation of hemoglobin in the injured tissue. We aim to understand the mechanisms of hemorrhage-induced secondary tissue after SCI. The acute phase protein hemopexin (Hx) sequesters heme, and has protective effects after CNS hemorrhage. We aim to better understand the mechanisms of hemorrhage-induced secondary damage after SCI and to modify these effects using Hx.

Hypothesis: Hemopexin is beneficial after SCI in reducing inflammation, tissue damage and improving functional recovery after SCI.

Methods: Contusive spinal cord injury was induced in female C57BL/6 wildtype and Hx deficient mice (8 weeks) or in wildtype mice for Hx treatment studies. Treatment was initiated immediately after injury by applying gel foam containing Hx (5µg) or a vehicle control (saline). All mice were assessed behaviorally using the Basso Mouse Scale (BMS) for locomotor recovery for 28 days. At the end of the experiment, mice were euthanized, and spinal cords were processed, cryoprotected, and sliced in 14µm thick serial sections for staining and lesion analysis. To assess the inflammatory response at day 3 and 7 after injury, tissue was harvested, RNA extracted and gene expression for IL-1b, IL-6, IL-10, TNF, CD163 and LRP-1 was quantified using Q-RT PCR.

Results: Hx-/-mice showed impaired recovery compared to wildtype mice, and significantly fewer Hx-/-mice were able to support their body weight on day 3 and 7 post injury. In Hx treated wildtype mice, a trend towards better weight support at day 3 was detected, while the BMS did not show any differences. mRNA expression of the pro-inflammatory genes IL-1b and IL-6 was significantly reduced in the early phase post SCI following treatment, while a significant reduction in TNF was found at a later phase. No significant difference in lesion size was detected.

Conclusions: Hx-/-mice showed significant behavioral and locomotor deficits compared to wildtype mice. Treatment with Hx showed a potential effect on reducing pro-inflammatory cytokine expression with a small effect on behavioral recovery.

Future Directions: Intrathecal injection of Hx will be administered at specific time points after injury to achieve higher concentrations of Hx at the lesion site.

Abstract #: 12

The antiviral transcription factor Interferon regulatory factor 3 (IRF-3) promotes chronic gammaherpesvirus infection

Sylvester, P*.; Johnson, K*.; Aurubin, C.; Tarakanova V.

Medical College of Wisconsin, Department of Microbiology and Immunology

Gammaherpesviruses are ubiquitous pathogens which infect over 95% of the human population. These viruses remain within an infected host for life and, while relatively quiescent in an immune-competent host, immunodeficiency can lead to the development of lymphoproliferative disorders. To understand how these viruses cause disease, we must study host and viral factors which keep these viruses controlled during infection. Our studies utilize the rodent gammaherpesvirus, murine gammaherpesvirus 68 (MHV68) to do *in vivo* infections in a genetically tractable host. Interferon Regulatory Factor-3 (IRF-3) is a host transcription factor involved in the type 1 interferon response, a major antiviral pathway. IRF-3 is required for the control of several acute viral infections including West Nile Virus, Murine Norovirus and MHV68. However, the role of IRF-3 during chronic MHV68 infection has not been established. We decided to investigate this role by infecting IRF-$3^{-/-}$ mice with MHV68. Surprisingly, despite the canonical antiviral function of IRF-3, we observed a significant decrease in viral latency in $IRF3^{-/-}$ mice, indicating a pro-viral role for IRF-3 during chronic gammaherpesvirus infection. Due to co-evolution with their host, gammaherpesviruses have developed machinery to counteract antiviral pathways such as the type 1 interferon pathway. For MHV68, the viral kinase ORF36 antagonizes IRF-3 function, *in vitro*. This viral kinase also facilitates infection *in vivo* as mice infected with an MHV68 mutant lacking ORF36 (N36S) have reduced viral latency. To determine whether the absence of IRF-3 will rescue the latency defect of the N36S mutant, IRF-$3^{-/-}$ mice were infected with the N36S virus. Interestingly, the latency defect of the ORF36 deficient mutant was further exacerbated in IRF-$3^{-/-}$ mice, indicating that ORF36 and IRF-3 have independent roles in promoting chronic infection. In summary, we have discovered a novel pro-viral role for IRF-3 in chronic gammaherpesvirus infection that is in part independent of ORF36 function.

Abstract #: 13

Absence of IL-12p40 mediates a beneficial effect on recovery after spinal cord injury

Jose Rosas, Nicolas Pelisch, Brandy Aperi, Kyle Stehlik, Karin Swartz, Antje Kroner

Department of Neurosurgery, Medical College of Wisconsin; Clement J. Zablocki Veterans Affairs Medical Center; Microbiology and Immunology Department, Medical College of Wisconsin

Traumatic spinal cord injury (SCI) is a relatively frequent event that imposes a massive burden on the health, quality of life and socioeconomic situation of affected persons and their caregivers. Both localization and extent of tissue damage in the injured cord influence the functional outcome. Tissue damage after SCI occurs in two phases: While primary damage describes tissue loss caused by the initial trauma, the lesion is expanded by secondary damage processes, including inflammation, hemorrhage, edema and production of reactive oxygen species.

A more complete understanding of individual contributors to inflammatory damage is necessary to specifically target and modify detrimental factors. Inflammation after SCI is exacerbated, with activated microglia and monocyte-derived macrophages being the main immune cell populations in the injured tissue.

Of particular interest are the pro-inflammatory cytokines IL-12 and IL-23, which share a subunit (p40) that is strongly upregulated after phagocytosis of red blood cells. IL-12 and IL-23 are expressed by a variety of cell types and have critical functions in regulating both the adaptive and innate immune system by inducing the production of pro-inflammatory cytokines.

We are assessing the role of IL-12 and IL-23 and their receptors after SCI, using a low thoracic (T11) moderate contusion injury (50 kDyne) in mice.

First, we quantified protein expression using Western blot. IL-12p40 is significantly upregulated 24h after injury and stays upregulated throughout the observation period (28 days). The receptor subunit IL12Rβ2 is also significantly upregulated, while IL-12Rβ1or IL-23p19, the second subunit of IL-23, are not significantly changed.

We utilized the IL-12p40 and IL-23p19 knockout mice and wildtype controls to quantify locomotor recovery after SCI using the Basso Mouse Scale (BMS). We observed improved recovery in IL-12p40 deficient mice compared to wildtype mice. IL-12p40 KO mice also demonstrate significantly more spared tissue and less iron accumulation at 28 days after SCI. In contrast, IL-23p19 KO mice did not differ from wildtype mice regarding their locomotor scores.

In addition, we sought to identify the effect of locally applied rIL-12 on locomotor recovery and inflammation after SCI. Our preliminary data suggest that local application of rIL-12 does not change locomotor recovery compared to vehicle treated controls.

In summary, these results suggest that the absence of IL-12p40, but not IL-23p19, mediates a neuroprotective effect after contusion SCI, serving as a new potential therapeutic target to ameliorate secondary damage and promote better functional recovery outcomes.

Abstract #: 14

Fibroblast-like synoviocytes produce NK cell-activating factors to promote the arthritogenic IFNγ response in Lyme arthritis

Joseph Rouse, Rebecca Danner, Amanda Wahhab, Sarah Timmler and Robert Lochhead, Ph.D.

Department of Microbiology & Immunology, Medical College of Wisconsin, Milwaukee, Wisconsin

Lyme arthritis (LA) is the predominant late-stage manifestation of Lyme disease, caused by the tick-borne spirochete *Borrelia burgdorferi* (*Bb*). LA is often associated with autoimmune T and B cell responses and a high IFNγ response. However, it is unclear how the IFNγ response is initiated in patients during infection. We aim to better understand the effects of the arthritogenic IFNγ response in LA. We isolated primary fibroblast-like synoviocytes (FLS) from C57BL/6J (B6) mice that develop mild arthritis and from B6 *Il10*$^{-/-}$ mice that develop severe, IFNγ-mediated LA. We stimulated FLS with *Bb*, IFNγ, or IFNγ+*Bb* to recapitulate inflammatory conditions in the joint microenvironment during LA. Cells were analyzed by flow cytometry, supernatants were collected for cytokine analysis, and RNA was isolated to assess gene expression. IL-10$^{-/-}$ FLS stimulated with IFNγ or IFNγ+*Bb* displayed increased surface expression of IL-15Rα, compared with B6 FLS. FLS also showed upregulation of innate cytokines (IL-1β and TNFα), IFNγ-inducing cytokines (IL-12 p35 and p40), as well as T cell and natural killer (NK) cell chemokines (CXCL9 and CXCL10) and growth factors (IL-15). Since IL-15 signaling is required for priming and proliferation of NK cells, an early innate source of IFNγ, our results indicate that IL-10$^{-/-}$ FLS may interact with NK cells to promote arthritogenic IFNγ responses in the synovial microenvironment.

Abstract #: 15

Mononuclear phagocytes respond to ceftriaxone-induced Enterococcus faecalis dissemination

Kevin Jennings, Rajrupa Chakraborty, Christopher Kristich, Nita Salzman

Department of Microbiology & Immunology, Medical College of Wisconsin

Enterococcus faecalis (EF) is a gram-positive commensal that acts opportunistically in immunocompromised hosts and contributes significantly to hospital acquired infections. EF is intrinsically resistant to cephalosporins and it has been proposed that systemic EF infections can be attributed to expansion of gut-residing EF populations during antibiotic treatment. We have developed a mouse model whereby intraperitoneal ceftriaxone injection leads to increases in intestinal EF populations and subsequent dissemination to peripheral organs. Experiments in Rag1-/- mice that lack adaptive immune cells effectively clear ceftriaxone-induced EF infection without pathology. Furthermore, we have shown that intestinal lamina propria and mesenteric lymph node adaptive immune cell numbers are not affected by EF dissemination; however, several subsets of intestinal mononuclear phagocyte (MNP) populations are significantly decreased. Interestingly, viable EF have been recovered by isolating MNPs following ceftriaxone treatment, while in vitro experiments show that EF can survive up to 72 hours within J774 macrophages. These data indicate that MNPs respond to EF dissemination and suggest evasion of phagocyte killing as a potential mechanism for EF pathogenesis in immunocompromised hosts.

Abstract #: 16

Gammaherpesvirus usurps host IL-17 signaling to support chronic infection

Jondle, C., Johnson, K., Xin, G., Aurubin, C., Sylvester, P., Mboko, W., Cui, W., Huppler, A., & Tarakanova, V.

Department of Microbiology & Immunology, Medical College of Wisconsin

Gammaherpesviruses are ubiquitous pathogens that establish lifelong infection and are associated with B cell lymphomas. These viruses infect naïve B cells and induce a polyclonal germinal center (GC) response to establish long-term infection in memory B cells. We showed that Interferon Regulatory Factor 1 (IRF-1), an antiviral and tumor suppressor transcription factor, selectively restricts the GC reaction driven by murine gammaherpesvirus 68 (MHV68). Further, latent MHV68 infection was poorly controlled in IRF-1$^{-/-}$ mice, including in the peritoneal cavity where the latency is not regulated by the GC response. In our search for the mechanism underlying the observed phenotypes, we unexpectedly found a significant increase in Th17 cells in MHV68 infected as well as naive IRF-1$^{-/-}$ mice compared to WT mice.

Intriguingly, Herpesvirus saimiri (HVS), a related simian gammaherpesvirus, encodes a viral IL-17, posing an intriguing possibility that IL-17 is proviral. MHV68 does not encode an IL-17 homologue, suggesting that MHV68 may rely on a host derived IL-17.

Interestingly, latent MHV68 infection was significantly attenuated in IL-17RA$^{-/-}$ mice establishing, for the first time, a proviral role of host IL-17 signaling during gammaherpesvirus infection. Further, there was a significant decrease in the GC response in IL-17RA$^{-/-}$ mice following MHV68, but not LCMV infection, indicating that IL-17 signaling is selectively required to support the GC response during gammaherpesvirus infection. Additionally, neutralization of IL-17A in IRF-1$^{-/-}$ mice during MHV68 infection significantly reduced the GC response, though infection of mice lacking both IL-17RA and IRF-1 resulted in a phenotype similar to what was found in IRF-1$^{-/-}$ mice alone. These data suggest, that IL-17 may signal though an alternative receptor, which is not highly expressed when IRF-1 is present. Altogether, our study establishes IRF-1 as a suppressor of Th17 differentiation, while also indicating that IRF-1 may suppress an alternative receptor for IL-17 to signal through in the absence of IL-17RA and reveals a novel proviral role of IL-17 during gammaherpesvirus infection.

Abstract #: 17

IRF-7 restricts gammaherpesvirus infection in the peritoneal cavity by altering viral tropism

Johnson, K.E.*, Aurubin, C.A.*, Jondle, C.N., Lange, P.T., Tarakanova, V.T.

Department of Microbiology & Immunology, Medical College of Wisconsin

Gammaherpesviruses are ubiquitous lymphotropic pathogens that establish lifelong infections and are associated with a variety of malignancies, including lymphomas. To maintain lifelong infection of their host, gammaherpesviruses will primarily target B cells and Macrophages for latency. While little is known about risk factors for these virus-driven cancers, it is clear that elevated reactivation of gammaherpesviruses from latency contributes to viral oncogenesis. Previous work in our lab has demonstrated the importance of Interferon regulatory factor 7, (IRF-7), a transcription factor largely known for its role in the induction of interferons, in the context of acute infection. However, no studies have examined the importance of IRF-7 during chronic infection until now.

Here we show that IRF-7 restricted gammaherpesvirus latency and reactivation in the peritoneal cavity, but not in the spleen, during peak latent infection. Interestingly, the virus-specific CD8+ T cell response that is typically critical for control of viral reactivation during chronic infection was not decreased in IRF-$7^{-/-}$ mice, and there was no notable change in expression of IFN induced genes that are known for restricting gammaherpesvirus. Further, IRF-$7^{-/-}$ macrophages appear to be just as responsive to IFN treatments during infection compared to wild type macrophages. Finally, and most notably, IRF-7 expression appears to control viral tropism in the peritoneal cavity, but the mechanism through which it does so remains a mystery. These data for the first time demonstrate an important IFN independent antiviral role of IRF-7 during chronic gammaherpesvirus infection.

Abstract #: 18

NOS1 provides a molecular mechanism from which neutrophils respond to inflammation

Katy M. LaFond, MA, Diego Rodrigues Coelho, PhD, Marcelo Bonini, PhD, Benjamin Gantner, PhD

Department of Medicine, Endocrine Division, Medical College of Wisconsin

Polymorphonuclear neutrophils (PMNs) are key components of the immune system, responding to threats like pathogens and tissue-injury. Neutrophils are recruited into tissue and activated by cytokines. These inflammatory messengers program neutrophils to target, manage, or destroy the threat using an array of effector responses. Many disease states involve a “cytokine storm”, which triggers excessive neutrophil responses and presents a cost to the host since PMNs are primarily responsible for inflammatory damage to host tissue. Our research shows that with a change in inflammatory output, PMN responses divert from the expected destructive pathway to a more focused, repair response. In addition, we discovered a novel way from which PMNs alter the release of chromatin, a response called NETosis; relevant to subverting damage to host and promoting repair. Our lab uses macrophage-secreted cytokines to model the in vivo microenvironment that neutrophils experience to respond to four inflammatory states: non-inflammatory (medium alone), septic (LPS), pro-injury inflammation (NOS1+/+ cytokines), pro-repair inflammation, and (NOS1-/- cytokines). In some experiments, we also model an acute bacterial infection using formylated peptide. Under inflammatory conditions, PMNs increase the release of ROS and lytic granules; all of which result in collateral damage to the host. Our lab discovered that NOS1-/- cytokines promote a distinct form of NETosis that is only recently described. In this so-called “vital NETosis”, chromatin is exposed and yet the cells surprisingly maintain metabolic activity. We observed that PMNs not only remained mobile, but they cleaned up debris and dead cells in their environment. These findings are consistent with our hypothesis that NOS1 controls the neutrophil’s decision to promote tissue injury or tissue repair, and therefore, may serve as a therapeutic target for tailoring specific inflammatory responses.

Abstract #: 19

Inhibition of Oxidative Phosphorylation Modulates Anti-Tumor Immunity and Abrogates Melanoma

Mahmoud Abu Eid, Laura McOlash, Donna McAllister, Gang Chen, Balaraman Kalyanaraman, Michael Dwinell

Department of Microbiology & Immunology, Medical College of Wisconsin

Melanoma remains one of the deadliest cancers with a poor response to chemotherapeutics. Recent immunotherapy approaches have 20-30% response rate. Poor chemotherapeutic outcome is partially due metabolic reprogramming of cancer cells from aerobic glycolysis ,Warburg effect, to oxidative phosphorylation (OXPHOS) is increasingly recognized as a mechanism of chemoresistance. The potential contribution of metabolic changes in accessory immune cells in chemoresistance remain poorly understood. Mito-magnolol, a novel mitochondria-targeted analog of the natural polyphenolic compound magnolol was designed as a novel probe to dissect roles for OXPHOS metabolism in melanoma progression and immune evasion. As a first step, immunocompetent B16 melanoma-bearing mice treated with 0.25. 0.5 or 1 doses of Mito-MGN showed increased survival concurrent with profound changes in the tumor immune microenvironment. Specifically, anti-cancer doses of Mito-MGN decreased levels of suppressive regulatory T cells, myeloid-derived suppressor cells (MDSC) and type 2 tumor associated macrophages (TAMs) with a concomitant elevation in activated CD8 and CD4 T cells. In culture, Mito-MGN inhibited proliferation, mitochondrial complex I-mediated oxygen consumption of B16-F0 and B16-F10 melanoma cells. Consistent with its effects in vivo, Mito-MGN inhibited the differentiation and viability of bone marrow-derived MDSCs in culture. Moreover, T-cells treated with Mito-MGN exhibited a more active phenotype, defined as elevated CD69intCD44hiCD62Llo. Cumulatively, these data indicate that OXPHOS metabolic reprogramming in melanoma cancer cells and myeloid and lymphoid cells can be inhibited with mitochondria-targeted complex I inhibitor Mito-MGN.

Abstract #: 20

Harnessing the IL-21-BATF pathway in the CD8 T cell anti-tumor response

Paytsar Topchyan[1], Gang Xin[2], Weiguo Cui[1,2]

[1]Department of Microbiology and Immunology at Medical College of Wisconsin [2]Versiti's Blood Research Institute

Advanced melanoma is a major target of cancer immunotherapy research due to its poor survival rate even with recent treatment advances. Current strategies focus on CD8 T cells which enter a dysfunctional state in the tumor microenvironment. Recent studies have found that intratumoral CD8 T cells consist of a heterogeneous population of memory-like progenitor, effector and terminally exhausted cells that exhibit differing functional and self-renewal capacities. However, the cellular and molecular processes involved in the differentiation of these subsets within melanoma is not well understood. Exploring the intricacies of CD8 T cell differentiation towards an effector profile can identify novel immunotherapeutic targets. Thus, we elucidate the mechanisms regulating effector CD8 T cell differentiation and function in melanoma, by utilizing various adoptive cell transfer therapy models in B16 tumor-bearing mice.

CD4 T cell production of cytokines provide help to CD8 T cells. Recent studies have uncovered the cytokine IL-21 as a critical signal produced by CD4 T cells to help promote CD8 T cell maintenance in chronic infection. However, the direct effects of IL-21 produced by CD4 T cells on CD8 T cell differentiation and function in cancer remains unknown. Preliminary data presented in this abstract suggest that IL-21 producing CD4 T cells induce an effective antitumor response dependent on CD8 T cells. Additionally, it has been previously found that IL-21 signaling induces the transcription factor BATF, which is known to cooperatively bind with IRF4 to induce changes in the chromatin landscape. This led to our hypothesis that the IL-21-BATF pathway enhances melanoma-infiltrating effector $CX3CR1^{+}$ CD8 T cells and their function.

This project will enhance our understanding of how CD8 T cells differentiate and function in melanoma. The IL-21-BATF axis may provide a novel therapeutic mechanism to enhance effector CD8 T cell differentiation to fight cancer.

Abstract #: 21

T Cells Armed with Novel anti-CD30/anti-CD3 Bispecific Antibodies for Immunotherapy of CD30+ Malignancies

Oldham, Robyn AA[1,2]; Faber, Mary[2]; Thakur, Archana[4], Lum, Lawrence[4]; Medin, Jeffrey A[1,2,3]

[1]Department of Medical Biophysics, University of Toronto; Departments of [2]Pediatrics and [3]Biochemistry, MCW; [4]Department of Medicine, UVA Cancer Center

Chimeric antigen receptors (CARs) have delivered striking clinical successes for patients with leukemia and lymphoma. However, gene-modified autologous T cell products can be expensive and complex to produce. Bispecific antibodies (biAbs) can similarly redirect T cells to kill tumor cells, and may be more straightforward to produce.

CD30 is a promising immunotherapeutic target due to its expression on a number of malignancies and limited expression on normal tissues. Several approaches targeting CD30 are currently being evaluated preclinically and clinically, including an antibody-drug conjugate and CAR-T therapies. We have developed novel CD30/CD3 biAbs (and CARs) and are assessing efficacy of the former against CD30+ tumor cells.

We developed 5 novel anti-huCD30 hybridoma cell lines and confirmed antibody binding specificity to CD30 by flow cytometry and ELISA. All antibodies (Abs) were characterized by epitope mapping, along with DNA and protein sequencing. Two candidate Abs, which bind different epitopes of CD30, were selected for development into biAbs (named bi8D and bi10C) by heterconjugation with an anti-CD3 Ab (OKT3). BiAb-armed human CD3+ T cells were assessed by flow cytometry to measure binding and conjugation ability. *In vitro* cytotoxicity was analyzed by ^{51}Cr release. IL-2 and IFN- γ production was measured by ELISA.

Bi8D and bi10C bind to both tumor cells and T cells. Bi8D T cells show efficient conjugation with CD30+ tumor cells and effectively kill all CD30+ cell lines tested. Bi10C-T cells are less effective. When co-cultured for 24 hours with CD30+ tumor cells, both bi8D-T cells and bi10C-T cells robustly produce IL-2 and IFN- γ. No killing or production of cytokines was observed in response to $CD30^-$ or $CD30^{low}$ cells.
Overall, bi8D-T cells are more effective than bi10C-T cells at conjugation and lysis of CD30+ cells. Importantly, our CD30 biAbs do not kill $CD30^{low}$ T cells, allowing for lysis of tumor cells without fratricide. *In vivo* studies of bi8D T cells are underway.

Abstract #: 22

Myeloperoxidase (MPO) inhibition through administration of KYC reduces skin inflammation severity in a mouse model of plaque psoriasis

Savannah Neu, Tien Vo, Cody Gurski, Kirkwood Pritchard, Bonnie Dittel

Versiti Blood Research Institute & Medical College of Wisconsin

Over 100 million people suffer from plaque psoriasis, a condition of the skin where epithelial cell overgrowth and inflammation causes itchiness, redness, and general discomfort across the surface of the body. Plaque psoriasis can be episodic and triggered by factors such as acute stress, allergens, and concomitant illness; but for many, the cause is unknown. Variability between cases makes designing personal, inexpensive, and noninvasive therapies difficult. Many modern treatments inhibit members of adaptive immunity including B and T cells, often leading to unintended, systemic consequences. Innate immune cells may also contribute to the pathophysiology of plaque psoriasis, but less is known about their exact function in this disease. Neutrophils are first-responder, innate cells that localize early to inflammation sites. They secrete attractants to recruit and engage the immune system and can meanwhile protect target tissues by producing antimicrobial enzymes. Myeloperoxidase (MPO) is the most abundant enzyme found in neutrophilic granules and is released into the inflammatory microenvironment during wounds and infections. MPO kills invading pathogens by many mechanisms (NETosis, chlorotyrosination) but can inadvertently damage surrounding host tissue. We hypothesize that neutrophils and MPO contribute to plaque psoriasis inflammation and are novel targets for topical psoriasis treatment. Using the Imiquimod (IMQ) inducible model of plaque psoriasis in mice, we inhibit MPO by administering KYC, a tripeptide compound that ablates MPO enzymatic activity in vitro. KYC was developed and tested in partnership with the Pritchard lab for treating many inflammatory models. During IMQ psoriasis, we score inflammation severity of the affected skin using a modified psoriasis-area severity index (PASI). We observed that KYC decreased IMQ psoriasis severity at a topical dose of 0.3 mg/kg. The mechanism whereby MPO contributes to plaque psoriasis remains unknown, but in future studies we hope to assess MPO effects on vascular permeability and immune cell migration into the skin.

Abstract #: 23

Antibody Cloning Identifies Pathogenic and Non-Pathogenic Antibodies in Heparin-Induced Thrombocytopenia and Defines the Molecular Signatures That Differentiate the Two Groups

Wen Zhu, Yongwei Zheng, Ph.D, Mei Yu, PhD, Yaling Wu, Jianhui Wei, Nicholas Schneider, Anand Padmanabhan, MD, PhD, Richard H. Aster, MD, Renren Wen, PhD and Demin Wang, PhD

Department of Microbiology & Immunology, Blood Research Institute

Heparin-induced thrombocytopenia (HIT) is a common adverse drug reaction associated with frequent life-threatening thrombotic complications. The hallmark of HIT is polyclonal antibodies (Abs) that recognize platelet alpha granule chemokine PF4 when it binds to heparin (PF4/H). These Abs can be detected in PF4/H ELISA, but only a minority of patients testing positive actually have HIT. In patients who have clinical HIT, Abs that activate platelets can be detected using a platelet-activation assay like PEA. Thus, there are at least two distinct types of heparin-induced Abs - those that cannot activate platelets are seemingly "non-pathogenic" and those that can activate platelets are "pathogenic". To date, the molecular basis for the differing Ab behaviors is uncertain. To address this issue, we performed single cell culture to clone B cell receptors from IgG_1+ B cells from HIT patients. Clones positive only in PF4 ELISA, positive in both PF4 ELISA and PEA, or negative in PF4 ELISA were designated NA (non-platelet-activating), PA (platelet-activating) and NB (non-binding), respectively. To date we have cloned 7 PA, 48 NA and 40 NB clones from 8 HIT patients. The following findings were made when sequences in the 3 groups were compared:

1. PA clones preferentially used JH6 and the VH3/JH6 combination;
2. The PA and NA Abs all employed κ chains, whereas κ chain usage for NB clones was 61%;
3. PA Abs had longer heavy chain CDR3s (HCDR3) than NA or NB; more positively charged amino acid residues compared to NA or NB; more tyrosine residues compared to NA or NB;
4. Five of 7 PA clones contained an $RX_{1-2}K/RX_{1-2}R/H$ (RKH) motif in HCDR3; the remaining 2 PA clones contained a string of at least 5 tyrosine (Y_5 motif) in HCDR3. Substitution of alanine for basic Aas of the RKH motif or of tyrosine residues in the Y_5 motif in PA clones reduced PF4/H binding and platelet activation, arguing for functional significance of both motifs.

High throughput sequencing of IgG H chains was performed on peripheral blood mononuclear cells (PBMC) from 7 HIT patients and 3 healthy donors. 0.69% H chain from HIT patients contained the RKH and 1.1% contained the Y_5 motif. In 3 healthy donors, 0.28% H chain contained RKH and none contained Y_5. The findings reflect amplification of B cells with receptors containing RKH and Y_5 motifs in HIT patients.

$F(ab')_2$ fragment of either RKH or Y_5 containing PA clone can (partially) inhibit platelet activation induced by HIT plasma. Indicating the functional relevance of these motif containing abs in the HIT pathogenesis.
These observations provide the first characterization of Ig structural motifs that are favored for selection in the humoral immune response leading to HIT and suggest that the RKH and Y_5 motifs in particular may contribute importantly to Ab pathogenicity. Findings made are expected to facilitate further work to define features specific to "pathogenic" HIT Abs and, possibly, to identify genetic variants that predispose individuals to experience HIT and to provide an alternative way for HIT treatment.

Abstract #: 24

Engineered Fluorescently Labeled Human and Murine Chemokines and Cytokines with Full Biological Activity

C.A. Koplinski, A.E. Getschman, M. Schnepppfister, M.B. Dwinell, B.F. Volkman, and F.C. Peterson

Protein Foundry, LLC, and Medical College of Wisconsin

Recombinant human and murine chemokines and cytokines remain in demand as reagents for preclinical and fundamental biomedical research. In vivo administration in preclinical disease models requires milligram to gram quantities of pure, biologically active material that are not widely available. Over the last 20 years, our research programs have developed a series of specialized molecular tools to confirm the structural integrity and function of chemokines, with particular attention to preserving the native N-terminal amino acid sequence, ensuring full biological activity. Building from this robust protein engineering platform, we have now produced and tested in reductionist cell culture and preclinical mouse systems, recombinant chemokines containing fluorescence and biotin labels. Chemokines with a carboxy-terminal label have been used for the real-time detection of chemotactic gradients in 2D cell migration assays while near-infrared-labeled chemokine has been detected in mouse models of cancer. Protein Foundry also specializes in custom chemokine production, including site-directed mutagenesis and stable isotopic labeling for NMR or mass spectrometric applications. New product additions include functionally validated human and murine IL2, IL3, IL7, and IFNgamma. Protein Foundry's entire catalog adheres to the highest standards of quality and is the preferred choice for many investigators in the US, Europe, and England. Our products are available for purchase at www.proteinfoundry.com.