Autoimmunity Centers of Excellence: 2018 Achievements

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Overview

The Autoimmunity Centers of Excellence (ACE) Program consists of three interrelated research components: (i) clinical projects led by four UM1 sites; (ii) basic science projects being conducted by seven U19 sites; and (iii) collaborative translational projects that arose from the original peer-reviewed grants but that were subsequently integrated to take maximal advantage of the complementary expertise within the ACE group. As described below, these three components are not independent, but rather represent a coherent approach to several of the most important challenges in the field of autoimmune diseases. The ACE is led by a Steering Committee formed with the Principal Investigators (PIs) and chaired by Dr. David Wofsy and Dr. Ignacio Sanz.

Clinical Projects (Clinical Trials)

The clinical trials component of the ACE program consists of four programs that reflect distinct, complementary approaches to the treatment of autoimmune diseases. As summarized briefly below, one program is exploring therapeutic strategies designed to inhibit inflammation (Feinstein Institute); one program is exploring strategies designed to inhibit pathologic immune mechanisms (University of Michigan); one program is exploring strategies designed to augment protective immune mechanisms (University of California, San Francisco); and one program is exploring strategies designed to prevent the development of autoimmune disease in people at high risk (University of Colorado).

Feinstein Institute, PI: Cynthia Aranow

ALE09: JBT-101 in Systemic Lupus Erythematosus (SLE)

This program is predicated on the notion that tissue injury in autoimmune disease is the end result of multiple and often redundant inflammatory pathways and mediators (ClinicalTrials.gov Identifier: NCT03093402). Therefore, an approach modulating multiple inflammatory mediators will have broad applications, and may offer safer and better-tolerated interventions than those that are currently available for treatment of inflammatory autoimmune diseases. Toward this end, Feinstein is conducting a project (ALE09) to evaluate the efficacy, safety, and tolerability of ajulemic acid/JBT-101 in 100 patients with systemic lupus erythematosus (SLE) in a 12-week, phase 2 double-blind, randomized, placebo-controlled multicenter study. Ajulemic acid is a synthetic, non-psychotropic cannabinoid that possesses multiple anti-inflammatory properties. Mechanistic studies evaluating the biologic effects of the agent are an integral component of this ACE supported study. Enrollment for this study is proceeding significantly ahead of the original projections, with randomization of 31 subjects to date (Nov 2018).

University of Michigan, PI: David Fox

This program has two clinical/translational projects and is also involved in the collaborative projects. The two trials are summarized in this section, and the collaborative work is summarized below in the section devoted to collaborative projects.

AMS04: Mechanistic Studies of Phase III Trial With BAF312 in Secondary Progressive Multiple Sclerosis

Immunological Profile Changes in Patients with Secondary Progressive Multiple Sclerosis (SPMS) Treated with BAF312 (AMS04) – PI Yang Mao-Draayer, MD, PhD (ClinicalTrials.gov Identifier: NCT02330965). This project is a multi-faceted immune mechanistic study of EXPAND trial, which is a multi-center, randomized, double blind, placebo-controlled Phase III secondary progressive multiple sclerosis study with siponimod (BAF312). Siponimod (BAF312) is a novel sphingosine-1-phosphate ($S1P_{1,5}$)-specific

receptor modulator developed by Novartis. EXPAND trial results were published in Lancet in March 2018 and the paper demonstrated that the primary efficacy endpoint was met with BAF312 (siponimod).

A total of 17 US sites participated in this ACE substudy (AMS04). Following unblinding of the data, our flow cytometry assays showed that BAF312 significantly reduced absolute number and frequency of peripheral lymphocytes (including CD4+ T cells, CD8+ T cells, B cells) at month 6 and month 12. In the siponimod treatment arm, CD4 and CD8 TN, TCM and TEM absolute counts were reduced; Tn and Tcm cells were preferentially depleted. With siponimod treatment, anti-inflammatory Th2 and Treg as well as Breg subsets were proportionally increased.

Microarray analysis of whole blood RNA showed nice separation on the PCA plot between placebo and BAF312-treated SPMS patients; 1,531 differentially expressed genes were identified out of a total of 13,399 genes with measured expression. The majority of differentially-expressed genes were decreased after 12 months of BAF312 treatment compared to the baseline untreated or placebo-treated patients (e.g., CD28, CCR7, ICOS, CARD11, IGHG1, IGHD, IGHA2, CD40LG, S1P1 etc.). The functions of these genes are involved in T/B cell interaction and receptor signaling pathway, co-stimulation of T/B cells, antibody production, antigen receptor-mediated signaling pathways (e.g., AKT-mTOR, JAK-STAT, and NFκB signaling), and cytokine-cytokine receptor interaction. Currently, Luminex assays on chemokine, cytokine, and innate immune factor changes are underway. We will continue comprehensive analysis to correlate the changes of subsets of lymphocyte, chemokine/cytokine, and gene array to clinical outcome measures.

ASSET: A Study of Subcutaneous Abatacept to Treat Diffuse Cutaneous Systemic Sclerosis

A Phase 2 Study of Abatacept versus Placebo in Diffuse Cutaneous Systemic Sclerosis (SSc) – PI Dinesh Khanna, MD, MPH (ClinicalTrials.gov Identifier: NCT02161406). The clinical protocol upon which this project is based is funded by Bristol-Myers Squibb; the mechanistic studies are supported by the ACE. The ASSET trial (abatacept in diffuse systemic sclerosis) has completed enrollment and the final results were presented as an oral presentation at the 2018 annual rheumatology meeting. Eighty-eight participants were randomized to abatacept or placebo (1:1 randomization) for 12 months. We have a rich biological database with skin tissue and whole blood collected in the trial. The highlights so far include: 1. Clinical evidence of efficacy of abatacept on certain outcome measures and no safety concerns, 2. In collaboration with Dr. Pillai (MGH], we have shown activated, clonally-expanded and tissue-infiltrating CD4+CTLs are abundant in early scleroderma and may cause apoptotic death of endothelial cells, 3. We have confirmed the findings of elevated CTLs (expressed as CD319+CD4+T cells) in the blood, 4. Baseline inflammatory skin gene expression subset is associated with greater response to abatacept in the skin score and composite end point (our original hypothesis) and may help enrich patients who are likely to respond to abatacept (this study is performed in collaboration with Dr. Whitfield, Dartmouth). We are currently collaborating with Dr. Utz at Stanford to complete autoantibody profiling of the baseline samples and actively conducting additional analyses on the blood and skin samples with Dr. Berrocal, statistician on the study. A manuscript is in preparation.

University of California San Francisco, PI: David Wofsy

ALE08: Autologous Polyclonal Tregs for Lupus

This program is designed to examine the potential for using *ex vivo* expanded autologous regulatory T cells (Tregs) in the treatment of autoimmune diseases (ClinicalTrials.gov Identifier: NCT02428309). Initially, we sought to examine Treg therapy in patients with cutaneous manifestations of systemic lupus erythematosus (SLE), but enrollment proved to be very challenging due to complicating features of SLE. One patient with SLE underwent the protocol and provided very tantalizing findings. Twelve weeks after the Treg infusion, there was a 75% increase in percent Tregs in affected skin, with documentation that

the increase in Tregs reflected the presence of the infused cells. Moreover, there was a profound impact on the local microenvironment, characterized by a selective 75% decline in IFN-γ-producing T cells. RNA-seq analysis confirmed a dramatic decrease in IFN-γ-response genes. A similar pattern was observed in the peripheral blood. This work constitutes the first analysis of the effects of Treg therapy at the site of autoimmune tissue injury. The clinical and mechanistic results have been published in *Arthritis & Rheumatology* (Dall'Era et al., 2018).

APG01: Polyclonal Regulatory T Cells (PolyTregs) for Pemphigus

Due to enrollment challenges with the lupus trial, we initiated our alternate project, in which we are examining the effects of autologous *ex vivo* expanded Tregs in patients with pemphigus (ClinicalTrials.gov Identifier: NCT03239470). This trial is being conducted as a multicenter trial to ensure adherence to the enrollment timeline.

University of Colorado, PI: Michael Holers

ARA08: Strategy to Prevent the Onset of Clinically-Apparent Rheumatoid Arthritis (StopRA)

There is an early stage of RA development called 'Preclinical RA' that is defined by elevations of RA-related autoantibodies in the absence of, and prior to, the inflammatory arthritis (IA) that characterizes classifiable RA. Importantly, when present, these autoantibodies, and specifically antibodies to citrullinated protein/peptides (ACPAs), are highly predictive of the future development of RA. Based on these observations, the Colorado ACE has developed and implemented a clinical trial entitled 'Strategy for the Prevention of Onset of Clinically-Apparent Rheumatoid Arthritis', or StopRA (ClinicalTrials.gov Identifier: NCT02603146). StopRA's primary hypothesis is that intervention with the pharmacologic agent hydroxychloroquine (HCQ) in individuals who have serum ACPA elevations in the absence of IA at baseline will delay or prevent the future development of classifiable RA. Secondary hypotheses include that HCQ will act to delay or prevent RA through mechanisms including abrogation of epitope spreading and inflammation in the preclinical stage of RA.

StopRA opened in April 2016 and is currently active at 18 sites across the United States; as of November 30, 2018, StopRA has enrolled 82 (41%) of the 200 planned subjects. There have been no major safety issues identified, and individuals are developing RA at expected rates. The primary challenge in the study has been difficulty in identifying individuals who are ACPA positive without IA. However, over the first ~2 years of the study, multiple mechanisms have been developed and improved to identify and enroll ACPA positive subjects. These methods include population-based screening for ACPA positivity through means such as health-fairs and blood donor screening, screening of established biobanks, as well as improved methods to identify ACPA positivity individuals without IA in rheumatology clinics and to test and identify ACPA positive first-degree relatives of patients with RA. These activities have resulted in an increased rate of randomizations over the past ~8 months of the study. Based on current activity, it is anticipated that the study will reach full enrollment in 2020.

Once completed, StopRA should provide critical data regarding the role that HCQ can play in the delay and/or prevention of RA in at-risk individuals. Furthermore, StopRA will provide a rich source of clinical data and biospecimens including DNA/RNA, PBMCs, serum and plasma that can be used to improve the understanding of the evolution of preclinical to classifiable RA. Specifically, findings from this trial should improve predictive models for RA as well as potentially identify new targets for future prevention studies that can utilize the extensive clinical trial infrastructure that has been developed for StopRA.

Principal Projects

Baylor / Weill Cornell Medical Center, PI: Virginia Pascual

The overall theme of our ACE is to define novel pathogenic amplification loops for type I IFN and autoantibody generation in systemic autoimmune diseases.

Project 1 focuses on uncovering novel pathogenic players in pediatric SLE. Work generated during the first three years of award revealed the mechanisms responsible for the release of interferogenic (oxidized) DNA of mitochondrial origin (Ox mtDNA) by SLE neutrophils exposed to TLR7-agonist autoantibodies. We next turned to study the adaptive immunity consequences of exposing plasmacytoid DCs (pDCs) to Ox mtDNA and showed that this type of activation skews naïve CD4+ T cells towards $IL2^{low}$, $IFN\gamma^{high}$, $IL10^{high}$ secreting B helper cells different from follicular helper (Tfh), T peripheral helpers (Tph) and Type 1 regulatory (Tr1) CD4+ T cells. During the past year, we focused on understanding the full set of factors responsible for conferring MtDNA-activated pDC-induced CD4+ T cells with a B cell helper phenotype and determining if CD4+ T cells with these characteristics are present in SLE patients. Our studies confirmed the expansion of $CD4^+$ T cells within the $CXCR5^{neg}$ compartment of SLE blood that express high levels of IL10, IFNγ, and IL3 (Th10) upon anti-CD3/CD28 stimulation. As their *in vitro* counterparts, Th10 cells provide potent B cell help through the synergism between IL10 and the metabolite Succinate, which is produced as the result of mitochondrial reverse electron transport. Indeed, blocking IL10 or the succinate receptor, which is highly expressed on human B cells, blocks the B cell helper capacity of Th10 cells.

In order to confirm that Th10 cells expanded in SLE blood are different from professional T cell helpers (Tfh), we performed RNAseq and ATACseq experiments and confirmed a distinct transcriptional program that separates these cells according to cytokine, chemokine receptor and cytolytic molecule profiles. In addition, chromatin accessibility patterns are different in these cell types and correlate significantly with their respective gene expression profiles. Finally, we found cells with the characteristics of TH10 cells (IFNγ+ IL10+ CD4+ T cells) infiltrating the peritubular areas of Proliferative Lupus Nephritis patient's kidney sections and in the proximity of B cells. These cells might play a role in extrafollicular B cell responses and perhaps contribute to tissue damage through the production of ROS and Succinate. A manuscript summarizing this work was recently published (Caielli et al., *Nat Med*. 2018).

During the past year, our Bioinformatics Core has provided integrative and robust bioinformatics support. We have made several important upgrades to the Biostatistics Analysis Reporting Tool (BART) to provide scalable, efficient and user-friendly interface for RNA-Seq research. Notably, we removed the dependency of the previous version of BART on commercial software and migrated our statistical computing to an R-based environment. We also developed key functionalities to facilitate the analysis of RNA-Seq data and developed a new R package, genBart, to help users assemble results pooled from multiple statistical analyses, as recently implemented in the context of Systemic Juvenile Idiopathic Arthritis studies (Cepika et al., *J Exp Med*. 2017 Nov 6;214(11):3449-3466). The manuscript describing the first-generation BART is under review. A free, open-source package is available for the scientific community to access from CRAN (https://cran.r-project.org/). In addition, a web-based BART is hosted through Amazon Web Services.

While continuously maintaining and supporting the current BART software for integrative immunology data analysis, we have also initiated the next phase of BART. Several improvements have been made to tailor the BART for unique requirements from next generation sequencing data analysis, including a new interface in BART to interactively load, visualize, and export quality control (QC) reports from the RNA-Seq pipeline.

In addition to BART, we have developed and published a new statistical software called Phantom (Pareto Heterogeneity Analysis of Time-resolved Omics data on Modules) to model heterogeneity in gene sets and modules for time course data analysis (Gu et al., *Bioinformatics*, 2017). Phantom uses state-of-the-art techniques from multi-objective optimization to model transcriptional heterogeneity. We have demonstrated that Phantom can significantly improve the power to detect heterogeneous gene expression trends in gene sets for time course datasets. Phantom is available through CRAN and Baylor hosted web-services.

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Massachusetts General Hospital, PI: Shiv Pillai

While we have established that CD4+CTLs are the dominant T cell in IgG4-RD tissues and are not prominent in non-fibrotic diseases, our more recent work has been in systemic sclerosis. CD4+CTLs are the most prominent T cells in the tissues of patients with diffuse systemic sclerosis. We interrogated a total of 350 sections from 35 patients with untreated early diffuse systemic sclerosis from the ASSET

trial (kindly provided to us by Drs. Dinesh Khanna and Robert Lafyatis). This collaboration would not have occurred but for the frequent bi-annual face-to-face interactions engendered by the ACE mechanism. We have also shown that SSc tissues exhibit an increase in apoptosis of endothelial cells and that CD4+CTLs are in the vicinity of apoptotic endothelial cells.

Apart from "activated naive" B cells and plasmablasts we see 4 other B cell subsets in IgG4-RD. We have visualized the accumulation of some of these cells in tissues, and also their physical interaction with CD4+CTLs in disease tissue sites. Given that B cell depletion is very effective in IgG4-RD and that CD4+CTLs decline after B cell depletion, these activated B cell populations are of great interest.

We have identified a subset of human T_{FH} cells that expresses IL-4 and the BATF transcription factor (Maehara et al., *Life Science Alliance* 2018). These IL-4+ T_{FH} cells make up 5% or less of T_{FH} cells in human tonsils, cervical and mesenteric lymph nodes but in secondary and tertiary lymphoid organs in IgG4-RD this subset constitutes more than 50% of all T_{FH} cells. We have used a cytokine capture approach to purify this subset of T_{FH} cells and also purify non-IL-4 secreting T_{FH} and non-T_{FH} IL-4 secretors (mainly T_{H2} cells) from tonsils. RNA-seq has revealed distinct differences in these various cell types.

We have used Next Gen sequencing of Ig heavy chain genes to define the dominant B cell clones in patients, used single cell cloning to identify the peak matched heavy and light chain genes, generated recombinant monoclonal antibodies and used three different antibodies to identify the same antigen, Galectin-3, in one patient, using mass spectrometry. Screening of 140 patient samples revealed that IgG4 antibodies to Galectin were specific to IgG4-RD and were seen in approximately 30% of all subjects (Perugino et al., *Journal of Allergy and Clinical Immunology* 2018). This process is being repeated in a number of patients and is helping us subset the disease. With Bill Robinson at Stanford we have shown that about 17% of IgG4-RD subjects have antibodies to the IL-1receptor antagonist (IL-1RA).

University of Chicago, PI: Marcus Clark

Project Leader: Patrick Wilson

Understanding the balance between immune tolerance and protection: The long-term goal of our research is to understand the pathways that regulate the balance between autoimmunity and protection. Our work has found that antigenically novel influenza viruses induce protective and broadly reactive antibody responses derived from memory B cells targeting the highly-conserved stalk region of the viral surface glycoprotein hemagglutinin (HA). The majority of stalk-specific monoclonal antibodies (mAbs) are polyreactive, the property of non-specifically binding to structurally unrelated antigens, including autoantigens. These data suggest that novel influenza strain exposure induces polyreactive antibodies against conserved HA-epitopes. The goal of the proposed research is to investigate the role of polyreactive antibodies in viral binding and protection against infection. We hypothesize that poly-reactivity acts as a stop-gap for the rapid production of antibodies to novel antigens. These polyreactive cells would then be at a selective disadvantage due to immune tolerance mechanisms such as anergy, allowing specific responses to develop and become dominant.

Analysis of human intestinal plasma cells in the context of inflammatory bowel diseases: Surprisingly, patients deficient in IgA have only a modest increase in rates of intestinal infections. However, they do have an increased risk for Celiac Disease and Inflammatory Bowel Disease (IBD), suggesting that plasma cells may play a role in the pathogenesis of intestinal disease. Both IBD and the often-associated autoimmune disease Primary Sclerosing Cholangitis (PSC) have strong HLA-II associations on GWAS, which suggests B cell involvement in pathogenesis. Our preliminary findings show that the plasma cells found in the PSC-IBD colon have a recently-arrived phenotype and are predominantly of the IgG isotype. Additionally, we recently identified that this plasma cell phenotype is associated with an IL-17 signaling gene signature. To investigate the significance of these findings we have been sorting plasma cells and T-cells in bulk for transcriptional analysis. This analysis will allow us to identify any repertoire biases within

these populations that may exist in diseased individuals. We have also single cell sorted plasma cells from PSC subjects and controls to perform single-cell transcriptional analysis as well as cloning of the antibody from each cell. These antibodies will be tested for both autoantigen binding and will capture microbiota extracts for 16s sequencing. We expect to see a correlation between microbiota reactivity and IBD syndromes, particularly where the unusual IgG plasmablasts are concerned.

Oklahoma Medical Research Foundation, PI: Judith James

The Oklahoma ACE has contributed important new insights to the genetic and molecular underpinnings of systemic autoimmune rheumatic diseases, particularly systemic lupus erythematosus (SLE), Sjögren's syndrome, and related disorders. Recent accomplishments led by the Oklahoma ACE have elucidated the genetic architecture of autoimmune diseases (Fu et al, Arthritis Res Therapy, 2018), defined clinical characteristics in unique populations of patients with lupus and incomplete lupus (Aberle et al, Arthritis Care Res 2017; Kheir et al, Lupus Sci Med 2018), identified prognostic associations in lupus and Sjögren's syndrome (Young et al, Lupus 2018; Bagavant et al, Clin Exp Rheumatol, 2018; Arriens et al, Clin Immunol 2017), and demonstrated new modes of B cell involvement in Sjögren's syndrome pathogenesis (Koelsch et al, Arthritis Rheumatol 2018).

The Oklahoma ACE has also developed a novel, unbiased analysis pipeline that integrates ChIP-seq, ATAC-seq, RNA-seq and genotyping data. Using this pipeline, we identified 6,261 genetic variations that cause allele-specific imbalances in post-translational histone modifications (histone quantitative trait loci; hQTLs), and 386 of these associated with autoimmune disease (Pelikan, et al. Nature Commun 2018). In addition, we identified 245 haplotypes containing a gEUVADIS expression QTL (eQTL) and at least one hQTL in strong LD. Of these hQLTs, 36% were found to significantly increase the gene expression variance of the eQTL target gene relative to the eQTL alone or other non-hQTLs on a shared haplotype. This provides valuable knowledge for deconstructing the linkage disequilibrium that defines a haplotype and prioritizing the SNPs that are most likely to contribute to locus-specific functionality. To further explore the potential modifying effects of hQTLs, we aligned the hQTL to 3D chromatin topology maps of H3K27ac that were developed independently using HiChIP. We uncovered significant physical interactions between distant hQTLs and eQTLs located on different haplotype blocks, which may explain, in part, the gene expression variability of eQTL data. In addition, we discovered that hQTLs were enriched in an enhancer on the HLA class II locus, resulting in differential 3D chromatin conformation and gene expression between two of the most common HLA class II autoimmune disease risk haplotypes, HLA-DR3 and HLA-DR15. In summary, our novel approach provides a priori evidence about the potential regulatory functions of genetic variants located within chromatin networks that 1) are more likely to influence gene expression phenotypes than eQTLs and non-hQTL variants in LD with them, 2) influence gene expression through long-range interactions, and 3) expose hidden substructure within gene expression data. Together, these data clarify the genetic architecture of autoimmune diseases in ways that inform follow-up studies of functional modeling and have profound implications for precision medicine initiatives.

In addition to these projects within the Oklahoma ACE, we have cultivated productive collaborations with other ACE sites. The outcomes of our collaborative work with Michigan and Stanford are detailed below. Additional collaborative studies have characterized the function of a plausibly causal lupus-associated risk variant in the STAT1-STAT4 locus (Patel et al, *Hum Mol Genet* 2018) and identified mechanisms of clonal lymphoproliferation in Sjögren's syndrome (Glauzy et al, *Arthritis Rheumatol* 2018). Finally, the Oklahoma ACE has helped lead and been a major contributor to the ACE clinical trial of baminercept in Sjogren's syndrome (St Clair et al, *Arthritis Rheumatol* 2018) and the associated mechanistic analyses for this study (manuscript submitted).

Emory University, PI: Ignacio Sanz

The Emory ACE U19 Principal Project pursues the following specific aims

Aim 1. Participation and antigenic selection of different B cell compartments in SLE flares

Aim 2. Relative contribution of short-lived and long-lived antibody secreting cells to the SLE serum autoantibody proteome.

Aim 3. To understand the epigenetic and transcriptional programs of SLE B cells (Collaborative Emory Project – Collaborative ACE Agenda)

Major progress has been made on all aims over the last 4 years. Main Center accomplishments include:

1. Delineating the human B cell extra-follicular pathway
    - Cellular components
    - Drivers
    - Role in SLE autoimmune responses
2. Defining the heterogeneity of human plasma cells and the identity of the long-lived plasma cell compartment
3. Establishing a comprehensive B cell immunomics approach
    - Multi-dimensional cytometry
    - B cell receptor repertoire Next Generation Sequencing (BCR-Seq)
    - Serum autoantibody proteomics sequencing (Ig-Seq)
    - Ultra-high throughput single cell mAb generation
    - Integrated transcriptional and epigenetic analysis
    - Integrated BCR, transcriptional and ATAC-Seq analysis of single B cells using the 10X Genomics platform
4. Defining B cell heterogeneity in SLE
5. SLE B cell epigenetic landscape (Collaborative Project with Dr. Boss further delineated below)
    - Molecular pathways of activation/differentiation
    - Poised state of "resting" B cells
    - Biomarkers of SLE B cells

Specific scientific progress can be summarized as follows:

- **Newly activated naïve B cells contribute a major fraction of antibody-secreting cells (ASC) and serum autoantibodies in lupus flares** [1]

We demonstrated that lupus flares are characterized by the expansion of a novel activated naïve B cell population (aN; CD19hi IgD+ CD27- MTG+ CD21- CD23- CD38lo) containing a large fraction of clonal expansions with high frequency of VH4-34 clones. Both aN and plasmablasts (PB) display a high degree of clonal connectivity and clonally expanded ASC contain a high frequency of unmutated and scarcely mutated sequences and interconnected aN and PB clones account for dominant serum autoantibodies. Unmutated VH4-34 PB clones display significant reactivity against lupus antigens, in some cases with cross-reactivity against DNA, chromatin and Ro RNA-binding proteins. Our work defines a distinct population of activated naive cells of considerable clonality that contributes a major fraction of newly generated PB and serum autoantibodies during lupus flares even in the absence of somatic hypermutation, a finding consistent with direct naïve differentiation through an extra-follicular pathway.

These findings identify a separate immune response in lupus flares; suggest the participation of triggering antigens separate from conventional lupus antigens; discern activation pathways amenable to specific targeting; and identify biomarkers and predictors of reactivation.

— **Definition of the human B cell extra-follicular pathway and its TLR7 regulation in SLE[2-4]**

We initially described a prominent expansion of class-switched B cells lacking IgD and CD27, considered a universal marker of human B cell memory [5] (double negative; DN). However, the clinical and immunological significance of DN cells in SLE and their ontogenic relationship to other B cells remained poorly understood. We performed a comprehensive phenotypic, immunological and transcriptional interrogation of SLE DN cells. Our results indicate that the expansion of DN cells in SLE is accounted for by a CXCR5- CD21- CD11c+ subset (DN2) lacking FcRL4, a marker of B cell exhaustion in HIV infection. DN2 expansion is most prominent in African American patients with active disease and active nephritis and strongly correlates with anti-Smith, anti-RNP and anti-RNA autoantibodies. DN2 cells and aN cells share multiple phenotypic markers that separate them from other B cells, require IL-21 for differentiation into PB and have a highly similar transcriptome lacking the BACH-2 repressor of effector function and the essential B and T cell central memory TCF7 transcription factor (TF). In contrast, they express high levels of both T-bet and Zeb2, a T-bet regulated TF that induces terminal differentiation of effector CD8 T cells. Notably, aN and DN2 cells are the main SLE B cell populations with high levels of T-bet and CD11c and therefore, represent the human equivalent of the so-called Age-Associated B cells (ABCs), a TLR-7 dependent population critical for viral clearance and B cell-mediated autoimmunity in the mouse

Importantly, DN2 cells are poised for PC differentiation as indicated by high levels of IRF4-induced genes in plasma cells; low levels of Ets1 which combined with high IRF4 promotes extra-follicular PB differentiation; accessible BLIMP-1 chromatin; and a high degree of clonal connectivity with circulating PB. Finally, DN2 cells express high levels of TLR7 and the lowest levels of negative TLR regulators including TRAF5, with enhanced TLR7 responsiveness. Moreover, DN2 cells express increased IFNλ-R1 and enhanced responsiveness to IFNλ, a type III IFN that synergizes with TLR7 and is highly expressed in SLE serum and kidneys. aN and DN2 cells are also defective in other regulators of pro-inflammatory responses to TLR and TNF, two pathogenic pathways in SLE, including NFKBIA and TNFAIP3, which is associated with genetic SLE susceptibility.

Our work defines a novel extra-follicular differentiation pathway responsible for the generation and expansion of effector B cells (aN and DN2 cells) as precursors of PB in active SLE in a TLR-7-dependent fashion enhanced by defective TLR negative regulation and hyper-responsiveness to IL-21 and IFNλ[4].

— **Transcriptional and epigenetic regulation of human SLE B cells**

SLE B cells are hyper-responsive to innate immune signals through enhanced stimulation and defective negative regulation [2,6].

In the Principal Project, we investigated the transcriptional regulation of SLE B cells through RNA-seq [2,6]. Our results provide the first description of the confluence of the extrinsic and intrinsic factors underlying B cell dysregulation in SLE. Across all populations, SLE B cells are characterized by higher IFN-regulated genes (both type I and type II modules). Notably, SLE B cells are significantly enriched for innate sensors of viral RNA, including TLR7 and the RIG-I-like receptors (RLRs) MDA5 and RIG-I. Also increased are several innate dsDNA sensors that induce inflammatory pathways relevant to SLE including STING and the NLPR3 inflammasome. Moreover, SLE B cells have a deficiency of negative signaling regulators that is particularly profound in the activated components of the effector extra-follicular pathway (aN and DN2 cells).

SLE B cells display distinct molecular programs underpinning skewed differentiation [7-9]

We have worked with Dr. Boss through the Collaborative Project and this aspect is further discussed below and under the corresponding section in this document.

SLE resting naïve B cells are epigenetically imprinted for activation and differentiation [10]

Our demonstration that naïve B cells represent major participant in disease flares [1], suggests that B cells harbor pathogenic alterations at an early stage. We used the Assay for Transposase Accessible Sequencing (ATAC-seq) to examine the accessibility landscape of resting naive B cells to identify an SLE specific epigenetic signature. Differentially accessible regions (DARs) mapped to 988 distinct genes, including 66 genes with >1 DAR. Of these genes, 98% (65/66) displayed concordant changes in accessibility, suggesting coordinated changes in accessibility of potential cis-regulatory elements associated with disease. Notably, higher accessibility in SLE was also present in the STAT4 promoter. STAT4 polymorphisms are highly

associated with autoantibody production and disease susceptibility and changes in accessibility could result from higher IFN-alpha signaling or suggest that SLE B cells are epigenetically predisposed for activation of the STAT4 pathway. In contrast, HC specific accessible loci were associated with genes involved in transcriptional regulation including RXRA, whose deficiency results in increased antinuclear antibodies and nephritis.

We have extended these studies in a larger number of patients and additional B cell subsets to integrate transcriptional and epigenetic studies. The data show that lupus resting naïve cells over-express genes associated with B cell activation through different stimulatory pathways including BCR and TLR.

— **B cell profiling in human SLE. Implications for disease heterogeneity and segmentation [11,12]**

Extant studies of B cells in SLE have been hampered by univariate analysis of pauci-color flow cytometry (FCM) datasets that fail to capture the complexity of human B cells and to incorporate extended B cell phenotypes [13] and did not integrate complex immunological and clinical data. We addressed these shortcomings through the use of multicolor FCM [13-19]; automated multidimensional analysis [19]; and cutting-edge bioinformatics for systems-scale analysis [13,20-23]. These methods have been extensively applied by our laboratory to ACE and Immune Tolerance Network studies [13,16,19,24-28]. We conducted a large SLE study of patients (N=150) from the University of Rochester and Johns Hopkins (**Fig. 4**) [29,30]. The data show that B cell signatures segment SLE into 3 major clusters with different clinical, racial and immunological attributes using gap statistics. **Cluster 1** is highly enriched for active disease and severe flares; AA patients; IFN activity; autoantibody load (dsDNA, 9G4 and Sm/RNP); and multiple clinical manifestations including a history of nephritis. Its B cell profile includes large expansions of DN2 and aN cells as well as PB. In contrast, **cluster 3** is mostly inactive with a healthy-like profile. **Cluster 2**, is driven by milder expansions of the activated fractions of CD27+ switched memory and DN cells. Longitudinal analysis of non-active patients within all clusters indicates that a more active B cell profile at baseline correlates with a higher rate of flaring disease over 3 months of follow-up. Of interest, no significant changes were demonstrated in patients with inactive B cell profile (cluster 3) at the time of disease flare. Our data demonstrate that SLE is heterogeneous with disease clusters defined by B cell profiles that correlate with race, disease activity, immunological features and flare risk. The lack of B cell changes at the time of flare in patients with a normal baseline profile suggests a B cell-independent disease segment. These findings bear significant implications for the design of clinical studies and the treatment with B cell targeting agents.

— **Identification of human long-lived plasma cells and their survival factors [31,32]**

The existence of plasma cells that survive for prolonged periods of time (long-lived plasma cells; LLPC) has been postulated in humans on the basis of the half-lives of serum antibodies ranging from 10 years (tetanus) to hundreds-thousands of years (mumps: >500 years; measles: 3,000 years). Indirect evidence for LLPC also derives from the persistence of anti-microbial antibodies and some lupus autoantibodies (RBP) but not others (dsDNA), after prolonged B cell depletion. We have directly defined the identity of human LLPC through cellular and BCR repertoire analysis of different fractions of bone marrow PC and coordinated proteomics of anti-mumps and measles serum antibodies that persisted in high titers for over 40 years after the original infection[31]. We demonstrated that CD19−CD38hiCD138+ PCs represent the exclusive reservoir of these antibodies. This population has a distinct transcriptome and a VH repertoire that is largely uncoupled from other BM PC subsets and likely represents the "historical record" of B cell responses. In all, our studies provide the first identification of human LLPCs and provide the basis for mechanistic studies of the cellular origin of different lupus autoantibodies. More recently, we have identified unique factors of the BM microenvironment that support *in vitro* survival of human PB for over 50 days [*32*]. Critical components consist of products from primary BM MSC (fibronectin and YWHAZ), APRIL, and hypoxic conditions.

— **Development of a comprehensive B cell Immunomics approach to human autoimmune diseases[33]**

Understanding the properties and fates of autoreactive B cells is critical to understand SLE and other autoimmune diseases; to develop better biomarkers for segmentation and personalized treatments; and to develop new assays to measure restoration of B cell tolerance. To that end, we have developed a comprehensive experimental toolbox that integrates cellular and molecular analyses with a detailed characterization of the antigenic targets recognized by different antibody repertoires. Our approach is

high-throughput and enables the study of large numbers of cells and antibodies from multiple samples. A detailed discussion of this approach has been published[33].

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University of Michigan, PI: Bruce Richardson

Epigenomics of Systemic Autoimmunity

The University of Michigan Autoimmunity Center of Excellence (ACE) characterizes a previously unrecognized, epigenetically altered CD4+ T cell subset found in patients with active lupus and related systemic autoimmune diseases including rheumatoid arthritis, systemic sclerosis and Sjogren's Syndrome. Similar epigenetically altered CD4+ T cells cause lupus-like autoimmunity in animal models, suggesting that the subset may contribute directly to flares of lupus. The goals of the ACE are to use genomics approaches to identify the genes aberrantly overexpressed by the epigenetically altered cells generated in vitro as well as similar cells isolated from patients with active lupus, and test if antibodies to the gene products identified can inhibit the effects of the altered T cells on immune function. We have now completed comprehensive transcriptome sequencing of the epigenetically altered T cells generated in vitro as well as of the epigenetically altered T cell isolated from patients with active lupus. The subset is characterized by expression of multiple pro-inflammatory, cytotoxic genes. Importantly, the cells also expressed the entire killer-cell immunoglobulin-like receptor (KIR) gene family. The KIR genes are clonally expressed on NK and NKT cells but not on normal T cells. This means that antibodies to a single KIR protein will deplete the pathologic subset but only a clone of NK and NKT cells, indicating a safe but effective way to deplete the cells for the treatment of lupus. Using other funding we have now confirmed therapeutic efficacy in a mouse model in which lupus flares are caused by the same mechanism as human lupus flares. Our ACE results also demonstrate that our epigenetically altered CD4+KIR+CD28+ T cell subset closely resembles the epigenetically altered CD4+KIR+CD28- T cell subset found in the same patients as well as patients with other chronic inflammatory diseases. In contrast to the CD4+KIR+CD28+ subset though, the CD4+KIR+CD28- T cell subset invades atherosclerotic plaques, promoting their growth and rupture. Current studies are comparing the transcriptomes of the two subsets, and testing if anti-KIR antibodies will also prevent atherosclerosis development in mouse models, again with other funding.

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Stanford University, PI: Paul J. Utz

Principal Project (Leader: W. Robinson)

The Stanford Principal Project is focused on the following objectives:

1. Sequencing the antibody repertoire in RA, to identify antibody profiles associated with clinical subtypes or response to therapy.

Profiling the evolution of the antibody repertoire in RA. The Robinson lab used our cell barcode-enabled antibody repertoire sequencing method to sequence the blood CD19+CD3-IgD-CD14-CD20-CD27+CD38++ plasmablast antibody repertoires from multiple time points over several years in anti-CCP+ individuals with established RA. The resulting datasets were used to construct phylogenetic trees to provide an overview of the plasmablast antibody repertoire, including identification of clonally expanded families/lineages. By combining bioinformatic analysis of the sequence data with flow-based staining data of citrullinated peptide-multimers, we identified ACPA-expressing clonal lineages and singletons. Across eight subjects, we identified 164 persistent lineages, which included multiple lineages that contained plasmablasts expressing ACPA of both the IgA and IgG isotypes. Together, these observations suggest persistent mucosal antigen stimulation of the plasmablast response in RA, resulting in divergent affinity maturation and class-switching to IgG within lineages over months in RA.

Analysis of persistent lineages across patients. We performed clustering analysis, which revealed multiple clusters containing shared sequence motifs across subjects (70% identity) within the identified persistent lineages. Two highly related clusters included clonal lineage members and were grouped into a single cluster. Within the clusters, we identified sequences produced by plasmablasts that bound the citrullinated peptide-multimers, revealing three clusters consisting of sequences from two subjects, each with at least one ACPA-producing member. Thus, we demonstrated that individuals with established RA generate persistent B cell lineages that contain IgA and IgG members and exhibit shared amino acid motifs across individuals.

Affinity maturation drives epitope spreading. In-depth characterization of persistent lineages from different subjects revealed mutation patterns suggesting that affinity maturation drives epitope spreading. ELISA analysis of antibodies from a lineage derived from Subject 1 confirmed that the later timepoint-derived antibody, rAb66, bound specific epitopes of H2B and H2A, while the earlier member, rAb65, did not bind these epitopes. We also observed substantial binding differences for rAb65 and rAb66 to full-length, in vitro citrullinated H2A, H2B and PAD, the auto-citrullinating enzyme used to citrullinate these antigens. Similarly, for a Subject 8 derived lineage, the later timepoint, more mutated rAb82 bound full-length, citrullinated H2B while rAb81 did not. In both cases, IgTree-based lineage tree analysis revealed a bifurcation from a common parent with 37 and 21 shared alterations for rAb65/rAb66 and rAb81/rAb82, respectively, followed by continued, divergent evolution. Interestingly, direct alignment with germline HC and LC VJ sequences also demonstrated substantial differences between the lineage members in regions outside of CDR3 (e.g. CDR1, CDR2, framework.

2. Characterizing key RA recombinant plasmablast clonal family antibodies to uncover mechanisms by which they arise and contribute to autoimmune inflammation.

Increased stimulation of macrophage TNF-α production by affinity-matured recombinant antibodies. To examine the ability of representative, persistent plasmablast lineage-derived, recombinant antibodies to stimulate macrophage TNF-α production, we generated plate-bound Immune Complexes (ICs) of these antibodies with citrullinated H2A, H2B, or PAD. Human monocyte-derived macrophages produced significantly higher levels of TNF-α in response to ICs generated with recombinant ACPAs representative of persistent lineages from two different subjects compared to citrullinated antigen alone. Additionally, citrullinated H2A and H2B significantly increased TNF-α production compared to cells alone; however, plate-bound PAD (that included all *in vitro* citrullination reagents but lacked H2A or H2B) resulted in a similar level of TNF-□ production as compared to cells alone. To investigate the role of FcγRII and TLR4, we pre-incubated cells with blocking reagents prior to incubation with immobilized ICs. Blocking FcγRII (anti-CD32, clone IV.3) reduced the levels of TNF-α produced compared to IC alone, with significant reductions observed for rAb81 and rAb82 complexed with citrullinated H2B. Additionally, blocking FcγRII significantly reduced the level of TNF-α produced by rAb66 in complex with citrullinated H2A or PAD. Further, the TLR4 small molecule inhibitor (TAK-242) significantly reduced TNF-α levels stimulated by citrullinated antigens alone or in complex with recombinant antibody. Together, these findings suggest a key role for mucosal antigens in promoting the development and persistence of ACPA responses, including class switching from IgA to IgG and affinity maturation that gives rise to ACPA that more potently induce production of TNF-α and other inflammatory mediators that promote joint destruction. Further, these findings have the potential to transform not only our mechanistic understanding of RA but also the therapeutic approach – perhaps targeting microbial mucosal antigen drivers could provide a more fundamental and effective treatment approach that targeting TNF-α and other downstream inflammatory mediators.

Collaborative Projects

Aim 1 – Characterize peripheral adaptive immunity in autoimmune disease subjects in the context of disease flares/relapses compared to periods of disease quiescence and before/after cell depleting or other immunosuppressive therapies.

Aim 1A: Pathogenic B Cells, Leader: J Bennett (Colorado)

The translational analysis of the B cell and antibody responses in active and quiescent Neuromyelitis optica (NMO) has completed enrollment of the 20 NMO subjects (10 relapsing and 10 quiescent) planned for the study. We are working to complete our modified analyses of B cells in Aims 1-3 and have sent additional aliquots of cryopreserved B and T cells to ACE collaborators at Emory [Sanz, Boss] and Mt Sinai [Ueno]. Plasma samples collected from the same 20 enrolled subjects have been quantitatively analyzed for serum IgG glycovariants in Aim 4. Subaim progress is as follows:

Aim 1A1: We used a lentiviral M23AQP4-eGFP transduced cell line to capture peripheral blood plasmablasts and memory cells. The experiments were hindered by the low abundance of circulating AQP4-specific cells, mediocre enrichment using the M23AQP4-eGFP target cell, and inefficient VH and VL sequence amplification from single memory cells (Stanford ACE collaboration). None of the recombinant antibodies (rAbs) constructed from over-represented single cell VH/VL sequences bound to AQP4. Despite this setback, we have identified an expanded population of CD11c+CXCR5- B cells that correlates with NMO disease activity. This B cell population is Tbet+CD38loFcRL5+CD21-CD11c+CXCR5-. We are currently analyzing whether this B cell population is enriched in AQP4-specific cells using in vitro stimulation, B cell receptor sequencing, and rAb production. In addition, we are completing high throughput VH repertoire analysis of the CD11c+CXCR5- B cell compartment from 4 active and 4 quiescent NMO patients. These repertoires will be compared to the IL10+ B regulatory and anergic VH repertoires generated from the same patients.

Aim 1A2: IL-10-producing B10 and B10Pro regulatory cells were induced from the peripheral blood of active and quiescent NMO patients using CpG and CD40L followed by PMA and ionomycin. The percentage of circulating B10 and B10Pro cells showed no statistical difference between patient populations. In collaboration with the Stanford ACE, individual IL10-secreting B10 cells were collected for high throughput single cell deep sequencing. Unfortunately, VH/VL sequences were not amplified. Instead, we are completing VH repertoires from IL10-secreting B10 cells recovered from 4 active and 4 quiescent patients. The repertoires will be compared to the repertoires generated from the CD11c+CXCR5- and anergic B cell compartments of the same patient.

Aim 1A3: The abundance of CD19+IgD+CD21loCD27-IgMlow anergic B cells in active and relapsing NMO patients were quantified by FACS. The analysis is near completion and it appears that the percentage of anergic B cells are lower in active than quiescent NMO subjects. Anergic B cells were collected for bulk VH sequencing. Patient-specific anergic VH repertoires will be compared with the same subject's repertoires obtained in Aims 1 and 2.

Aim 1A4: Glycovariants of IgG1 AQP4-specific rAbs were produced at MGH (Dr. Anthony). Agalactosylated and sialylated glycoforms of most AQP4-specific rAbs demonstrated normal complement dependent cytotoxicity (CDC); however, a lower affinity AQP4 rAb exhibited reduced CDC. All AQP4-specific rAbs showed reduced ADCC following the removal of galactose or introduction of sialic acid residues. FcR binding is normal for IgG1 antibodies; the binding of various glycoforms is being completed. There are no differences in the abundance of serum IgG glycovariants in active and quiescent NMO patients. AQP4-specific rAbs with FcR mutations impaired transplacental transport offering new opportunities for designing antibody therapeutics for use during pregnancy.

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Aim 1B: B cells and T cells, Leaders: P. Utz (Stanford), J. James (OMRF), A. Sawalha (Michigan)

Major Accomplishments:

- Established the new EpiTOF experimental platform that is broadly applicable to several areas of chromatin and immunology research (Cheung et al. Cell 2018);
- Completed EpiTOF analysis on 20 SLE patients from diverse ethnic backgrounds, with or without an interferon signature, and with varying degree of disease severity;
- Developed a giant magnetoresistive (GMR) sensor capable of detecting >60 SLE autoantigens, with strong potential for a smartphone-controlled point of care platform;
- Performed EPiTOF analysis of samples from Michigan suggesting distinct epigenetic regulation in the CD4+CD28+KIR+CD11ahi demethylated T cell subset, in addition to DNA methylation;
- Identified a novel CD4+CD28+KIR+CD11ahi T cell subset that is expanded in lupus and other related autoimmune diseases (Strickland et al. Lupus Sci Med 2016);
- Characterized in detail the epigenetic and transcriptional features of the CD4+CD28+KIR+CD11ahi T cell subset in lupus patients, identifying novel therapeutic targets for lupus (Gensterblum et al. J. Autoimmunity 2018);
- Demonstrated higher genetic risk in African-American lupus patients compared to European-American patients, establishing a genetic reason that might contribute to differences in disease susceptibility and severity between the two ethnicities (Gensterblum et al. J. Autoimmunity 2018);
- Identified immunophenotypes and signaling alterations associated with SLE disease activity in African American and European American patients;
- Defined impact of hydroxychloroquine treatment on immune cell abundance, immunophenotypes, and soluble mediator levels in SLE patients;
- Demonstrated modulation of STAT3 pathways by mycophenolate mofetil through CyTOF, xMAP assays, ELISAs, and flow cytometry of PBMCs from SLE patients taking or not taking mycophenolate mofetil.

This sub-aim involves current collaborations between the Stanford, OMRF, Colorado, Emory, Chicago and Michigan Basic ACE programs. The Stanford group has made significant progress on the EpiTOF (Epigenetic landscape profiling using cytometry by Time-Of-Flight) project in the last year. The first manuscript describing key findings from EpiTOF studies on healthy subjects was published in Cell. This published work presents a new experimental platform that is broadly applicable to several areas of chromatin and immunology research, identifies new molecular signatures of human aging at the level of chromatin, and unveils the heritability of chromatin marks and the relative contributions of nature and nurture on chromatin dynamics in aging human cells.

The Utz lab has further investigated dysregulation of the epigenome in patients with immune-mediated diseases in collaboration with groups in multiple ACE centers. In collaboration with OMRF, EpiTOF analysis has been performed on 20 SLE patients from diverse ethnic backgrounds, with or without an

interferon signature, and with varying degrees of disease activity. Preliminary results (analyzed with P. Khatri lab, Stanford) indicate that SLE patients have distinct epigenetic landscapes when compared with healthy individuals. In particular, we identified a number of chromatin marks that are differentially regulated across several immune cell subtypes. Additionally, in order to better understand the effects of type I interferon and other cytokines on the epigenome, we collected EpiTOF data from healthy PBMCs at baseline and after treatments with various cytokines at short (15 min) and longer (24 hour) time points. Comparison of this dataset with the SLE data will facilitate understanding of the contribution of type I interferon to the altered epigenomic landscape in SLE patients. We are finalizing the analysis comparing differential chromatin marks and SLE disease activity, as well as with the type I interferon signature. The results from this pilot study will provide the basis for the design of a larger EpiTOF experiment performed on an expanded ACE SLE cohort. The overarching goal of this project is to identify an epigenetic signature shared by SLE patients to better understand the roles of dysregulated epigenetic mechanisms in the pathogenesis and disease progression in SLE.

In collaboration with Dr. Michael Holers' group at University of Colorado at Denver, the Utz lab performed a pilot study with EpiTOF analysis of 20 PBMC samples from six new-onset RA patients, seven CCP+ at-risk subjects, and seven CCP- healthy individuals. Preliminary results on 40 chromatin marks in 22 major immune cell populations showed significant epigenetic changes among the three stages of RA development which suggest interesting changes in PADI4/citrullination and arginine methylation. We are in the process of expanding our study to a larger cohort, in which we will determine if we can confirm our preliminary findings and obtain publication-quality datasets.

As part of the SCOT collaborative project, we performed CyTOF analysis to measure the viability of SCOT clinical samples. In the same experiment, we recycled the leftover cells and performed EpiTOF analysis on 36 SCOT samples. We found a clear separation between systemic sclerosis (SSc) patients and healthy individuals in their epigenome, despite poor viability of PBMCs from the SCOT trial. This pilot study provides a basis for studying epigenetics and chromatin modification changes in SSc.

In collaboration with Dr. Mellins' group at Stanford, the Utz lab performed EpiTOF analysis on 15 patients with systemic juvenile idiopathic arthritis (sJIA) and five healthy controls. We identified both a chromatin mark that best separates sJIA patients from healthy controls and a new chromatin-modifying enzyme that catalyzes this histone modification. We are in the processing of employing ChIP-seq to ask how the changes of this chromatin mark affect gene expression (and potentially other DNA-templated biological functions). Working with the Mellins' group, we are expanding EpiTOF analysis to additional new-onset patients where we observed the most striking changes in epigenome compared to patients with stable disease.

The Utz lab has performed EpiTOF analysis on a cohort of 20 PBMC samples derived from four patients with active Crohn's disease, four with stable Crohn's disease, four with active ulcerative colitis (UC), four with stable UC, and four healthy controls, in collaboration with the Inflammatory Bowel Disease Registry led by Dr. Aida Habtezion at Stanford. EpiTOF analyses on several other immune-mediated diseases (e.g. infection) have been run or are being planned.

In addition, the Wang lab as part of the Stanford ACE collaborative project team has developed a real-time giant magnetoresistive (GMR) sensor capable of detecting >60 SLE autoantigens (or autoantibodies) and is now developing a point of care platform for preclinical and clinical applications. We performed preliminary reactivity patterns of >40 SLE monoclonal antibodies provided by Sanz (Emory) and Clark (Chicago), using an 80-feature next generation GMR chip. The GMR biosensor chips, spotted with autoantigen probes, have been demonstrated to detect purified autoantibodies from SLE patients (Emory) in a multiplexed manner. Importantly, different antibodies have very different reaction patterns with spotted antigens. For example, "56 B6" antibody associated with SLE is found to readily react with Histone 1002 (Histone H2A &H4), Histone 1003 (Histone H2B), Ribo-P (Ribosomal

Phosphoprotein P0), and Ro60 (Ribonuclearprotein 60kDa). In contrast, “627 E6” antibody (also associated with SLE) is found to readily react with Histone 1003, Histone 1004 (Histone H3), and La (Sjögren syndrome type B (La/SSB)) only. The multiplexed assays reveal rich information on the reactivity of autoantibodies in a simple a workflow. Further, the Wang lab has made great strides in realizing a fully automated point-of-care GMR biosensor reader platform. The reader is fully controlled with a smartphone, and the GMR chip is inserted into a small slot for readout. The biochemical reactions inside the user-friendly box are automated with miniature valves. The device will be well suited for aiding autoimmune disease diagnosis in physicians’ offices or for rapid assessment of antibody reactivity in research labs.

This sub-aim also addresses the hypothesis that the size of the demethylated CD4+CD28+KIR+CD11ahi T cell subset interacts with total genetic risk to determine disease activity in lupus, and that this subset can serve as a biomarker for disease progression and remission in lupus patients. The strategy to address these questions includes a cross sectional and a longitudinal approach. We have completed the analysis for data in the cross-sectional phase of this study, and recruitment for the longitudinal phase has been completed. For the cross-sectional study, we determined the size of the demethylated CD4+CD28+KIR+CD11ahi T cell subset by flow cytometry in 105 lupus patients including 68 European-American patients and 37 African-American patients. Genotyping across 43 confirmed lupus susceptibility loci was performed to calculate total genetic risk. Genetic risk score was significantly higher in African-American compared to European-American lupus patients in our cohort (7.81±0.12 versus 7.31±0.12 (mean±SEM), P= 0.0079). As expected, African-American patients had a higher mean SLEDAI score compared to European-American patients (4.19±0.86 versus 1.94±0.29 (mean±SEM), P= 0.003). The CD4+CD28+KIR+CD11ahi T cell subset size correlated with disease activity in European-American (r=0.25, P=0.038), but not in African-American lupus patients (r=-0.18, P=0.29). Linear regression models suggest that this correlation is largely independent of genetic risk, as the subset size was only a slightly better predictor of disease activity when normalized for total genetic risk in each individual (European-American: r=0.27, P=0.024; African-American: r=-0.15, P=0.37). To determine if age influenced the relationship between SLEDAI score and subset size, we assessed age-dependent correlation between the subset size and SLEDAI scores, and showed that the CD4+CD28+KIR+CD11ahi T cell subset size, whether normalized to genetic risk score or not, is a better predictor for disease activity in European-American lupus patients ≤ 40 years of age. Indeed, we found no correlation between disease activity and the subset size in patients > 40 years of age. No correlation between the subset size and disease activity was detected in any age group in African-American patients. We have recently demonstrated that the CD4+CD28+KIR+CD11ahi T cells are polyclonal, demethylated, and characterized by a pro-inflammatory transcriptional profile.

The Michigan Basic ACE is also analyzing data from the Study of Subcutaneous Abatacept to Treat Diffuse Cutaneous Systemic Sclerosis (ASSET) trial, in collaboration with the Clinical ACE program. These analyses will determine if demethylated T cells in peripheral blood correlate with response to therapy using samples collected and processed from 46 patients at multiple time points in the ASSET trial.

Collaborative studies of the OMRF Basic ACE program have evaluated the immune cell phenotypes that contribute to increased lupus disease activity, and the impact of lupus treatments on these phenotypes. We analyzed immune cell phenotypes and phospho-proteins by CyTOF in peripheral blood samples from healthy individuals (n=18) and SLE patients with either high disease activity (SLEDAI≥4; n=20) or low (SLEDAI<4; n=20) disease activity. Patients with high disease activity were distinguished by differences in cellular frequencies, cellular activation markers, and responses to stimulation. In African American patients, higher disease activity was associated with elevated frequencies of memory B cells ($p<0.05$); increased expression of activation markers on neutrophils ($p<0.05$), plasmacytoid dendritic cells (pDCs; $p=0.005$), CD8+ T Cells ($p=0.0003$) and NKT cells ($p=0.0033$). In addition, high disease activity in African American patients was associated with reduced responses to interferon α stimulation (by pSTAT5 in

almost all major cell populations; by pSTAT3 in monocytes, granulocytes, and B cells) and TLR7/8 stimulation (by cCASP3, p-p38, pCREB, and Syk in granulocytes and antigen presenting cells). In European American patients, higher disease activity was associated with reduced frequencies of peripheral B cells, specifically naïve B cells (p=0.0101) and double negative B cells (CD27-IgD-) (p=0.0220); increased expression of activation markers on B cells (p=0.0350) and pDCs (p=0.0435); and increased signaling in B cells and DCs following TLR4 and TLR7/8 stimulation, specifically in p-p38, pERK1/2, and pCREB ($p<0.05$).

Mycophenolate mofetil (MMF) is commonly used to treat major organ involvement in SLE. To define the impact of MMF treatment on immune cell subsets, cell activation, and soluble mediator pathways, we compared PBMCs and plasma samples from 5 SLE patients taking MMF and 10 SLE patients not taking MMF (n=10) by mass cytometry, xMAP assays, ELISAs, and flow cytometry. Compared to patients not taking MMF, patients taking MMF had significantly fewer transitional B cells (p=0.0077), plasmablasts (p=0.0480) and T cells (p=0.0486), specifically CD4+ Th17-type cells (p=0.0260) and CD4+ Treg-type cells (p=0.0469). In addition, patients taking MMF showed reduced activation of dendritic cells (p=0.0080) and B cells (p=0.04), specifically naïve (p=0.0127) and memory B cells (p=0.04), and lower levels of activated CD4+ T cells (p=0.0483). Plasma soluble mediators were decreased in MMF treated SLE patients, including chemokines (MIG/CXCL9 and SDF-1a/CXCL12) and growth factors (VEGF-A and PDGF-BB) ($P<0.05$). Cytokines, chemokines and significant cell populations grouped by STAT-pathways, cell lineage and functional properties revealed significant modifications associated with the STAT3 and B cell pathways. Healthy PBMCs treated with IL-6 showed a significant downregulation of pSTAT3 following MMF addition (p=0.0313), but no alterations in pSTAT5 or caspase3/7 levels were observed following a 3-hour incubation. Our results indicate that MMF suppressed STAT3 phosphorylation in response to IL-6 with associated decreases in antigen presentation, lymphocyte activation, and pro-inflammatory soluble mediators of SLE patients. Together these data suggest immunologic changes in the STAT3 pathway are critical for MMF-driven disease remission in SLE patients.

Aim 1C. Adaptive Epigenetics, Leader: J. Boss (Emory)

Major Accomplishments:

- Developed protocols for epigenetic analyses on low numbers of cells and biobanked samples.
- Determined lineage relationships between healthy control (HC) and SLE subject B cell subsets and described the overall pathway leading to the production of double negative 2 (DN2) cells, which are now being observed in multiple immune settings, including autoimmune diseases and chronic infections.
- Showed that resting naïve B cells in SLE patients are already epigenetically and transcriptionally programmed to follow a disease state.
- Showed that the activated naive (aN) and DN2 subsets display similar epigenetic programming in SLE patients compared to HC.
- Identified transcription factor family member signatures associated with SLE that appear to drive the transcriptional state of SLE B cells.
- Identified a series of epigenetic biomarkers that correlate with SLE disease severity that could be used as prognostic or diagnostic tools.
- Showed that ex vivo expanded Tregs from HC and T1D patients are similar to each other and exhibit no disease specific programming.
- Identified NMO-specific and NMO-SLE shared transcriptional programming of B cells from NMO patients.

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The major goals of this program were to elucidate the epigenetic programming that controls cell fate decisions and phenotypes associated with autoimmune disease. To achieve this goal, we developed a series of low cell-number assays that provided robust data from specific subsets of FACS isolated peripheral cells. Three assays were developed that include reduced representation bisulfite sequencing (RRBS) to determine the landscape of DNA methylation events; ATAC-seq to determine chromatin accessibility and provide a readout of epigenetic marks and regulatory element potential; and RNA-seq to assess the transcriptome of cells and link the potential role that epigenetic modifications may have on gene expression.

Collaborative Project with the Sanz group (Emory U). In this project, we applied the above assays to an African American cohort of SLE patients who were exhibiting active disease and an age and sex matched cohort of healthy control subjects (HC). Five specific B cell subsets representing resting/naïve, activated, and memory compartments were isolated. A limited number of plasma cell subsets were also processed as these cell types provide the terminal differentiation effector stage of the B cell program and function. Using the three established technologies, comprehensive datasets were collected and analyzed. The epigenetic relationships among the B cell subsets revealed a hierarchy/order in B cell differentiation. When comparing HC and SLE states, multiple SLE disease specific signatures were identified. Importantly and somewhat surprisingly, a disease signature was already manifested in naïve B cells from SLE subjects and that this signature persisted throughout B cell differentiation. This suggests that the microimmune environment, possibly through cytokines, TLR, and BCR signaling, shapes the epigenome of pathogenic cells. A large number of epigenetic markers were found to follow the severity of disease and could potentially be used for prognostic/diagnostic purposes. Advanced integration of the diverse epigenetic and transcriptional datasets highlighted novel transcription factor networks that were altered in SLE. The paths converged on signaling networks and revealed external environmental cues that contribute to expansion of pathogenic B cell subsets. Together, our results suggest that the SLE environment predisposes B cells to a pathogenic phenotype that is epigenetically propagated through B cell differentiation. The data further suggests that such signals in SLE prime extrafollicular naïve B cell differentiation into antibody secreting cells.

Collaborative Project with the Bluestone group (U. California San Francisco) was initiated to develop an epigenetic/molecular diagnostic tool to determine the quality of *ex vivo* expanded Tregs and predict through the detection of molecular biomarkers their ultimate efficacy for use in type 1 diabetes (T1D) patients. We have completed comparisons between ex vivo expanded Tregs from HC and patient

samples using RNA-seq and ATAC-seq. Although there was a signature of ex vivo expansion, there was no difference between HC and T1D Tregs. These data provided a molecular roadmap of ex vivo expanded Tregs that can be used to define biomarkers of successful ex vivo expansion.

Collaborative Project with the Bennett group at U. Colorado seeks to determine if the expanded B cell populations in neuromyelitis optica (NMO) are similar to those in SLE. Several B cell subsets from NMO patients were FACS isolated, including the DN2 population. RNA-seq and ATAC-seq have been completed and the data show that there are both SLE-shared and NMO-unique signatures in the DN2 subset from NMO patients.

Collaborative Project with Mao-Draayer group at U. Michigan sought to determine the epigenetic and transcriptional programming of B regulatory cells associated with healthy and multiple sclerosis patients. Samples have been collected and RNA-seq and ATAC-seq have been performed. The data are being analyzed and preliminary results indicate that B regulatory cell subsets harbor distinct epigenetic programming.

Aim 1D: Microbiome, Leader: Ramnik Xavier (MGH)

Collaborators: John Stone (MGH), Shiv Pillai (MGH), and Dinesh Khanna (Michigan)

In a large number of autoimmune disorders there is growing evidence for a mucosal trigger. During the course of the ACE we used metagenomics to compare the intestinal microbiome between patients with IgG4-RD and SSc and healthy controls. Remarkable similarities were observed in the microbial species that accumulated in these two very different diseases, that both however share an autoimmune fibrosis phenotype, and a close similarity in immunological mechanisms that appear to involve CD4+CTLs and activated B cells. Close similarities exist at the species and strain levels in the microbiome in IgG4-RD and SSc.

We obtained data on IgG4-RD and SSc patients. After excluding patients with more than one diagnosis our analyses were compared from 59 MGH patients with IgG4-RD and 66 Michigan patients with SSC. Stool samples were collected at home by recruited patients at MGH and Michigan. At each study visit, subjects received a kit with fecal collection tube, and accompanying instructions and questionnaires. Questionnaires and de-identified study ID number was sent to the Broad with each stool sample.

Total DNA and RNA were extracted and the Nextera Illumina platform was used to generate metagenomic data from DNA and stored the paired RNA samples at -80C for future studies. Although at the phylum level no broad differences in alpha or beta diversity were observed, interesting species differences were noted when comparing disease subjects to controls. Broad similarities were observed in SSc and IgG4-RD and each of the diseases could be clearly distinguished from controls. In general, *Proteobacteria* species were increased in the autoimmune fibrotic disease patients while *Firmicutes* were decreased, indicating similar taxonomic changes in both diseases.

One of the really interesting differences between SSc and IgG4-RD was the enrichment of specific strains of *Bacteroides dorei* that induce pathways related to integrin signaling in SSc patients. More detailed studies are proposed to follow up on this and many other observations.

Aim 2 - Characterize in situ adaptive immunity in autoimmune disease.

Aim 2A: *In situ* TFH cell function in inflammation, Leaders: M. Clark (Chicago), H. Ueno (Mount Sinai)

Since the inception of this Aim, the scope has expanded beyond *in situ* T follicular help (Tfh) and B cell function to developing and implementing approaches to study all aspects of *in situ* adaptive immunity.

Major accomplishments:

1. Development of Cell Distance Mapping (CDM) as a novel method to identify and quantify adaptive immune cell networks in human tissue.
2. Defined the mechanisms of selection of anti-vimentin antibodies (AVAs) in lung sarcoidosis.
3. Characterized the origin and selection of AVAs in lupus nephritis.
4. Defined the molecular and antigen-driven mechanisms by which B cells are selected *in situ* in inflammation.
5. Demonstrated the importance of TLR7 and OX40L in lupus autoantibody production.
6. Defined the role of cTfh1 cells in modulating disease activity in lupus.
7. Demonstrated that IRF4 is key transcription factor defining Th1/Tfh cell differentiation.
8. Developed a novel assay for detecting and quantifying antigen specific Tfh cells.
9. Applied CDM to identify major antigen presenting cells in rheumatoid arthritis (RA).
10. Demonstrated that the transcription factor AIRE controls gene expression in synovial fibroblasts.
11. Identified a novel citrullinated autoantigen in RA.
12. Demonstrated that soluble CD13 is a novel angiogenic and arthritogenic factor in RA.

University of Chicago

The University of Chicago ACE has accomplished the principal goals of its ACE to develop new technologies to characterize in situ adaptive immunity, to identify antigens driving in situ adaptive immunity and the molecular mechanisms by which lymphocytes are selected in situ in inflammation and autoimmunity.

1. **Development of Cell Distance Mapping.** While techniques to count and characterize cell subsets in human tissue have rapidly evolved, there are few techniques to determine which cells interact in human tissue. Such knowledge is critical for understanding *in situ* adaptive immunity which depends upon linked antigen recognition and cell:cell interactions. In previous publications (1-2), we have demonstrated that distance between nuclear edges can be used to discriminate between when T and B lymphocytes are in close proximity and when they are engaged in cognate interactions. However, such a simple approach is not sufficient to capture interactions between complex cells such as dendritic cells.

Two-photon excitation microscopy (TPEM) has revolutionized our understanding of adaptive immunity and is the gold standard for understanding dendritic cell:T cell interactions. However, TPEM usually requires animal models and is not amenable to the study of human disease. Recognition of antigen by T cells requires cell contact and is associated with changes in T cell shape. We postulated that by capturing these features in fixed tissue samples, we could quantify *in situ* adaptive immunity. Therefore, we used a deep convolutional neural network to identify fundamental distance and cell shape features associated with cognate help (Cell Distance Mapping, CDM). In mice, CDM was comparable to TPEM for discriminating cognate from non-cognate T cell-dendritic cell (DC) interactions. However, unlike TPEM, CDM can be applied to the study of human tissue (3).

CDM confirmed that myeloid DCs present antigen to $CD4^+$ T cells and identified plasmacytoid DCs as an important antigen presenting cell in human lupus nephritis. Interestingly, these *in situ* plasmacytoid DCs did not have surface markers typical of those subsets that present antigen in the periphery. Therefore, function, rather than surface markers, is a superior approach to characterize APCs *in situ*. These data

reveal a new approach to study human *in situ* adaptive immunity broadly applicable to autoimmunity, infection and cancer (3).

2. **Selection of AVAs in the lung of sarcoidosis patients.** *In situ* autoimmunity to vimentin is not a specific feature of lupus. In DR3+ sarcoidosis patients, lung resident antigen presenting cells display vimentin peptides what are recognized by T cells expressing the Vα2.3β22 TCR. These T cell populations are commonly expanded in the lungs of DR3+ sarcoidosis patients. In collaboration with Dr. Johan Grunewald at the Karolinskia Institutet, we have now characterized *in sit*u humoral immunity to vimentin in sarcoidosis patients. These studies demonstrated that DR3+ patients had high titers of AVAs in broncheoalveolar lavage fluid (BALF) and that titers correlated with the frequency of CD4+ T cells expressing the Vα2.3β22 TCR. The strongest correlations were with BALF C-terminal AVAs. These and other data demonstrate a coordinated adaptive immune response to vimentin in the lungs of sarcoidosis patients. Interestingly, high BALF AVAs were not reflected in the serum. This suggests that the immune response in the lung is remarkably self-contained and is not in equilibrium with that in the periphery (4)

3. **Origin and selection of AVAs in lupus nephritis**. Originally, we used laser capture microscopy to clone expanded expressed immunoglobulin repertoires from the tubulointerstitium of patients with lupus nephritis (Arth Rheum, 2014). Eleven of these 25 initially characterized antibodies were reactive with the cytoplasmic intermediate filament vimentin. We have now reverted the anti-vimentin antibodies and have extensively compared their binding characteristics. Initial *in vitro* binding indicated that these AVAs were selected to be highly polyreactive. However, *in vivo* binding, either to cells or tissue, suggested that they were highly selective for vimentin. Single amino acid reversions, and assessing binding *in vivo* for vimentin revealed both strong selection for vimentin affinity and selection against polyreactivity. In one striking example, the germline antibody was highly reactive for histones. Through selection, histone reactivity was lost and high vimentin reactivity conferred. In another, high vimentin affinity arose from low vimentin germline reactivity. These studies reveal selection for both affinity and specificity *in vivo* in human lupus nephritis. Furthermore, our results suggest that commonly used *in vitro* assays, based on purified single antigens, are misleading (manuscript in preparation).

4. ***In situ* selection of B cells in inflammation.** This project has been a full collaboration between U Chicago with MGH who has performed the single cell RNA-seq. Using protocols similar to those used by the Accelerating Medical Partnership (AMP) consortium, we have developed and validated protocols to isolate single B cells from renal biopsies and subject them to single cell RNA-seq. Preliminary studies have been done on renal allograft rejection as these biopsies are readily available. Initial studies of approximately 800 B cells from five renal biopsies and two human tonsils have demonstrated that the transcriptome of infiltrating IgG+ B cells have an upregulation of innate sensing pathways compared to tonsil B cells. We have now gone on to characterize the antibody specificity of 100 of these B cells. Unexpectedly, 24% of renal *in situ* selected antibodies are against HLA class I or class II alleles. Not all antibodies were specific for donor specific allotypes and, furthermore, many different allotypic antibodies were present in the serum of these patients. These results indicate that there is a general loss of tolerance to polymorphic HLA allotypes *in situ* in mixed allograft rejection.

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Mount Sinai School of Medicine

We have made many significant achievements under the U19 ACE Collaborative Projects.

1. Identifying the role of the TLR7-OX40L axis in autoantibody production in systemic lupus erythematosus (SLE). We have demonstrated that 1) OX40 ligand (OX40L) was overexpressed by myeloid APCs in blood and in inflammatory tissues of SLE patients, 2) OX40 signal promoted human naive and memory $CD4^+$ T cells to become Tfh-like cells, and 3) RNA-containing ICs present in SLE sera induced monocytes to express OX40L in a TLR7-dependent manner (1). We propose that RNA-containing ICs in SLE patients activates the TLR7-OX40L axis, and promote the generation of autoantibodies by promoting Tfh cell response. Our findings provide a rationale to target TLR7 and OX40L for therapeutic approaches for SLE. We have authored several reviews on Tfh cells in autoimmunity (2-5).

2. CD57+ cTfh1 cells correlate with disease activity and severity in pediatric SLE. We have found that the frequency of CD57+ cTfh1 cells showed a strong positive correlation with SLEDAI score and a negative correlation with serum C3 complement level. To our surprise, the expression of ICOS or PD-1 on cTfh cells did not correlate with any clinical parameters in pediatric SLE. The manuscript is under preparation.

3. Tfh1cells as suppressor of Tfh and germinal center (GC) responses in human lymphoid organs. We have found that GC Tfh1 cells display suppressive functions against Tfh cells and GC B cells. Our discovery suggests that the quality and the magnitude of germinal center response is governed by the balance between Tfh1 and non-Tfh1 subsets. The manuscript is under revision.

4. Tfh1 cells as efficient helpers for memory B cells. Interestingly GC Tfh1 and Pre Tfh1 cells provide efficient help to memory B cells to become Ab-producing cells. This is consistent with our previous observation that blood cTfh1 cells provide efficient help to memory B cells, but not naïve B cells (6). The manuscript is under revision. Collectively, Tfh1 cells promote or suppress B cell responses depending on their location and their B cell counterpart. How this Tfh subset contributes to SLE pathogenesis will be an important future research topic.

5. IRF4 as a key transcription factor defining the early differentiation pathway between Th1 and Tfh cells. We have found that high IRF4 expression was required to promote early CD4+ T cell differentiation programs towards Tfh and away from Th1 cells (7). Low IRF4 promoted the expression of Eomes, and accordingly the population producing IFN-□. This suggests that factors promoting IRF4 expression, e.g., stronger TCR signals, facilitate the differentiation of Tfh cells in inflamed lymphoid organs.

6. A novel method to detect Ag-specific cTfh cells. Currently there is no method to detect self-antigen-specific cTfh cells in blood samples of human autoimmune diseases. We have developed a novel assay that sensitively detects rare Ag-specific CD4+ and CD8+ T cells in human blood samples. The preliminary results are promising, and our new assay can detect rare Ag-specific T cells with high specificity and

sensitivity. The manuscript is under preparation. We plan to analyze AQP4-specific cTfh cells in NMO blood samples by using this method.

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Aim 2B: Role of Organ-Specific Parenchymal Cells in Human Organ-Targeted Autoimmune Diseases, Leader: D Fox (Michigan)

Progress in the past year has been highlighted by several discoveries related to molecular mechanisms by which synovial fibroblasts orchestrate RA synovitis

A. Interaction of T cells with stromal cells and APCs in rheumatoid arthritis synovium (University of Michigan Clinical ACE and University of Chicago ACE). The Chicago ACE has developed a new algorithm that optimizes cell distance imaging for studying lymphocyte-APC interactions involving both myeloid and stromal cell APC's. This is being applied to analysis of RA synovium.
B. Control of gene expression in synovial fibroblasts (FLS) by the autoimmune regulator protein (AIRE):

   We have discovered during the past year that, similar to the recent results from studies of Graves' Disease fibrocytes by co-investigator Terry Smith, the secreted protein Slit2 controls AIRE expression in FLS. Neutralization of Slit2 is now allowing us to compare FLS with high versus low levels of AIRE expression to analyze AIRE regulation of gene expression in FLS.
C. A new citrullinated RA autoantigen, cit-Id1, has been discovered. Aliquots of cit-id1 and Id1 have been sent to the Stanford ACE for inclusion in their autoantigen microarrays. A manuscript has been submitted (Arthritis Rheum, in revision) that reports the effects of Id1 knockdown on FLS cytokine production and proliferation, and also describes the autoantigenic properties of cit-Id1.
D. We have discovered a new ligand for CD6, and identified it as CD318, which is expressed in a cytokine-inducible manner in vivo on FLS, is chemotactic in a shed, soluble form, and which participates (along with CD166, also a CD6 ligand) in T cell adhesion to FLS.
E. We have recently shown that soluble CD13, shed from the FLS surface, is chemotactic for endothelial cells and monocytes and is strongly angiogenic (J Immunol in revision). Our prior published work showed that it was chemotactic for activated T cells. Moreover, it is arthritogenic in vivo as a single stimulus, inducing synovitis in rodent knees within 24 hours

of intra-articular injection.

All of these actions occur at physiologic concentrations of sCD13, and are mediated by a G-protein-coupled receptor.

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