

**National Cancer Institute
National Institute of Arthritis and Musculoskeletal and Skin Diseases
National Institute of Allergy and Infectious Diseases
Office of Research on Women's Health
National Institutes of Health**

In cooperation with the Lupus Foundation of America, Inc.

**Systemic Lupus Erythematosus:
From Mouse Models to Human Disease
and Treatment**

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EXECUTIVE SUMMARY

Systemic lupus erythematosus (SLE) is an autoimmune disease that is more common in women than in men, but more severe in men. At present there is no cure, and therapies only seek to relieve the symptoms of lupus. Recent developments in genomics, molecular genetics, and bioinformatics, as well as studies in lupus-prone mouse models, have contributed to advances in the understanding of the mechanisms underlying lupus. In addition, several promising therapeutics have been developed, and some are undergoing clinical trials. Yet more work is clearly needed to further understand the biology of lupus, determine how existing therapies work, and characterize the heterogeneity seen among patients with lupus.

On September 2–3, 2010, the National Cancer Institute (NCI), the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), the National Institute of Allergy and Infectious Diseases (NIAID), and the NIH Office of Research on Women's Health (ORWH) convened a conference, bringing together basic and clinical scientists from academia, industry, and government to discuss clinical and molecular similarities and differences between human lupus and mouse models. The conference also aimed to encourage discussion of clinical markers and their utility in assessing the relevance of mouse models and the efficacy and effectiveness of treatment. Drs. Howard Young, of NCI, Silvia Bolland, of NIAID, and Juan Rivera, of NIAMS, organized the conference and served as conference leaders.

Following an opening lecture by Dr. Betty Diamond, of the Feinstein Medical Research Institute, were four sessions on interferon and innate responses in SLE, autoantibodies and lymphocyte activation, new advances and clinical challenges in the treatment of SLE, and genetic analysis of lupus disease in mouse and human. Dr. Timothy Behrens of Genentech gave a closing lecture, and the conference ended with a panel discussion in which Drs. Joe Craft of Yale University, Mary Crow of the Hospital for Special Surgery, and David Close of MedImmune summarized themes of the conference and highlighted important areas that had not been discussed (see Conference Themes, Areas for Further Discussion and Study, and Suggested Resources, below). Each lecture and session included opportunities for discussion. Many speakers presented unpublished data, thus highlighting the cutting edge of lupus research. As a result, discussions often focused on technical aspects of the speaker's work.

Opening Lecture

Dr. Diamond presented four vignettes to illustrate the varying degrees of success in translating results from mouse studies to studies of human health. She pointed out that although mouse models have been instrumental in the current understanding of SLE, no existing model mimics the spontaneous flares and remissions seen in human lupus.

Session 1: Interferon and Innate Responses in SLE

The first half of this session focused on the type I interferon (IFN) signature of gene expression, which is quite marked among patients with SLE. Dr. Crow introduced this signature by discussing her laboratory's work on IFN- α , a key type I IFN, at various stages in lupus disease. She also noted that the primary inducer of IFN- α remains unknown. Dr. Virginia Pascual, of the Baylor Institute, presented evidence of the involvement of neutrophils in lupus. These cells interact with plasmacytoid dendritic cells, which stimulate IFN- α production through the creation of neutrophil extracellular traps (NETs), a neutrophil-specific type of cell death induced by exposure to anti-ribonucleoprotein antibodies. Dr. Mariana Kaplan, of the University of Michigan, then discussed a role for type I IFNs in the impaired vascular repair seen in patients with lupus. The work she presented suggested that type I IFNs interfere with repair partly by downregulating interleukin (IL) 1 and pro-angiogenic factors such as vascular endothelial growth factor and peroxisome proliferator-activated receptor-gamma.

The second half of the session took a broader view of IFN signaling in lupus. Work presented by Dr. Young showed that deletion of conserved elements in the 3' untranslated region of the *IFN- γ* gene leads to constitutive IFN- γ expression, which exerts immune and metabolic effects similar to those seen in patients with SLE. Dr. Bolland then introduced the role of Toll-like receptor (TLR) signaling by presenting mouse studies showing the importance of TLR7 in both the inflammatory and autoreactive components of the lupus immune system. The session ended with a presentation by Dr. Westley Reeves, of the University of Florida, who discussed the formation of ectopic lymphoid tissue, in the form of lipogranulomas, in mice treated with pristane. Pristane treatment induces an accumulation of immature monocytes in the peritoneum, and these monocytes serve as a source of IFN and are unable to terminally differentiate. Thus pristane treatment induces a vicious cycle of inflammation.

Session 2: Autoantibodies and Lymphocyte Activation

The session began with a presentation by Dr. Iñaki Sanz of the University of Rochester, who presented cutting-edge technologies in B-cell profiling and touted the value of B-cell profiling in identifying patient subsets and disease subtypes. Dr. Judith James of the Oklahoma Medical Research Foundation discussed the temporal relationship between viral infection, the production of autoantibodies, and the development of lupus disease. Dr. Mark Shlomchik of Yale University presented evidence of a TLR7-, TLR9-, and MyD88-dependent extrafollicular B-cell response to rheumatoid factor and suggested that autoimmunity might result from an immune dominance of chromatin or rheumatoid factor, rather than a breakdown of T-cell tolerance. Dr. Rivera closed the session by discussing a role for basophils and the Lyn kinase in immunoglobulin E-mediated autoimmunity.

Session 3: New Advances and Clinical Challenges in the Treatment of SLE

Dr. Roland Kolbeck of MedImmune discussed potential therapeutic approaches that interfere with the IFN- α pathway, and Dr. Douglas Hough of Human Genome Sciences discussed BLYS, which is involved in B-cell proliferation and differentiation and might thus serve as a target for lupus therapies. Dr. Franck Barrat of Dynavax discussed approaches to interfere with TLR7 and TLR9, particularly in the cutaneous form of lupus. Dr. Anne Davidson of the Feinstein Medical Research Institute discussed the importance of testing potential therapies in several different lupus-prone mouse models, as well as in humans, to account for strain differences, avoid mouse-specific markers, and obtain clues on how to better subtype patients with SLE.

Session 4: Genetic Analysis of Lupus Disease in Mouse and Human

Dr. John Harley of the Cincinnati Children's Hospital Medical Center introduced the session by discussing the need for large numbers of patients and healthy controls in genetic association studies. He also described the utility of physical chromosome mapping in increasing the likelihood of identifying accurate candidate gene associations. Dr. Gary Gilkeson of the Medical University of South Carolina presented genetic studies exploring the role of IFN regulatory factor 4 in the lupuslike disease seen in a lupus-prone mouse model. Dr. Herbert (Sandy) Morse III of NIAID described mouse models of the link between autoimmunity and lymphoma development, and Dr. Derry Roopenian presented evidence that interleukin-21 (IL-21) serves as an early signal to B cells before other evidence of autoimmunity is present.

Closing Lecture

Dr. Behrens focused his lecture on the future of SLE research and drug development. He began with a discussion of advances in SLE genetics, then briefly discussed clinical trial results from Genentech. He closed by describing the challenges facing SLE research and the drug development pipeline, and he discussed what will be needed for future research.

Conference Themes

- **A systems biology approach.** The linear model of immune activation involving mediators, B cells, T cells, and antigen-presenting cells (APCs) is giving way to a systems model that comprises several feedback pathways and some components beyond the immune system. Although more work is needed to better understand each component of the lupus system, researchers should take care not to overcommit to one component with respect to therapeutics, as there are several points for intervention.
- **Regulation of immune response.** Lupus research has traditionally emphasized immune tolerance, but increasing evidence suggests that autoimmunity might also arise from dysregulation of normal immune response. More study is needed to understand the levels and points of immune regulation, the events between autoantibody production and lupus disease manifestation, and various aspects of immune tolerance.
- **Mouse versus human studies.** As illustrated by presentations at this conference, the use of mouse models has contributed to significant progress in what is known about the mechanisms of lupus, how they might change over time, and potential ways to block these mechanisms. Both mouse models and human clinical studies will be needed to further drug development and understand how best to treat patients with active disease.

- **Disease and patient heterogeneity, and the need to better characterize SLE patient subtypes.** Patients with SLE exhibit variability both in disease manifestations and response to treatment. Mechanistic studies are needed to further understand this heterogeneity, and clinical trials must account for it. Investigators are encouraged to think of the “global patient,” encompassing sex, racial/ethnic, genetic, environmental, and other differences.
- **A better understanding of current lupus therapies.** Mouse studies can be used to better understand how the current standard of care works, which will aid in the design of new therapies and be critical if current therapies are to be used as controls in future clinical trials.
- **The potential for cost-effective and combination therapies.** Combination therapies have been suggested for patients with SLE, and cost effectiveness is a goal, but high toxicity levels remain a concern. On the basis of new understanding of how current therapies work, future clinical trials could define the risks and benefits of cost-effective approaches in which therapies are used sequentially. The tools exist to move these studies forward.
- **The importance of communication.** Meetings such as this conference remain vital in sharing information. Foundations and associations such as the Alliance for Lupus Research, the Lupus Research Institute, and the Lupus Foundation of America have been instrumental of bringing together basic and clinical scientists from different areas of expertise, emphasizing innovative science, and identifying ways to make clinical drug development more efficient.
- **The need to think creatively and work with other disciplines.** The current understanding of lupus has resulted in part from advances in physics, chemistry, and bioinformatics, and one of the most effective existing treatments for lupus emerged from the field of transplantation science. Lupus research will continue to benefit from interactions with other medical specialties and several scientific research disciplines.
- **The risky business of drug development.** Industry will continue to need data from mouse and clinical studies to justify the average \$900 million investment in drug development. Like academic researchers, researchers in industry must also write scientific and clinical justifications similar to grant applications, and as with the grant application process, the risk for failure is high.

Areas for Further Discussion and Study

- Environmental factors that trigger or amplify the genetic substrate underlying SLE manifestations, particularly flares and remissions.
- The role of the following in SLE:
 - Sex
 - Nonimmune cell types
 - Epigenetics
 - The gut microbiome
 - Stress
- Natural mechanisms for downregulating the immune response.

Suggested Resources to Aid Future Research

- Continuation of the Lupus Registry and Repository at the Oklahoma Medical Research Foundation and expansion of the registry to 50,000 patients. This is considered a high priority as funding for continuation of the registry is not certain.
- A mechanism to help centers explore existing repositories, registries, and databases and identify appropriate samples and patient populations for their studies.
- A mechanism to fund small, collaborative studies that would help investigators share innovative assays or procedures more widely. At present, many investigators agree to test others' samples, but this constitutes a financial burden. It is envisaged that such mini-grants would fall into the \$10,000 to \$40,000 range.
- A mechanism to allow investigators to quickly access and explore new technologies. At present, with flat funding levels, investigators do not have the purchasing power to access technologies and enter into new collaborations. As a result, creativity is stifled.
- An analysis of the metabolome of 20 different mouse models of lupus, including both sexes and two different time points with appropriate controls. This data would be made freely available to the research community. The cost of such an analysis is estimated to be \$250,000.

LIST OF ABBREVIATIONS AND ACRONYMS

3'UTR	3' untranslated region
APC	antigen-presenting cell
ARE	adenine-uridine-rich element
BAFF	B-cell activating factor belonging to the TNF family
BCR	B-cell receptor
BLyS	B-lymphocyte stimulator
CAC	circulating angiogenic cell
CSF	cerebrospinal fluid
DiD	1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine,4-chlorobenzenesulfonate
DoD	Department of Defense
ds	double-stranded
EAE	experimental autoimmune encephalitis
EBV	Epstein-Barr virus
EPC	endothelial progenitor cell
HLA	human leukocyte antigen
ICOS	inducible co-stimulator
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IRF	IFN-regulatory factor
IRS	immunoregulatory sequences
LDG	low-density granulocyte
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
NCI	National Cancer Institute
NET	neutrophil extracellular trap
NIAID	National Institute of Allergy and Infectious Diseases
NIAMS	National Institute of Arthritis and Musculoskeletal and Skin Diseases
NIH	National Institutes of Health
NK	natural killer
NMDA	N-methyl-D-aspartic acid
NMHCII-C	non-muscle myosin heavy chain II-C

OA	Osteoarthritis
ORWH	Office of Research on Women's Health
PBMC	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cells
PPAR	peroxisome proliferator-activated receptor
RA	rheumatoid arthritis
RF	rheumatoid factor
RNP	ribonucleoprotein
SIAE	sialic acid acetyltransferase
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
ss	single-stranded
TLR	Toll-like receptor
TNF- α	tumor necrosis factor alpha
Treg	regulatory T cells
VCA	viral capsid antigen
VEGF	vascular endothelial growth factor
Yaa	Y-linked autoimmune accelerator

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Conference Summary

INTRODUCTION AND OVERVIEW

On September 2–3, 2010, three Institutes within the National Institutes of Health (NIH), along with the NIH Office of Research on Women's Health (ORWH) and the Lupus Foundation of America, Inc., held a conference to discuss clinical and molecular similarities and differences between human systemic lupus erythematosus (SLE) and animal lupus models. The conference encouraged discussion of clinical markers and their utility in assessing both the relevance of animal models and the effectiveness of treatment. Drs. Howard Young of the National Cancer Institute (NCI), Silvia Bolland of the National Institute of Allergy and Infectious Diseases (NIAID), and Juan Rivera of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) organized the conference and served as conference chairs.

Following an opening lecture by Dr. Betty Diamond of the Feinstein Medical Research Institute, conference participants heard presentations focused on interferon and innate responses, autoantibodies and lymphocyte activation, advances and challenges in treating SLE, and genetic analysis in mouse and humans. Dr. Timothy Behrens of Genentech gave a closing lecture, and the conference closed with a panel discussion by Drs. David Close, Joe Craft, and Mary Crow. The conference aimed not only to encourage further discussion, but also to develop some consensus regarding the most important features of lupus and the best models for developing new markers and treatments.

OPENING LECTURE: UNDERSTANDING HUMAN LUPUS: FROM MOUSE TO HUMAN AND BACK

Betty Diamond, M.D., Feinstein Medical Research Institute, Manhasset, New York

Dr. Diamond highlighted four vignettes from her laboratory to illustrate the various relationships between animal models and clinical studies. In the first, researchers in her laboratory identified a peptide mimotope that triggered the production of anti-DNA antibodies and the deposition of immunoglobulins in the glomerulus in BALB/c mice, but not in DBA/2 mice. Through backcross analysis, the laboratory identified three regions in chromosome 9 that confer susceptibility to

lupus following peptide exposure, then generated congenic mice (C9) carrying these regions. C9 mice generated anti-DNA antibodies similar to those seen in BALB/c mice, and B cells from these mice showed attenuated response to B-cell receptor (BCR) signaling and less BCR-mediated apoptosis in the transitional population, suggesting that B-cell hyporesponsiveness contributes to lupus.

Among the several candidate genes located within the identified susceptibility regions, *CSK*, which encodes a kinase that phosphorylates the *Lyn* gene, was highly expressed in BALB/c and C9 cells, compared with DBA/2 cells. Overexpression of *CSK* led to less phosphorylation of an inhibitory site on the *Lyn* gene, as expected, but it also led to a weaker BCR signal and less anti-immunoglobulin M (IgM)-mediated apoptosis. Yet the more highly expressed *CSK* allele appeared to confer only a modest effect on the predisposition to lupus. Further study of the B haplotype revealed that the TT allele of *CSK* conferred a twofold higher risk for lupus compared with the CC allele or the CT heterozygote. Indeed, in normal individuals with the TT allele, BCR signaling was attenuated and B cells were hyporesponsive, allowing the maturation of autoreactive antibodies. Thus Dr. Diamond's laboratory was able to identify a potential susceptibility gene in mice and demonstrate that that gene can predispose humans to the production of autoantibodies. In addition, *CSK* appears to have the same function as *PTPN22*, which also reduces BCR signaling and predisposes individuals to lupus. Thus *CSK* might be an appropriate target for therapies.

A second vignette from the laboratory has not yet shown a similar success. Sex bias is the most salient feature of SLE; the disease is more common among women than among men. To determine whether sex hormones play a role in this disparity, Dr. Diamond and colleagues treated the R4A mouse model, a transgenic BALB/c strain that carries the IgG2B heavy chain of anti-DNA antibodies, with estrogen. Estrogen-treated mice exhibited anti-DNA antibody production, transgene expression in B cells, and attenuated BCR signaling as demonstrated in reduced calcium flux, Erk phosphorylation, and apoptosis. These mice further showed a failure to negatively select against transitional B cells but no loss of high-affinity B cells. However, C57Bl/6 mice did not show the same pathogenic effects in response to estrogen, and the literature offers conflicting reports on the effects of hormones on disease risk or exacerbations.

Dr. Diamond and colleagues performed microarray analysis to identify expression patterns that are altered in BALB/c but not in C57Bl/6 mice in response to estrogen. They found that expression of *p202*, a gene that is part of the interferon (IFN) signature, plays a critical role in the NZB/W mouse model, and interferes with the expression of pro-apoptotic molecules, increases in transitional B cells in BALB/c but not C57Bl/6 mice. They also found that expression of *ITPKB*, a kinase that regulates calcium flux and B-cell selection and activation, is increased selectively by estrogen treatment in BALB/c but not C57Bl/6 mice. However, further study of these genes in humans yielded no evidence of regulation by estrogen. Thus, the use of mouse models has raised interesting questions with respect to B-cell regulation, but these models have not yet translated to humans, and it is not clear how useful they will be.

In the third vignette, experiments in a conditional Blimp-1 knockout showed that dendritic cell development appears normal, even though Blimp-1 expression is downregulated. Yet female Blimp-1 conditional knockouts develop anti-nucleic acid antibodies by 3 months of age, and all these antibodies are IgG and somatically mutated. Further studies in these mice showed higher

levels of IL-21 expression, activated dendritic cells that expressed increased amounts of IL-6, and large germinal centers resulting from large numbers of follicular T cells. However, offspring resulting from a cross between Blimp-1 conditional knockout and IL-6 haploid mice showed no antinucleic acid antibodies, increases in follicular T cells, or germinal centers. Although it is not yet clear whether the Blimp-1 conditional knockout will translate to humans, these studies suggest that a subset of lupus patients might benefit from IL-6 benefit.

The final vignette focused on neuropsychiatric lupus, which is a major cause of morbidity in patients with SLE and results not only in acute events but also in insidious behavioral changes and cognitive impairment. The abovementioned peptide, which can trigger autoantibody production, contains a consensus sequence that appears in subunits of the N-methyl-D-aspartic acid (NMDA) receptor. Cross-reactive anti-DNA antibodies triggered by this peptide can enhance excitement of postsynaptic potentials and promote neuronal death. Cross-reactive antibodies are found in the serum of 40 percent of lupus patients and can be eluted from their brain; serum antibodies from these patients induce neuronal death among mouse hippocampal cells. Cerebrospinal fluid (CSF) antibody titers, but not serum antibody titers, correlate with neuropathy; declines in CSF titers are accompanied by decreased symptoms. Studies have demonstrated that antibodies target hippocampal neurons following lipopolysaccharide infection, resulting in memory deficits, and that the use of epinephrine to breach the blood-brain barrier exposes amygdala neurons to serum antibodies, resulting in behavioral impairments.

On the basis of these results, Dr. Diamond and colleagues hypothesized that a peptide might protect glomerular and cognitive function. Indeed, soluble D peptide appears to reduce glomerular Ig deposition and bind antibodies, systematically protecting neurons from the effects of epinephrine and high antibody titers. However, this peptide has a half-life of only 6 to 8 hours, and it would have to be administered subcutaneously at least daily. Dr. Diamond and colleagues thus synthesized a small-molecule mimotope that binds mouse anti-DNA antibodies and antibodies from lupus patients with much higher affinity. As expected, the small molecule inhibits DNA binding and protects against glomerular binding and neuronal death. However, there is no metric for assessing neuroprotection over an extended period of time, and mouse models cannot be used to develop one. Thus clinical trials of this peptide appear to be impossible.

These vignettes illustrate the importance of assessing what can be accomplished with animal models. Although they have been useful in increasing knowledge of the immunology underlying SLE, this understanding has not yet translated fully to improved therapies for SLE. Researchers must understand what organs are involved and how those organs contribute to what is seen systemically and in patients' serum, what genes are involved in or predispose to lupus, whether there is a sexual dimorphism, and whether therapeutic interventions in the models can predict response in patients with SLE. It should be noted that no mouse model exhibits the spontaneous remissions and flares seen in human disease and that organs involved in human disease might not be involved in mouse models. Few available mouse models exhibit the female bias of SLE, and although concordance in monozygotic twins is about 30 percent, models where less than 30 percent of individuals develop disease are ignored. In addition, data from mouse models are derived from only one genetic background, and the effects of genetic heterogeneity are not assessed. Moreover, the targets and metabolism of drugs might differ between mice and humans. More study is needed to determine the utility of mouse models in assessing gene-environment

interactions, developing biomarkers of response to therapy, understanding the transition from inflammation to organ damage, and measuring the effects of polypharmacy.

SESSION 1: INTERFERON AND INNATE RESPONSES IN SLE

IFN- α in Lupus Pathogenesis

Mary K. Crow, M.D., Hospital for Special Surgery, New York, New York

Shortly after the discovery of type I IFNs, Steinberg and colleagues conducted the first murine study of the relationship between this IFN class and lupus.¹ Since then, studies in mouse models have yielded evidence that genetic backgrounds or environments that induce type I interferons, particularly IFN- α , can induce or accelerate lupuslike disease and contribute to its autoimmune and organ manifestations.² Microarray analyses in the lupus-prone NZB/W mouse strain show a type I IFN signature: expression of genes induced by type I IFNs is markedly higher in this mouse strain, compared with that seen in BALB/c or MRL-LPR mice.³ Further work in lupus-prone mice reveals a picture of fibrosis accelerated by the induction of type I IFNs.⁴ Thus, although IFN- γ , a type II IFN, has received more attention historically, type I IFNs also play a role in SLE.

Studies in human patients have yielded results consistent with observations from mouse studies. In the first study of patients with SLE, disease activity and the presence of anti-DNA antibodies were correlated with higher levels of circulating IFN- α .⁵ Administration of IFN- α for the treatment of hepatitis C can induce lupuslike disease, and IFN- α in serum from lupus patients can activate the immune system, enhancing the ability of dendritic cells to drive T-cell proliferation. Like lupus-prone mouse models, peripheral blood from patients with SLE also shows a type I IFN gene expression signature.⁶ In addition, several gene variants associated with lupus are associated with the activation or increased production of IFN- α . The IFN- α -inducible gene *MXA* is expressed more highly in skin lesions from patients with SLE or discoid lupus, compared with healthy donors,⁷ and gene expression patterns are enriched for type I IFN-inducible genes in the synovium from lupus patients, compared with patients with RA or osteoarthritis (OA).⁸ Functional studies in vitro indicate that type I IFNs inhibit the differentiation of monocytes into osteoclasts and that this effect might be mediated by the chemokine CXCL11,⁹ and some evidence suggests that the type I IFN signature is also involved in Degos disease, a syndrome that does not involve immune complexes or lupus autoantibodies but focuses on the vasculature (Magro and Crow, submitted).

Data from these studies suggest that IFN- α is likely active at multiple stages in SLE pathogenesis and that it exerts its effects on local tissue not only through the immune system but

¹ Steinberg et al. Proc Natl Acad Sci U S A 1969.

² Mathian et al. J Immunol 2005; Santiago-Raber et al. J Exp Med 2003; Braun et al. J Autoimmun 2003.

³ Lu et al. Genes Immunity 2007.

⁴ Triantafyllopoulou et al. Proc Natl Acad Sci U S A 2010.

⁵ Hooks et al. N Engl J Med 1979.

⁶ Crow et al. Autoimmunity 2003.

⁷ Farkas et al. Am J Pathol 2001.

⁸ Toukap et al. Arthritis Rheum 2007.

⁹ Leomil Coelho et al. Proc Natl Acad Sci U S A 2005.

directly as well. The primary inducers of IFN- α production are not known, but there are several candidates. Several studies have shown that immune complexes containing RNA can induce IFN- α production through Toll-like receptor (TLR) signaling.¹⁰ Infection, retrotransposons,¹¹ and environmental triggers such as the hydrocarbon pristane¹² are also candidates.

Interplay Between Neutrophils and Plasmacytoid Dendritic Cells in Human SLE

Virginia Pascual, M.D., Baylor Institute, Dallas, Texas

Data from pediatric studies indicate a role for IFN- α in dendritic cell differentiation.¹³ As has been observed in other studies, blood and leukocytes from children with SLE exhibit an IFN- α signature¹⁴ that can be extinguished by treatment with high-dose glucocorticoids.¹⁵ Moreover, patients with lupus develop autoantibodies and immune complexes that induce plasmacytoid dendritic cells (pDCs) to produce even more IFN. In particular, immune complexes containing chromatin activate pDCs and B cells, but the nucleic acid in these complexes is not enough. Proteins such as LL37 and HMGB1 intercalate with these acids, facilitating uptake and activation of these cells in endosomal compartments and giving rise to downstream effects.

Neutrophils, which are abundant in the immune system, express receptors for LL37 and HMGB1, as well as TLRs such as TLR7 and TLR9. Dr. Pascual's laboratory has identified a neutrophil-specific gene-expression signature that correlates positively with SLE disease activity.¹⁶ Although this signature is expressed in all patients with SLE, it is expressed more highly in patients with active disease. The genes within the signature are neutrophil-restrictive genes, but peripheral blood mononuclear cells (PBMCs) from SLE patients also copurify with neutrophils. How neutrophils contribute to SLE disease pathogenesis and particularly to SLE-associated nephritis is not clear.

Mature neutrophils from SLE patients die through apoptosis and necrosis.¹⁷ These neutrophils also die from the creation of neutrophil extracellular traps (NETs), a form of death specific to neutrophils.¹⁸ In response to exposure to anti-ribonucleoprotein (RNP) antibodies, nuclear membranes in the neutrophils are disrupted, resulting in exposure of the nucleus, a mixture of nuclear and cytoplasmic material, and the release of DNA, histones, and cytoplasmic neutrophilic proteins into the extracellular space. Work in Dr. Pascual's laboratory has yielded evidence that neutrophils are activated and induced to die in a manner dependent on TLR7, Fc γ receptor IIA, and NADPH oxidase; that NETs are loaded with LL37 and HMGB1; and that NETs stimulate pDCs and induce IFN production in a TLR- and NET-DNA-dependent manner (Garcia-Romo et al, submitted).

¹⁰ Stein et al. Cell 2005; Kambach et al. Cell 1999; Koyama et al. Cytokine 2008.

¹¹ Cordaux et al. Nature Rev Genetics 2009.

¹² Reeves et al. Trends Immunol 2009.

¹³ Blanco et al. Science 2001.

¹⁴ Pascual et al. Ann Rev Immunol 2010; Bennett et al. J Exp Med 2003.

¹⁵ Guiducci et al. Nature 2010.

¹⁶ Bennett et al. J Exp Med 2003.

¹⁷ Brinkman et al. Science 2004; Martinelli et al. J Biol Chem 2004; Zychlinsky Trends Immunol 2007

¹⁸ von Kockritz-Blickwede et al. J Mol Med 2009.

Type I Interferons and the Development of Organ Damage in SLE

Mariana J. Kaplan, M.D., University of Michigan, Ann Arbor, Michigan

Patients with SLE experience increased vascular damage and atherosclerosis, and thus a much higher risk for cardiovascular disease, compared with healthy individuals or even patients with other inflammatory conditions such as RA.¹⁹ Renal disease is also a significant complication of SLE, but what events lead to progressive loss of renal function in these patients is unclear. Evidence from other diseases suggests that loss of vascular density in the kidney might be a key driver of SLE-associated renal disease. However, the traditional mechanisms associated with vascular damage in other diseases have not been observed in patients with SLE. Various groups have suggested that an imbalance between cell damage or apoptosis and vascular repair or regeneration affects the development and acceleration of atherosclerosis in other patient populations.²⁰

Vascular repair involves bone-marrow-derived endothelial progenitor cells (EPCs), whose low numbers or activity predict cardiovascular events, and myeloid circulating angiogenic cells (CAC), which are found in the CD14⁺ cell population; differentiate into endothelial-like cells *in vitro*; and promote EPC migration, release, and homing to the vasculature.²¹ Patients with lupus show increased levels of circulating apoptotic endothelial cells, which correlate with endothelial dysfunction, tissue factor generation, and severity of carotid atherosclerosis. Both lupus patients and mouse models of lupus show decreased vascular repair of the damaged endothelium, as illustrated by lower numbers of EPCs, an abnormal capacity of EPCs to differentiate into mature endothelial cells and incorporate into the vasculature, and lower levels of proangiogenic factors such as vascular endothelial growth factor (VEGF) or hepatic growth factor.²²

Type I IFNs have been implicated in the development of abnormal vasculogenesis in SLE.²³ Expression and synthesis of these IFNs are increased in EPC and CACs cultured from SLE patients, and IFN- α induces apoptosis of these cell types. Moreover, recombinant IFN- α interferes with vasculogenesis by skewing progenitor CACs toward nonangiogenic pathways. Neutralization of IFN- α , the type I IFN receptor, TLR7, or TLR9 restores EPC and CAC function in SLE. Although mice are generally resistant to atherosclerosis, studies focused on endothelial dysfunction as a surrogate have yielded results consistent with human or *in vitro* studies. Wild-type lupus-prone mice that depend on type I IFNs for disease progression show significant endothelial dysfunction, abnormal levels and function of EPCs, and increased exposure of EPCs to type I IFNs *in vivo*.²⁴ Endothelial function and the vasculogenesis show significant improvement in lupus-prone mice deficient in the type I IFN receptor. A potential role for type I IFNs in platelet activation and thrombosis has recently been proposed.

The pathways through which type I IFNs interfere with vascular repair are not clear, and most studies of pathways have been done with cancer cell lines. However, a recent gene array analysis

¹⁹ Manzi et al. *Am J Epidemiol* 1997; Roman et al. *N Engl J Med* 2003; El Magadmi et al. *Circulation* 2004; Urowitz et al. *Am J Med* 1976.

²⁰ Werner et al. *Arterioscler Thromb Vasc Biol* 2006.

²¹ Kawamoto. *Trends Cardiovasc Med* 2008.

²² Rajagopalan et al. *Blood* 2004; Denny et al. *Blood* 2007; Thacker et al. *Lupus* 2010.

²³ Denny et al. *Blood* 2007.

²⁴ Thacker et al. *Lupus* 2010.

in healthy and lupus EPCs following treatment with IFN- α revealed significant repression of IL-1, an important player in inflammation and angiogenesis, as well as significant upregulation of the antiangiogenic factor IL-1RN.²⁵ Consistent with these results, treatment of lupus EPCs with IL-1 improved differentiation capacity. A comparison of kidneys from lupus patients, healthy controls, and patients with ANCA-positive vasculitis showed this pathway to be operational *in vivo*, with significant downregulation of VEGF and upregulation of IL-1RN in renal blood vessels associated with decreased capillary density and renal vascular rarefaction. Molecules involved in peroxisome proliferator-activated receptor (PPAR) pathways are also downregulated by type I IFNs, and treatment of lupus-prone mice with a PPAR agonist improves endothelial relaxation, EPC function, and nephritis.²⁶

Neutrophils also appear to play a role in vascular damage and endothelial repair. Low-density granulocytes (LDGs) have been observed in the PBMCs of adult SLE patients.²⁷ Lupus LDGs are cytotoxic to the endothelium, and they secrete increased levels of proinflammatory cytokines and type I IFNs. Thus these cells appear to interfere with vascular repair through type I IFN, and their depletion abrogates abnormalities in EPC differentiation. In addition, they also release NETs in the absence of exogenous stimulation. Further studies of the mechanisms by which LDGs impair vascular repair and are cytotoxic are under way.

Chronic IFN- γ Expression as a Model for SLE: A New/Old Mouse Model of Lupus

Howard A. Young, Ph.D., National Cancer Institute, Bethesda, Maryland

The IFN- γ gene has been cloned from several species and shows a tremendous amount of evolutionary conservation in the 3' untranslated region (3'UTR), particularly in an adenine-uridine-rich element (ARE). Dr. Young's laboratory has generated a C57Bl/6 transgenic mouse strain in which the ARE has been replaced with random nucleotide sequences while other regulatory elements in IFN- γ are conserved. Mice expressing this replacement show a low but persistent expression of IFN- γ and higher levels of IFN- γ protein than those seen in wild-type mice. IFN- γ mRNA is increased in several organs, including the lymph nodes, kidney, spleen, and thymus. Persistent expression of the mutant IFN- γ results in induction of IFN- γ -responsive genes such as *CXCL10* and complement factor C3. ARE-deleted mice also show upregulation in genes that are specifically upregulated in patients with lupus. A sex effect has been observed: expression is altered for a larger number of genes in the female mouse than in the male mouse. This effect might be consistent with reports that the IFN- γ promoter contains an estrogen receptor response element.

Blood from ARE-deleted mice shows a significant increase in the number of neutrophils and a modest increase in the number of eosinophils and monocytes. These mice also have a skewed Ig repertoire: IgG1 and IgG3 levels are significantly lower, whereas IgG2A and IgM levels are higher. The Th1 response is stronger and the Th2 response is suppressed, as demonstrated by increase production of IFN- γ by the T cells and decreased IL-4 production. In addition, ARE-deleted mice produce antibodies against single- (ss) and double-stranded (ds) DNA at higher

²⁵ Thacker et al. J Immunol 2010.

²⁶ Zhao et al. J Immunol 2009.

²⁷ Denny et al. J Immunol 2010.

titers. ARE-deleted mice also appear to have membranoproliferative glomerulonephritis, as illustrated by complement and IgG deposition in the kidneys.

Dr. Young's laboratory has bred mice homozygous for the ARE deletion to assess the effects of chronic IFN- γ expression on host metabolic processes. Serum concentration of bile acids, an indicator of hepatic damage, is higher in patients with lupus, and bile acids serve not only as metabolic regulators, but also as immune modulators by inhibiting cytokine gene expression, monocyte and neutrophil chemotaxis, and calcium mobilization. Mice homozygous for the ARE deletion show extremely high levels of bile acid metabolism. However, these mice also exhibit significantly decreased levels of serum adenosine deaminase and cytidine deaminase, indicating an overall adenine deficiency, whereas patients with SLE show higher levels of these enzymes.²⁸ ARE-deleted mice also have higher levels of 3-nitrotyrosine, yet another marker in lupus.²⁹ Consistent with these results, indoleamine 2,3-dioxygenase expression is higher and tryptophan levels are lower, indicating chronic production of nitric oxide synthase. Tryptophan degradation has been observed in patients with SLE.³⁰

The ARE-deleted mouse thus represents a new-yet-old mouse model. Previous papers in the 1970s and 1980s noted higher circulatory levels of IFN- γ in patients with autoimmune disease³¹ and IFN- γ antibodies in the lupus-prone NZB/W mouse.³² In a subset of patients with SLE, symptoms might thus arise from higher circulating levels of IFN- γ . Consistent with this hypothesis, both IFN- α and IL-18 are involved in induction of IFN- γ . ARE-deleted mice show increased numbers of natural killer (NK) cells and CD4+ and CD8+ lymphocytes, as well as altered myeloid populations and more suppressive regulatory T cells (Treg).

Role of Viral RNA-Sensing Pathways in Autoimmune Disease

Silvia Bolland, Ph.D., National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

Fc γ receptor IIB (Fc γ RIIB), an inhibitory receptor for IgG, regulates antibody production and immune complex-induced inflammation. The Fc γ RIIB-knockout mouse presents a model of chronic, long-term disease, with increased serum anti-nucleic acid antibodies, vascular infiltration in the lung, and glomerular inflammation in the kidney. The Y chromosome from mice expressing Y-linked autoimmune accelerator (Yaa) exacerbates the pathology and changes in autoantibody specificity in this model. Antibody titer is slightly lower and different, and B-cell selection is specific to RNA and nucleolar antigens, rather than DNA. This exacerbation involves TLR7, which behaves synergistically with RNA-specific BCR signaling to drive autoreactivity. TLR7 recognizes viral single-stranded RNA in the endosome and induces an inflammatory response, and doubling the copy number of the TLR7 gene in Yaa mice promotes RNA-specific autoreactivity. These effects likely involve more than B-cell activation.

Dr. Bolland's laboratory has generated a transgenic mouse that carries multiple copies of *Tlr7* gene in the C57Bl/6 background. While duplication of the *Tlr7* gene exacerbates the pathology

²⁸ Taysi et al. Clin Chem Lab Med 2002.

²⁹ Khan et al. Scand J Immunol 2006.

³⁰ Widner et al. Adv Exp Med Biol 1997.

³¹ Hooks et al. N Engl J Med 1979.

³² Jacob et al. J Exp Med 1987.

seen in the Fc γ RIIB model, a *Tlr7* copy number of eight or more is lethal by itself. Increased dosage of TLR7 results in upregulation of proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), MCP-1, and IL-12p40; reduced platelet counts and hematocrit; and splenomegaly. Transgenic mice carrying multiple copies of *Tlr7* also exhibit spontaneous germinal centers, lymphocyte activation, myeloid expansion, and an increase in monocyte-like activated cells. The activated B and T cells and spontaneous germinal centers appear before and independently of the appearance of inflammatory cells and cytokines.

These results suggest that the entire immune system is activated in transgenic mice carrying multiple copies of *Tlr7*. Increased TLR7 drives autoreactivity via activated CD4⁺ T cells, germinal center reactions, and autoantibody production. However, TLR7 also appears to drive inflammation via IFN activation and myeloid expansion. These inflammatory responses are likely a consequence of chronic autoreactivity and perhaps an attempt by the immune system to shut down responses. Further work in Dr. Bolland's laboratory suggests that these responses also involve more than immune complex formation. B cells and NK1.1+CD11c⁺ cells appear to be the primary responders to TLR7 overexpression, and T-cell assistance is necessary for B-cell activation and the inflammatory pathology seen in these mice. All of these points suggest targets for intervention.

Thus far TLR7 appears to be the only TLR that exerts a gene-dosage effect on lupus susceptibility. This phenomenon might arise from the highly inducible expression of TLR7, its sensitivity to IFN, possible activation by viral RNA ligands that might be present spontaneously, and expression of TLR7 in APCs. To assess the effects of other inducible innate immunity pathways, Dr. Bolland's laboratory has generated a transgenic mouse strain that overexpresses MDA5. These mice show strong IFN signature expression, high serum levels of MCP-1 and memory lymphocyte features, but only mild splenomegaly and no inflammatory pathology. As is the case with TLR7 overexpression or spontaneous IFN induction, MDA5 overexpression can accelerate lupus disease. It does not trigger autoimmunity or autoreactivity, however, most likely because it does not stimulate T cells or induce germinal center reactions. This is similar to chronic type I IFN responses, which also do not stimulate autoreactivity.

Innate Immune Mechanisms in Experimental Lupus

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Injection of pristane into the peritoneum of BALB/c mice triggers an inflammatory response through the formation of granulomas and, later, plasmacytomas.³³ Although this model has been used primarily to study multiple myeloma, injection of pristane without hybridoma cells gives rise to lupus, as illustrated by a classic IFN signature after 1 to 2 weeks, a high titer of antinucleic acid antibodies after 2 to 3 months, and glomerulonephritis at later time points.³⁴ Pristane-induced granulomas appear to be ectopic lymphoid tissue, but they are not true granulomas, as demonstrated by immunohistochemistry.³⁵ They are organized into B-cell, T-cell, and dendritic cell zones that express several lymphocytic chemokines and are vascularized by

³³ Anderson and Potter. Nature 1969.

³⁴ Satoh and Reeves. J Exp Med 1994; Satoh et al. Proc Natl Acad Sci U S A 1995; Nacionales et al. Am J Pathol 2006; Nacionales et al. Arthritis Rheum 2007.

³⁵ Nacionales et al. Am J Pathol 2006.

endothelial venules. Pristane does not induce autoantibodies or lupus nephritis in mice deficient in the type I IFN receptor.

Humans appear to develop a similar hydrocarbon-induced immune response, as illustrated by the case of a patient taking mineral oil as a laxative.³⁶ This patient developed pneumonia and underwent a lung biopsy, which revealed lymphocytic infiltration and holes containing mineral oil. Thus hydrocarbons can induce a chronic inflammatory response leading to the formation of lipogranulomas in both human patients and mouse models. Several B-cell-mediated autoimmune diseases, including Hashimoto's thyroiditis, Sjögren's syndrome, and RA, as well as B-cell lymphomas, arise from de novo formation of organized, ectopic lymphoid tissue during chronic inflammation.³⁷ Thus, chronic inflammation appears to predispose individuals to B-cell lymphomas. Key pathological features of these diseases include lymphoid chemokine expression by stromal cells, development of high endothelial venules, and compartmentalization into T- and B-cell zones.

Pristane treatment results in the disappearance of B1 cells, the expansion of CD11+ cells, a large number of Ly6G-positive neutrophils and Ly6C-high monocytes, and a smaller number of mature monocytes.³⁸ The presence of Ly6C-high monocytes appears to be chronic and a major source of IFN- α and IFN- β production, and these monocytes are similar in appearance to monocytes isolated from mice deficient in the IFN receptor. They home to the peritoneum and fail to terminally differentiate in the presence of IFN-I. In wild-type and CCR2-deficient mice, Ly6C-high monocytes appear in the circulation, but no mature or Ly6C-high monocytes appear in the peritoneum. However, the introduction of liposomes loaded with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine,4-chlorobenzenesulfonate (DiD) results in the migration of DiD-positive cells to the peritoneum and, in the absence of IFN, rapid downregulation of DiD and monocyte maturation. Thus pristane treatment gives rise to a vicious cycle of inflammation. Ly6C-high monocytes are induced by IFN-inducible chemokines and go on to stimulate further IFN production, which blocks their maturation and further induces chemokine production.

TLR7 signaling appears to be central to the pathogenesis of lupus in the pristane-treated mouse model. Pristane-induced IFN production is abolished in TLR7-deficient mice, but not in TLR9-deficient mice. Likewise, IFN production is greatly enhanced, and the number of Ly6C-high monocytes doubled, in response to treatment in male Yaa mice, which carry two active copies of TLR7. Pristane injection also accelerates the disease course in male C57Bl/6 mice. Further work has shown that the IFN response to pristane depends on MyD88 and the TRIF, IPS-1 and TBK-1 pathways of IFN production.³⁹ There also appears to be a correlation between Ly6C-high monocytes and accumulation and the production of anti-Sm/RNP antibodies.⁴⁰

Both B cells and T cells proliferate in pristane-induced lipogranulomas, as demonstrated by immunization studies with OVA-specific T cells.⁴¹ Likewise, studies involving lipogranuloma transplantation from pristane-treated mice that produce antibodies against the U1A small nuclear

³⁶ Berg and Saenger. *N Engl J Med* 1998; Reeves et al. *Trends Immunol* 2009.

³⁷ Kratz et al. *J Exp med* 1996.

³⁸ Lee et al. *Am J Pathol* 2009; Lee et al. *J. Immunol* 2008.

³⁹ Lee et al. *J Exp. Med* 2008.

⁴⁰ Lee et al. *J Immunol* 2008.

⁴¹ Weinstein et al. *J Immunol* 2008.

RNP demonstrate that antibodies in recipient mice derive from donor plasma cells. However, activation of B and T cells in lipogranulomas is lost, and B cells and plasma cells in the transferred lipogranuloma are replenished by recipient cells. Moreover, lipopolysaccharide treatment in pristane-treated mice results in the activation of B cells and the secretion of anti-U1A antibodies in the bone marrow and spleen, but not in the lipogranulomas, suggesting a possible role for memory B cells in the pristane-induced inflammatory response.

On the basis of this work, pristane appears to deliver a danger signal that induces chronic TLR7 activation, unremitting IFN- α/β production, lymphoid neogenesis, and germinal center-like reactions. Pristane treatment also results in antibody-producing plasma cells and autoreactive memory cells. The role of autoreactive memory B cells is not yet clear. Nor is it clear why depletion or inactivation of autoregulatory B cells is defective in the setting of chronic inflammation or lymphoid neogenesis.

SESSION 2: AUTOANTIBODIES AND LYMPHOCYTE ACTIVATION

Understanding B Cells in Human SLE: Complex System and Reductionist Approaches

Iñaki E. Sanz, M.D., University of Rochester, Rochester, New York

B cells play multiple opposing functions, and the balance among these functions appears to be critical to disease characterization, outcomes, and response to treatment. As demonstrated primarily by mouse studies, B cells have a dual nature, promoting either autoimmunity or pathogenicity.⁴² Despite the conventional wisdom that B-cell deletion does not occur in SLE, treatments that promote a balance toward regulatory or otherwise favorable B cells might lead to positive outcomes. The conventional lupus profile involves increased switched memory, increased numbers of effective B cells, and the presence of unswitched cells in the peripheral blood. A dominance of naïve cells is present in patients who respond well to rituximab, but a higher number of CD38+ and CD24+ cells is present in patients who fail this treatment. Thus disease profiles differ across patients, and B-cell profiling might provide insight into disease complexity and aid in diagnosis, prognosis, clinical study design, treatment choice, and outcome measurement. Such profiling might also enhance the value of isolated analyses in predetermined populations.

Although the conventional phenotypic profile classifies B cells into naïve, unswitched, or switched memory cells, the reality is more complex, and current approaches have failed to address this complexity. In addition, B-cell analysis is so subjective, operator dependent, and labor intensive that it is prohibitive and unreproducible. Thus analytical, automated tools are needed. Dr. Sanz's laboratory has begun to employ tools such as a 13-color flow cytometry panel, which has revealed more than 1,000 possible B-cell subsets. The laboratory has also collaborated with others to develop a multidimensional, automated tool, FLOCK, which offers complexity, quantitation, and reproducibility (Qian et al., manuscript in press). Complex systems analysis is under way to analyze and visualize FLOCK data, and Dr. Sanz's collaborators aim to identify natural divisions within the data. In one example, a multicolor analysis of B-cell samples, combined with an examination of cell number or frequency, from a cross-section of

⁴² Manjarrez-Orduño et al. *J Invest Dermatol* 2009; Sanz and Lee. *Nat Rev Rheumatol* 2010.

lupus patients has aided investigators in identifying three classifications of patients:⁴³ one distinguished by the presence of increased T1, T2, and naïve B cells; another characterized by significant increases in T3 cells and decreases in T1, T2, and naïve cells; and a third distinguished by an increase in memory cells as demonstrated by 9G4 staining. However, it should be noted that some cells that appear to be 9G4 positive are merely absorbing 9G4, not expressing it.

A reductionist approach can be used to decrease the complexity of multidimensional data to focus on cells of interest. In this way, investigators have discovered that the number of IgD/CD27 double-negative effector cells correlates with the intensity of lupus flares.⁴⁴ These cells predominantly express markers of activation and trafficking to systemic tissues. An examination of CD21+ versus CD21- cells has yielded evidence that SLE might involve a defect in inhibiting or exhausting activated B cells. Other analyses have yielded evidence that effector B cells, but not memory B cells, depend on B-cell activating factor belonging to the tumor necrosis factor family (BAFF) for survival, suggesting different regulatory pathways for the two cell populations.⁴⁵ In addition, the doubling time for effector cells is longer, compared with that for memory cells.

This work demonstrates a larger degree of B-cell heterogeneity than previously recognized. Multiparameter flow cytometry can be used to identify discrete subsets and functional correlates, and automated, multidimensional analysis can be used for reproducible quantitation and adjudication of subsets. These approaches in B-cell profiling might be effective in other diseases, conditions, and outcomes. For example, recent work has shown the utility of B-cell immunoprofiling in differentiating outcomes of renal transplantation.

Environmental Influences and Early Events in Lupus Autoimmunity

Judith A. James, M.D., Ph.D., Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

Some individuals produce autoantibodies but never develop the clinical manifestations of lupus, whereas others develop pathologic autoimmunity. What distinguishes one subset of patients from another is not clear, although environmental factors, such as vitamin D, viral infection, or IFN production, might be involved.⁴⁶ In collaboration with the Walter Reed Medical Center and National Naval Hospital, Dr. James' laboratory has assessed samples from a Department of Defense (DoD) serum repository and identified a cohort of 130 patients who developed lupus while on active duty and have serial samples available before diagnosis.⁴⁷ They have found that autoantibodies occur before disease onset in linked subsets and that some autoantibodies subsets occur closer to disease onset than other specificities. A screen of 13 common autospecificities found that the most common initial targets were 60kd Ro and nRNP A,⁴⁸ although patients developed other autoantibodies over time. More than half the patients developed antibodies targeting one or two specific autoantigens early in the course of SLE, but a small subset of patients developed antibodies to several lupus-specific antigens first detected in the same sample.

⁴³ Sanz and Lee. *Nat Rev Rheumatol* 2010.

⁴⁴ Wei et al. *J Immunol* 2007.

⁴⁵ Jacobi et al. *Arthritis Rheum* 2010.

⁴⁶ James and Harley. *Arthritis Rheum* 2010.

⁴⁷ Arbuckle et al. *N Engl J Med* 2003.

⁴⁸ Heinlen et al. *PLoS One* 2010.

Work is under way to further establish the temporal relationship, if any, between SLE-associated IFN activity and lupus onset. Dr. James' laboratory is also investigating temporal relationships between viral seroconversion and lupus. Epstein-Barr virus (EBV) seroconversion is strongly associated with lupus. Patients with SLE exhibit higher responses to EBV viral capsid antigen (VCA) than do healthy controls, and EBV-VCA titers tend to increase the closer individuals get to a diagnosis of lupus. The appearance of these EBNA-1 antibodies precedes the production of autoantibodies in the pre-clinical military cohort. A study in pediatric patients has revealed that lupus patients are also more likely to produce antibodies against EBNA-1.⁴⁹ In addition, the anti-EBNA-1 antibodies are specific to regions not normally targeted in healthy individuals. These data might explain how a virus that infects almost everyone causes lupus in only a small subset of infected individuals.

Studies in a cohort of individuals from lupus genetic studies and the Lupus Family Registry and Repository suggest that serum from SLE patients and female first-degree relatives is more likely to have antibodies against EBV and herpes simplex virus 2, and sera from select family members are enriched for antibodies against autoantigen- and EBV-derived peptides.⁵⁰ However, only some unaffected family members show anti-viral responses that cross-react with known lupus autoantigens. Studies are under way in unaffected relatives to determine whether those with select autoantibodies or epitope profiles are at highest risk for developing SLE, as well as to identify serologic biomarkers, genetic profiles, environmental factors, or collective algorithms that help identify family members at highest risk for transition to clinical lupus. Identifying those at highest risk might uncover mechanisms of disease pathogenesis and make prevention trials possible. Unfortunately, not all viral infections, including EBV, can be modeled in mice at this time.

Activation of Autoreactive B Cells

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Several systemic autoimmune disorders involve an immune dominance of DNA, RNA, and rheumatic factors rather than a breakdown of tolerance to antigens. Dr. Shlomchik's laboratory has studied this dominance using an AM14 cell line and a transgenic BCR "knockin" mouse model (Weigert laboratory 1991). AM14, a rheumatoid factor (RF) isolated from MRL/lpr mice, is specific for IgG2A but binds only to the "a" allotype. This specificity facilitates the creation of a unique mouse model that expresses or lacks the "a" autoantigen. Whereas B cells reside in a "clonally ignorant" state in normal mice, 1 percent of B cells in these transgenic mice exhibit an RF specificity conferred by the AM14 heavy chain. Thus B-cell activation can occur without cell transfer and be tracked by increases in cell frequency.

The offspring of a cross between this model and MRL/lpr mice show spontaneous activation of RF-specific B cells on the border of the T-cell zone. These activated cells do not reside in the germinal centers, but are extrafollicular. Further examination of these cells reveal expanded clones in the white pulp, each different from the others, with ongoing cell division and somatic mutations. The extrafollicular response is the predominant type of B-cell response in many murine lupus models and represents the first line of adaptive immune defense in some

⁴⁹ McClain et al. *Arthritis Rheum* 2006.

⁵⁰ Arbuckle et al. *Scand J Immunol* 1999; McClain et al. *Nature Med* 2005.

pathogenic infections. Thus the extrafollicular space is likely the first and major site for interactions between B cells and T cells, which could induce the breakdown of tolerance in the T-cell compartment, thus leading to clinical disease. However, the mechanisms governing this response are not clear.

To begin to identify factors that might control the extrafollicular B-cell response, Dr. Shlomchik's laboratory has employed a technique to stimulate an AM14 B-cell response de novo by producing protein immune complexes or by cross-linking immune complexes that contain chromatin. Injection of protein immune complexes into BALB/c mice results in a large AM14 germinal-center response, whereas injection of the chromatin immune complex results in an extrafollicular T-zone response in the red pulp, similar to that seen in aged MLR/lpr mice. The extrafollicular response is dampened in offspring from a cross of AM14-transgenic mice with TLR7- or TLR9-deficient mice. However, a cross between AM14-transgenic mice with mice deficient in both TLR7 and TLR9 or with mice deficient in MyD88 signaling yields offspring with no extrafollicular response at all. Thus TLRs contribute, and MyD88 signaling is essential for extrafollicular response.⁵¹ Likewise, the stimulation of AM14-specific B cells with ligands containing TLRs produces an exclusively extrafollicular response, suggesting that B-cell intrinsic MyD88 is required. It is likely then that with stimulation by protein immune complexes, the BCR escorts the immune complex into the cell to interact with TLR7 or 9, whereas antichromatin B cells specifically recognize free chromatin and become reactive.

Further studies in this system have yielded evidence that T cells are not required for an extrafollicular response but that they do optimize the quality of response and play a role in isotype switching and somatic mutations. Neither $\alpha\beta$ T cells, nor CD40L, nor the IL-21 receptor is required, although they might play a role in the ability of B cells to respond to specific cytokines. Likewise, the removal of CD4+ T-cell help in a spontaneous model reduces the extrafollicular response only twofold. These results are consistent with other studies showing autoimmunity independent of T cells.⁵²

On the basis of these studies, the extrafollicular response pathway appears to be a high-affinity response pathway that is TLR-dependent and T-cell independent, and loss of tolerance by this pathway might represent a primary event in lupus. Although this mechanism does not require a breakdown in T-cell tolerance, it likely causes it.

Basophils and the Th2 Environment in the Development of Lupus Nephritis

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Lyn is a kinase and a key player in IgE-mediated cellular responses.⁵³ A key component of several immune receptors,⁵⁴ Lyn promotes effector functions, regulates basophil and mast-cell function, and contributes to the maintenance of homeostasis. It is expressed in most

⁵¹ Shlomchik. *Immunity* 2008.

⁵² Groom et al. *J Exp Med* 2007; Tsao et al. *J Immunol* 2008; Mumtaz. Keystone Symposium 2009, Abstract 278; Berland et al. *Immunity* 2006; Hermiston. Conference on B Cells and Autoimmunity, presentation 2009.

⁵³ Charles et al. *Immunity* 2009; Odom et al. *J Exp Med* 2004; Parravicini et al. *Nature Immunol* 2002.

⁵⁴ Hibbs and Harder. *Growth Factors* 2006.

hematopoietic cells but not in T cells.⁵⁵ Recent evidence indicates that Lyn expression is decreased in B lymphocytes from some patients with SLE and that Lyn is associated primarily with autoantibody production in SLE. Mice deficient in Lyn⁵⁶ develop SLE-like disease later in life and show an altered peripheral B-cell population, B-cell hypersensitivity to IL-4 and CD40L, and immune complex deposition. These mice go on to die from lupus nephritis. Early in life, however, Lyn-deficient mice develop an allergic hypersensitivity and exhibit increased numbers of peritoneal mast cells, hyperreactive mast cells, increased levels of circulating histamine, and eosinophilia. Immune responses are skewed toward the Th2 response in the absence of Lyn, and this skewing is driven by IgE and by basophils, which produce large quantities of IL-4.

To further assess the relationship between allergy in early life and autoimmunity in late life in these mice, Dr. Rivera's laboratory has generated double-deficient strains of mice: one deficient in Lyn and the heavy chain of IgE, one deficient in Lyn and IL-4, and one deficient in Lyn and mast cells.⁵⁷ In the absence of IL-4 or IgE, Lyn-deficient mice show little glomerulonephritis, compared with mice deficient in Lyn alone or in both Lyn and mast cells. Indeed, the absence of IgE or IL-4 appears to rescue kidney function when Lyn is absent. These results are consistent with other studies in which offspring from a cross of IL-4-deficient mice with young MRL/lpr mice show a rescued kidney phenotype and dampened, abnormally elevated levels of IgE and IgG1 production. In addition, loss of IgE or IL-4 in the absence of Lyn also results in a marked decrease in autoantibody production, along with reduced levels of BAFF.

Basophils appear to be essential to the autoimmune phenotype seen in Lyn-deficient mice. Dr. Rivera's laboratory has adapted a protocol that employs monoclonal antibodies to the alpha chain of FcεRIα to determine the role of basophils in driving autoantibody production and the proinflammatory environment in the kidney. Depletion of basophils results in a marked reduction of autoantibody production and a reduction of both IL-4 and IFN-γ in the kidney. CD62L expression is upregulated, and the number of basophils in the secondary lymphoid organs of Lyn-deficient and Lyn/mast-cell-deficient mice is increased. In contrast, few basophils reside in the lymphoid organs of IgE and Lyn or IL-4 and Lyn double-deficient mice, and basophils from these mice exhibit a reduced ability to home to secondary lymphoid organs. Thus basophils home to the secondary lymphoid organs in lupus. They also express major histocompatibility complex (MHC) II genes and membrane-bound BAFF, and they support short-lived plasma cells in the spleen. Thus basophils drive and sustain autoantibody production through their ability to promote the production of short-term plasma cells.

Data from previous studies have suggested that some lupus patients produce autoreactive IgE antibodies. Consistent with these results, Lyn-deficient mice exhibit marked increases in the production of autoreactive IgE antibodies, but this increase is dampened in Lyn-deficient mice that are also deficient in IL-4 or IgE. Dr. Rivera's laboratory has also demonstrated that immune complexes containing IgE, but not IgG, can induce basophils to produce IL-4. In collaboration with other laboratories, they have also found that patients with the highest levels of disease activity show higher levels of anti-dsDNA IgE antibodies. The level of autoreactive IgE

⁵⁵ Flores-Borja et al. *Arthritis Rheum* 2005; Lu et al. *Genes Immunity* 2006.

⁵⁶ Odom et al. *J Exp Med* 2004; Parravicini et al. *Nature Immunol* 2002; Yu et al. *Curr Biol* 1998; Hibbs et al. *Cell* 1995; Nishizume et al. *Immunity* 1995,

⁵⁷ Charles et al. *Nature Med* 2010.

antibodies also correlates with active nephritis. In addition, basophils are present in the lymph nodes and spleens of patients with SLE, and basophils from these patients exhibit an activated phenotype, even in cases of inactive disease. However, the ability of basophils to home to secondary lymphoid organs is inhibited in patients with inactive SLE, compared with those with active disease.

These data support a model in which autoreactive plasma cells produce not only IgG, IgM, or IgA autoantibodies, but also IgE autoantibodies, which then activate basophils to home to secondary lymphoid organs and induce increases in circulating immune complexes, leading to kidney injury and disease. However, it is not clear whether the relationship between autoreactive IgE and autoantibody production is specific to the Lyn-deficient mouse model or can be reproduced in other models. The relationship between basophils and short-lived plasma cells is also unclear.

SESSION 3: NEW ADVANCES AND CLINICAL CHALLENGES IN THE TREATMENT OF SLE

Targeting the Innate and Adaptive Immune System for the Treatment of Lupus

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IFNs have been established as major players in the underlying pathogenesis of lupus. TLRs 7, 8, and 9 are all upregulated in skin biopsies from patients with cutaneous lupus, and peripheral blood from these patients shows an elevated type I IFN signature. Polymorphisms in the type I IFN pathway, elevated serum IFN- α levels in patients, the appearance of lupuslike symptoms in patients undergoing IFN treatment, increased IFN expression of type I IFN-induced genes in the blood and involved tissues in SLE, and correlations between the type I IFN signature and disease activity all support a key role for type I IFNs in lupus.⁵⁸ Thus MedImmune is exploring ways to interfere with the type I IFN pathway.

On such approach involves sifalimumab is a fully human IgG1 monoclonal antibody with broad selectivity for several IFN- α subtypes but no selectivity for other type I IFNs and no detectable effector function. Results from phase I clinical trials suggest that sifalimumab inhibits the type I IFN signature, both in blood and in skin, as demonstrated by the downregulation of TLRs 7 and 9 and UNC93, which is involved in the translocation of TLRs from the endoplasmic reticulum to the endosome. Sifalimumab also appears to reduce the incidence of flares. MedImmune also has developed a monoclonal antibody against ILT7, an IFN-regulatory receptor expressed exclusively on pDCs. Results from phase I trials demonstrate that administration of this antibody can be used to specifically deplete pDCs and downregulate production of IFN- α .

MedImmune is also targeting other pathways involved in lupus, for example by modifying T helper cells using antibodies against inducible co-stimulator (ICOS). ICOS is a member of the CD28 superfamily that binds a unique B7 family member ligand, B7RP-1; is expressed on recently activated T cells and on follicular T helper cells; and provides a signal for B-cell maturation, isotype switching, and differentiation of germinal centers and plasma cells. The

⁵⁸ Hooks et al. *N Engl J Med* 1979; Ioannou and Isenberg. *Arthritis Rheum* 2000; Crow. *Arthritis Rheum* 2003; Dall'era et al. *Ann Rheum Dis* 2005.

antibody generated by MedImmune has been engineered to increase its affinity for Fc γ RIIIA by tenfold. In nonhuman primates, this antibody depletes ICOS+CD4+ T cells from the periphery and secondary lymphoid organs in a reversible and dose-dependent manner. Four weeks of anti-ICOS antibody administration results in reduced expression of markers associated with splenic and follicular T cells and in the dissolution of established germinal center B cells.

An Anti-B-Lymphocyte Stimulator Therapy From Bench to Bedside

Douglas Hough, M.D., Human Genome Sciences, Rockville, Maryland

B-lymphocyte stimulator (BLyS), a member of the TNF superfamily, is a type II membrane-bound protein that can be expressed by monocytes, macrophages, neutrophils, and dendritic cells.⁵⁹ Upon activation by the presence of antigen, monocytes express membrane-bound BLyS, which is cleaved to form an active, soluble homotrimer that binds three receptors, primarily BR3, to activate B cells and induce them to proliferate and differentiate (Human Genome Sciences).⁶⁰ BLyS-transgenic MRL/lpr or NZB/W mice develop autoimmune disease, and BLyS antagonism by soluble receptors slows disease in these models.⁶¹ In addition, patients with autoimmune diseases show elevated BLyS levels, and these levels correlate with disease activity.⁶² On the basis of these studies and the role BLyS plays in B-cell development, BLyS is a promising target for lupus therapies.

Belilumab is a fully human IgG1 monoclonal antibody that specifically targets soluble BLyS.⁶³ A single dose of belilumab inhibits splenocyte proliferation in mice.⁶⁴ Belilumab has shown promise in a phase I trial in SLE patients with stable disease for 2 months or longer. Although a phase II trial in patients with active SLE and a history of autoantibodies did not meet its primary endpoint, seropositive patients did show a statistically significant reduction in disease activity and a decrease in autoantibody levels over time in response to belilumab treatment.⁶⁵ Two large phase III trials, one based in the United States and Western Europe and the other based in Latin America and Asia/Pacific, have been designed based on lessons learned from the phase II trial. Both of these studies have met their primary endpoints, and data suggest that belilumab is efficacious, as illustrated by declines in anti-DNA antibodies, naïve B cells, and plasma cells over time. Results will be presented at the American Association for Cancer Research conference.

Role of TLR Recognition of Self Nucleic Acids in Inflammation

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Upon activation, immature pDCs produce large amounts of IFN with low antigen presentation. If these cells are activated through TLR7 and TLR9, however, they mature with low IFN- α

⁵⁹ Oren et al. *Nature Struct Biol* 2002.

⁶⁰ Cancro et al. *J Clin Invest* 2009.

⁶¹ Moore et al. *Science* 1999; Schneider et al. *J Exp Med* 1999; Gross et al. *Nature* 2000; Batten et al. *J Exp Med* 2000; Groom et al. *J Clin Invest* 2002; Cheema et al. *Arthritis Rheum* 2001; Mackay et al. *J Exp Med* 1999; Ramanujam et al. *J Clin Invest* 2006; Khare et al. *Proc Natl Acad Sci U S A* 2000.

⁶² Petri et al. *Arthritis Rheum* 2008.

⁶³ Do et al. *J Exp Med* 2000; Ammana et al. 2003.

⁶⁴ Baker et al. *Arthritis Rheum* 2003.

⁶⁵ Wallace et al. *Arthritis Rheum* 2009.

production and high antigen presentation.⁶⁶ CpG-C activation of pDCs through TLR9 induces two signaling pathways, one driven by IRF-7 and the other driven by NF- κ B,⁶⁷ but the response to TLR9 activation by other CpGs is compartmentalized, with CpG-A stimulating immature pDC to produce IFN- α and promote an innate response, and CpG-B stimulating pDC maturation and an adaptive response.⁶⁸ In addition, injection of TLR9 into mice induces a response that produces IL-6, IL-12, and TNF- α , with little IFN, whereas injection of CpG into humans stimulates the production of IFN. Thus the two TLR9-dependent pathways are specialized. What governs that specialization is not clear.

Unwanted stimulation of TLR7 and TLR9 has been observed in a wide array of autoimmune diseases, as well as in some acute infections, liver fibrosis, and acute liver injury. pDCs are the source of elevated IFN- α in patients with lupus, and they appear to act in a feedback loop with B cells, which also express TLR7 and TLR9. These receptors thus represent additional therapeutic targets in lupus. Indeed, immunoregulatory sequences (IRS), highly stable oligonucleotides⁶⁹ specific to TLR7 and TLR9, inhibit both receptors equally, and they increase survival and reduce autoantibodies, proteinuria, and glomerulonephritis in NZB/W mice.⁷⁰

Patients with lupus often receive large doses of glucocorticoids, but this treatment does not prevent flares, and aggressive, high-dose glucocorticoid pulse therapy only induces a transient reduction in the type I IFN signature and the number of pDCs.⁷¹ On the basis of in vitro data, activation of pDCs through TLR7 and TLR9 changes their sensitivity to glucocorticoids, and glucocorticoids thus fail to inhibit IFN- α production by these cells. Likewise, activation of TLR7 or TLR9 by self nucleic acids induces lupuslike symptoms in lupus-prone mice, and cells bearing these receptors are more resistant to glucocorticoid-induced cell death. Yet this resistance to glucocorticoids can be abrogated by pretreatment of pDCs with IRS. Blocking TLR7 and TLR9 therefore shows promise for corticosteroid-sparing therapy.

The roles of TLR7 and TLR9, as well as pDCs, have been explored in autoimmune skin diseases. Patients with cutaneous lupus show a strong IFN- α signature in and recruitment of pDCs to the skin, as well as immune complex deposition at the junction between the epidermis and dermis and skin lesions arising from chemokine production and cytotoxic lymphocyte recruitment induced by dysregulated IFN- α production.⁷² In addition, infiltration by activated neutrophils likely perpetuates inflammation by promoting tissue destruction. Studies in a tape-stripping model in normal and lupus-prone mice have yielded evidence of pDC and neutrophil activation, their contribution to increased expression of proinflammatory genes and sustained inflammation, and an effect of IRS on the inflammatory gene signature, but not on cellular infiltration, in the skin (Guiducci et al., manuscript submitted). Other work has demonstrated that acetaminophen hepatotoxicity depends on TLR9 and can be reduced by IRS treatment. Thus work with IRS not only support TLR7 and TLR9 as potential therapeutic targets; it also supports a model in which

⁶⁶ Colonna et al. *Nature Immunol* 2007; Duramad et al. *Blood* 2003.

⁶⁷ Guiducci et al. *J Exp Med* 2008.

⁶⁸ Guiducci et al. *J Exp Med* 2006, 2008.

⁶⁹ Latz et al. *Nature Immunol* 2007.

⁷⁰ Barrat et al. *Eur J Immunol* 2007.

⁷¹ Guiducci et al. *Nature* 2010.

⁷² Wenzel and Tüting. *J Invest Dermatol* 2008.

mammalian and microbial nucleic acids are potent ligands for these receptors, but only when efficient delivery to endosomes takes place.

Can We Predict Responses to Therapeutics for SLE Using Murine Models?

Anne Davidson, M.D., Feinstein Medical Research Institute, Manhasset, New York

The use of diverse mouse models is needed to gain a better understanding of lupus and possible therapeutic approaches. Dr. Davidson’s laboratory has studied two potential therapies using three lupus-prone mouse models—NZB/W, NZW/BXSB, and NZM2410—that differ in immunology (see table) and target organs. The laboratory has also investigated these potential therapies, both alone and in combination, at three different disease stages. By using multiple models and looking at various time points, Dr. Davidson’s laboratory aims to determine the stage at which each drug is most effective, whether that drug is effective in several models, and whether the mechanisms of action are the same across models.

Mouse Model	Antibodies	Antibody Subtype	Age at Onset of Proteinuria (weeks)	Type of Nephritis	Germinal Centers	Plasma Cells
NZB/W	DNA	IgG2A	37	Proliferative	Spontaneous	Short- and long-lived
NZB/W + IFN	DNA	IgG2A and IgG3	16	Crescents	Large, induced	Short-lived, appear primarily in the spleen
NZW/BXSB	SmRNP, cardiolipin	IgG2A	18	Proliferative	Disappear with age	Short- and long-lived
NZM2410	Chromatin	IgG1, IgE	22	Sclerotic	Spontaneous	Short- and long-lived, appear primarily in the spleen

CTLA4-Ig, a competitive antagonist of CD28/B7 interactions, prevents lupus by preventing anti-DNA antibody production, somatic mutations, and class switching.⁷³ However, the window of opportunity for this potential therapy appears to be short.⁷⁴ Administration of CTLA4-Ig antibodies to NZW/BXSB mice at 9 weeks improves survival. However, this improvement is lessened with administration at 10 weeks, when these mice show evidence of proteinuria, and

⁷³ Mihara et al. J Clin Invest 2000.

⁷⁴ Akkerman et al. Autoimmunity 2004.

survival is even further reduced with fusion protein administration at 12 weeks, when the mice have already developed autoantibodies. Treatment with CTLA4-Ig at 9 weeks also prevents T-cell activation at later time points, as shown by reduced percentages of naïve and memory cells, and treatment between 9 and 12 weeks reduces the percentage of cardiolipin-positive hybridomas. Thus, in NZW/BXSB mice, CTLA4-Ig must be administered before 12 weeks to prevent disease onset. CTLA4-Ig therapy has been studied further in an IFN- α -induced NZB/W model, in which administration of adenovirus at 12 weeks accelerates the lupus phenotype (Liu et al, manuscript in press). Dr. Davidson's laboratory has also used this model to study the effect of TACI-Ig, an inhibitor of the TNF- α superfamily members BAFF and APRIL (Liu et al, manuscript in preparation). IFN confers relative resistance to both CTLA4-Ig and TACI-Ig in NZB/W mice, suggesting that one should take the IFN signature into account when designing and interpreting the results of clinical trials.

Dr. Davidson's laboratory has also begun remission-induction studies to identify pathways that are activated in inflamed kidneys, to identify potentially pathogenic molecules that are re-expressed during impending relapse, and to identify targets that are shared by the three mouse models and humans and can further be modeled in mice. They have found that response to remission reduction depends on strain and context.⁷⁵ For example, antibodies against BAFF efficiently induce remission in NZM2410 mice, in which little inflammation occurs compared with NZB/W or NZW/BXSB mice. These results emphasize the importance of strain differences and the need to subtype patients with SLE. Remission-induction studies also have identified a correlation between interstitial macrophage infiltration in the nephritic kidney and poor prognosis,⁷⁶ as well as a correlation between the onset of proteinuria and changes in gene expression in the endothelial and macrophage/dendritic cell activation pathways.⁷⁷ Further study of activated renal macrophages and alterations in gene expression are under way in the three mouse models and humans.

SESSION 4: GENETIC ANALYSIS OF LUPUS DISEASE IN MOUSE AND HUMAN

Lupus Genetics: An Emerging Matrix of Complex Risk

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Before 2006, genetic analyses of lupus relied on linkage and candidate gene studies based on biological queries. More than 150 gene associations were reported, but of these, only seven are now reproduced and considered established, and the associations are small. Because lupus is a complex disease, genetic association studies aim for an odds ratio of 1.2, and power calculations for this ratio, which must account for additive or multiplicative mechanisms of inheritance, indicate that study samples in the tens of thousands are needed to yield significant results.⁷⁸ Recessive mechanisms of inheritance are particularly problematic. Large case-control studies have no power to detect or replicate recessive mechanisms of disease. The discovery and replication of these mechanisms is highly unlikely, leaving a large area of lupus genetics

⁷⁵ Ramanujam et al.. Arthritis Rheum 2010.

⁷⁶ Hill et al. Kidney Int 2001.

⁷⁷ Schiffer et al. J Immunol 2008.

⁷⁸ Harley. Nature Genetics 2007.

unapproachable by case-control studies. The advent of high-throughput technologies has increased genotyping capacity in an extraordinary way, from less than 300 genotypes a day before 2006 to more than 100 million genotypes a day in 2010. These technologies have thus increased the potential for identifying case controls and modes of inheritance and for describing the genetics of SLE.

Because of the large amount of data generated by new technologies, the data must be organized in a meaningful way. Physical mapping allows investigators to look for potential contributing genes and then further explore the biology to identify mechanisms, targets, and potential preventive strategies. However, these approaches only yield candidate pieces of DNA. Further study is required to determine what in that piece is causative, and the assignment of a gene or polymorphism sometimes requires some amount of guesswork and confidence in the assignment of causation. The likelihood that an assignment is correct increases with the ability to narrow physical mapping to a single polymorphism or very small haplotype. In addition, investigators must be careful in their selection of cases and controls, as intrinsic differences between the two populations might yield differences that are irrelevant to the lupus phenotype. Large collections of well-characterized cases and controls, with nucleic acid samples, cell specimens, and genetic information, are needed for future genetic studies.

In one example of physical mapping, the International Consortium for SLE Genetics has conducted a genome scan of a piece of the human leukocyte antigen (HLA) region in Northern European women, found a significant peak at *MSH5*, and generated data suggesting multiple effects arising from the HLA region.⁷⁹ These studies have been followed by several other genome scans in European and Asian patients that have identified more than 35 significant associations, representing genes involved in immune complex processing, IFN pathways, and TLR signaling.

In another example, Lee and Nath have conducted a meta-analysis⁸⁰ of all lupus linkage studies done before 2005. They have further studied chromosomal region 16p13-q13, genotyped 10 candidate genes, and identified a powerful association between a polymorphism in *ITGAM* (*CD11b*) and lupus in European Americans. The association between lupus and this polymorphism, which changes Arginine 77 to Histidine, was first established in African-American patients, with the Gullah being particularly helpful. This is an example of trans-ancestral mapping providing causal allele identification, because the haplotype block in this region of the European genome involves a large haplotype (more than 50kb), from which the causal allele cannot be identified. *ITGAM* has adhesion properties and more than 50 ligands, and it binds complement. Thus, these studies yield evidence of a link between cell adhesion and the complement system in lupus pathogenesis. In yet another example, Sawalha and colleagues have found an association between *MECP2* and lupus.⁸¹ Further studies employing a candidate gene approach have shown that the *IRAK1* gene, which is involved in TLR signaling,⁸² resides in the same region as *MECP2*. A large study is under way to distinguish which gene is more closely associated with lupus, leaving open the possibility that both genes, which happen to be neighbors, are important in lupus genetic risk.

⁷⁹ International Consortium for Systemic Lupus Erythematosus Genetics (SLEGEN). *Nature Genetics* 2008.

⁸⁰ Lee and Nath. *Human Genetics* 2005.

⁸¹ Sawalha et al. *Autoimmunity* 2008.

⁸² Zhang et al. *Immunol Rev* 2007.

In a recent study a sentinel piece of DNA was moved to a mouse model then deleted to show an association between a noncoding interval in region 9p21 and risk for coronary artery disease.⁸³ This type of model might be useful in further exploring the biology of lupus. Studies⁸⁴ demonstrating multiple effects of single nucleotide polymorphisms (SNPs) in IRF5, which is expressed by pDCs and B cells, mediates TLR7 signaling, and induces type I IFN production, might also provide important clues.

Dr. Harley's group has also investigated relationships between SLE and Klinefelter's syndrome, in which phenotypical males have an XXY genotype, to help reveal the basis of the sex bias in lupus. The incidence of SLE among men with Klinefelter syndrome is nearly identical to that among women. Although hormone and physiologic differences between women and Klinefelter syndrome patients do not affect SLE risk, studies suggest that two X chromosomes confers higher risk than one X chromosome, and that the Y chromosome exerts no effect.

Lessons Learned From Genetic Knockouts in Lupus-Prone Mice

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IFN-regulatory factors (IRFs) are critical for type I IFN production, TLR signaling, and T-cell differentiation. IRF4, a transcription factor expressed in T cells, B cells, macrophages, and dendritic cells,⁸⁵ is involved in Th2 and Th17 differentiation⁸⁶ and necessary for B-cell development and function.⁸⁷ Although IRF4 appears to play a role in regulatory and follicular T-cell development, its specific role is not yet clear. A knockout mouse model deficient in IRF4 lacks Th17 cells and fails to develop experimental autoimmune encephalomyelitis (EAE). The incidence of inflammatory bowel disease is reduced in the IRF-knockout mice, and IRF4 expression is increased in the colons of patients with ulcerative colitis and Crohn's disease.

On the basis of studies of mice deficient in complement factor B, a potential therapeutic was developed to block the complement factor B pathways for lupus and many other diseases. However, complement factor B is part of the MHC. Dr. Gilkeson's laboratory has backcrossed complement factor B-deficient mice with MRL/lpr mice and found that H2b MRL/lpr mice are deficient in IgG3. This deficiency results from low expression of MSH5, which plays a role in class switching and perhaps IgA deficiency.⁸⁸ Further breeding of these mice has yielded an MRL/lpr H2b/k mouse, which produces no IgG. Genetic mapping reveals that this deficiency results from a spontaneous deletion of the IRF4 gene on chromosome 13.

Dr. Gilkeson's laboratory has backcrossed IRF4-deficient mice with MRL/lpr H2b/k or C57Bl/6 mice for nine generations and found decreases in epithelial cell activity, vasculitis, and interstitial inflammation in IRF4-deficient MRL/lpr mice. However, these mice still experience mesangial proliferative renal disease, with a hypercellularity identical to that seen in wild-type MRL/lpr mice, and they undergo back skin erosion and ear necrosis similar to that seen in wild-

⁸³ Visel et al. Nature 2010.

⁸⁴ Graham et al. Proc Natl Acad Sci U S A 2007.

⁸⁵ Lohoff and Mak. Nature Rev Immunol 2005; Tamura et al. J Immunol 2005.

⁸⁶ Mudter et al. J Clin Invest 2008.

⁸⁷ Mittrucker et al. Science 1997.

⁸⁸ Watanabe et al. J Immunol 2000; Sekine et al. J Immunol 2006; Sekine et al. Proc Natl Acad Sci U S A 2008; Sekine et al. J Immunol 2009.

type or IRF1-deficient MRL/lpr mice. These mice also show an absence of CD4+ T cells that produce IL-17 and an increase in the number of CD4+ T cells producing IFN- γ or IL-4. Thus IRF4 is required for the full manifestation of lupuslike renal disease in MRL/lpr mice, most likely by maintaining and modulating autoreactive B cells, and the pathogenesis underlying this and skin disease appears to be independent of autoantibody or Th17 mechanisms. It is likely then that the renal and skin disease seen in MRL/lpr mice is mediated by Th1 and Th2 cells.

Autoimmunity as a Precursor to Lymphoma

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Despite the rarity and heterogeneity of lymphomas and autoimmune diseases, several population-based studies have shown an increased risk for lymphomas among patients with these diseases. Patients with SLE, Sjögren's syndrome, and highly active RA are at especially increased risk, with Sjögren's patients being at highest risk for salivary gland mucosa-associated lymphoid tissue (MALT) lymphomas. Autoimmunity contributes to the development of these malignancies through chronic antigenic stimulation, immunodeficiencies, local and systemic inflammation, and genetic mutations.

The link between autoimmunity and lymphomas has also been observed in mice, where autoimmune strains develop specific B-cell lymphomas as determined by flow cytometry or histological studies. Disease in these mice begins in the peritoneum, then spreads to the spleen and becomes leukemic in later life. BALB/c and C3H strains homozygous for *Fas* or *Fas* ligand mutations show autoreactivity and class switching and go on to develop plasmacytoid/anaplastic lymphomas. Likewise, SJL/J mice develop anti-nucleic acid antibodies at about 3 months of age, are susceptible to EAE, and show a decrease in immune competence and an increase in the number of regulatory T cells with age, and they go on to develop diffuse large B-cell lymphomas. In this mouse strain, lymphoma development depends on CD4+ T cells. Similar to MRL/lpr mice, SJL/J mice homozygous for the *Fas*^{lpr} mutation show marked proliferation of B220+D4-CD8- T cells. However, unlike MRL/lpr mice, SJL/J.lpr mice exhibit minimal renal disease and vasculitis, have a shortened life span, and die of pulmonary insufficiency or heart failure. SJL/J mice heterozygous for the *Fas*^{lpr} mutation also show accelerated mortality and a lymphoma incidence of 100 percent.

In another mouse model, BALB/c or C3H mice homozygous for *Fas*^{gld} develop B-cell tumors with clonal rearrangements of IgH at 9 to 15 months. These tumors are plasmacytoid and preceded by expansions of B220+CD23-CD21- B cells that are class-switched and hypermutated and secrete polyactive, rheumatoid factor-positive, anti-DNA antibodies. The tumors display histologic features similar to plasmacytic phenotypes seen in other mouse strains, and they carry gene expression profiles similar to that seen with memory B cells. Although no mutations appear in *Myc*, these tumors carry duplications of a chromosomal region containing *Myc*, indicating a role for *Myc* in their development.

In yet another mouse model, lymphoproliferation occurs in 40 percent of mice carrying an engineered point mutation in the non-muscle myosin heavy chain II-C. These mice also show an accumulation of *Fas*^{lpr}-like B220+CD4-CD8- T cells, elevated blood urea nitrogen, and early glomerular disease, and they develop clonal, transplantable lymphomas. Lymphoproliferation

also occurs in 40 percent of a second strain of mice carrying this mutation, but this strain does not show the same abnormalities. Instead, these mice exhibit lymph node enlargement, splenomegaly, cellular infiltration, and a destruction of the normal splenic architecture. Similar to *lpr* or *gld* mice, these mice show expansions of CD3+B220+ T cells in the lymph node, spleen, and thymus. Unlike T cells from unaffected mice, however, those from affected mice do not undergo apoptosis. Yet the levels of *Fas* expression are similar between affected and unaffected mice, suggesting a defect in the transport of *Fas* from the endoplasmic reticulum to the cell surface. The mechanism is not yet understood.

Interleukin 21 and Lupuslike Autoimmunity

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IL-21 is produced primarily by activated T cells, particularly follicular T cells. It activates the Jak/Stat signaling pathways, increases the cytotoxic activity of the CD8/NK axis, and exerts potent effects on osteoclasts and immature monocytes and dendritic cells. Genetic associations between lupus and IL-21 and its receptor have also been found in human autoimmune diseases, but these associations are relatively weak. Yet on the basis of studies in animal models, IL-21 signaling appears to play a critical role in lupus.

In the T-cell-dependent antibody response, naïve T cells respond to dendritic cell stimuli to become activated CD4+ T cells, which go on to become peripheral effector cells or follicular cells. This choice between effector and follicular cell determines which cytokines are secreted. In lupus, activated T cells undergo upregulation of Bcl6 and CXCR5 and interact with the B-cell follicle, promoting germinal center development, plasmablast production, and IgG production. Activated CD4+ T cells produce IL-21, but although some evidence suggest that IL-21 acts on these cells to reinforce signals, IL-21 appears to exert a more pronounced effect on B cells.

The BXS^B.Yaa mouse model⁸⁹ develops severe but episodic SLE-like disease, with lymphoid hyperplasia, anemia, hyper IgG, antinuclear antibodies, and monocytosis, and these mice often die from immune complex-mediated nephritis with nephrotic syndrome. Only male mice are affected. Autoimmunity arises from a duplication of TLR7; B cells are hyperresponsive to BCR and other stimuli and spontaneously secrete IgM, and dendritic cells appear to be dysregulated. BXS^B.Yaa mice go on to develop substantial germinal center and extrafollicular plasmablastic responses. Studies in this model demonstrate a requirement for direct IL-21 receptor signaling on the B cells, but not the T cells, for lupuslike disease to occur.⁹⁰ Indeed, IL-21 receptor signaling on T cells might even reduce the degree of autoimmune disease. IL-21 receptor expression by B cells is elevated before any evidence of autoimmune disease appears, and this elevation depends on both the TLR7 duplication and the presence of T cells. These results suggest that IL-21 serves as an early signal in lupus pathogenesis, and it is likely that TLR7 induces CD4+ T cells to produce this early signal, which then upregulates IL-21 receptor expression on B cells and dendritic cells. Further studies suggest a role for the CD8+/natural killer axis in preventing even more aggressive disease.

⁸⁹ Roths and Murphy, c 1975.

⁹⁰ Bubier et al. Proc Natl Acad Sci U S A 2009.

CLOSING LECTURE: THE FUTURE OF LUPUS RESEARCH AND THERAPY

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Mouse models and human studies have yielded several lines of evidence regarding the fundamental pathophysiology of lupus and provided some clues on potential therapeutic targets. Yet more work is needed. It is likely that lupus, as well as other autoimmune diseases, comprises several distinct disease or patient subsets, and thus one therapy is unlikely to work for all patients with lupus. The identification of biomarkers that correlate with these distinct subsets would facilitate the development of targeted therapies.

The construction of biorepositories and databases that include target tissue, gene markers, blood cells, serum, plasma, clinical test results, images, and other clinical data has been helpful in this regard. For example, the NIAMS-supported Autoimmune Biomarkers Collaborative Network (ABCON) contains samples drawn from 300 lupus patients over the course of 1 year. Patients are now undergoing longitudinal follow-up, and more samples are added to the repository on an ongoing basis. ABCON has banked RNA, serum, PBMC, urine, and DNA samples, which are available for collaborative studies.

With the development of new technologies and new hypotheses, investigators can now look at their results in the context of broader datasets. For example, a sensitive assay has allowed investigators to find significantly increased levels of BAFF expression in IFN signature-positive individuals, compared with signature-negative individuals, consistent with the higher levels of BAFF seen in lupus patients than in healthy controls. A luciferase-based IFN reporter assay has shown, however, that IFN activity is elevated even in signature-negative individuals. Further study of a subset of ABCON participants has found large differences, between patients with active disease and those with inactive disease, in the expression of IFN-regulated chemokines at baseline,⁹¹ and the risk for disease flares over the next year can be stratified by level of chemokine expression.⁹² Thus, ABCON data have facilitated a better understanding of what the IFN signature might mean and suggest a drug trial design in which the clinical population is enriched for patients most likely to experience a disease event. More work is needed to confirm and expand these results across several sites.

SLE genetics has benefited both from careful studies of candidate genes and from unbiased genomic screens, and more than 30 genes have been confirmed as SLE genes⁹³ Studies in mouse models are now receiving confirmation in human studies, and many of the genes identified have mapped to the TLR and IFN pathways,⁹⁴ further establishing these pathways as a potential Achilles' heel for lupus. Most importantly, these studies are yielding rarer lupus-associated alleles, which can be more informative and predictive in a general population. For example, deficiencies in sialic acid acetyltransferase (SIAE), which may play a role in recruiting negative

⁹¹ Bauer et al. PLoS Med 2006.

⁹² Bauer et al. Arthritis Rheum 2009.

⁹³ Gateva et al. Nature Genetics 2009; Horn et al. N Engl J Med 2008; Cunninghame et al. Nature Genetics 2007; Remmers et al. N Engl J Med 2007; Graham et al. Proc Natl Acad Sci U S A 2007; Graham et al. Nature Genetics 2006; Kyogoku et al. Am J Hum Gen 2004.

⁹⁴ Thibault-Fletcher et al. Expert Rev Clin Immunol 2010.

regulatory molecules to the BCR, have been associated with excessive BCR signaling, production of anti-chromatin antibodies, and lupus-like disease in mice.⁹⁵ Germline SIAE variants defective in esterase activity or secretion are enriched in patients with autoimmune diseases, including SLE, and they behave in a dominant-negative manner. These variants have been found in only 2.6 percent of autoimmunity cases and 0.3 percent of controls. These and other rare variants can be used to generate mouse models for further study.

Advances in sequencing have allowed investigators to tap even further into the genetic complexity of SLE. For example, whole transcriptome sequencing, followed by digital transcript analysis, of PBMC RNA from SLE patients has been used to identify differences in expression of IFI44L and RSAD2, with a 12- to 13-fold difference between IFN signature-positive and signature-negative individuals. However, these differences are related not to differences in gene expression, but to differences in isoforms, suggesting that some genes that do not appear to be regulated by IFN might in fact be regulated via alternative RNA splicing.

Translating these advances into therapies for SLE will require a recruitment of the best and brightest scientists to SLE research and clinical development. A large number of these scientists have been attracted to the field over the past 10 to 15 years, and all are passionate about helping patients. Yet the current funding environment is challenging and threatens further recruitment. With NIH paylines as low as 8 percent, many in the scientific community are concerned about sustaining existing investigators, and the implications of the health care reform legislation are unclear. Although foundations have stepped up their support, they can only do so much. More support is needed from industry to push basic research forward.

SLE research must continue to focus on furthering the basic science and understanding of human disease. More studies on the basic immune system, epidemiology, genetics, human pathophysiology, and biomarkers are needed, and the best targets should be identified. More compelling preclinical models, not only those focused on the pathophysiology of SLE in mice, but new ones that better reflect human disease, are needed. Mouse models have been helpful in increasing our understanding of the pathophysiology underlying human disease, but models are also needed to test potential therapeutic targets and mimic what is already known to work in lupus.

The clinical drug development path and its challenges must also be addressed. Once research yields a clinical candidate, the timeline involves 19 months to develop that candidate via toxicity studies, molecular formulations, and written submissions in preparation for phase I trials. Phase I trials take an average of 21 months, phase II trials another 23 months, and phase III trials another 44 months. Thus, on average, potential drugs take 9 years to move through the pipeline. Moreover, out of all the candidates that enter into phase I trials, only 20 percent eventually reach FDA approval, and the total estimated cost per each approved drug is more than \$900 million. With this time and cost commitment, it is unlikely that academic institutions or NIH can succeed alone in taking candidate molecules through the entire process. Clinical development is further hampered by the clinical heterogeneity of SLE, the lack of a standard regulatory path, problems with concomitant medications, and problems with quantifying disease activity. We should celebrate the successes of drug development for lupus, but also work to understand the failures.

⁹⁵ Suroliia et al. Nature 2010.

PANEL DISCUSSION: WHAT WE HAVE LEARNED AND FUTURE DIRECTIONS

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Conference Themes

Drs. Craft, Crow, and Close closed the meeting by summarizing the conference and identifying several themes:

- **A systems biology approach.** The linear model of immune activation involving mediators, B cells, T cells, and APCs is giving way to a systems model that comprises several feedback pathways. Genetics appears to form the scaffold the entire system, and a lot has been learned by observing and characterizing genes associated with innate immune response, generation of antigen, autoimmunity, and tissue response. As demonstrated by several talks at this conference, the lupus system goes even beyond the immune system. Neutrophils serve as a source of self-antigen and DNA, endothelial cells serve as targets for antibodies and IFN, and stromal cells interact with B cells and contribute to the production of IFN. All the various steps and components are important to the system of pathogenesis, and the lupus research community should take care not to overcommit to or focus solely on one component. This is especially true for the development of therapeutics, as there are several points of intervention as illustrated by promising therapies such as rituximab, IFN blockade, and corticosteroids.
- **Regulation of immune response.** Over the years lupus research has emphasized the concept of immune tolerance, but several talks during this conference have suggested that autoimmunity might also involve normal immune response that is persistent and chronic. Self-antigens and autoreactive antibodies play a positive role in wound repair and addressing damage, but autoimmunity likely results from dysregulation of these processes. More study is needed to understand the levels and points of immune regulation, and the events between autoantibody production and lupus disease manifestation remain unclear. However, various aspects of immune tolerance also need further study.
- **Mouse versus human studies.** As illustrated by presentations at this conference, the use of mouse models has contributed to significant progress in what is known about the mechanisms of lupus, how they might change over time, and potential ways to block these mechanisms. The role of the IFN signature, additional functions for B cells in lupus pathophysiology, and potential therapies such as rituximab and BLyS blockade are examples. Both mouse models and clinical studies will be needed to further drug development and understand how best to treat patients with active disease.
- **Disease and patient heterogeneity.** Large, global studies have yielded evidence of variability among patients with SLE, for example differences in the degree of response to treatment and the side effects experienced with that treatment. Future studies, particularly clinical trials, will need to account for and address this heterogeneity. Mouse and human studies that explore the mechanisms underlying these differences might increase understanding of the complexity of lupus, aid in understanding the variable responses to standard of care, and help to avoid a “one-size-fits-all” approach. Investigators are encouraged to think of the “global patient,” encompassing sex, racial/ethnic, genetic, environmental, and other differences.

- **A better understanding of current lupus therapies.** Mouse studies can be used to better understand how the current standard of care works. Although these therapies appear to relieve signs and symptoms, the mechanisms underlying their success are not clear. Further understanding of how these therapies work will aid in the design of new therapies and will be critical if existing therapeutics are to be used as controls in future clinical trials.
- **The potential for cost-effective and combination therapies.** The safety of therapeutics is just as important as the benefit associated with them. Combination therapies have been suggested for patients with SLE, but high toxicity levels remain a concern. On the basis of new understanding of how current therapies work, future clinical trials could define the risks and benefits of cost-effective approaches in which therapies are used sequentially. The tools exist to move these studies forward.
- **The importance of communication.** Meetings such as this conference remain vital in sharing information. Foundations and associations such as the Alliance for Lupus Research, the Lupus Research Institute, and the Lupus Foundation of America have been instrumental of bringing together basic and clinical scientists from different areas of expertise, emphasizing innovative science, and identifying ways to make clinical drug development more efficient.
- **The need to think creatively and work with other disciplines.** Mycophenolic acid, one of the most effective existing drugs for treatment of lupus, emerged from the field of transplantation science, and its mechanism of action in lupus is still poorly understood. As more is learned about the pathophysiology underlying lupus, more agents used for other conditions might prove to be useful in SLE.

The current understanding of lupus has been advanced by the advent of high-throughput technologies and bioinformatics, which have arisen from disciplines not traditionally affiliated with lupus research. Even though most lupus researchers have not been trained in chemistry, physics, or informatics, they have been able to exploit these advances to gain a better understanding of innate immune response, the inflammasome, and TLR signaling pathways.

- **The risky business of drug development.** Industry will continue to need data from mouse and clinical studies to justify that \$900 million investment in drug development. Like academic researchers, researchers in industry must also write scientific and clinical justifications similar to grant applications, and as with the grant application process, the risk for failure is high.

Areas for Further Discussion

Although these research questions were not discussed in detail during the conference, the panel speakers recognized their importance.

- **Environmental factors that trigger or amplify the genetic substrate underlying SLE manifestations.** Most patients with SLE experience periods of flares and remissions, whereas most lupus-prone mouse models show progressive disease. Thus it is still unclear what accounts for exacerbations and remissions. Careful characterization of patients and

biologic samples from clinical studies might increase understanding of potential environmental triggers.

- **The role of sex in SLE.** Lupus is more common in women than in men, but investigations of estrogen suggest that other factors might be involved. Studies of the X chromosome, epigenetics, ovarian biology, and meiosis are needed or under way.
- **Other topics for further discussion and study:**
 - Non-immune cell types
 - Epigenetics
 - The gut microbiome
 - Natural mechanisms for downregulating the immune response
 - Stress

Suggested Resources for Future Lupus Research

- Continuation of the Lupus Registry and Repository at the Oklahoma Medical Research Foundation and expansion of the registry to 50,000 patients. This is considered a high priority as funding for continuation of the registry is not certain.
- A mechanism to help centers explore existing repositories, registries, and databases and identify appropriate samples and patient populations for their studies.
- A mechanism to fund small, collaborative studies that would help investigators share innovative assays or procedures more widely. At present, many investigators agree to test others' samples, but this constitutes a financial burden. It is envisaged that such mini-grants would fall into the \$10,000 to \$40,000 range.
- A mechanism to allow investigators to quickly access and explore new technologies. At present, with flat funding levels, investigators do not have the purchasing power to access technologies and enter into new collaborations. As a result creativity is stifled.
- An analysis of the metabolome of 20 different mouse models of lupus, including both sexes and two different time points with appropriate controls. This data would be made freely available to the research community. The cost of such an analysis is estimated to be \$250,000.