THE DENDRITIC CELL RESPONSE TO EXOGENOUS AND ENDOGENOUS DANGER SIGNALS

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ABSTRACT

Systemic lupus erythematosus (SLE) is complex autoimmune disease in which autoantibodies form against double stranded DNA (dsDNA) and nuclear antigens. Autoantigen immune complexes form, deposit in the vasculature, and cause multisystem organ damage. Both genetic and environmental factors contribute to the development of SLE. This thesis will explore three major themes found in the study of SLE: 1) Bacterial infection as an environmental trigger, 2) cytokine dysregulation in immune cells, and 3) the treatment of end organ damage in the form of lupus nephritis.

Viral infections have long been associated with the development of systemic autoimmune disease, but the mechanisms by which chronic bacterial infections may promote autoimmunity remain unclear. In chapter three we show that a component of bacterial biofilms, the amyloid-like protein “curli”, irreversibly forms fibers with bacterial or eukaryotic DNA during biofilm formation. This interaction accelerates amyloid polymerization and creates potent immunogenic complexes that activate immune cells, including dendritic cells, to produce cytokines such as type I interferons, which are pathogenic in SLE. When given systemically, curli/DNA composites trigger immune activation and production of autoantibodies in lupus-prone and wild type mice. We also found that infection with curli-producing bacteria triggered higher autoantibody titers in lupus-prone mice compared to curli-deficient bacteria. These data provide a mechanism by which the microbiome and biofilm-producing enteric infections may contribute to the progression of SLE and point to a potential molecular target for treatment of autoimmunity.

Cytokine dysregulation is also common in SLE patients. Serum cytokines are often elevated during active disease, including type I IFNs and IL-10. In chapter four we demonstrate that *Il10* is a type I IFN response gene and has increased basal expression in dendritic cells (DCs) derived from pre-disease lupus-prone Sle1,2,3 mice. We show that Sle1,2,3-derived DCs overproduce IL-10 in response to TLR ligands and that this is the result of autocrine signaling though the type I IFN receptor (IFNAR). These results suggest that dysregulation of cytokine signaling in the myeloid compartment may contribute to IL-10 dysregulation in SLE.

Renal disease remains a major cause of morbidity and mortality in SLE. A number of mouse models of chronic kidney disease have implicated the EGFR-family receptors in the progression of renal fibrosis and dysfunction. In chapter five we show that renal expression of ErbB2 is increased in murine lupus. We therefore asked if EGFR-family inhibition could prevent murine lupus nephritis. To test this possibility we used lapatinib, an EGFR-ErbB2 dual kinase inhibitor, in an IFN-accelerated model of murine lupus. We found that lapatinib administration lowered autoantibody levels but worsened renal disease. Lapatinib failure to treat murine lupus nephritis despite lowered autoantibody levels suggests EGFR-family signaling is required for tissue repair in the acute phase of kidney injury.

Together this thesis clearly demonstrates the complexity of systemic autoimmune disease – bringing us to the crossroads of immunity and tolerance. The combination of both environmental triggers (e.g. bacterial infection) and genetic susceptibility (e.g. intrinsic cytokine dysregulation) leads to end organ damage (e.g. lupus nephritis). Here we sought to explore each aspect of disease progression in the hopes to develop better interventions for systemic autoimmune disease.

ACKNOWLEDGEMENTS

"Science is an enterprise that should be cherished as an activity of the free human mind because it transforms who we are, how we live, and it gives us an understanding of our place in the universe."

-Neil deGrasse Tyson

"Remember kids, the only difference between screwing around and science is writing it down"

-Adam Savage

Science is not an exact science. Most of our days in the lab are spent moving small volumes of liquid from one tube to another or poring over a literature so vast we need entire computer programs to manage the hundreds of PDFs. It is frustrating work and fraught with uncertainty. But hidden away are moments of clarity, flashes of inspiration, and ideas to be tested. The joy of science comes in developing those ideas and testing them. Sometimes, if you are immensely lucky, one idea might even be *right*. As anyone who has tried it will tell you, investigating the natural world requires patience and imagination – and for that I am deeply indebted to all of those who have supported and inspired me in my life and work.

When I first chose to enter the MD/PhD program at Temple, I was in the lab of Dr. David Mosser at the University of Maryland. It was in the Mosser lab that I first found my interest for immunology and I am deeply indebted to the Mosser lab for instilling in me a solid foundation in the scientific method. I turned to the post-docs for advice as I looked through the Temple website in search of possible lab rotations. One of the first pages I came across was that of Dr. Stefania Gallucci. The post-docs agreed, the Gallucci lab would be an excellent choice.

I have been extremely lucky to be a part of the DC lab. Dr. Gallucci has been an exceptional mentor, striking the delicate balance between patience and innervation – always demanding the best of her students. But perhaps more importantly, Dr. Gallucci understands that being a scientist is a privilege and should above all be fun. All the members of the DC lab have taught

me a great deal about the technical aspects of science. However, I think the most important thing that I have received during my PhD is the encouragement and the guided freedom to flow my instincts and enjoy doing science. So thank you to Dr. Gallucci for being a great role model. Thank you for letting me walk into your office whenever I felt the need; for understanding that students need a vacation every now and then; for encouraging your students to go to meetings; and for showing us that doing science should be fun, collaborative, and amenable to other parts of life.

It has been a pleasure to work in the DC lab for many reasons, chief among them however is the lab members. From the very beginning I was welcomed into the lab like a family member. Birthdays were strictly observed with cake and conversation. New publications are celebrated and time was always made for banter – understanding that there is more to life than business. Competition is not uncommon in science and some labs will pit students against each other (a misguided strategy in my opinion). This was never the case in the DC lab. The culture of the lab has always been one of a team. Special thanks to Uma and Linda for first teaching me PCR and dendritic cell culture; to Jun for teaching me the intricacies of Flow cytometry and western blot; to Marita for helping me troubleshoot and for spending late nights harvesting organs with me; to Rob for lightening the number of oral gavages done with lapatinib; to Annette Palladino for reminding me that what we do is really cool; and to Mike for taking an active role in lab management and bringing new ideas to my work. Thank you all for making my PhD years so enjoyable.

It's said that it takes a village to raise a child – but the same could be said of a scientist. Interaction with other scientists brings new perspectives on the same problem and accelerates scientific progress. I've been lucky that Dr. Gallucci does not shy away from collaboration and I've learned a great deal in the process of building scientific relationships. I'd like to thank Dr. Cagla Tukel and all the members of the Tukel lab. Special thanks to Glenn Rapsinski for working with me from the beginning. The curli project took off early on and has been a fascinating dive into the pathogenesis of lupus. Thanks to Gertrude, Sarah, and Paul for helping me with curli and infection experiments. Thanks also to Dr. Cohen for taking an active role in my training from the beginning, for asking tough questions, and for always welcoming me into the clinic. Unfortunately

the work with MerTK hit a number of obstacles, but it has sparked my interest in cell death and clearance – an area I plan to revisit in the future. Thanks to Dr. Roberto Caricchio for helping me interpret results and for keeping me thinking about the clinical relevance of my work. Thanks to Drs. Wenhai Shao, Brendan Hilllard, and Mike Denny for sharing reagents and invaluable knowhow. Thanks to Drs. Patricia Costa Reis and Kathleen Sullivan at U. Penn for including me on the lapatinib project. It was a great experience to perform a large, clinically driven animal experiment and I hope to continue to work together in the future (I still have a lot of ideas). Thanks to Dr. Doina Ganea for sharing mice and for always showing interest and support for my work. Thank you to Dr. Gamero and the Gamero lab for being so generous with sharing STAT-KO mice. Big thanks to the others on the 11th floor: Kirsten, Wiemin, Katie, Hangfei, Chelsea, and Andrea. Thanks for always being friendly and for always being willing to share supplies and reagents.

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Being a physician-scientist is not an easy undertaking. Fortunately, I have had outstanding role models including Dr. Cohen and Dr. Caricchio. Their commitment to their patients and to the study of rheumatologic disease has been inspirational. I hope that I may be similar one day. I would also like to thank the rheumatology faculty and fellows for always making me feel welcome in the rheumatology clinic. I have learned a great deal from the patients we have seen together and look forward to my time in the hospital when I return to medical school.

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CHAPTER 1- REVIEW OF THE LITERATURE

Systemic Lupus Erythematosus

Overview: Clinical Aspects

Systemic lupus erythematosus (SLE) is complex autoimmune syndrome in which autoantibodies form against double stranded DNA (dsDNA) and nuclear antigens – leading to immune complex formation and multisystem organ damage.[1] The current understanding of lupus pathogenesis is multifactorial including many genetic and environmental factors.[2,3] However, the molecular triggers that initiate disease have yet to be elucidated.

SLE is likely not a single entity but rather a spectrum of related syndromes. The "classic" form of lupus has a strong sex bias, with about 90% of lupus patients being female (although the female to male ratio falls in older adults and pediatric forms). Clinically overt disease usually presents after puberty in the second or third decade of life. The incidence of SLE is approximately 5 in 100,000 and the prevalence in the continental USA is about 52 in 100,000 – although estimates may vary widely as milder forms may be difficult to diagnose.[4] In the United States SLE is more prevalent among women of African, Asian, and Hispanic descent – with higher disease burden in patients from disadvantaged socioeconomic groups.[5] Although differences may exist, the idea that SLE is much more common in Americans of African descent compared to their African counterparts is likely to be partly myth.[6]

Patients may present with a wide variety of manifestations. Constitutional symptoms (e.g. fatigue), musculoskeletal pain (i.e. arthritis), and cutaneous involvement (e.g. rash, often photosensitive) are among the most common presenting complaints, but disease may manifest in the renal, neuropsychiatric, pulmonary, gastrointestinal, circulatory, or hematologic systems.[7] Sexual dysfunction is also common in women with SLE.[8] The kidneys are commonly involved in SLE and will be discussed in greater detail in later sections.

Genome wide association studies (GWAS) have demonstrated that SLE is a complex polygenic disorder of the immune system in which gene interactions predispose an individual to disease (reviewed in [9,10]). Twin studies have confirmed the notion that both genetic and environmental factors contribute to the development of SLE.[11] However, there are forms of lupus

that are caused by single gene mutations, such as TREX1 mutations and complement deficiencies. These forms of lupus are rare and generally present early in life. In pediatric forms of lupus the male-to-female ratio tends to be less drastic, with the ratio approaching closer to one. Clinical manifestations of pediatric lupus are also somewhat different from adult onset SLE – with children having greater incidence of malar rash, mucocutaneous involvement, renal disease, seizures, and hemolytic anemia.[12]

In SLE, the accumulation of autoantibodies to dsDNA and chromatin leads to immune complex-mediated tissue damage (type III hypersensitivity). However, the presence of autoantibodies does not necessarily indicate a disease process. A low level of low-affinity autoantibodies (particularly IgM) is common in healthy individuals and some healthy patients can have a positive antinuclear antibody test (ANA).[13] So although the ANA is very sensitive for SLE, it is not very specific – leading to the general recommendation that an ANA not be performed unless the clinical picture is suggestive of an autoimmune disease process. In patients destined to develop clinically overt SLE, autoantibodies are often present long before diagnosis – slowly increasing before becoming symptomatic.[14] There seems to be a progression by which autoantibodies appear before the onset of SLE, with anti-phospholipid, anti-Ro, and anti-La being detected early; and anti-dsDNA, anti-Sm, and anti-RNP coming closer to the time of diagnosis.[14] This phenomenon suggests an underlying process in which autoantibodies are a side effect. What that process might be will be explored in this thesis.

History

The term “lupus” is derived from the Latin for “wolf.” The term was originally used to describe any destructive skin lesion that supposedly resembled the bite of a wolf (reviewed in [15]). This gross description included a number of dermatologic diseases including cutaneous tuberculosis (lupus vulgaris) up to the 19th century. In the 1830s Pierre Louis Alphee Cazenave published the work of his teacher Laurent Theodore Biett, who is credited with the first clear description of lupus.[15,16] Cazenave coined the term “*lupus erythematosus*” and described it as ““*a rare condition, which appears most frequently in young females who are otherwise healthy, attacking*

the face chiefly. Round red patches, slightly elevated, about the size of a shilling, gradually increase in size and sometimes spread over the greater part of the face. The edges of the patches are prominent, and the center, which retains its natural color, is depressed. There is heat and redness but no pain or itching. It is essentially a chronic affection though its appearance would indicate otherwise."[16] This seems to be a description of what we would now call cutaneous discoid lupus.

In 1866 Ferdinand von Hebra described the malar rash, first using the butterfly metaphor to describe its distribution over the bridge of the nose and malar eminences.[17] In the 1870s Moriz Kaposi (of Kaposi sarcoma fame) described the constitutional and systemic manifestations of lupus.[18] Kaposi made the distinction between lupus erythematosus and cutaneous tuberculosis, maintaining that they were unrelated disorders – an idea that was not universally accepted at the time.[19] William Osler is credited with the addition of "systemic" to the disorder's name and described the periodic nature of lupus flares, which could exacerbate visceral disease.[20] In 1923, Emanuel Libman and Benjamin Sacks described the non-infectious endocarditis associated with SLE,[21] and it would take another two decades before Malcolm Hargraves, Helen Richmond, and Robert Morton would describe the LE cell in 1946.[22]

The lupus erythematous (LE) cell is an abnormal cell found in the bone marrow of some patients with lupus-like disease. Eventually it was found that these cells are phagocytes distended with cellular debris and nuclear protein immune complexes.[23] Although the importance of their discovery was not immediately understood, this observation would be the first indication that failure to properly dispose of dead cells may be the underlying cause of SLE. However, in the early 20th century the antibody was still largely a mystery and autoimmunity was generally considered impossible. Paul Ehrlich famously wrote "*It would be dysteleologic in the highest degree, if under these circumstances self-poisons of the parenchyma – autotoxins – were formed*" (cited in [24]). Many of his students would become the leading figures in the new field of immunology and held to the belief that autoimmunity was exceedingly rare, if not impossible (i.e. horror autotoxicus). It wasn't until 1982 that the American College of Rheumatology added the antinuclear antibody test and anti-dsDNA titers to the classification criteria for SLE.[25]

Murine Models of Lupus

Systemic lupus erythematosus is a complex autoimmune disease. The study of lupus in humans is difficult because of its protean manifestations and its difficult, largely clinical diagnosis. Fortunately, there are a number of useful mouse models of lupus which have facilitated our understanding of this multifaceted disease. The work found in this thesis will make use of two lupus-prone mouse strains, the NZB/W-F1 and the B6.NZM Sle1/Sle2/Sle3 (B6.Sle1,2,3). In chapter 5, I will also describe the use of a model in which spontaneous murine lupus is accelerated using an adenovirus vector which produces murine IFN-α.

NZB/W-F1

The first animal model of autoimmunity was the inbred mouse strain called the New Zealand Black (NZB). Originally developed for cancer research,[26] the NZB strain was first described in 1956 by Bielschowsky et al as developing spontaneous hepatosplenomegaly and a fatal autoimmune hemolytic anemia.[27] Interestingly, NZB mice also develop a marked lymphoproliferative phenotype that precedes autoimmunity.[26] It's unclear if the lymphoproliferation found in NZB mice contributes to the breakdown of tolerance and development of autoimmunity, as it does in the MRL/*Fas*^{lpr} strain.

More than a decade later, Helyer and Howie reported that crossing the NZB line with other inbred strains, including the New Zealand White (NZW), changed the phenotype to one consistent with a lupus-like disease, including the development of anti-dsDNA autoantibodies[28] and glomerulonephritis.[29] It was later confirmed that nephritis in these mice was immune-mediated and thus a true model of SLE.[30] The F1 generation of the cross between NZB and NZW (NZB/W-F1) subsequently became one of the most widely used models in research of systemic autoimmunity.

Aimed at defining the defect in NZB/W-F1 mice, early work suggested that they had decreased antibody avidity[31] and defective phagocytosis in macrophage.[32] In the 1990s the NZB/W-F1 mice were used to test CTLA4-Ig fusion proteins[33] – with the rationale that soluble CTLA4 could block costimulatory molecule signaling and thus limit autoimmune T-cell

responses.[34] Daikh et al showed that CTLA4-Ig could prevent fatal autoimmune disease in NZB/W-F1 mice.[33] Although this strategy has had limited success in human disease, it illustrates the important role the NZB/W-F1 mouse model has played in the development of potential therapeutics for autoimmune disease.

The appeal of this mouse model is that it closely resembles disease seen in humans. Unlike some models, the NZB/W-F1 model has a polygenic, progressive disease that recapitulates most aspects of human SLE. This similarity includes a significant difference between male and female NZB/W-F1 mice, where 100% of females, but less than 40% of males, show significant renal disease by 1 year of age.[35]

Sle1,2,3

One limitation of the NZB/W-F1 mouse model is that it requires the maintenance and crossbreeding of two separate mouse strains (i.e. the NZB and NZW). To circumvent this, researches in the field developed inbred strains derived from the NZB/W-F1 generation, termed New Zealand mixed (NZM) strains.[36] One such strain, the NZM2410, was found to have highly penetrant lupus-like disease. This inbred mouse strain made it possible to dissect the genetic determinants contributing to disease susceptibility – found to be localized to four chromosomal segments.[37] Three of these intervals (later termed Sle1, Sle2, and Sle3) were independently introgressed into a B6 background to produce three congenic mice.[38] Creating these congenic models on a B6 background also solved another limitation: that the NZB/W-F1 did not have a strict wild type control. Individually these intervals contribute to different aspects of lupus-like disease: B6.Sle1 mice develop IgG anti-nuclear antibodies, B6.Sle2 mice have polyclonal B cell hyperactivity, and B6.Sle3 mice are susceptible to lupus nephritis.[38] Crossing these individual mice creates B6 mice carrying multiple Sle intervals – generating murine strains with highly penetrant disease.[39,40] The Sle1,2,3 mouse is therefore a B6 mouse carrying the Sle1, Sle2, and Sle3 genetic intervals (located on chromosomes 1, 4, and 7 respectively).

Similar to lupus patients, Sle1,2,3 mice produce an IFN signature that precedes the onset of disease.[41] Exaggerated proinflammatory cytokine production from the myeloid cell compartment

has also been described in single congenic B6.Sle3 mice, with macrophages and DCs producing higher levels of IL-12, IL-6, and TNF in response to LPS.[42] This inflated cytokine response seems to drive T cell hyperactivity and disease progression in these mice.[42] Therefore in chapter four we will use Sle1,2,3 mice to explore cytokine dysregulation in innate immune cells – with particular attention to IL-10, an anti-inflammatory cytokine that is less well studied in this mouse model.

IFN-α adenovirus-accelerated lupus

Murine strains that develop spontaneous lupus (e.g. NZB/W-F1) generally do so gradually and later in life. Therefore the study of disease in these mice may require them to be aged for extended periods. To overcome this limitation, disease may be accelerated in lupus-prone mice. One powerful strategy for accelerating disease in lupus-prone mice is with the use of an IFNα-expressing adenovirus (IFN-adeno), which dramatically accelerates disease progression (see below). Although acceleration strategies may be useful, they may also change the way results are interpreted.

Many patients with active lupus will have an "IFN signature" – that is the overexpression of genes induced by type I IFN.[43,44]. Lupus prone mice also demonstrate this IFN signature, even before the onset of autoimmunity.[41,45] Further evidence for the importance of type I IFN in the progression of lupus comes from lupus-prone mice with a defective IFN alpha receptor (IFNAR). Multiple groups have shown that defective IFNAR signaling in lupus-prone strains largely prevents disease progression.[46-49] In contrast, treating lupus-prone mice with type I IFN can accelerate disease, although the resultant disease may be slightly different compared to non-accelerated disease (reviewed in [50]).

Accelerating murine lupus is often done using an adenoviral vector that expresses IFNα (IFN-adeno).[51] A single i.v. dose of this IFN-expressing adenovirus is able to drastically accelerate disease in lupus prone mice in a T cell-dependent manner.[52] IFN-adeno will induce splenomegaly, bone marrow suppression, lymphopenia, and B cell activation in both B6 and B6.Sle1,2,3 mice.[53] However, treating B6 mice with IFN-adeno does not lead to proteinuria or

glomerular disease.[53] In chapter five we will use this strategy to accelerate lupus-like disease onset in NZB/W-F1 mice.

TLR-accelerated lupus

Pathogen-associated molecular patterns (PAMPs) (e.g. LPS, flagellin, peptidoglycans) are recognized by pattern recognition receptors (PRRs)[54-57] and are released by both pathogens and other microbes associated with the natural flora.[58] Among the PRRs, Toll-like receptors (TLRs) are a well characterized group (reviewed in [59,60]). The Toll protein was first linked to the immune defense in Drosophila, in which it recognizes the endogenous ligand Spatzle to initiate antifungal responses.[61] It is now well established that the TLRs are critical to the function of mammalian innate immune cells – recognizing a wide variety of conserved pathogen products from bacteria, viruses and parasites.[54,62,63] Other PRRs include the RIG-I-like receptors (RLRs), recognizing nucleic acids; [64] C-type lectin receptors (CLRs), binding β-glucans;[65] Nod-like receptors (NLRs), recognizing several bacterial products; and the intracellular DNA sensor AIM2 – the final two being activators of the inflammasome.[66]

Some of the exogenous ligands for these PRRs have been extensively studied in autoimmune diseases. For example, many reports support a still controversial association between infection with Epstein-Barr virus (EBV) and SLE,[67,68] in part because of the antigenic mimicry between Epstein-Barr nuclear antigen 1 (EBNA-1) and the ribonucleoprotein autoantigen (RNPs).[69] Additionally, EBV also contains a number of PAMPs. Among these viral PAMPs, molecules like nucleic acids can induce, via TLR7 and TLR9 mediated recognition by pDCs,[70] the production of Type I Interferons (IFNs), the endogenous danger signals pathogenic in lupus.[71] Moreover, the TLR7 and TLR8 ligand imiquimod can exacerbate the skin disease psoriasis[72] and TLR2 and NLR ligand peptidoglycans have been found in affected tissues of patients with Crohn's disease, rheumatoid arthritis (RA)[73] and multiple sclerosis (MS).[74] Because polymorphisms in *TLRs* are associated with autoimmune diseases such as type 1 diabetes[75] and Crohn's disease,[76] and mutations in *NOD2* are also associated with Crohn's disease,[77] it has

been proposed that infections promote autoimmunity by providing acute or chronic immune stimulation via PAMPs.

Spontaneous murine models of lupus can be accelerated by administering TLR ligands. Polyinosinic:polycytidylic acid (PolyI:C), a ligand for TLR-3, is known to accelerate the onset of lupus nephritis in susceptible mice through the induction of type I IFNs.[78] Early work showed that LPS could also induce mild lupus-like traits in non-autoimmune mice.[79] Later it was found that LPS can promote disease progression in lupus-prone mice.[80-83] The mechanisms by which LPS promotes nephritis are not well understood, but may involve mesangial cell inflammation and cytokine production.[84,85]

Imiquimod and resiquimod (i.e. R848) are small molecules used in cream-based medication for skin malignancies and genital warts. Imiquimod and imiquimod-like compounds modulate the immune response largely though activation of TLR-7 and -8, leading to production of pro-inflammatory cytokines. Application of imiquimod-like compounds to murine skin is a commonly used model to study psoriasis-like disease. However, a recent study used these TLR-7 agonists topically to induce systemic autoimmunity in wild-type mice, including BALB/c and C57BL/6.[86] In this study, 7 to 9 week old female mice were treated 3 times a week with topical imiquimod or resiquimod to the right ear. They demonstrate that, unlike i.p. injection with imiquimod, topical treatment induces production of autoantibodies to anti-dsDNA after 4 weeks. After 4 weeks of topical imiquimod, BALB/c mice develop proteinuria and show histological evidence of immune-mediated glomerular disease.[86] It is not clear if this disease phenotype improves when the drug application ceases. This work supports the notion that increased TLR-7 activity can lead to systemic autoimmunity. In this thesis we will explore the use of a bacterial product called curli (see Amyloid section) to accelerate autoimmunity.

A Word on Sex

Sex is a major biologic determinant and can have profound effects, even on the most “basic” of cellular phenomena. However, sex has not always been treated as an important biological variable in basic science research. Indeed it seems that in some areas pre-clinical research is

done using only male cells and male animals[87] (with results not always being reproducible in female animals). What's more, a surprisingly large proportion of basic science research fails to report the sex of animals and cells used in their investigations – even in fields like immunology where mostly female mice are used. This has led to a number of initiatives from the NIH and journal editors to encourage the use of both sexes in experimental design and increase reporting sex as a biological variable.[88]

Fortunately for those of us working in the field of autoimmunity, the idea that sex influences even "basic" biology is not a new one[89] – and in the case of SLE stares us directly in the face. The sex bias in SLE is a major clue to its pathophysiology and is an active area of research in many labs, including the Gallucci lab. Because SLE is predominantly a female disease we largely make use of female animals to explore new areas that do not require direct comparisons between the sexes. For that reason, the majority of the work in this thesis uses female animals and cells. The sex of animals and cells for individual experiments will be reported throughout the thesis.

Infection and the Microbiome in SLE

Infection in SLE

One of the major causes of morbidity and mortality in patients with SLE is infection.[90,91] This may be largely due to the use of potent immunosuppressive therapy, but may also stem from inherent immune dysregulation particularly in the context of renal dysfunction.[92] Patients with SLE often encounter bacterial infections including urinary tract infections, pneumonia and sepsis.[93] Bacterial infections are thought to contribute to SLE propagation by inducing cell death and inflammation, or by introducing pathogen-derived antigens which promote lupus flares.[94] However, the exact mechanisms by which bacterial infections contribute to SLE pathogenesis remains unclear.

The Microbiome and SLE

The human body is an ecosystem. In and on it lives a staggeringly diverse population of commensal microorganisms – including bacteria, viruses, and fungi. A healthy microbial flora is critical to good health, leading some to describe the human condition as a supra- or meta-organism. The microbiome generally comprises the commensal microbial flora found on mucosal surfaces (i.e. the skin and GI tract). "Pathogens" are often regular members of the normal microbial flora – requiring disturbances in tissue and commensal homeostasis before causing disease. Recent work has shown that microbial ecosystems on mucosal surfaces can have dramatic effects on disease – even in visceral locations. Recently, Zhang et al used lupus-prone MRL/*lpr* mice to describe how sex, age, and the onset of autoimmunity affects the gut microbiome.[95] They show that the gut microbiota of MRL/*lpr* mice had significant reduction in *Lactobacillaceae* and an increase in *Lachnospiraceae* compared to MRL controls.[95] Interestingly, oral administration of *Lactobacillaceae* species has been shown to suppress the development of experimental arthritis in rats,[96] suggesting that exposure to these bacteria may be protective in inflammatory disease. Zhang et al also show that vitamin A treatments could correct the *Lactobacillaceae* deficiency seen in MRL/*lpr* mice and thus improve disease activity.[95] This work suggests the importance of the microbiome in the development of autoimmunity.

Exposure to Gut Microbes

The microbes used in this thesis to explore infection-accelerated autoimmunity are enterobacteriaceae. Specifically, we used *E. coli* as a prototypic commensal and *Salmonella* Typhimurium as a prototypic pathogen – both of which commonly colonize the GI tract. Therefore one might ask if SLE patients are more prone to abnormalities in the GI tract that would make them more likely to encounter these microbes systemically. The answer is multifaceted because although isolated GI complaints are rare in SLE, vasculitis is common and can affect multiple organ systems including the GI tract.[97]

Lupus mesenteric vasculitis (LMV) is a severe and life threatening form of vasculitis that usually occurs during active disease and can lead to intestinal ischemia, infarction, and

perforation (reviewed in [98]). Mortality rates can be as high as 50%.[99] Estimated prevalence of mesenteric vasculitis among lupus patients ranges from 0.2% to 9.7% depending on the study and region of interest,[98] although it seems to be much more common in pediatric forms of lupus.[100] In one recent study from China the prevalence of LMV was 2.5% in hospitalized lupus patients and 49.5% in those lupus patients that presented with abdominal pain.[99] Compromised colonic blood supply may cause damage to the mucosal barrier and expose patients to microbial products, although this possibility remains largely unexplored in experimental models.

Germ Free Mice

As described above, research on how the microbiome influences human disease has become an active area of investigation in the last two decade. However, as early as the late 1960s some work was indicating that the microbiome could influence the development of murine lupus. In 1969 East et al found that NZB mice under germ-free conditions had smaller spleens and smaller lymph nodes compared to conventional, aged-matched NZB mice.[101] NZB mice under germ-free conditions also have attenuated renal disease, showing a decreased incidence and milder disease activity.[102] These studies suggest that commensal microbiota contribute to the natural development of lupus-like disease.

Similarly, pristane-induced lupus is significantly influenced by the presence of microbes. Balb/c mice injected with pristane under pathogen-free conditions had significantly lower levels of total IgG, attenuating resultant hypergammaglobulinemia, compared to conventionally housed mice.[103] Pathogen-free mice also had lower titers of antinuclear antibodies,[103] suggesting that the microbiome amplifies pristane-induced autoimmunity and promotes the breakdown of tolerance.

In contrast to NZB and pristane models, disease progression in MRL/*lpr* mice does not seem to be influenced by germ-free conditions – MRL/*lpr* mice under germ-free conditions have similar lymphoproliferation and autoantibody production compared to conventional MRL/*lpr* mice.[104] These results indicate that the microbial influence on lupus-like disease is dependent on the mouse strain, having less of an impact on monogenic models like the MRL/*lpr*.

Murine Lupus Susceptibility to Infection

Lupus-prone mouse strains may also be susceptible to some kinds of infections. In 1985 Nickell et al. found that MRL/*lpr*, NZB, and BXSB mice were all susceptible to *Trypanosoma curzi* infection.[105] NZB mice are particularly susceptible to T. *cruzi* – with all NZB mice succumbing to infection. Interestingly, the NZB/W cross (F1) were less susceptible to T. *cruzi*.[105] They also show that greater susceptibility to T. *cruzi* infection is correlated with higher levels of anti-*cruzi* antibodies.[105] This apparent paradox, we now know, is a strategy used by Trypanosomes to use host antibodies to ligate Fc receptors, enter phagocytes, and modulate the immune response.[106]

Mechanisms of Infection-induced Autoimmunity

In theory, chronic exposure to infectious agents may propagate autoimmune disease in susceptible individuals by causing inflammation and cell death, leading to flares and exposure to cellular autoantigens. To explore the role of chronic infection in the onset of autoimmunity Chowdhary et al chronically exposed mice to staphylococcal enterotoxin B (SEB) using osmotic pumps.[107] Mice chronically exposed to SEB produced autoantibodies and developed multiorgan dysfunction mimicking autoimmune disease.[107] It is unclear how much of the organ pathology was the direct result of SEB and how much is the result of autoimmunity – although this is also often difficult to differentiate clinically. Recently, Schuster et al demonstrated that chronic infection with murine cytomegalovirus can lead to a Sjogren's-like autoimmune disease in mice lacking the TNF-family member TRAIL. They found that NK cells expressing TRAIL are required to limit CD4+ T cell responses to the virus and failure to limit T cell responses led to autoimmunity.[108] This work suggests a direct link between autoimmunity and a failure to limit anti-viral responses. But infection is not always associated with acceleration of disease. Some viral infections may in fact be protective – as is the case with gammaherpesvirus 68 infection in Sle1,2,3 mice.[109]

With all the experimental evidence showing that infection can exacerbate autoimmunity, we might expect that patients with rheumatic diseases may be at higher risk for adverse reactions to common vaccines (reviewed in [110]). While research is limited and often contradictory, it seems

that vaccines are generally safe for use in patients with autoimmune diseases including lupus. Lupus patients seem to have normal responses to most vaccines and vaccination is not usually associated with disease flares.

Bacterial Biofilms as a Source of Chronic Infection

In chapter 3 of this thesis I will explore the potential role of biofilm-based infections in lupus pathogenesis using two enterobacteria as models. Therefore we will next explore the biology of these bacteria with special attention to the biofilm matrix.

Biofilm Biology

A biofilm is a group of bacteria (or other microorganism) that have aggregated to form a sessile community on a surface.[111] Single cell (planktonic) bacteria seed a surface or air-liquid interface, proliferate, and form a community under the control of quorum sensing pathways. The bacterial community then builds an extracellular matrix made of proteins, saccharides, and nucleic acids. The biofilm facilitates aggregation between bacteria, adhesion to both biotic and abiotic surfaces, and protection from environmental stresses. An immense amount of literature has explored bacterial biology in their planktonic state. However, it has become clear that the vast majority bacteria in the environment live in biofilms, spurring a tremendous growth in our knowledge of biofilm biology (reviewed in [112]). Interestingly, commonly used laboratory strains may lose their ability to create robust biofilms after years of passaging in enriched liquid media.[113-115] Under some conditions these “domesticated” bacteria may have very different biology compared to their wild counterparts.

Bacterial genetic signatures change drastically during the process of biofilm formation. The transformation from a planktonic lifestyle to a biofilm community has major implications for bacterial physiology. These changes can also have impacts on human disease. For example, biofilm formation not only promotes survival of *V. cholera* in the environment,[116] but allows *V. cholera* to better resist acidic conditions like those found in the stomach.[117] Biofilm-protected bacteria are then better able to colonize the intestine[117] and produce quorum-associated

virulence factors.[118,119] A similar event occurs in group A Streptococcus – community behaviors and biofilm formation change gene expression, leading to the production of multiple virulence factors including streptococcal pyogenic exotoxin B (reviewed in [120]).

Chronic Biofilm Infections

The ability to form biofilms is common to most environmental strains of bacteria. In one survey 90% of 289 *Streptococcus pyogenes* clinical isolates (invasive and non-invasive) were able to form biofilms.[121] The ability to form a biofilm acts as a virulence factor by supporting the development of a chronic infection. This has been well studied in cystic fibrosis (CF), an autosomal recessive disease in which there are abnormally thick secretions and mucous plugging in the lungs. This environment is conducive to *Pseudomonas aeruginosa* biofilm formation, with the vast majority of CF patients developing chronic *P. aeruginosa* infection in the lung.[122] Biofilm-mediated chronic *P. aeruginosa* infection likely contributes to disease by producing a chronic inflammatory state that prevents proper wound healing and tissue repair (reviewed in [123]). Similarly, *P. aeruginosa* has been found to aggregate in wounds from patients with chronic venous leg ulcers, suggesting that bacterial communities may play a role in preventing proper tissue repair.[124]

In vivo biofilm formation seems to protect the bacterial community from clearance. In one study using group A Streptococcus, robust biofilm formation was positively associated with antibiotic treatment failure.[125] Similarly, chronic middle ear infections,[126] severe periodontal disease,[127] urinary tract infections,[128] and indwelling medical device infections[129] are often the result of bacterial biofilms. Interestingly, when grown in synovial fluid *Staphylococcus aureus* will aggregate and form a biofilm (a process that does not occur in serum) – suggesting biofilm formation may also contribute to chronic joint infections.[130] Together this wide range of evidence suggests that biofilm-based chronic infections are likely understudied and underdiagnosed.

Although biofilm-based infections are now known to be associated with human disease, the interaction between individual biofilm components and the immune system is still largely unstudied. Recently, nucleic acids have been shown to be an important part of the microstructure

of some biofilms.[131,132] Extracellular DNA (eDNA) acts as a molecular glue to hold biofilm components together. Our collaborators in the lab of Dr. Cagla Tukel, in the Department of Microbiology-Immunology at Temple University School of Medicine, have recently found that bacterial nucleic acids are an integral part of *Salmonella* and *E. coli* biofilms. This observation led us to ask if chronic biofilm infection and exposure to bacterial nucleic acids could promote autoimmunity.

Amyloid

In chapter 3 of this thesis I will demonstrate that amyloids derived from bacterial biofilms potently activate innate immune cells and accelerate autoantibody production *in vivo*. Below I will consider what we know about amyloid structure and pathophysiology, as it may inform the interpretation of our results.

Overview: Structure and Fibril Formation

The proteinaceous cross- β-sheet structure is the defining element of an amyloid.[133] Amyloid units stack to from non-branching fibrils in which the individual polypeptide chains run perpendicular to the long axis of the fibril.[134] This fibrillization produces a characteristic X-ray diffraction[133] and enables anisotropic binding of Congo red.[135] All amyloid fibrils are relatively consistent in diameter, having a lateral width of approximately 10 nm.[136]

Amyloid fibrils are insoluble fibrous aggregates that share a regular cross-β quaternary structure.[137] A number of human diseases are associated with the pathologic accumulation of amyloids, including neurodegenerative diseases, malignancies, and chronic inflammatory diseases. The products of more than 30 different genes can give rise to amyloid fibrils, each being defined by its precursor protein.[138] Common examples are: amyloid β (Aβ) which is derived from Amyloid β precursor protein and is associated with Alzheimer's disease and aging; Amyloid A (AA) which is derived from serum amyloid A and is associated with sporadic and reactive amyloidosis; and ATTR which is derived from Transthyretin and is associated with familial and sporadic systemic amyloidosis. Although amyloids share essentially the same structure,

amyloidogenic peptides are generally unrelated, with little to no sequence homology. Amyloidogenic sections of a protein are often hydrophobic and reside in the protein's interior structure.[139,140] Cleavage or unfolding events are often required for the transition to an amyloid structure.

The fibrillization of amyloids follow a predictable course, which is time and concentration dependent.[141,142] The higher the concentration of amyloid units and the longer they persist the more likely fibrils will form. Local metal ion concentrations can also influence the rate of fibril formation.[143] After the formation of amyloid units, a lag phase occurs in which a nucleation event ensues. The mechanism of this nucleation event is still enigmatic, but may be largely stochastic, increasing in likelihood with higher concentrations and longer incubation periods. A pre-formed amyloid seed or other chemical substances (e.g. DNA or polysaccharides) can replace and accelerate this nucleation event.[144] After a seed has been made, amyloid units fibrilize rapidly during the exponential growth phase. A plateau occurs once all the amyloid units have been consumed. The formation of amyloid fibrils can be tracked in real time using spectrophotometer techniques. It's important to note that amyloid fibrils formed *in vitro* are not necessarily the same as those formed *in vivo*. Amyloids deposited or fibrilized within tissues may incorporate other chemical substances into the fibril, including glycosaminoglycans and lipids.[144,145] Aβ amyloid deposits in Alzheimer's disease aggregate with other compounds, including nucleic acids (reviewed in [146]).

Amyloid Recognition and Clearance

Amyloids fibrilize to form particulate matter with properties not unlike crystals or silica. For example, type 2 diabetes (T2D) is a multifactorial disease characterized by insulin resistance and β-cell destruction. Genetic susceptibility exacerbates the result of poor diet and a sedentary lifestyle to cause this metabolic disease. One hallmark of T2D is the deposition of amyloid within the pancreatic islet, derived from islet amyloid polypeptide (IAPP).[147] Amyloid deposition in the pancreas is not the underlying cause of T2D, but seems to exacerbate disease and correlates with β-cell loss.[148] Amyloidogenic IAPP triggers NLRP3 inflammasome activation and IL-1β

production, a process that seems to contribute to activation of innate immune cells within the pancreas.[149] Similar observations have been made in Alzheimer's disease in which Aβ amyloid activates the inflammasome and may contribute to disease progression.[150]

In addition to the inflammasome, there are a number of receptors that have been reported to recognize amyloids. The receptor for advanced glycosylation end products (RAGE) seems to bind a number of amyloids and contribute to their pathology. In mouse models of systemic amyloidosis RAGE is upregulated and binds amyloid A.[151] Exogenous soluble RAGE (sRAGE) was able to prevent disease progression in these mice.[151] RAGE has also been well studied in Alzheimer's disease, where it is thought to be one of the major contributors of receptor-mediated entry of β Amyloid into the CNS.[152] Interestingly, RAGE has also been implicated as a receptor for both DNA[153] and apoptotic cells[154] – therefore playing a targetable role in the progression of lupus nephritis.[155]

Amyloid-like protein aggregates are not necessarily static deposits. Although aggregates may at first function to sequester toxic protein, work from a number of fields has shown that they can be cleared if the initial insult is removed. For example, transgenic mice expressing the causative mutated protein for Huntington's disease (huntingtin) will develop amyloid-like protein deposits and Huntington's-like neurodegeneration.[156] However, if abnormal huntingtin is conditionally expressed and then turned off, abnormal huntingtin aggregates will be cleared and brain function restored[157] – suggesting that healthy cells can clear toxic protein aggregates. In the brain there is no lymphatic system, but pulsatile flow through the paravascular system is able to replace lymphatic function and promote interstitial solute clearance (reviewed in [158]). Solute clearance in the brain through this pathway seems to be important in the clearance of Aβ and reduced flow through this pathway might explain the accumulation of Aβ aggregates in the aging brain and Alzheimer's disease.[159]

The PDAPP transgenic mouse overexpresses mutant human APP and develops Alzheimer's-like neurodegenerative disease. In 1999 Schenk et al. demonstrated that immunizing PDAPP mice with Aβ could drastically attenuate Alzheimer's-like disease pathology.[160] This observation was replicated in other transgenic mouse models[161,162] – raising hopes that a vaccine might be

developed to treat Alzheimer's disease. Immunization with prions is also protective in mouse models of prion disease.[163] Unfortunately, after successful Phase I clinical trials, the Phase II trial for Aβ immunization was stopped due to several cases of meningoencephalitis.[164] It's possible that Aβ immunization caused an autoimmune-like reaction in the brain,[165,166] leading to encephalitis – but the exact cause of this side effect is still largely unknown. Despite this setback, this work suggests that directed immune responses are able to clear abnormal amyloid accumulation.

Pathologic Amyloids in Rheumatic Disease

An exhaustive analysis of amyloid-related pathologies is beyond the scope of this review. However, exploring the role of amyloid in autoimmune and autoinflammatory diseases is important for our interpretation of findings reported in this thesis. Reactive AA amyloidosis is associated with chronic inflammation and infection (particularly tuberculosis). It was the most prevalent form of systemic amyloidosis before the advent of modern medicine and became relatively rare with the control of bacterial disease like tuberculosis.[167]

Chronic inflammatory diseases became the leading cause of systemic AA amyloidosis, especially rheumatoid arthritis[168] but including ankylosing spondylitis, psoriatic arthritis, and Crohn's disease. Amyloidosis is also a common manifestation of autoinflammatory diseases like familial Mediterranean fever.[169] Serum Amyloid A (SAA, the precursor of AA amyloid) is an acute phase protein that is expressed during inflammation and reaches persistently high plasma concentrations in these diseases. It is thought that persistently high concentration of protein and chronic inflammation lead to misfolding, amyloid deposition, and organ dysfunction. As we have become better at treating patients with these inflammatory diseases, the occurrence of systemic AA amyloidosis has become rare (reviewed in [170]).

Although amyloidosis may be common in some untreated rheumatic diseases, SLE patients do not seem to develop amyloidosis.[171,172] Only limited case reports can be found in the literature describing amyloidosis in lupus patients.[173,174] Interestingly, however, some SLE patients (as well as RA patients) seem to develop antibodies to AA amyloid.[175] This would suggest that SLE

patients are exposed to misfolded amyloid-like proteins. The periodic nature of lupus (i.e. flares followed by disease inactivity) may prevent the development of amyloidosis, as AA amyloid may be cleared by immunological means[176] between flares. How the immune response to endogenous amyloids may contribute to disease progression and the utility of these anti-amyloid antibodies as biomarkers remain to be explored. Consistent with an autoimmune response to endogenous amyloids, Clark et al found that patients with type 2 diabetes had increased autoantibody titers to IAPP, the precursor to islet amyloid which characteristically forms deposits in the pancreas.[177] It is important to note that antibodies to one amyloid may cross-react with other amyloids, suggesting that conclusions about exposure to a specific amyloid must be made carefully.[178]

Curli as a Functional Amyloid

As described above, it is thought that abnormal protein folding leads to formation of cytotoxic amyloid deposits, resulting in disease progression. Amyloids were therefore once thought to be pathologic by definition. However, it is now clear that amyloids or amyloid-like structures may have functional roles in both bacterial and mammalian cells (reviewed in [179]).

Curli is a functional amyloid in that it is used in bacterial biofilms for its amyloid-like properties and is produced in a highly regulated manner. Named for its coiled filamentous structure, curli was first described as a virulence factor in *Escherichia coli* (*E. coli*) isolates from cases of bovine mastitis.[180] Later, curli was also identified in *Salmonella Typhimurium* serovars.[181] Curli enables bacterial communities to form robust biofilms and adhere to both biotic and abiotic surfaces.[182] The cross-β quaternary amyloid structure makes curli very stable and highly resistant to mechanical and chemical stresses. Indeed, dissolving curli aggregates into peptide monomers requires powerful reagents such as concentrated formic acid. Curli thus provides protection to the bacterial biofilm. For example, biofilms containing curli are resistant to hydrogen peroxide[183] and sodium hypochlorite treatment.[184] Curli-producing bacteria are relatively common. *E. coli* isolates from healthy individuals represent commensal organisms which commonly contain the curli-producing operon.[185] Patients are also exposed to curli during urinary tract infections[186] and

sepsis.[187] The Tukel lab has recently found that bacterial nucleic acids enhance curli fibrillization and promote biofilm formation. Nucleic acids are therefore complexed with bacterial amyloids within biofilms.

The development of strategies aimed at combating bacterial biofilms has rightly received a significant amount of attention. However, the microstructures and biomaterials found within bacterial biofilms have benefited from millions of years of evolution – leading some researches to engineer biofilm products like curli into functionalized nanotechnology.[188,189] Despite this work, the systemic immune response to curli is still largely unexplored and will be the focus of investigation in chapter 3.

The Immune Response to Nucleic Acids

Preventing the Recognition of Self-Nucleic Acids

The immune system is finely tuned to recognize a wide range of signals that initiate inflammation and/or antibody generation (reviewed in [190]). However, the discrimination between pathogen- and self-nucleic acids can be particularly problematic. Some of the known mechanisms by which immune cells prevent an immune response to self-nucleic acids include: (1) the recognition of chemical differences between microbe and self-nucleic acids, (2) the degradation of extra-nuclear nucleic acids, and (3) the compartmentalization of nucleic acid receptors.

One of the major chemical differences between human and bacterial DNA is methylation. Unmethylated CpG motifs are much more common in bacterial DNA and activate immune cells.[191] The endosomal receptor TLR-9 recognizes these unmethylated CpGs as foreign DNA.[192,193] Sequence differences are also important in the recognition of DNA (reviewed in [194]). CpG-A (also called D-type) contains one palindromic CpG motif with a phosphodiester (PO) backbone and a phosphorothioate (PS) poly(G) tail. CpG-B (also called K-type) contains multiple non-palindromic CpG motifs and is completely phophorothioated. The sequence dependence of TLR recognition is dramatically demonstrated by CpG oligonucleotides (ODNs) that actively inhibit TLR signaling. In 1998 Krieg et al. found that some adenovirus-derived CpG sequences

could inhibit stimulatory CpGs.[195] Later, inhibitory motifs capable of inhibiting TLR stimulation were found in other organisms.[196,197] Recently, it has been shown that while TLR-9 will dimerize when bound to agonist CpG sequences, inhibitory ODNs fail to dimerize TLR-9 – remaining in the monomeric state.[198]

Another measure that prevents the recognition of nucleic acids is active degradation. When a cell dies by apoptosis nuclear contents stay sequestered inside apoptotic bodies and nucleases digest chromatin to prevent the buildup of self-DNA. Indeed, impaired clearance of apoptotic cells is known to be associated with the development of autoimmunity (reviewed in [199]). Specifically, caspase-activated DNase (CAD) cleaves chromatin during apoptosis.[200,201] Lupus-prone mice lacking this DNase have higher levels of autoantibodies and worse lupus nephritis, demonstrating that failure to degrade nucleic acids may promote autoimmunity.[202]

A third safeguard against the immune response to nucleic acids is the sequestration of nucleic acid sensors to the interior of the cell, where they are only exposed to nucleic acids from ingested pathogens. Indeed, forced expression of TLR-9 at the cell surface leads to uncontrolled TLR-9 signaling and autoimmunity.[203] Compartmentalization of TLRs in the endosome also allows TLR recognition of nucleic acids to be pH dependent. TLRs 7, 8, and 9 contain a long insertion loop (Z-loop) between leucine rich repeat (LRR) 14 and 15. Proteolytic cleavage of the Z-loop is required for TLR signaling,[198,204] ensuring that only nucleic acids found in the endosome stimulate immune cells. This safeguard may fail when self-nucleic acids are complexed with autoantibodies or inflammatory proteins – as discussed in the following sections.

The Breakdown in Tolerance to Self-Nucleic Acids

Nucleic acids play a complex role in the pathogenesis of lupus since they are the primary self-antigens and, either as part of immune complexes with autoantibodies[205] or carried by chaperones like heat shock proteins and HMGB-1, are DAMPs that activate DCs through TLR7 and TLR9 stimulation and induce the production of Type I IFNs.[206,207] Oligonucleotides that inhibit the response to TLR7 and TLR9 ameliorate disease in lupus prone mice.[208] Similarly the genetic deficiency of TLR7, or TLR7 and TLR9 combined prevent disease, while the single

deficiency of TLR9 renders the disease worse, suggesting a double-edge effect of TLR9.[46] In our hands, DCs from lupus prone mice express constitutively high levels of Type I IFNs and IFN responsive genes and this IFN Signature was diminished by treatment with oligonucleotides inhibitory for TLR7 and TLR9, supporting a role for the response of DCs to nucleic acids in the excessive production of Type I IFNs in lupus.[41] The importance of TLR7 was further highlighted by discovery that the Yaa translocation of a piece of chromosome X, containing 16 genes including TLR7, to the Y chromosome induces in male mice a severe lupus-like disease by creating a duplication of TLR7.[209]

Necrotic cells are a source of immunogenic nucleic acids[210] as well as neutrophils undergoing NETosis.[211] Neutrophils are critical to innate immunity and may play an important role in the pathogenesis of SLE (Reviewed in [212]). When neutrophils encounter activation signals (e.g. a microorganism, inflammatory cytokines) they may undergo a specialized form of cell death called NETosis, whereby the neutrophil releases its intracellular and granular contents into the extracellular space.[213] DNA from the neutrophil forms a net-like scaffold for antimicrobial granule components, acting as a trap for foreign material. Some SLE patients seem to have a defect in the clearance of NETs[214] – leading to the proposal that NETs may be a source of autoantigens in systemic autoimmune disease like SLE. The formation of NETs requires peptidylarginine deiminases (PAD) and NADPH oxidase (Nox2). Some mouse models support the notion that NETs may be involved in the pathogenesis of lupus. Mariana Kaplan's group found that neutrophils from lupus-pone NZM2328 mice are more sensitive to NET-inducing stimuli, producing more NETs compared to Balb/c and C57BL/6 controls.[215] They also show that NZM mice produce anti-NET autoantibodies and that a NETosis inhibitor (a PAD-inhibitor) could improve some vascular outcomes in these mice, although proteinuria was not significantly improved.[215] Recently, Knight et al performed a similar trial in MRL/*lpr* mice with a novel PAD inhibitor. They show that, similar to the NZM2328, MRL/*lpr* neutrophils are primed for NET formation and that MRL/*lpr* mice have anti-NET autoantibodies. They demonstrate that their new PAD inhibitor can improve vascular function, reduce the IFN signature in the kidney, and decrease proteinuria in MRL/*lpr* mice.[216]

Conversely, work from Mark Shlomchik's group has suggested that NETosis is not involved in murine lupus. They demonstrate that MRL/*lpr* mice with a Nox2 null allele developed markedly worse disease compared to MRL/*lpr* controls – with increased autoantibody titers and exacerbated renal disease.[217] Together these data show that neutrophil biology is an important aspect of SLE pathogenesis, although the exact mechanisms are still unclear.

It's important to note that recognition of nucleic acids is not always pathogenic. This is particularly true in the gut where basal IFN signaling is required for the maintenance of the gut epithelium.[218] A healthy microbiome in the GI tract promotes gut fitness through the activation of TLRs[219-221] and is thought to modulate immune cells that circulate in the gut epithelium. Recently it has been proposed that, similar to commensal bacteria, enteric viruses can promote gut health and stimulate tissue repair in a type I IFN-dependent manner.[222]

Nucleic Acid Immune Complexes

One of the hallmarks of lupus is the formation of autoantibody-autoantigen immune complexes (auto-IC). These auto-ICs have profound effects, both perpetuating disease and causing end organ damage (see section Lupus Nephritis). The formation of relatively large immune complexes promotes the recognition of host nucleic acids, which intensifies the antigen-specific immune response to nuclear components. Early work suggested that large immune complexes are not processed as efficiently in SLE patients[223] – an observation similar to that of impaired cell death clearance.[224] Therefore, in addition to causing end organ damage, auto-ICs actively modulate immune cell function. IgG-based ICs activate DCs through the FcγRs, leading to CCR7 up-regulation and migration to lymph nodes – a process that seems to be overactive in lupus.[225] Auto-ICs containing apoptotic cell material can promote IFN-α production from pDCs in a FcγR-dependent manner,[226] and in cDCs auto-ICs activate both TLR-9-dependent and independent pathways.[227] B cells are also activated by auto-ICs, although surface IgM also seems to play a role.[205] This work demonstrates that the immune response to nucleic acids is enhanced when in complex with autoantibodies.

Protein-Nucleic Acid Composites

Double stranded DNA (dsDNA) is a major target for autoantibodies in SLE. Therefore the immune response to both bacterial and host nucleic acids are important areas of study. A number of reports have suggested nucleic acids in the context of a protein antigen can induce the production of autoantibodies to DNA. Indeed, naked DNA is poorly immunogenic, as T cell-dependent antigens are often protein. For example, in 1993 Tony Marion's group complexed DNA with a trypanosomal protein Fus1 and demonstrated that the DNA-Fus1 composite could induce autoantibodies.[228] In 2007 Lande et al. showed that the antimicrobial peptide LL37 forms complexes with host DNA which activates pDCs and results in inflammatory disease.[229] Neutrophil NETs may be an important source of protein-DNA composites that over-activate pDCs in SLE[230] (See above section: Preventing the Recognition of Self-Nucleic Acids). More recently Domizio et al. found that Balb/c mice immunized with amyloid fibrils complexed with nucleic acids produce autoantibodies with 12 weeks and go on to develop immune complex-associated kidney disease.[231]

Similar to proteins, the properties of nucleic acids also change when in complex with polysaccharides. CpG ODNs complexed with the soluble β-glucan schizophyllan creates a rod-shaped nanoparticle that enhances production of type I IFN and acts as a potent vaccine adjuvant.[232] In their study of schizophyllan-CpG composites, the Ishii group showed that schizophyllan binds Dectin-1, a receptor known to interact with other β-glucans like Zymozan. The adjuvant effect of this complex is dependent on TLR-9.[232]

High mobility group box protein 1 (HMGB1) is a well-established endogenous danger signal[233] – released by necrotic cells that have lost the membrane integrity[234] as well as secreted by phagocytic cells as a late mediator of inflammation.[235] HMGB1 is actively secreted by macrophages in response to LPS and TNF-α, and autocrine HMGB1 mediates and sustains DC activation and ability to induce Th1 polarization upon LPS stimulation.[236] HMGB1 is significantly increased in the serum of patients with brain and myocardial ischemia[237] and in septic patients in which it mediates the late phase of septic shock.[238] HMGB1 is recognized by the receptor for advanced glycation end products (RAGE)[239] as well as by TLR2 and TLR4.[240] Serum HMGB1

levels, as well as anti-HMGB1 autoantibody titers, positively correlate with disease activity in SLE patients.[241,242] It is thought that HMGB1 may contribute to autoimmunity by binding nucleic acids and promoting their recognition by innate immune cell.[243,244] Indeed, HMGB1-containing nucleosomes from apoptotic cells have been shown to induce secretion of pro-inflammatory cytokines and expression of costimulatory molecules in macrophages and DCs and the administration of HMGB1-nucleosome complexes in mice induces lupus-like autoantibodies.[245,246] Reactivity to nucleosomes in the kidney may be involved in the development of lupus nephritis.[247] Together this work suggests that exposure to immunogenic protein-DNA composites may be a mechanism by which peripheral tolerance is broken in genetically susceptible individuals.

Dendritic Cells in the Context of Autoimmunity

In chapters 3 and 4 we will use dendritic cells (DCs) as prototypic innate immune cells in order to model immune responses to inflammatory stimuli in the context of autoimmunity. Below I will review DC biology with special attention to DCs in autoimmune disease.

Dendritic Cell Subtypes

Dendritic cells play a critical role in immune surveillance – recognizing invading micro-organisms via a variety of PPRs and regulating the immune response via antigen presentation and cytokine production. In the absence of pathogens or tissue damage, DCs tolerize lymphocytes reactive for self-antigens, thereby preserving immunologic tolerance.[248,249] There are a number of DC subsets with characteristic functions and surface markers including myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).[249,250] The mDC subpopulation is the major subset involved in inducing T cell responses, whereas pDCs are the major source of type I IFNs – often in response to viral infection.[251]

Dendritic cells are specialized antigen-presenting cells (APCs) that help maintain peripheral tolerance[252] and orchestrate the adaptive immune response by presenting both endogenous and exogenous antigens, and through the production of immune modulatory factors, such as costimulatory/inhibitory molecules and cytokines.[253,254] The DC lineage comprises a complex

population of several subsets – with recent advances demonstrating intricacies of gene transcription [255] and tissue localization [256] that define each population. Indeed, DC subsets vary greatly in terms of lineage markers, cytokine production, and ability to stimulate different immune responses. DC phenotypic variability is largely dependent on the tissue in which they reside or emigrate from [i.e. Langerhans cells from the skin [257] or CD103+ DCs from the gut [258]]. For simplicity, DCs are generally categorized as conventional DCs or plasmacytoid DCs. DCs are either resident within the secondary lymphoid organs like lymph nodes and spleen, or migratory, coming into the lymph nodes from the peripheral tissues.[254]

Conventional DCs (cDCs) are professional antigen-presenting cells. They reside in virtually all the tissues of our body in a predominantly antigen-capturing state. In the absence of danger signals, DCs maintain immunologic tolerance by routinely migrating to the draining lymph nodes and presenting self-antigens to lymphocytes in a tolerogenic manner.[249,259] Upon exposure to danger signals, DCs undergo a process of maturation/activation, which allows them to present in an immunogenic fashion the antigens captured in the periphery.[260,261] Immunogenic DCs stimulate T cells by presenting antigen as peptide-MHC complex (Signal 1) along with a repertoire of costimulatory molecules (Signal 2) and cytokines (Signal 3), which influence the specific type of immune response (i.e. Th1 vs Th2 vs Th17).[254,262] Plasmacytoid DCs (pDC) are relatively rare DCs that probably play less of a role in antigen-presentation, but are the major producers of Type I Interferons (IFNs). Type I IFNs act as danger signals during viral infections and contribute to the characteristically high levels of IFN responsive genes produced by the blood mononuclear cells of patients with SLE (i.e. the IFN signature).[263,264]

Dendritic Cells in Systemic Autoimmunity

The importance of DCs in the pathogenesis of autoimmune diseases is supported by the effects of their manipulation in experimental animal models. Indeed, the constitutive genetic depletion of DCs has been reported to induce spontaneous development of autoimmunity characterized by autoantibodies against nuclear and tissue-specific antigens, multi-organ lymphocyte infiltration, and severe tissue damage, especially in the intestine.[265] Therefore, DCs

may be necessary for the establishment and maintenance of immunological self-tolerance.[259] Paradoxically, DCs also seem to be powerful inducers of autoimmunity. Mice in which DCs accumulate as a result of a DC specific defect in apoptosis also develop chronic lymphocyte activation and lupus-like systemic autoimmunity.[266] Consequently, uncontrolled DC functions seem to be sufficient to break immunological tolerance and prime for autoimmune reactions. In many experimental models of autoimmunity, it has been shown that DCs indeed accumulate in the secondary lymphoid organs and in the tissues targeted by the autoimmune process,[267-270] although the causative mechanism for this accumulation remains unclear. Further support comes from studies demonstrating that priming with immunogenic (highly activated) DCs loaded with self-antigen induces or accelerates autoimmunity,[271,272] while the administration of tolerogenic DCs reduces disease and has consequentially been proposed as a potential therapeutic strategy in many models of autoimmune disease, such as type I diabetes [273] and experimental autoimmune encephalomyelitis (EAE).[274,275]

DCs may play a pathogenic role in autoimmunity by presenting self-antigens to T cells in an immunogenic fashion and by collaborating in the activation of autoreactive B cells. To do so, DCs have to be activated and express immunogenic costimulatory molecules and produce pro-inflammatory cytokines. Indeed, much evidence shows abnormally activated DC phenotypes in patients with different autoimmune diseases, as well as in murine models of autoimmunity (reviewed in [276]). In some autoimmune strains of mice, DCs generated in culture show abnormalities even when they are removed from the autoimmune microenvironment (i.e. DCs from young mice, before the onset of the disease).[41] These results suggest the existence of a genetic defect intrinsic to DCs, leading to their excessive activation, possibly through an uncontrolled production of danger signals.[71] In other cases, abnormalities were present only in DCs *in vivo* or isolated *ex vivo* from diseased mice, therefore pointing at a direct association with the autoimmune process[269,277] and suggesting that in some cases DC abnormalities are a consequence rather than the cause of the autoimmune environment.

Cytokine Dysregulation in SLE

In chapter 4 we will explore cytokine dysregulation in DCs derived from Sle1,2,3 mice, with particular attention to the crosstalk between type I IFN and IL-10. Below I will review the biology of type I IFN and IL-10 in the contest of autoimmunity.

Type I Interferon

Recent advances in our understanding of innate immunity have demonstrated that over-expression of both type I interferons (IFN) and IFN-stimulated genes (ISGs) contribute to the immunologic alterations seen in SLE.[44] Evidence from SLE patients[43] and lupus-prone mice[41] suggests that the increased IFN signature could be the result of shifts in dendritic cell function, particularly in response to environmental triggers and cell death (reviewed in references[278-280]).

Type I IFNs activate DCs *in vitro* and act as adjuvant *in vivo*.[281] They are secreted by virally infected cells[282] as a primary danger signal to alert both neighboring tissue cells and local immune cells to the presence of a viral infection. They are also secreted by immune cells as an amplifier of innate immunity.[283] Type I IFNs include IFN-alpha (α), -beta (β), -kappa, -delta, -epsilon, -tau, -omega, and -zeta. In this thesis "Type I IFNs" will refer to IFN-α and β unless otherwise specified. Type I IFNs mediate the activation of DCs to promote adaptive immune responses like cross-priming and isotype switching of responding murine B cells toward IgG2.[263] Type I IFNs are particularly important in autoimmunity and especially in SLE in which an IFN signature is present (the IFN signature is an abnormally high expression of Type I IFN Responsive genes in immune cells and tissues).[43,44,71,284] The hyper-activation of Type I IFNs may play a role in the early and most acute phases of disease because the IFN signature is common in pediatric patients[44] and in adults with central nervous system involvement and nephritis.[43] Supporting this notion, we have recently discovered that myeloid DCs and pDCs from lupus prone Sle1,2,3 mice express an IFN signature before the onset of autoimmunity. Even when generated in culture from bone marrow precursors, depleted of mature T and B cells, macrophages, and dendritic cells, and thus, severed from the *in vivo* pro-autoimmune

environment, DCs from Sle123 mice expressed an IFN signature.[41] These results indicate that DCs are an independent cellular source of the pathogenic danger signals implicated in lupus.[41,71] Since polymorphisms in genes that are part of the signaling pathway of Type I IFNs, such as IRF5, IRF7 and STAT4, are associated with a higher risk of developing SLE,[9] the over-expression of Type IFNs in these patients may be the result of the combination of genetic predisposition to produce high levels of Type I IFNs and exposure to danger signals that stimulate IFN production.[71,285]

Interleukin-10

Interleukin (IL)-10 has broad anti-inflammatory properties and is critical to the resolution of inflammation (reviewed in [286]). IL-10 largely signals through STAT-3 and was first recognized for its ability to suppress production of pro-inflammatory cytokines.[287,288] Furthermore, IL-10 deficient mice are highly susceptible to inflammatory disease, particularly in the gastrointestinal tract. In apparent contradiction, serum IL-10 levels are often elevated in lupus patients with active disease[289,290] and relatives of SLE patients have elevated circulating IL-10.[291] Polymorphisms in the IL-10 promoter are associated with SLE.[292,293] IL-10 is a B cell growth factor (reviewed in [294]) and may therefore promote autoantibody production.

Autoantibody-autoantigen immune complex formation is a hallmark of SLE. Immune complexes are known to increase IL-10 production crosslinking Fcγ receptors.[295-297] Indeed, immune complexes from SLE sera can induce IL-10 production.[298] Auto-immune complexes, composed of autoantibody and SLE auto-antigens DNA and ribonucleoproteins, also induce type I IFNs,[299] possibly as a result of immune complex shuttling of nucleic acids to TLR-9 and -7.

Mouse models of SLE show apparently conflicting roles for IL-10. In MRL/*lpr* mice, increased IL-10 limits disease pathology,[300] and IL-10-/- B6 mice develop more autoantibodies and nephritis upon injection with dead cell-loaded DCs than B6 mice.[301] However, in NZB/W-F1 mice administration of anti-IL-10 neutralizing antibody delayed onset of lupus-like disease.[302] This inconsistency may be due to the mechanistic differences in pathogenesis of these models. IL-10

may be protective in monogenic and inflammatory models, whereas it may be pathologic in polygenic models like the NZB/W-F1 and Sle1,2,3.

In DCs, IL-10 suppresses PI3K/Akt and IkappaB kinase activity[303] and enhances the production of pentraxin 3 in response to LPS.[304] DCs expressing IL-10 are also important in inducing regulatory T cell subsets.[305] These effects have not been evaluated in the pro-inflammatory context of the IFN signature, in which case the inhibition of NF-κB activity and the stimulation of Tregs may be altered. Additionally, although IL-10 may be cytoprotective for some cell types,[306] IL-10 promotes activation-induced cell death of lymphocytes in SLE[307] and is a potent pro-apoptotic signal for DCs.[308,309] How IL-10 modulates DC survival is still unclear, but may involve suppression of pro-survival factors like Bcl-2.[309]

The Crosstalk between Type I IFN and IL-10

Interestingly, type I IFNs may affect IL-10 production and function, although this may be context dependent. In one report, IFN-α stimulates T cells and monocytes to produce IL-10.[310] Another report argued that type I IFN stimulates IL-10 production from T cells, but inhibits IL-10 production from monocytes.[311] A better understanding of these cytokine networks and their perturbations will improve our ability to develop next generation therapies for SLE.

Some evidence suggests that IL-10 may signal through pro-inflammatory pathways when cells are primed with Type I IFNs. In this case IL-10 gains the ability to signal through STAT-1 and promote pro-inflammatory gene expression.[312,313] Increased STAT-1 signaling may in turn oppose the pro-survival and anti-inflammatory properties of IL-10 STAT-3 signaling (reviewed in [314]).

The MAP kinase Erk can promote IL-10 production via remodeling at the *Il-10* locus.[296] In a comparison of B6 and B6.*Sle1ab* mice, Edward Wakeland's group found that splenic B cells from B6.*Sle1ab* mice had increased levels of phospho-STAT3 (p-STAT3) and phospho-Erk (p-Erk). This aberrant STAT3 and Erk signaling seems to promote autoantibody production in autoimmune B cell cultures and *in vivo*.[315] Increased STAT3 and MAP kinase activation in B cells from lupus-prone mice was corroborated in a study which also showed these proteins are

associated with human disease.[316] Consistent with these findings, increased p-Erk and p-JNK in peripheral blood mononuclear cells is associated with disease activity in lupus patients.[317] Recently, rare activating mutations in STAT3 were described in families with early onset multi-organ autoimmune disease.[318]

The role of Erk in lupus pathogenesis is not entirely clear however; as a significant line of evidence from Bruce Richardson's group has shown that T cells from lupus patients and lupus-prone mice have decreased Erk signaling which can contribute to spontaneous and drug-induced murine lupus. It is not clear if this defect is specific to T cells. To our knowledge, Erk specific inhibitors have not been tested in murine pre-clinical studies as a therapeutic strategy – although a spleen tyrosine kinase (Syk) inhibitor does limit disease progression in NZB/W-F1 mice[319] and Erk inhibition inhibits development of murine experimental arthritis.[320] In chapter 4 we explore the role of Erk in the crosstalk between Type I IFN and IL-10.

The Kidney

In chapters 3 and 4 we will explore the causes of SLE (i.e. environmental and genetic factors leading to autoimmunity). However, in chapter 5 we investigate therapeutic possibilities for the end organ damage characteristic of SLE, namely renal disease. Below I review the pathophysiology of lupus nephritis and the molecular pathways we intended to exploit as therapy for its development.

Lupus Nephritis

Renal disease remains a major cause of morbidity and mortality in lupus. It is thought that the majority of lupus patients have some form of renal pathology and as many as 50% will go on to develop clinically-evident Lupus Nephritis (LN).[321,322]

The pathogenesis of LN is complex and multifactorial but is thought to be largely mediated by immune complex deposition. A large body of literature demonstrates that autoantibody immune complexes and complement activation precipitate glomerulonephritis. However, some animal strains are more susceptible to kidney injury, suggesting that signals intrinsic to the kidney also

contribute to renal inflammation. Indeed, little is known about how resident renal cells themselves contribute to nephritis.

Different lupus-prone mouse strains seem to have slightly different responses to renal immune complex deposition. For example, it is thought that FcγRs play an important role in promoting inflammation in response to immune complex deposition within the kidney. This is supported by the observation that NZBW-F1 mice crossed with Fc $\gamma^{-/-}$ mice have largely ameliorated disease despite the presence of similar amounts of immune complex deposition in the kidney.[323] In contrast, Fc $\gamma^{-/-}$ MRL/*lpr* mice develop disease indistinguishable from that of Fc $\gamma^{+/+}$ MRL/*lpr* mice.[324] These results suggest that other underlying mechanisms variably contribute to kidney disease depending on the mouse strain. It is unclear why the absence of FcγRs would limit disease progression in one mouse strain but not the other.

Recently it has been shown that kidney-resident DCs constitutively present glomerular antigen in the renal lymph nodes and that these DCs are required to potentiate renal disease.[325] Additionally, it has been suggested that increased glomerular cell death in pre-nephritic kidneys from Lupus-prone mice directly provide autoantigen from the glomerulus.[326] This is supported by the observation that renal cells from B6 mice are potent producers of type I interferons (IFNs) and that the IFN response to nucleic acids leads to mesangial cell death.[327-329]

The Epithelial Growth Factor Family in Kidney Disease

The epidermal growth factor receptor (EGFR) family of transmembrane receptor tyrosine kinases is comprised of four members: EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4),[330] where the HER and ErbB nomenclatures generally refer to human and mouse EGFR-family receptors, respectively.[331] There are at least 11 ErbB ligands including epidermal growth factor (EGF) itself. Depending on the ligand-receptor combination, signaling can involve MAP/ERK, PI3K/Akt, and JAK/STAT pathways – leading to cell survival, growth, and proliferation. EGFR is up-regulated in human kidney disease,[332] and is particularly marked in lupus nephritis (unpublished observations, Kathleen Sullivan).

The EGFR family is important in the development and function of many organs including the GI tract. EGF is produced within the amniotic fluid, bathing the fetal GI tract, promoting gut development, and preventing TLR-4-mediated necrotizing enterocolitis[333] (NEC) (reviewed in [334]). Even after a full term birth, the GI tract remains functionally immature and immunodeficient. Breast milk provides components, including mucin, that contribute to the formation of gut mucous, creating a protective gel-like barrier and promoting osmotic homeostasis. Breast milk also contains bioactive peptides and cytokines that promote gut turnover and barrier function in the neonate.[335] EGF is a major component of breast milk that protects the neonate from NEC by stimulating cell growth and promoting intestinal barrier function.[336] The EGFR family is also important in the neonatal kidney development (reviewed in [337]) and has been associated with the development of polycystic kidney disease.[338]

The influence of EGFR-family receptors on murine kidney disease is complex, with conflicting results in various *in vivo* models. Some early work suggested that exogenous EGF could promote renal repair in renal ischemia-reperfusion injury,[339] mercury-induced renal damage,[340] and unilateral ureteral obstruction (UUO).[341] More recently, Chen et al found that either erlotinib (an EGFR kinase inhibitor) or renal proximal tubule cell-specific knockout of EGFR delayed renal recovery after ischemia-reperfusion injury, suggesting that EGFRs are required for tissue repair in the kidney.[342] EGFR may also have an important immune regulatory role as the EGFR ligand amphiregulin enhances regulatory T cell functions.[343] These studies suggest that EGFR signaling is protective in kidney disease.

In contrast, other work has suggested that EGFR-family signaling exacerbates kidney disease. EGFR inhibition with erlotinib limits angiotensin II-induced renal fibrosis[344]; EGFR blockade reduces UUO-induced renal fibrosis[345]; and transgenic mice expressing a dominant-negative EGFR have reduced pathology in two different murine models of renal injury (subtotal nephrectomy and renal ischemic injury).[346] Similarly, Bollee et al found that HB-EGF and EGFR signaling promotes nephrotoxic serum-induced rapidly-progressing glomerulonephritis.[347] This research suggests EGFR-family signaling promotes kidney disease. The disparate effects of EGFR-family signaling in murine kidney disease may have many technical causes, although

some have suggested that EGFR-family signaling is protective in acute kidney injury and becomes maladaptive in chronic renal disease.[348,349] In chapter 5 we will test the dual EGFR/ErbB2 dual kinase inhibitor lapatinib in the treatment of lupus nephritis. An overview of lapatinib will be provided in chapter 5 (see section “Study Design”).

CHAPTER 2- MATERIALS AND METHODS

Mice

C57BL/6 and 129 mice were bred and maintained in our colony. C57BL/6 mice were also purchased from Jackson Laboratory together with lupus-prone NZBxW/F1 mice and used for experiments at least two weeks after arrival to our animal facility. Female mice were used between 6 and 14 weeks of age for most of the experiments. Protocols for the use of animals were in accordance with the guidelines of and were approved by the Institutional Animal Care of Temple University, which is an American Association for the Accreditation of Laboratory Animal Care-accredited facility.

Bone Marrow-Derived Dendritic Cells

Mouse bone marrow-derived DCs (cDCs) were generated as previously described.[41] See Appendix Protocols for detailed method. Briefly, bone marrow precursors were flushed from femurs and tibias of indicated mice and cultured for 6 days in 24 well plates in complete IMDM (10% FBS, 1% penicillin/streptomycin, gentamicin and 2-mercaptoethanol) containing 3.3 ng/ml GM-CSF (BD Biosciences) or 1% conditioned media from GM-CSF-secreting cell line B7H1 (the generation of B7H1-conditioned media is descried in Appendix: Protocols). One milliliter of medium was added on day 3 and half of the medium was replaced on day 5 and subsequently each day until the culture was used. cDCs were used for experiments up to day 8.

In vitro Dendritic Cell Studies

DCs were generated as described above and stimulated with increasing concentrations of purified curli fibers or 20 μg/ml of polymerized CsgA R4-5, isolated genomic Salmonella DNA, or 20 μg/ml CsgA of R4-5 polymerized in the presence of 20 ng/μl genomic Salmonella DNA. Supernatants were collected at 6 hours post-stimulation for IL-6 and TNF-α production measurements and 24 hours for IL-12 production measurements. ELISA of supernatants were conducted using IL6, TNF-α, IL-12p70 ELISA kits (BD), according to manufacturer's instructions.

In chapter 4 resting DC cultures were stimulated at day 6 or 7 of culture with each of the following: 100 ng/ml LPS (Sigma-Aldrich), 10 µg/ml CpG-B 1826 (synthesized by IDT Biotechnologies), and 1 µg/ml R848 (Invivogen). B6 DCs were pretreated with 2500 U/ml IFN-α (Hycult Biotechnology) to perform IFN priming. When TLR7/9 inhibitors [ODN 2088, ODN 954, ODN 661 (20 µg/ml)[208]] were used (synthesized by IDT Biotechnologies), cells were treated with the inhibitors for 30 min prior to stimulation.

Dendritic Cell-Biofilm Co-Culture

To assess the production of pro-inflammatory cytokines by cDC exposed to biofilms, biofilms of S. Typhimurium wild type*, msbB*, and *ΔfliCfljB* strains were grown in 48 well tissue culture plates at an angle to allow for attachment of pellicle biofilms to the bottom of the wells. 10^6 DCs were placed on top of attached biofilms in 1ml tissue culture media containing streptomycin and penicillin. Supernatants were collected at 6 hours to measure IL-6 and TNF-α by ELISA, according to manufacturer's instructions (eBioscience).

For imaging, GFP-expressing DCs were generated from the bone marrow of global GFP knock-in mice (Generously provided by Dr. Ganea). GFP-DCs were then added to intact Salmonella biofilms stained with Congo Red or intact biofilms from an α-Tomato-expressing Salmonella (prepared by the Glenn Rapsinski under the direction of Dr. Tukel) and visualized by confocal microscopy (performed by Glenn Rapsinski).

Purification of Curli Fibers

Note: Preparation of curli was performed by members of the Tukel lab. However, the purification of curli fibers is described below as it may inform the interpretation of experiments exploring the immune response to curli.

Curli was purified as previously described from S. Typhimurium msbB mutant (RPW3).[350] Briefly, an overnight culture of msbB mutant S. Typhimurium is diluted 1:1000 in 500 ml of YESCA broth containing 4% DMSO to promote curli formation. Cultures were grown for 96 hours in 26^o C water bath with shaking. Cultures were then spun at 10,000 rpm for 15 minutes and

resuspended in 10 mM Tris HCl pH 8.0 supplemented with 0.1mg/ml of RNase A (Sigma) and 0.1 mg/ml of DNase I (Sigma). After addition of MgCl2 to a final concentration of 1 mM, the solution was incubated with shaking for 20 minutes at 37° C. Next lysozyme is added to a final concentration of 1 mg/ml then the preparation was incubated with shaking for 40 minutes at 37° C. Next, SDS was added to a final concentration of 1% SDS. Preparation was incubated with shaking for 30 minutes at 37° C and then centrifuged at 10,000 rpm for 15 minutes at room temperature. After the supernatant was discarded, the pellet was resuspended in 10 mM Tris HCl and the enzymatic treatments were repeated again as described above. Finally the pellet was washed twice with 10 mM Tris HCl. Pellet was resuspended in 2 ml of 2X SDS-PAGE Sample Buffer and boiled for 15 minutes. The sample is then loaded on to a preparative 12% SDS-PAGE Gel and run overnight. Curli was recovered from the well. The fibers are then washed twice with 95% ethanol and twice with water. Finally, curli preparation was sonicated for 15 seconds to break up any large clumps of fibers.

In Vivo Curli Stimulation

To test the stimulatory capabilities of amyloid/DNA composites, purified curli fibers (50µg) were injected intraperitoneally (i.p.) in 0.5 ml PBS. 24 hours later, mice were sacrificed and spleens harvested. Spleens from treated C57BL/6 mice were harvested and incubated in medium with collagenase/DNase for 45 min as previously described.[41] Single cell suspensions were made using 100micron cell strainer and RBCs were lysed using red cell lysis buffer. Flow cytometric analysis is described below.

For cell isolation, spleens were harvested as described above. Then, splenocytes were washed and sorted using magnetic beads (Miltenyi Biotech) – first depleting B cells with anti-CD19 magnetic beads and then positively selecting cells labeled by anti-CD11c magnetic beads in the CD19 negative fraction. The CD11c purity was >90% as assessed by flow cytometry analysis. The sorted cells were then processed for RNA extraction and qRT-PCR.

Cytokine ELISA

ELISAs for IL-12, IL-10, IL-6, and TNF-α were purchased from BD Bioscience and were performed according to manufacturer's instructions.

Acceleration of Lupus Onset

6-week-old NZBxW/F1 mice and B6 and 129 mice were injected i.p. with PBS, or 50μg curli/DNA composites or BSA in 0.5 ml of PBS either once a week, or three times a week. Serum was collected from mice approximately once a week via tail bleed to monitor for autoantibody production.

Autoantibody ELISA

Serum samples were analyzed for autoantibodies to dsDNA and chromatin as described by Cohen et al.[351] Briefly, 96-well PVC plates were coated with calf thymus dsDNA or chicken chromatin in borate-buffered saline (BBS) overnight at 4°C. Plates were blocked with BBS containing 3% BSA and 1% Tween 80 (BBT). Serum samples were diluted in BBT at 1:250 and added to plates in duplicate. A positive control derived from an aged MRL/lpr or an aged Sle1,2,3 mouse was used to confirm positivity. Four to six samples from young B6 mice were used as a negative control. Plates were washed with BBS and probed for bound autoantibodies using a biotinylated antibody to the Fc fragment of IgG diluted 1:5000 in BBT (Jackson ImmunoRes cat. No. 115-065-071). Other biotinylated secondary antibodies were used to assay for IgG subclasses and IgM. Avidin-alkaline phosphatase conjugate (Sigma, cat. No. A7294) was diluted 1:8000 and added to wells. Alkaline phosphatase pNPP liquid substrate (Sigma) was added and plates were read at 650nm and 405nm using ELISA plate reader.

Anti-curli ELISA

Described in detail in Appendix Protocols. Antibodies to curli amyloid were detected in serum samples using an ELISA that we developed and adapted from the autoantibody ELISA described above and in Cohen et al.[351] Curli was diluted in BBS to 3μg/ml and used to coat a 96-well PVC

plate overnight at 4°C. The ELISA for anti-curli proceeds following the same protocol as outlined for autoantibodies above.

Western Blot

We performed Western blotting using 30–50 mg total protein. In brief, protein samples were denatured by boiling for 5 min and loaded onto 10% Bis-Tris gels (Invitrogen). After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked for 1 h with blocking buffer (5% nonfat milk in PBS), then incubated overnight at 4˚C with the primary Abs diluted in blocking buffer with 0.1% Tween 20 simultaneously. Mouse anti-GAPDH (Santa Cruz Biotechnology) was used as a loading control. After incubating with primary Abs, the membranes were washed with PBS containing 0.1% Tween 20 (PBST) three times. Then, the membranes were incubated for 1 h with IR Dye 800 goat anti-rabbit and IR Dye 680 goat anti-mouse (LI-COR Biosciences) diluted in blocking buffer plus 0.1% Tween 20. The blots were then washed three times with PBST and rinsed with PBS. Proteins were visualized by scanning the membrane on an Odyssey Infrared Imaging System (LI-COR Biosciences) in both 700-nm and 800-nm channels.

Murine Urinalysis

See Appendix Protocols for detailed method. Urine was collected in 1.5ml Eppendorf tubes from individual mice at regular intervals by holding the mouse in one hand and gently massaging the abdomen/bladder with the free hand, taking care to direct the urine into the tube. Urine was analyzed for protein using a turbidity assay detailed in Seavey, et al.[352] Briefly, urine samples and a protein standard were added to a 96-well plate in duplicate. Samples and standards were diluted in PBS if necessary. Three percent sulfosalicylic acid (Ricca chemical company, cat. No. 8115-16) was added to the plate. The acid causes protein in the urine to precipitate – causing the solution to become cloudy. Plates were read on an ELISA plate reader at 450 nm. Alternatively, urine samples were added to urinalysis indicator strips (Siemens No 2309) and interpreted according to manufacturer's instructions. Urine sediment was analyzed by gently

spinning urine for 2 minutes at ~3rpm. Excess urine was removed, leaving about 10 to15 μl. Sediment was gently resuspended in leftover volume, transferred to a clean microscope slide, and viewed using an inverted microscope.

Blood Urea Nitrogen

Blood urea nitrogen (BUN) was measured in serum samples using colorimetric assays from BioAssay Systems (cat. No. DIUR-500) following manufacturer's instructions for 96-well plates.

Antinuclear Antibody Test

Pre-fixed Hep-2 seeded slides were purchased from MBL-Bion. Serum diluted 1:40 was incubated on Hep-2 cells for 30 minutes at room temperature, allowing autoreactive antibodies to bind Hep-2 cells. Cells were then rinsed with PBS and washed in Coplin jars for 5 minutes. Cells were stained with anti-mouse IgG-FITC (SouthernBiotech, Cat. No. 1030-04) for 30 minutes. After washing as above, cells were mounted and visualized using fluorescence microscopy (Olympus BX60 microscope with Diagnostics Instruments Camera and Spot Advanced Software).

Flow Cytometry

Splenocytes from *in vivo* experiments were washed and incubated with rat anti-mouse CD16/CD32 (clone 2.4G2) mAb for 15 min to block FcγRs. Cells were then stained for 30 min on ice using various combinations of antibodies. DC populations were stained using APC-conjugated anti-CD11c Ab, PerCP-Cy5.5-conjugated anti-CD19 Ab, PE-Cy7-conjugated-anti-CD11b Ab, FITC-conjugated-anti-B220 Ab, and either PE-conjugated- anti-CD86, or anti-CD80, or anti-CD40 Abs (eBioscience and BD Biosciences). DCs were designated as CD19- CD11c+ CD11b+. T cell populations were stained using FITC-conjugated anti-CD3 Ab, PE-Cy7-conjugated-anti-CD4 Ab, APC-conjugated anti-CD8 Ab, and PE-conjugated- anti-CD69 Ab (BD Biosciences). Cells were fixed in 1% formaldehyde and analyzed on a FACSCanto cytometer (BD Biosciences). FlowJo software was used for data analysis.

Quantitative RT-PCR

Gene expression in cDCs was analyzed by quantitative real-time RT-PCR (qPCR) using TaqMan probes. Briefly, RNA was extracted using ZymoResearch Quick-RNA MiniPrep (R1055). Complementary DNA (cDNA) was synthesized using a cDNA archive kit (Applied Biosystems). Pre-synthesized TaqMan primers and probes were purchased from Applied Biosystems and were used to quantify the expression of ifnb and IFN responsive genes (irf7 (Cat No.: Mm00516788), isg15 (Cat No.: Mm01705338)). Relative quantification of gene expression was calculated using the Ct value method ($\Delta\Delta$Ct)[41], and the normalized Ct values (against cyclophilin) were calibrated against the control sample (untreated B6 cDCs) in each experiment. Ct values were normalized within each sample to a housekeeping gene (cyclophilin) and samples within an experiment were normalized to the control sample, untreated or PBS treated cells.

Lapatinib in Murine Lupus Design

NZBW-F1 mice were used to test the efficacy of oral lapatinib for the treatment of murine lupus nephritis. Lupus-like disease was accelerated in all mice using a single i.v. injection of 10^9 particles of an adeno virus expressing murine IFN-α. Mice were randomly assigned to one of three groups: 1) vehicle, 2) prevention, and 3) treatment. The vehicle group received vehicle by oral gavage from the onset of the study. Vehicle was 0.5% hydroxypropyl methylcellulose (Sigma) and 0.1% Tween in sterile distilled water. The prevention group received drug in vehicle by oral gavage from the onset of the study. The treatment group received drug in vehicle by oral gavage only after the onset of confirmed proteinuria (indicative of lupus nephritis). The drug (lapatinib) was administered at 75mg per Kg body weight, twice daily, 6 days per week, by oral gavage. University biostatistician Dr. Huaqing Zhao was consulted for power calculations prior to starting this murine trial. We determined that 10 mice per group (three groups) would provide sufficient power to detect differences within groups. Mice were monitored for clinical signs of lupus-like disease as described elsewhere in the methods section.

Histology

Kidneys were collected from euthanized mice and placed in formalin. Formalin fixed kidneys were sectioned and stained by hematoxylin and eosin. Replicate sections were stained by immunohistochemistry using anti-HER2 (anti-ErbB2). Performed by collaborators at University of Pennsylvania. Slides were visualized using light microscopy.

Immunofluorescence

Kidneys were collected from euthanized mice and snap frozen in OCT. Frozen sections were cut approximately 12 microns thick and fixed on microscope slides (Temple histology core). Sections were blocked with 20% FBS and washed with PBS. Replicate sections were stained with FITC-conjugated antibodies: anti-mouse IgG, anti-mouse complement C3, or isotype control. Kidney sections from healthy C57BL/6 mice were used as negative controls. Slides were then washed, mounted, and visualized by fluorescence microscopy (Olympus BX60 microscope with Diagnostics Instruments Camera and Spot Advanced Software).

Primary Renal Cell Scratch Assay

Kidneys were excised and dissociated using a scalpel followed by incubation in DNase and collagenase for 1hr in FBS-free RPMI. Tissue/cells were then strained through a 100μm nylon strainer. Cells were spun and resuspended in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamine, and 1% insulin, transferrin, selenium. Cells were fed with fresh media on day 3 and 6 of culture and used by day 8.

Once confluent, cells were used to test tissue repair. Where desired, drug/IFN was added 30 minutes prior to scratching. A scratch was made in the renal cell monolayer using a 1000μl pipette tip. Pictures were taken for baseline scratch distance. After 24 hour incubation, representative pictures were again taken of the scratch in each condition.

Human Samples

Human plasma samples were generously provided by Dr. Roberto Caricchio and they were from the Temple Lupus Clinic cohort, approved by the IRB. All personnel were trained in the proper use of human samples and all assays were done in accordance with international law. Human samples were used to probe for anti-curli antibodies (as described above and in Appendix Protocols). Dr. Caricchio de-identified the samples before we could use them for the assays performed in this thesis. Once we had the results, Dr. Caricchio correlated the results of the anti-curli ELISA with the clinical status of the patients. A flare was defined as an increase of at least 3 points in the SLEDAI score from the prior visit. Recovery was defined as decreased SLEDAI score of at least 10 points. Inactive disease was defined as no change in SLEDAI score. De-identified plasma samples from age and sex matched healthy controls were generously donated by Dr. Jarrat Jordan (Janssen Pharmaceutical, PA).

Statistical Analysis

Statistical analysis completed for each figure was done as noted in the figure legends. Data were analyzed using Prism software (GraphPad, San Diego). University biostatistician Dr. Huaqing Zhao was consulted for power calculations and to determine appropriate statistical analysis. Sample t-test or two-tailed Student's t-test were used as appropriate. A p value of <0.05 was considered significant. One-way analysis of variance (ANOVA) with Bonferroni post-test was utilized to calculate statistically significant differences where indicated. Values of $p < 0.05$ were considered statistically significant.

CHAPTER 3- LUPUS PATHOGENESIS: THE IMMUNE RESPONSE TO CURLI

Results

Our colleagues in microbiology, under the direction of Dr. Cagla Tukel, have recently found that amyloid-based biofilms are abundant in nucleic acids (see chapter 1, sections on biofilms and curli amyloids). Here we investigated the immune response to curli-DNA composites.

Curli Potently Activates Innate Immune Cells

We first asked how bone marrow-derived DCs (BMDCs) would respond to a dose titration of curli isolated from *Salmonella* biofilms. Figure 1 shows proinflammatory cytokine production by BMDCs from B6 mice in response to a wide range of curli concentrations. Curli was able to induce a robust production of IL-6 and IL-12 from BMDCs even in the nanogram range (Figure 1). Similarly B6-derived BMDCs also expressed high levels of *Ifnb* and IFN-stimulated gene *Isg15*, indicating that curli stimulates production of Type I IFNs (Figure 2).

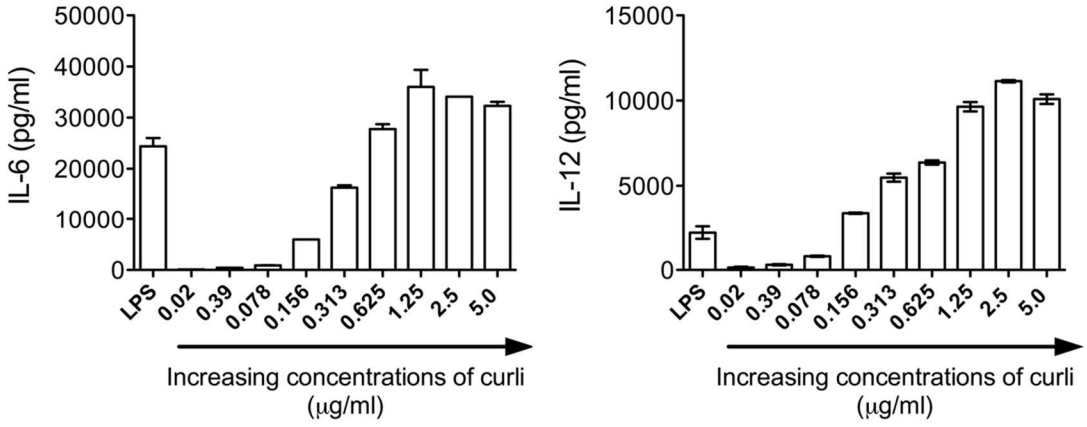

Figure 1: Curli-induced cytokine production by BMDCs
Proinflammatory cytokine production from BMDCs stimulated with a dose titration of DNA-containing purified curli isolated from *Salmonella msbB* mutant biofilms. Cytokine production was compared to LPS (100ng/ml) 24 hours after simulation.

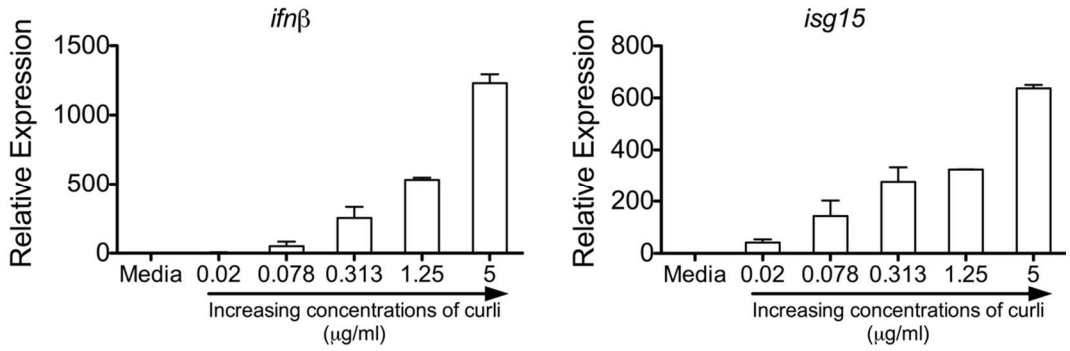

Figure 2: Curli-induced IFN gene expression in BMDCs
mRNA expression of interferon beta (*ifnß)* and IFN-stimulated gene *isg15*, from BMDCs generated *in vitro* from bone marrow of C57BL/6 mice stimulated for 6 hours with curli/DNA composites isolated from *Salmonella* biofilms. All of the conditions were normalized against the control (untreated DCs in medium only). Results are average of three independent experiments.

Next we sought to visualize the interaction between BMDCs and curli-based biofilms. To this end we used a global GFP-expressing B6 mouse to make BMDCs constitutively expressing GFP. GFP-DCs were then added to intact *Salmonella* biofilms stained with Congo Red or intact biofilms from an α-Tomato-expressing *Salmonella* and visualized by confocal microscopy (Figure 3). Dendritic cells interacted with the biofilm, sending dendrites into the biofilm and phagocytizing free bacteria, but were unable to penetrate into the biofilm matrix (Figure 3).

Curli and Nucleic Acids Synergize to Activate DCs

To determine how nucleic acids contribute to DC activation we used a nucleic acid-free synthetic curli peptide comprised of the 4th and 5th repeats of the major curli subunit CsgA (CsgAR4-5). This synthetic curli forms amyloid fibrils similar to that of wild type curli. Synthetic curli was fibrilized with and without *Salmonella* genomic DNA (gDNA). We stimulated BMDCs with *Salmonella* gDNA, fibrilized synthetic curli, or with synthetic curli fibrilized with *Salmonella* gDNA. We then measured proinflammatory cytokines IL-6 and IL-12 after 24 hours. BMDCs stimulated with synthetic curli or *Salmonella* gDNA alone produced minimal amounts of IL-6 and IL-12. However, BMDCs stimulated with synthetic curli fibrilized with *Salmonella* gDNA produced

significant amounts of IL-6 and IL-12 (Figure 4), suggesting that bacterial DNA complexed with curli represents a composite danger signal and synergizes to activate innate immune cells.

To confirm that biofilm isolated curli synergizes with nucleic acids we next complexed curli with CpG oligomers. We incubated curli and CpG together at 4°C for 24 hours, then washed curli of free floating CpG, leaving only curli-CpG composites. Stimulating BMDCs with curli-CpG composites induced higher levels of IFN gene expression compared to either curli or CpG alone (Figure 5). Together these results suggest that curli can facilitate the recognition of nucleic acids, acting as synergistic danger signals for innate immune cells.

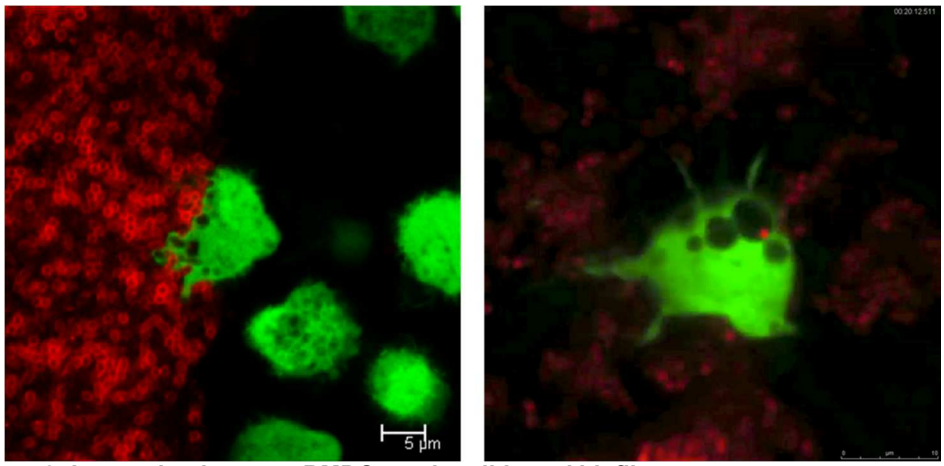

Figure 3: Interaction between BMDCs and curli-based biofilms
BMDCs were generated from B6.GFP mice and were incubated with intact curli-based *Salmonella* biofilms. Left) GFP-DCs in contact with the edge of a Congo Red-stained *Salmonella* biofilm is shown. Congo Red staining makes visible the curli amyloid corona surrounding each bacteria within the biofilm. Right) A GFP-DC within an α-Tomato-expressing *Salmonella* biofilm. Image shows a red bacterium within the DC phagolysosome. Cells were visualized by confocal microscopy (performed by Glenn Rapsinski).

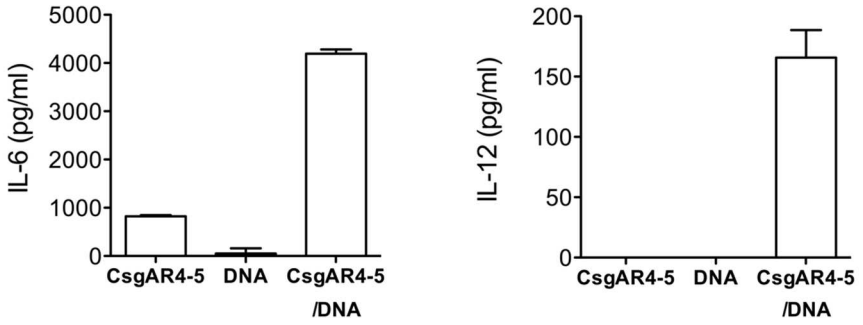

Figure 4: Cytokine production by BMDCs in response to synthetic curli
The 4th and 5th repeats of the main curli peptide (CsgA) was synthesized and fibrilized into amyloid in the presence of absence of genomic salmonella DNA. BMDCs were stimulated with the fibrilized synthetic peptide (CsgAR4-5), salmonella DNA (DNA), or synthetic peptide fibrilized in the presence of salmonella DNA (CsgAR4-5/DNA).

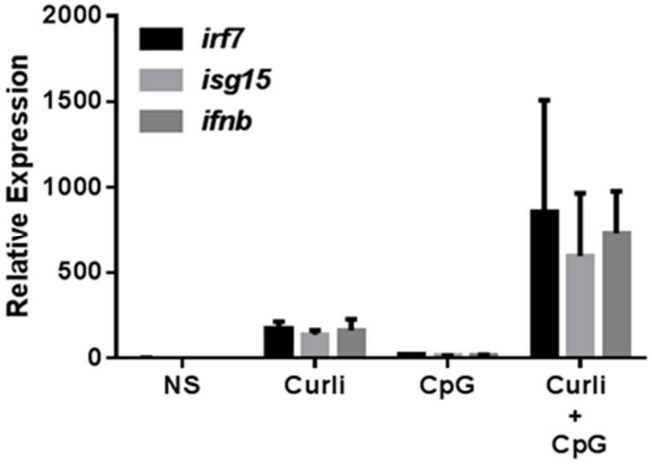

Figure 5: IFN-stimulated gene expression after curli-CpG complex stimulation
Biofilm-isolated curli was incubated with CpG at 4°C for 24hrs. Curli was washed of free CpG and BMDCs were stimulated with curli, CpG, or the curli-CpG composite. IFN gene expression was measured by qRT-PCR.

Effects of Curli on the Adaptive Immune System

To investigate the effects of curli/DNA composites on adaptive immune cells *in vivo*, we injected mice i.p. with either 50μg of curli/DNA composites purified from Salmonella biofilms or PBS and analyzed markers of activation in splenic populations 24 hours later. Injection of 50 μg/mouse of curli/DNA composites up-regulated the expression of the co-stimulatory molecule CD86 on CD19+ B cells and the activation marker CD69 on both CD4+ and CD8+ T cells after 24 hours (Figure 6), suggesting that curli/DNA composites stimulate polyclonal activation of T and B cells. It also caused an increase of CD11b+Ly6C+ inflammatory myeloid cells in the spleen (Figure 7). Moreover, curli/DNA composite injection induced DC activation in vivo, as splenic CD11c+ DCs up-regulated both co-stimulatory molecules CD86 and CD80 (Figure 6) and highly expressed the ISGs, irf7 and isg15, (Figure 6). These results suggest that bacterial amyloids, and in particular the curli/DNA composites, represent a new class of danger signals that stimulate innate immune cells and induce polyclonal activation of adaptive immunity.

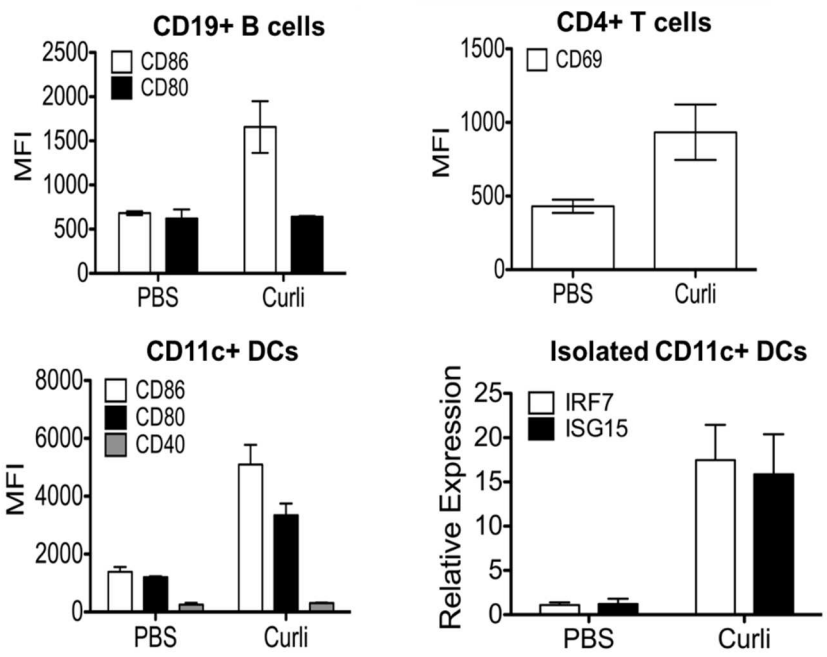

Figure 6: Curli activates immune cells *in vivo*
Flow cytometry analysis of the expression of activation markers in T-cells and B-Cells and in DCs from spleens of C57BL/6 mice 20 hours post i.p. curli injection; (lower right) mRNA expression of two interferon-stimulated genes (ISGs), irf7 and isg15, from C57BL/6 splenic CD11c+ DCs isolated with magnetic beads from the same mice. Results are shown as averages and STDEV, n=3 per group.

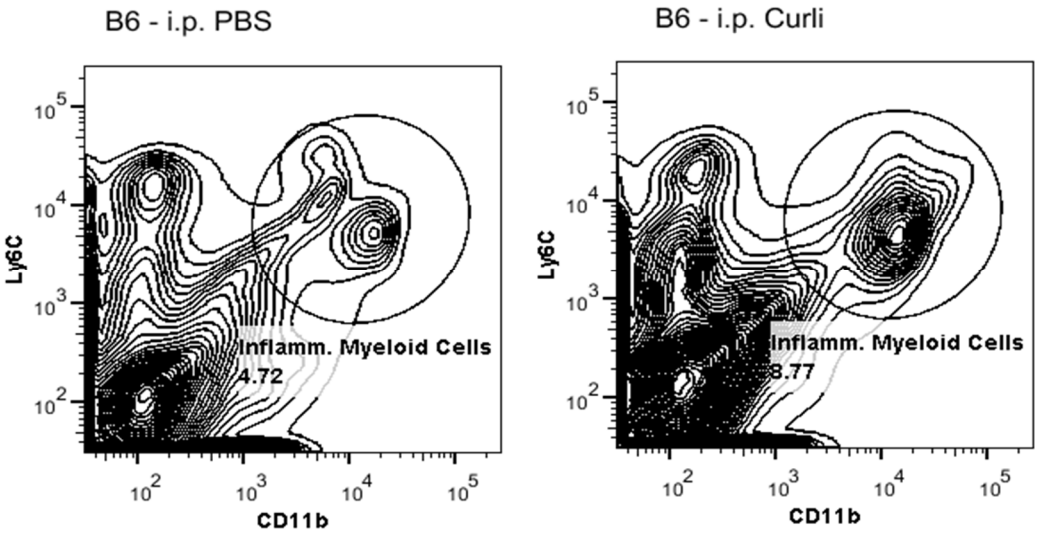

Figure 7: Curli induces myeloid cell activation in B6 spleens
Flow cytometry analysis of the expression of myeloid activation markers from spleens of C57BL/6 mice 20 hours post i.p. injection with 50µg of curli. Representative figures are shown, n=3 per group.

Curli/DNA Composites Promote Autoimmunity

Next, we asked whether the naturally occurring curli/DNA composites found in bacterial biofilms could induce autoimmunity in the absence of any added adjuvant. Since we found that curli/DNA composites stimulate the production of Type I IFNs (Figure 2), which accelerate disease onset in lupus-prone mice,[51] we hypothesized that exposure to curli/DNA composites may accelerate autoimmunity. We therefore injected 6-week-old, pre-diseased, female NZBxW/F1 mice i.p. with PBS or 50 µg of curli/DNA composites once a week, or three times a week; and monitored autoantibody production. Mice injected with PBS remained negative for autoantibodies up to 20 weeks of age. Mice injected with curli/DNA composites rapidly produced anti-dsDNA and anti-chromatin autoantibodies, a hallmark of lupus autoimmunity (Figure 8). As a protein control, we injected 50 µg of Bovine Serum Albumin (BSA) i.p. into NZBxW/F1 age and gender matched mice and found that injections with BSA did not induce development of autoantibodies (Figure 9), suggesting specificity for the autoimmunogenicity of curli.

Other adjuvants, including TLR ligands, can accelerate murine lupus.[78,353] However, curli/DNA composites induce autoantibody production very quickly and consistently, with all mice becoming positive for anti-dsDNA within two weeks of the first injection. We confirmed lupus autoimmunity in curli/DNA treated mice by antinuclear antibody test (ANA). PBS treated mice remained ANA negative while curli treated mice became ANA positive (Figure 10), with an initial perinuclear/endoplasmic reticulum staining pattern; this pattern then evolved into a nuclear staining pattern by 15 weeks of age, resembling the pattern expressed by aged diseased Sle1,2,3 mice, another strain of mice that spontaneously develop lupus.[38] The progression of positive ANAs suggest that curli/DNA treated mice develop true lupus autoimmunity.[354]

We next analyzed the subclasses of autoantibodies that are induced by curli/DNA composites. We found that anti-dsDNA and anti-chromatin autoantibodies were mostly of immunoglobulin G (IgG) subclass 2a and 2b, as in diseased Sle1,2,3 lupus-prone mice (Figure 11, Figure 12). Since IgG2a and IgG2b subclasses are generally considered pathogenic in systemic autoimmunity,[323,355] our results indicate that curli/DNA composites promote the production of autoantibodies pathogenic in lupus.

We next asked if curli/DNA composites could induce autoantibody production in mice that are not considered to be lupus-prone. We first injected curli/DNA composites into SvJ-129 mice, which do not develop lupus spontaneously but are genetically predisposed to autoimmunity.[356] We injected 6-week-old, female SvJ129 mice i.p. with PBS or 50 µg of curli/DNA composites three times a week and monitored autoantibody production. We found that these mice rapidly produced both anti-dsDNA and anti-chromatin autoantibodies (Figure 13), confirming that bacterial amyloid/DNA composites are sufficient to trigger autoimmunity.

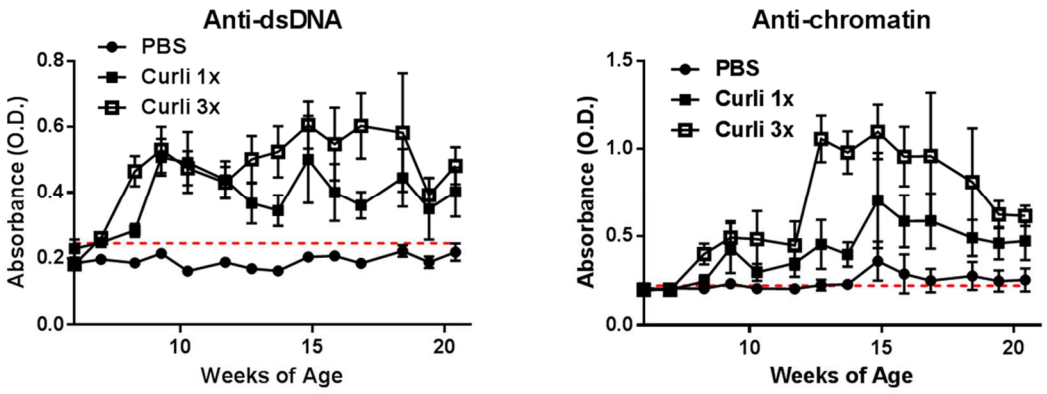

Figure 8: Curli-induced autoantibody production in NZBW-F1 mice
Anti-dsDNA and anti-chromatin autoantibodies were measured by ELISA in sera from NZBxW/F1 mice injected with PBS thrice a week, curli once a week (1x), or curli thrice a week (3x). Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA or anti-chromatin autoantibodies. Error bars indicate SEM, n=5 per group. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

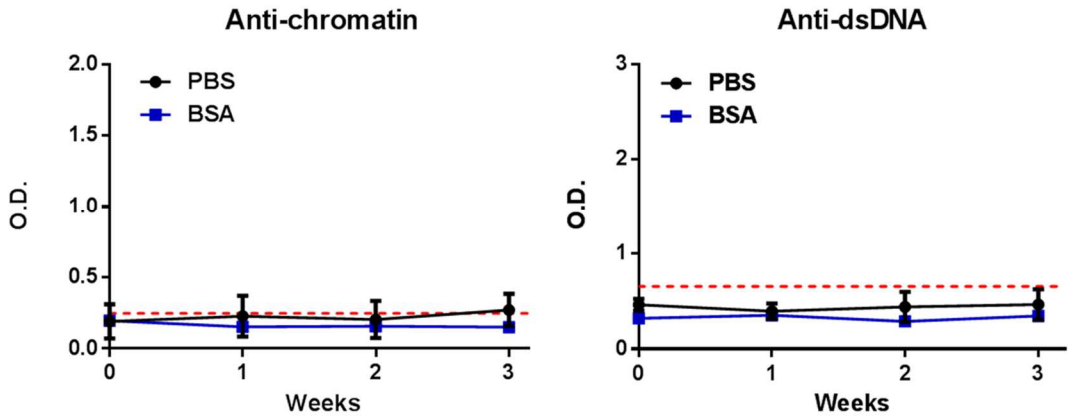

Figure 9: NZBW-F1 mice i.p. injected with BSA do not develop autoantibodies
Anti-dsDNA and anti-chromatin autoantibodies were measured by ELISA in sera from NZBxW/F1 mice injected with PBS or BSA (50µg/injection) three times per week. Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA or anti-chromatin autoantibodies. Error bars indicate SEM, n=5 per group. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

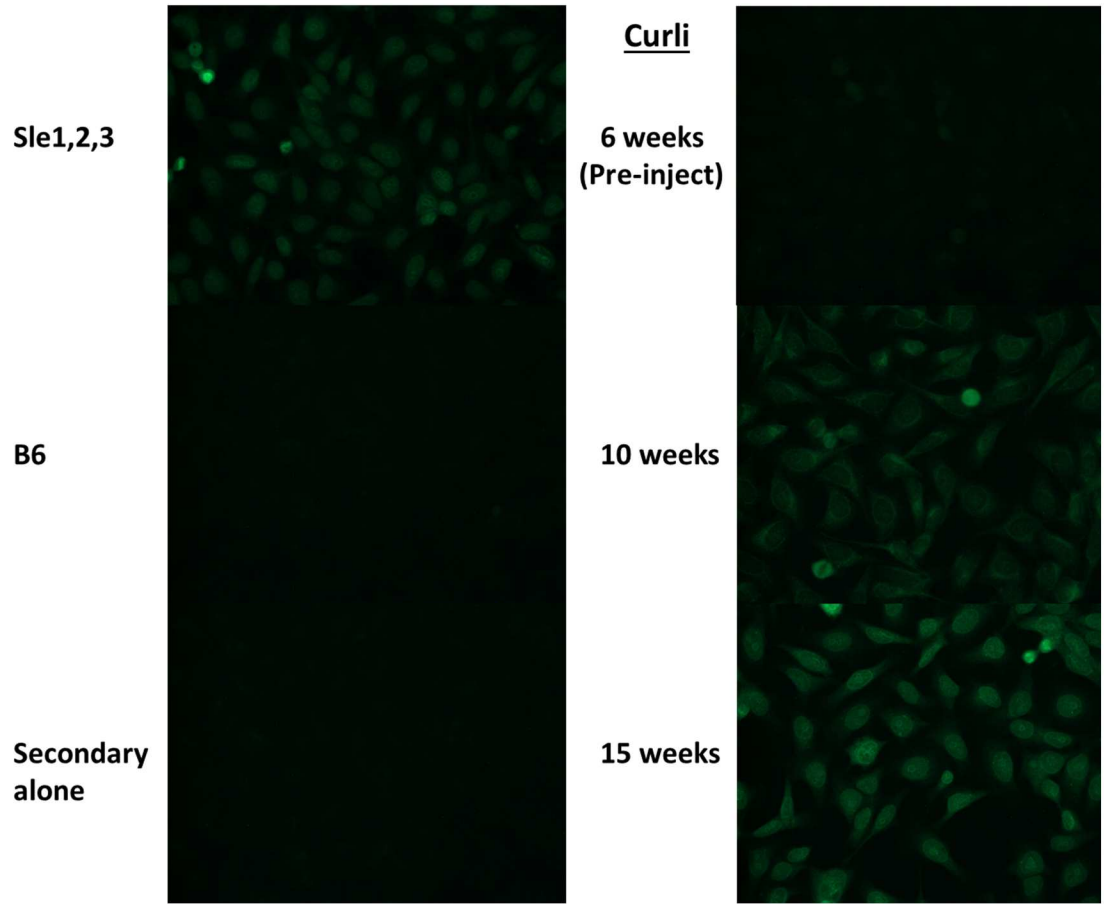

Figure 10: Antinuclear antibody test
Serum from 20 week old mice injected with PBS or curli (same mice as figure 6). Hep-2 cells were incubated with serum (diluted 1:40) and stained with FITC-conjugated anti-mouse IgG. Pictures are representative of 5 mice per group.

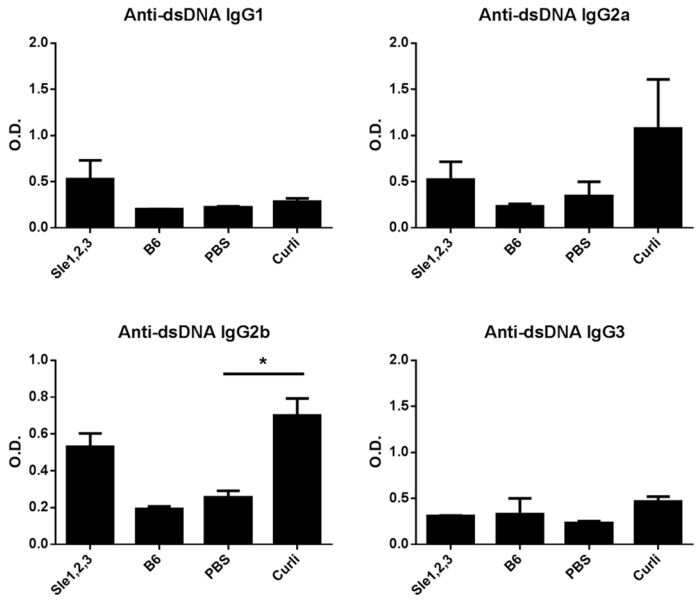

Figure 11: Anti-dsDNA subclasses from curli-treated NZBW-F1 mice
Isotypes for anti-dsDNA autoantibodies were measured by ELISA in sera from NZBxW/F1 mice injected with PBS or curli (50µg/injection) three times per week. Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA autoantibodies. Error bars indicate SEM, n=5 per group.

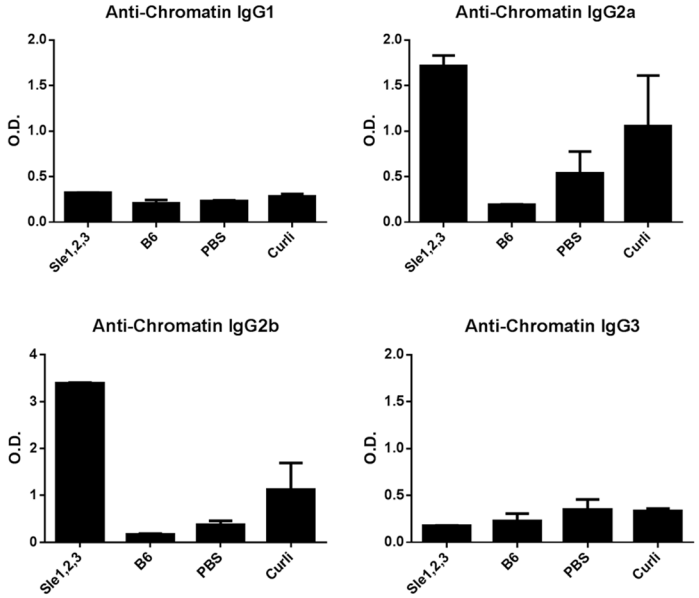

Figure 12: Anti-chromatin subclasses from curli-treated NZBW-F1 mice
Isotypes for anti-dsDNA autoantibodies were measured by ELISA in sera from NZBxW/F1 mice injected with PBS or curli (50µg/injection) three times per week. Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA autoantibodies. Error bars indicate SEM, n=5 per group.

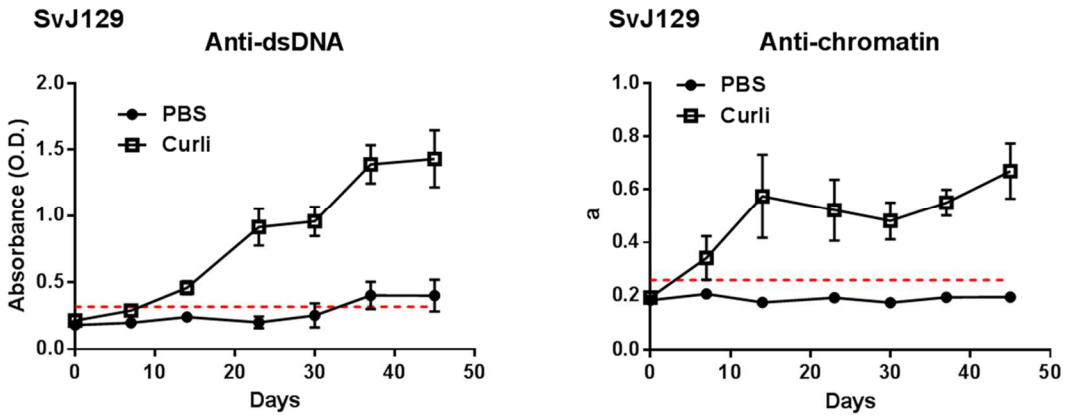

Figure 13: Curli-induced autoantibody production in SvJ129 mice
Anti-dsDNA and anti-chromatin autoantibodies were measured by ELISA in sera from SvJ129 mice injected with PBS or curli (50µg/injection) three times per week. Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA or anti-chromatin autoantibodies. Error bars indicate SEM, n=5 per group. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

Curli/DNA Composites Induce Autoimmunity in Wild Type Mice

Some stimuli, like chemical challenge with pristane, can induce systemic autoimmunity in non-autoimmune mice (i.e. B6). To test whether naturally occurring curli/DNA composites induce autoimmunity in mice not prone to lupus, we injected B6 mice i.p. with curli (50µg per injection) three times a week and found anti-dsDNA and anti-chromatin autoantibodies as soon as two weeks after injection (Figure 14), following the same kinetics seen in NZBxW/F1 and SvJ-129 mice. These results indicate that the direct exposure of the immune system to the danger signal curli/DNA composites is sufficient to break self-tolerance in non-autoimmune mice.

We next asked if autoantibodies would persist after stopping curli injections. We stopped injecting B6 mice from Figure 14 and followed their autoantibody levels for about two months. Autoantibody levels were variable between mice, but all stayed at least slightly positive for dsDNA. One mouse stayed highly positive for dsDNA, suggesting the process may be somewhat stochastic (Figure 15). After two months, most mice were close to negative for anti-chromatin – the one exception started low and increased over the two months period (Figure 15). This result suggests that curli may break tolerance in some mice.

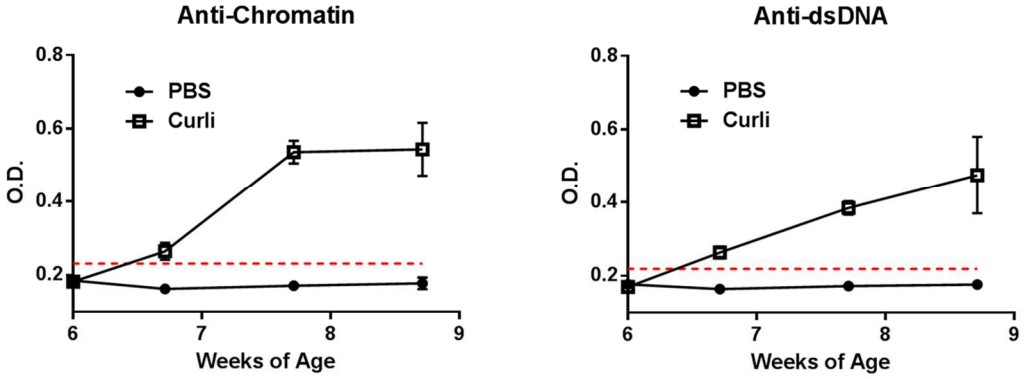

Figure 14: Curli-induced autoantibody production in B6 mice
Anti-dsDNA and anti-chromatin autoantibodies were measured by ELISA in sera from C57BL/6 mice injected with PBS or curli (50µg/injection) three times per week. Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA or anti-chromatin autoantibodies. Error bars indicate SEM, n=5 per group. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

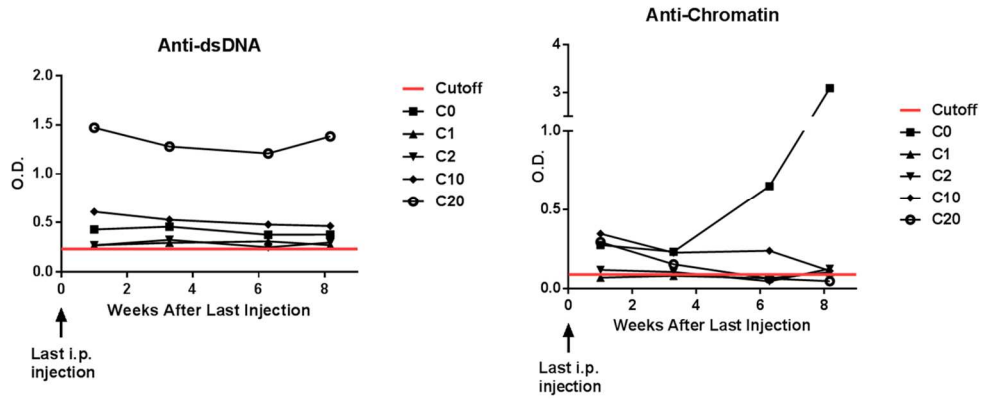

Figure 15: Autoantibodies in B6 mice after last curli injection
Serum Anti-dsDNA and anti-chromatin autoantibodies were measured by ELISA after final i.p. injection with curli (mice from Figure 12). Each line represents an individual mouse (5 mice injected with curli). Number after the letter C represents the ear tag. Optical density (O.D.) indicates ELISA color change and the presence of anti-dsDNA or anti-chromatin autoantibodies. The horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

Infection with Curli-competent Bacteria Accelerates Autoimmunity

We next asked whether curli-expressing bacteria, either commensal or pathogenic, promote autoimmunity in lupus-prone NZBxW/F1 mice. We injected young pre-diseased NZBxW/F1 mice i.p. with either wild type (curli-expressing) or curli mutant commensal E. coli or virulent S. Typhimurium. Systemic route of infection (i.p.) was chosen to mimic the propensity of SLE patients to develop systemic infections including bacteremia. We injected E. coli once a week for 4 weeks or S. Typhimurium once every two weeks for 8 weeks. We spaced Salmonella injections every two weeks to prevent death. We then stopped injections and let mice recover for at least 4 weeks. We found that mice infected with curli-competent bacteria, both E. coli and Salmonella, developed high titers of autoantibodies (Figure 16). Mice exposed to curli mutant strains also developed autoantibody titers albeit at lower levels compared to curli-competent strains, indicating that curli is the major autoimmunogen in these bacteria. The predominant autoantibody isotype in response to infection was IgG2a, although IgG2b was also positive to a lesser extent (Figure 17). This suggests that infection can promote the production of pathologic autoantibodies to nuclear antigens. Furthermore, mice infected with curli-competent wild type bacteria had significantly stronger staining of fixed Hep-2 cells (i.e. ANA) (Figure 18). Interestingly, ANAs from mice infected with *S*. Typhimurium were more likely to have both nuclear and cytoplasmic staining while mice infected with *E. coli* were more likely to show nuclear staining only. The reason for this difference is not clear. After sacrificing the animals, we analyzed blood, spleens, and livers for colony forming units (CFUs) and found no evidence of persistent infection at the time of euthanasia (data not shown). Together our results indicate that the exposure to curli or infection with biofilm-competent bacteria can promote the development of autoantibodies in susceptible mice.

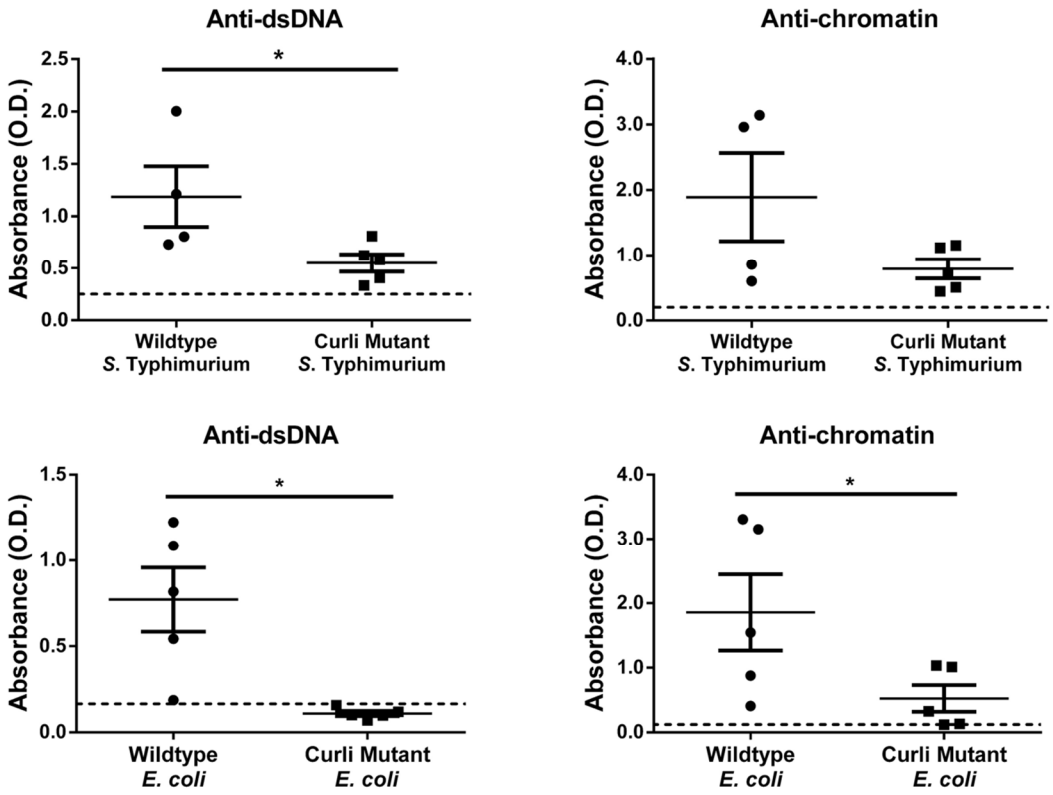

Figure 16: Bacterial infection in lupus-prone mice
Anti-dsDNA and anti-chromatin autoantibody ELISAs from NZBXW/F1 mice injected with live E. coli or S. Typhimurium strains. E. coli was injected i.p. into NZBXW/F1 mice at 10^5 CFU per injection once a week for four weeks (4 injections total). Live virulent S. Typhimurium was injected i.p. into NZBXW/F1 mice at 105 CFU per injection once every two weeks for 8 weeks (4 injections total). Mice were allowed to recover from infection for at least 4 weeks after the last injection. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

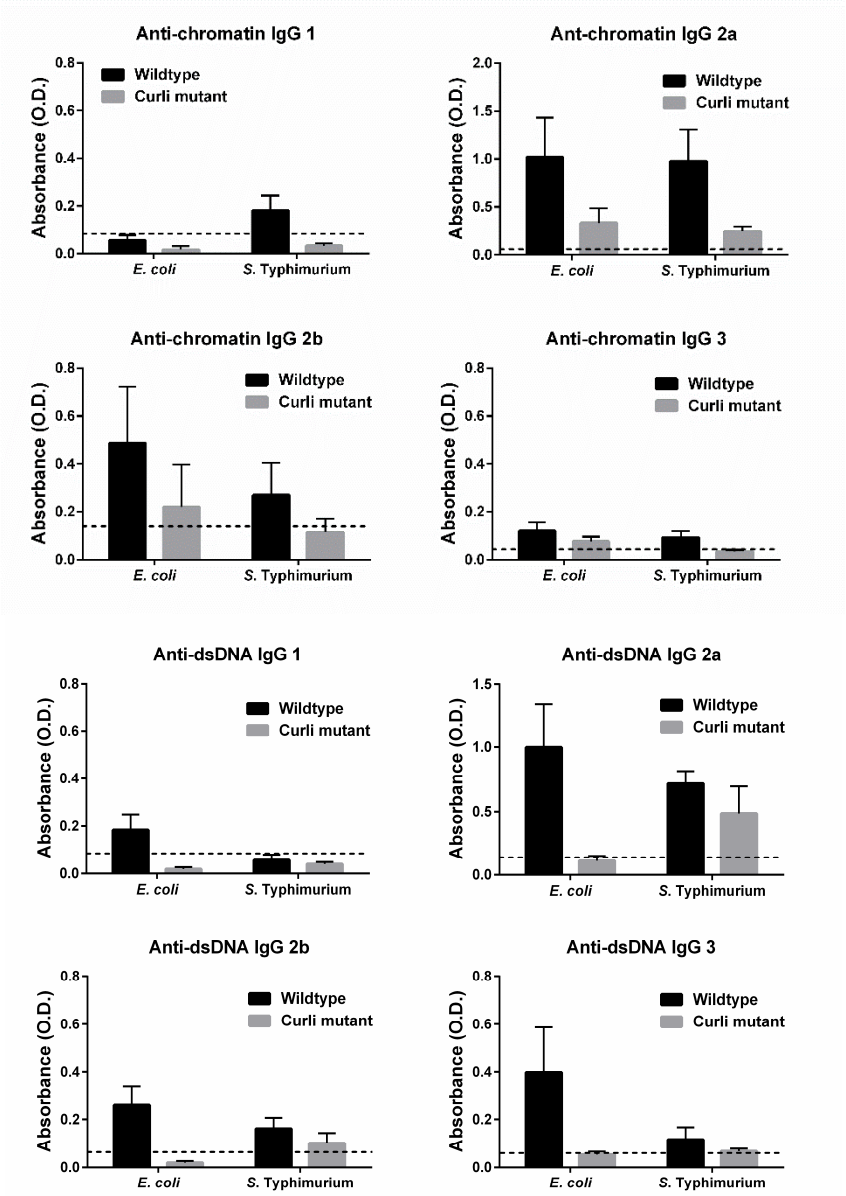

Figure 17: Isotypes from infected NZB/W-F1 mice
Anti-dsDNA and anti-chromatin autoantibody ELISAs from NZBXW/F1 mice injected with live E. coli or S. Typhimurium strains. E. coli was injected i.p. into NZBXW/F1 mice at 10^5 CFU per injection once a week for four weeks (4 injections total). Live virulent S. Typhimurium was injected i.p. into NZBXW/F1 mice at 105 CFU per injection once every two weeks for 8 weeks (4 injections total). Mice were allowed to recover from infection for at least 4 weeks after the last injection. The dotted horizontal line indicates cutoff for positivity, calculated as two standard deviations above the average of sera from naïve C57BL/6 mice.

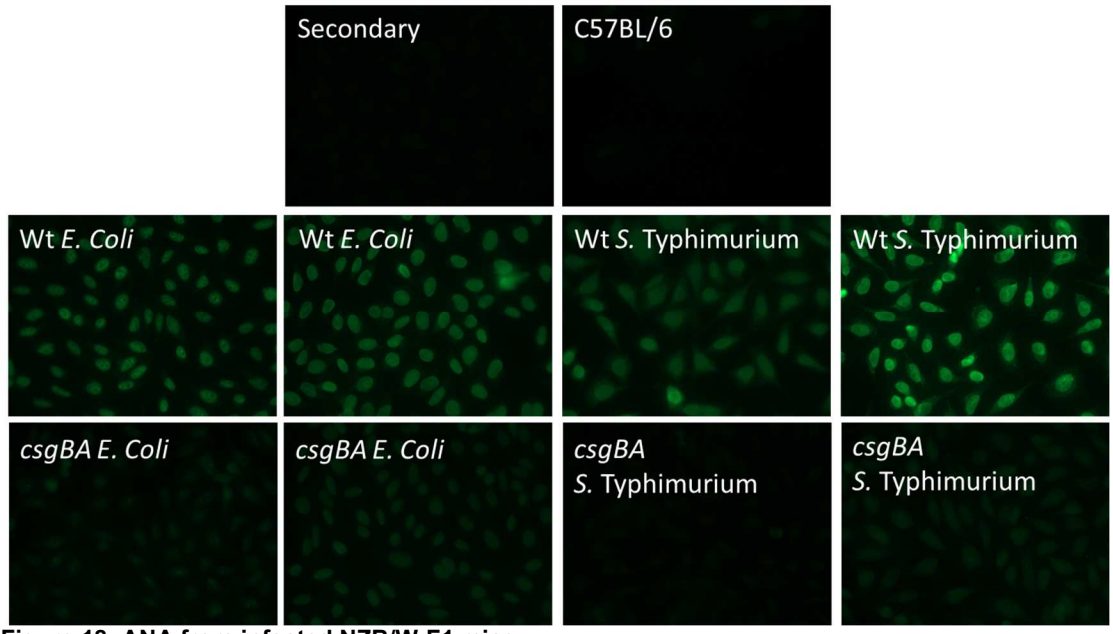

Figure 18: ANA from infected NZB/W-F1 mice

Antinuclear antibody tests using Hep-2 cells and serum from NZBXW/F1 mice injected with live E. coli or S. Typhimurium strains (diluted 1:40). E. coli was injected i.p. into NZBXW/F1 mice at 10^5 CFU per injection once a week for four weeks (4 injections total). Live virulent S. Typhimurium was injected i.p. into NZBXW/F1 mice at 10^5 CFU per injection once every two weeks for 8 weeks (4 injections total). Mice were allowed to recover from infection for at least 4 weeks after the last injection.

Curli Stimulates Antibody Production in the Absence of Adjuvant

To better understand the immune response to curli, we developed an ELISA to measure anti-curli antibodies in mouse serum (see Appendix: Protocols). The ELISA was adapted from that used to directly measure autoantibodies to dsDNA and chromatin.[351] A detailed description can be found in Appendix: Protocols. B6 or NZBxW/F1 mice were injected with PBS or 50 µg of curli per injection three times a week. All mice injected i.p. with curli developed robust anti-curli responses, whereas mice injected with PBS showed no evidence of anti-curli antibody production (Figure 19). Curli-injected mice developed anti-curli antibodies that peaked within two or three weeks without the use of an adjuvant. These results indicate that curli may act as both an antigen and an immunogen during bacterial infections.

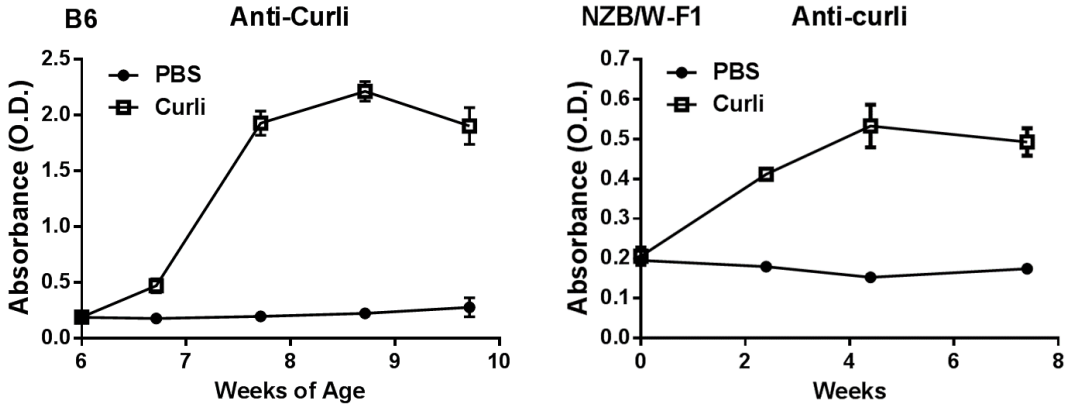

Figure 19: Anti-curli antibodies in B6 and NZB/W-F1 Mice
Anti-curli antibodies were measured by ELISA in sera from C57BL/6 or NZB/W-F1 mice injected with PBS or curli (50µg/injection) three times per week. Optical density (O.D.) indicates ELISA color change and the presence of anti-curli antibodies. Error bars indicate SEM, n=5 per group.

Curli-reactive Antibodies from Human SLE Patients are Associated with Disease Activity

In order to determine the relevance of our observations to human subjects, we next used the ELISA developed for murine studies to probe human serum/plasma for anti-curli antibodies. Human samples from healthy individuals and SLE patients were generously provided by Dr. Caricchio. Using the anti-curli ELISA, we found positivity for anti-curli Abs had a similar distribution between SLE patients and healthy controls (Figure 20). Indeed, we found that in both SLE and healthy individuals about a third seemed to be positive for antibodies reactive to curli (Figure 20). Analyzing the data from SLE patients alone, we found that anti-curli positivity was associated with the presence of a lupus flare (Figure 21). A flare was defined as an increase of at least 3 points in the SLEDAI score from the prior visit. Recovery was defined as decreased SLEDAI score of at least 10 points. Inactive disease was defined as no change in SLEDAI score.

This surprising result suggests an association between curli exposure and lupus disease activity. However, it is important to note that these are preliminary results that require further investigation. Although these antibodies seem to react with curli, they may have formed in response to a different amyloid. That is, anti-amyloid antibodies may cross-react because of structural similarities.[178] It may be more accurate to say that anti-amyloid antibodies are associated with disease activity. As discussed in chapter 1, antibodies may form to endogenous amyloids during some disease processes. Even so, ours would be the first evidence to show that anti-amyloid antibodies are associated with lupus disease activity.

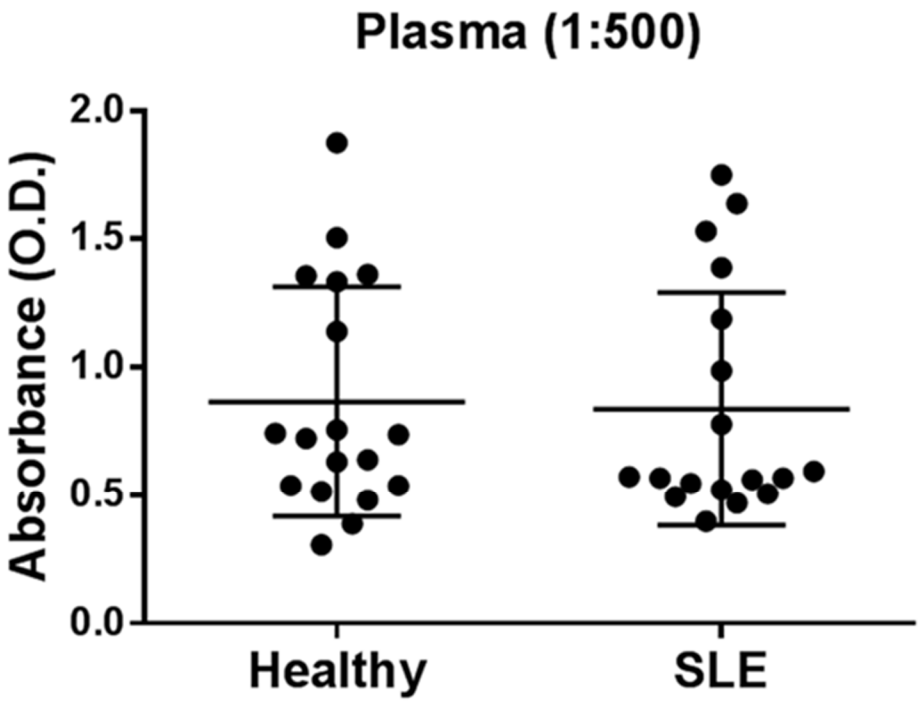

Figure 20: Anti-curli antibodies in healthy vs SLE patients
Human plasma samples were diluted 1:500 and probed for anti-curli antibodies by ELISA (described in Appendix: Protocols). Optical density (O.D.) indicates ELISA color change and the presence of anti-curli antibodies.

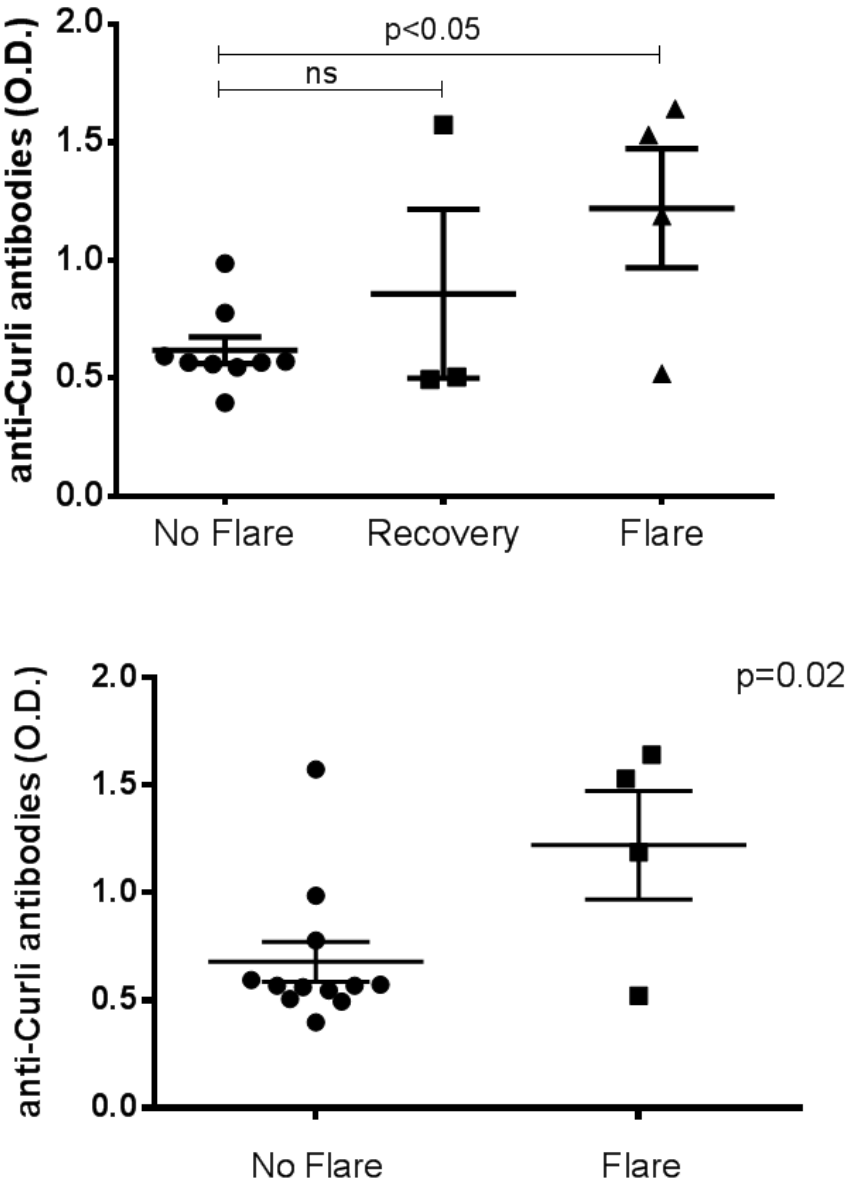

Figure 21: Anti-curli titers are associated with flares in lupus patients
Anti-curli ELISA results from Figure 18 were stratified by change in SLE disease activity index (SLEDAI) score. No flare was defined as no change in score, Recovery as a decrease in score, and a Flare as an increase in SLEDAI since the last visit (see methods section). Thank you to Dr. Caricchio for providing samples and clinical data.

Discussion

Curli/DNA Composites are Powerful Danger Signals

DCs are the sentinels of the immune system, which recognize pathogen associated molecular patterns (PAMPs) [60] and damage associated molecular patterns (DAMPs)[190] during infection or tissue damage and initiate the adaptive immune response by stimulating T cells.[357] We found that DCs are highly activated by biofilms and by curli/DNA composites in particular. The Tukel lab has also recently reported that the amyloid component of curli fibers stimulates the macrophages through the TLR2/TLR1 complex.[358,359] These results indicate that curli fibers are PAMPs, able to stimulate the innate immune system. Although studies on extracellular DNA (eDNA) and its sensors are limited, bacterial dsDNA can stimulate TLR9[193] and cytosolic STING-dependent DNA sensors resulting in a Type I IFN response.[360] The fact that curli/DNA composites are more immunostimulatory than curli or DNA alone suggests that different immune receptors might synergize to recognize these novel molecular patterns in bacterial biofilms.

Curli/DNA Composites are Strong Inducers of Type I IFNs and Autoimmunity

As many other PAMPs, curli/DNA composites strongly induce the expression of Type I IFNs and the IFN stimulated response in DCs in vitro and *in vivo*. Type I IFNs are pivotal in host defense against viruses but are also involved in antibody production.[361] A Type I IFN Signature is found up-regulated in PBMCs of SLE patients[284] and in DCs from lupus prone mice,[41] and administration of exogenous Type I IFNs accelerates autoimmunity in lupus prone mice.[51] Therefore, the results that curli/DNA composites accelerate the onset of autoimmunity in lupus-prone mice can be partially explained by the strong induction of Type I IFNs in DCs as well as by the polyclonal activation of the adaptive immune system, as curli up-regulated activation markers in splenic T and B cells.

SLE manifests with the production of antinuclear antibodies, including those directed against DNA, ribonucleoprotein complex (RNP), and nucleosomes.[362] Previous studies have shown that protein/DNA complexes can induce innate immune activation, production of autoantibodies to DNA, and autoimmunity.[229,231] Our findings that naturally occurring bacterial curli/DNA

composites, which can contain both bacterial and eukaryotic DNA, efficiently triggers Type I IFN stimulation and autoantibody production points to a novel bacterial stimulator of autoimmunity. The fact that eukaryotic DNA complexes with the bacterial amyloid curli raises the possibility that exposure of the lupus-prone immune system to autoantigen by the curli/DNA composites in presence of the adjuvant effects caused by TLR triggering and Type I IFN stimulation. Self-antigens can be released by NETs or by necrotic cells during tissue damage induced by bacterial infections. Moreover, defects in phagocytosis and clearance of apoptotic cells are associated with lupus[363] and may allow self DNA from post-apoptotic cells to be incorporated into biofilms and become immunogenic.

Because DNA is a major autoantigen in SLE, many studies have attempted to determine whether an excess of DNA in circulation may distinguish SLE patients. When accurate procedures were used to avoid in vitro artifacts – such as testing plasma rather than serum, because the in vitro coagulation procedure releases DNA[364] – it was found that SLE patients, like healthy individuals, had undetectable amounts of DNA in circulation, with the exception of SLE patients with vasculitis, who showed detectable levels of plasma DNA.[364,365] Recent studies using more sensitive techniques detected a low amount of DNA in normal individuals, but high levels of circulating DNA in patients experiencing major surgery, trauma, or cancer.[366] These results suggest that cell death is a source of extracellular DNA. In SLE patients, extracellular DNA may be released at the sites of tissue damage (e.g. infection), particularly when involving endothelial surfaces.[367]

Bacterial Biofilm-associated Infections and SLE

SLE has a multifactorial pathogenesis in which the genetic make-up and environmental triggers are considered the major players.[3] Among the environmental triggers, infections have been proposed as either initiators of autoimmunity or triggers of flares, the sudden increase in disease severity.[368] Several studies have tested this hypothesis, providing conflicting results. For example lupus-prone MRL/lpr mice bred in germ-free conditions develop lupus-like disease[104], suggesting that genetics trump environmental factors. However, the fact that SLE has a

concordance of 30-40% in identical twins[369], clearly indicate that the environmental triggers are required in human disease. Consistent with the idea that bacterial infections contribute to lupus pathogenesis, we found that infection with biofilm-competent, curli-producing bacteria exacerbates autoimmunity in lupus-prone mice. These results are possibly due to the direct exposure to curli itself but also to the fact that curli-expressing bacteria are better protected from the immune response – thereby increasing exposure and causing a greater flare in autoimmunity. In either case our work suggests that biofilm-based chronic infections are an important environmental trigger in lupus.

SLE patients often develop bacterial infections including those associated with bacterial biofilms such as urinary tract infections (UTIs), sepsis, and tissue abscesses caused by *S*. Typhimurium and *E. coli*.[93] However, how bacterial infections contribute to lupus pathogenesis and flares remain inconclusive. Our study suggests that biofilm-derived curli/DNA composites can accelerate autoimmunity through the induction of Type I IFNs, the activation of DCs, and the polyclonal stimulation of T and B cells. The fact that the commensal strain of E. coli could accelerate autoimmunity highlights the need to address how the interaction with pathogens and microbiota affects autoimmunity in genetically susceptible individuals.

Our results that curli/DNA composites can trigger autoimmunity even in non-autoimmune prone mice raise the question of why autoimmunity is not more frequent in the human population that is often exposed to biofilm-forming bacteria. A working hypothesis is that in non-lupus-prone individuals, or murine strains, natural infections do not normally result in the systemic release of curli/DNA composites in amounts equivalent to those we injected in this study; while in susceptible individuals, genetically determined dysfunctions may increase the systemic exposure during chronic infections. The more frequent outcome of salmonella infection in bacteremia and complications in soft tissues in SLE patients[370-372] and the recent identification of increased levels of circulating endotoxin in SLE patients[373], support this hypothesis. In addition, since inflammatory cytokines can decrease intestinal mucosal barrier function[374], the high levels of cytokines present in pre-disease stages of lupus[41,369], might compromise mucosal barrier function and allow the

biofilm material produced by microbiota at mucosal sites to access the immune system, act as danger signals and trigger lupus onset.

In conclusion, our study highlights a novel role of bacterial amyloid/DNA composites in stimulating the innate and the adaptive immune system, and suggests that chronic biofilm-producing bacterial infections may represent an important environmental contributor to SLE pathogenesis (Figure 22).

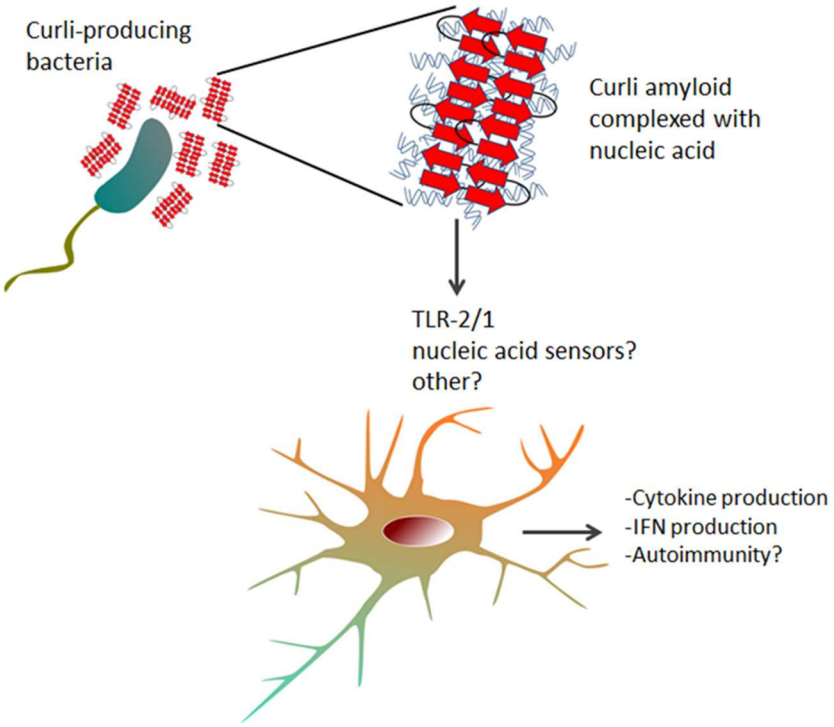

Figure 22: The immune response to curli
Curli is the main proteinaceous component of some bacterial biofilms, including those of enteric bacteria *E. coli* and *S.* Typhimurium. Curli forms an amyloid which polymerizes into non-branching fibrils. Within a biofilm, bacterial nucleic acids become incorporated into the curli fibril. The proteinaceous amyloid is recognized by TLR-2/1 heterocomplex. Curli/DNA composites are potent danger signals that induce high levels of pro-inflammatory cytokines. Curli/DNA composites or infection with curli-competent bacteria accelerate autoimmunity.

CHAPTER 4- CYTOKINE DYSREGULATION: IFN AND IL-10 CROSSTALK IN SLE

Results

Bone Marrow Derived DCs from Sle1,2,3 Mice Overexpress Il10

The lab of Dr. Gallucci has previously found that DCs derived from Sle1,2,3 mice express an IFN-signature which precedes disease onset.[41] In addition to classic IFN-signature genes (i.e. ifnb, isg15, and irf7), we have recently found that Sle1,2,3 bone marrow-derived DCs also overexpress basal levels of *Il10* in comparison to B6 controls as measured by qRT-PCR (Figure 23). Increased *Il10* gene expression in unstimulated DCs suggests that IL-10 is primed for translation in Sle1,2,3 DC cultures. Although basal mRNA levels of *Il10* seem to be increased in Sle1,2,3-derived DCs, we found that IL-10 protein is undetectable in supernatants from unstimulated cells, as measured by ELISA (data not shown).

Sle1,2,3 DCs Overproduce IL-10 in Response to TLR ligands

Sriram et al. have previously found that DCs from Sle1,2,3 mice overexpress type I IFN and IFN-stimulated genes in response to TLR stimulation.[41] Increased basal *Il10* expression suggested that Sle1,2,3 DCs may also overexpress IL-10 in response to TLR ligands. We therefore stimulated BMDCs from Sle1,2,3 and B6 mice with TLR ligands. We used LPS (100 ng/ml) to stimulate TLR4; R848 (1 μg/ml, an imiquimod-like compound) to stimulate TLR-7; and CpG (10μg/ml) to stimulate TLR-9. Cells were stimulated and supernatants were collected after 24 hours. IL-10 production was increased in DC cultures from Sle1,2,3 mice in response to all three TLR ligands (Figure 24). These results suggest that signals leading to IL-10 production are overactive in Sle1,2,3 DCs.

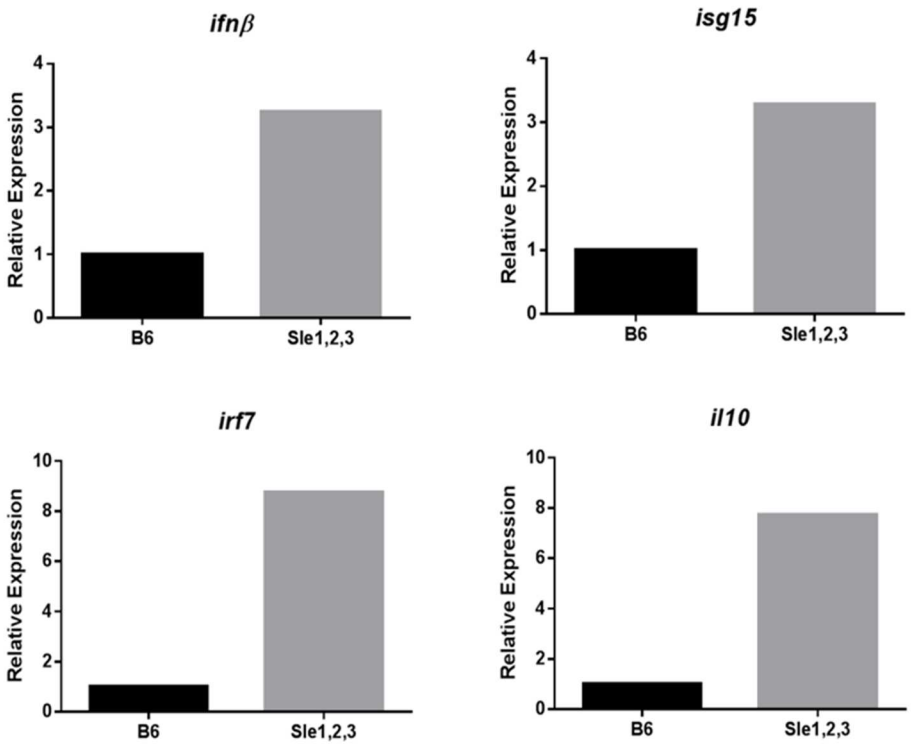

Figure 23: The IFN signature in Sle1,2,3 BMDCs
BMDCs were generated from the bone marrow of B6 and Sle1,2,3 females using GM-CSF-conditioned IMDM. Resting gene expression was measured by qRT-PCR and normalized to resting B6 BMDCs.

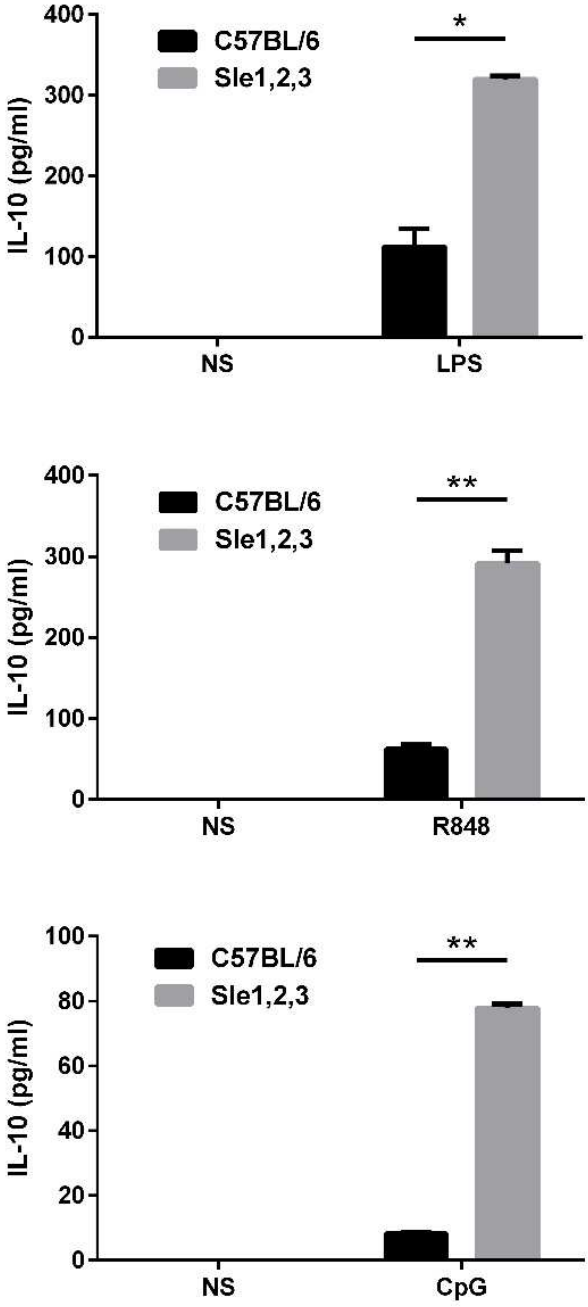

Figure 24: Increased TLR-induced IL-10 production in Sle1,2,3 BMDCs
BMDCs from B6 and Sle1,2,3 female mice were stimulated with TLR ligands LPS, R848, and CpG for 24hrs. We used LPS (100 ng/ml) to stimulate TLR4; R848 (1 µg/ml, an imiquimod-like compound) to stimulate TLR-7; and CpG (10µg/ml) to stimulate TLR-9. IL-10 was measured by ELISA.

Priming B6 DCs with Type I IFN Mimics IL-10 Overproduction in Sle1,2,3 DCs

Because Sle1,2,3 DCs overexpress type I IFNs, we next asked if type I IFNs could induce IL-10 overexpression. We first primed BMDCs from B6 mice with type I IFNs for 48 hours. After priming, cells were stimulated with TLR ligands. We found that priming B6 DCs with type I IFNs for 48hrs enhanced IL-10 production in response to TLR ligands LPS, R848, and CpG (Figure 25). IFN-priming alone sometimes induced low levels of IL-10 production, however this was not common and always at the lower limit of detection – suggesting that IFN requires the TLR signal in order to promote IL-10 production. Priming with type I IFN increased IL-10 production in B6 DCs to levels similar to that of Sle1,2,3 DCs, suggesting that the increased IL-10 production in Sle1,2,3 DCs is due to autocrine IFN.

IFNAR Blockade Prevents IL-10 Overproduction in Sle1,2,3 DCs

To determine if increased IL-10 production by Sle1,2,3 DCs is due to IFN feedback we used a blocking antibody to the type I IFN receptor (anti-IFNAR). DCs were pretreated with anti-IFNAR or isotype control (10μg/ml) 24 hours and 30 minutes prior to stimulation with LPS. IFNAR blockade significantly decreased IL-10 production from Sle1,2,3 mice (

Figure 26). These results suggest that overproduction of IL-10 from Sle1,2,3 DCs is the result of autocrine IFNAR signaling and that overexpression of type I IFN in Sle1,2,3 DCs leads to intrinsic IFN priming and overproduction of IL-10.

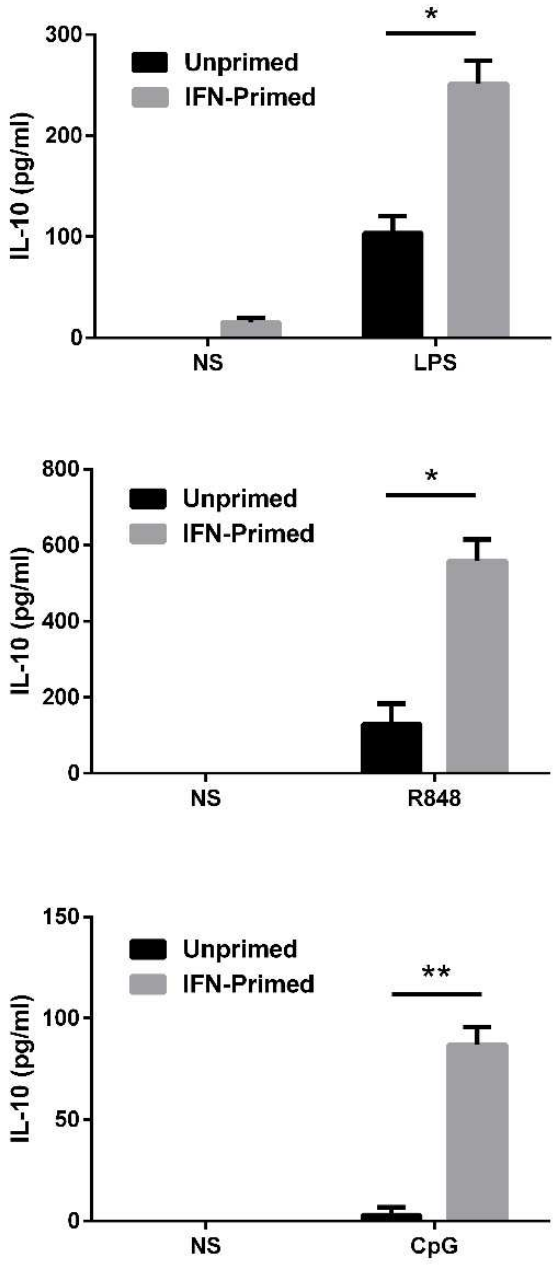

Figure 25: Increased TLR-induced IL-10 production in IFN-primed B6 BMDCs
B6 BMDCs were stimulated with TLR ligands with and without 48 hours priming with type I IFNs. We used LPS (100 ng/ml) to stimulate TLR4; R848 (1 µg/ml, an imiquimod-like compound) to stimulate TLR-7; and CpG (10µg/ml) to stimulate TLR-9.

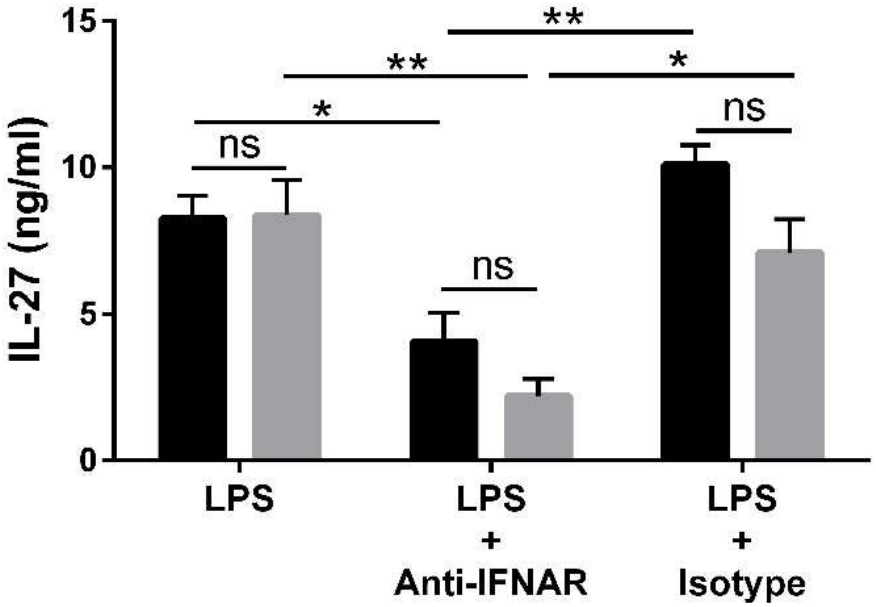

Figure 26: Overproduction of IL-10 by Sle1,2,3 BMDCs requires type I IFN
BMDCs were treated with anti-IFNAR antibody or isotype control 24 hours and 30 minutes prior to stimulation with LPS. Supernatants were collected 24 hours after LPS stimulation and IL-10 was measured by ELISA (ns = not significant).

Erk Activation Contributes to IFN-induced IL-10 Production

The MAP kinase Erk is known to contribute to IL-10 production in response to a number of stimuli.[296,297] Knowing that type I IFN can induce IL-10 production[375] (Figure 25), we asked if Erk activation could contribute to autocrine IFN-induced IL-10. First we used the Erk inhibitor PD98095 to determine if IL-10 overexpression in Sle1,2,3 DCs is Erk dependent. We found that Erk inhibition leads to a significant decrease in IL-10 production from Sle1,2,3 DCs (Figure 27). Erk inhibition was verified by western blot (Figure 28). This result suggests that Erk is required for maximal IL-10 production in response to LPS stimulation in both B6 and Sl1,2,3 DCs.

To determine if autocrine type I IFN leads to Erk activation, we stimulated DCs in the presence of an IFNAR blocking antibody. DCs were pretreated with anti-IFNAR for 24hrs and 30 minutes prior to LPS stimulation. Cells were harvested in ice cold western lysis buffer approximately 1.5 hours after LPS stimulation. We found that although they had similar constitutive levels of phosphorylated Erk, Sle DCs responded to TLR stimulation with higher levels of phospho-Erk than B6 DCs. Type I IFN blockade slightly reduced LPS-induced Erk phosphorylation, suggesting that autocrine Type I IFN partially contributes to Erk activation (Figure 28). Together our results demonstrate that IL-10 overexpression in Sle1,2,3 DCs is the result autocrine type I IFN and is mediated in small part by Erk activation.

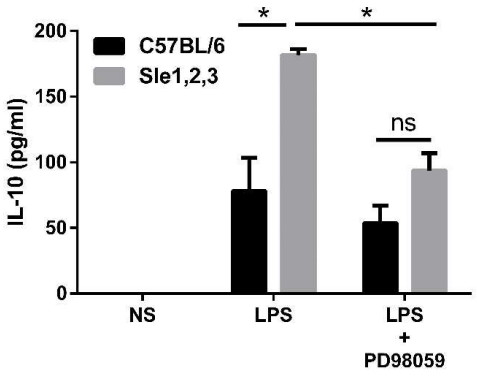

Figure 27: Overproduction of IL-10 by Sle1,2,3 BMDCs is reduced by Erk inhibition
BMDCs were pretreated with Erk inhibitor PD98059 for 30 minutes prior to LPS stimulation. Supernatants were collected 24 hours after LPS stimulation and IL-10 was measured by ELISA.

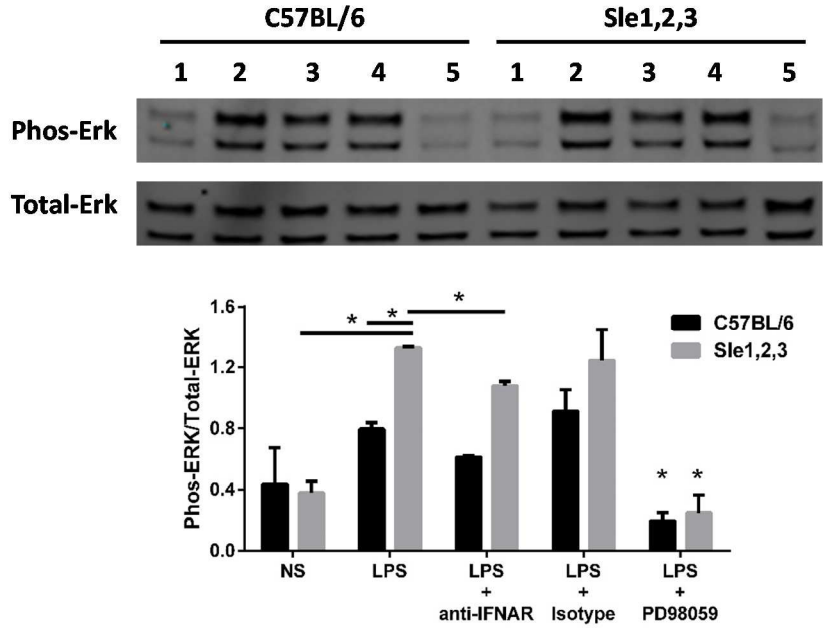

Figure 28: IFNAR blockade decreases Erk activation
Top: Western blot; Bottom: densitometry. BMDCs were pretreated with anti-IFNAR for 24hrs and 30 minutes or with Erk inhibitor PD98059 for 30 minutes prior to LPS stimulation. Cells were harvested in ice cold western lysis buffer 1.5 hours after LPS stimulation. Western blots were probed for total and phosphorylated Erk. Number code: 1) Not stimulated, 2) LPS, 3) LPS + anti-IFNAR, 4) LPS + isotype control, 5) LPS + PD98059

Discussion

IL-10 is Dysregulated in Sle1,2,3-derived DCs

We first observed IL-10 dysregulation in Sle1,2,3-derived DCs after stimulation with TLR-ligands. Bone marrow derived DCs from Sle1,2,3 mice consistently produced elevated IL-10 compared to DCs from B6 controls. This observation was made by multiple members of the Gallucci lab. If IL-10 is produced by resting (unstimulated) Sle1,2,3-derived DCs it is under the ELISA detection threshold. We therefore probed *Il10* mRNA expression levels in Sle1,2,3 and B6 BMDCs and found that basal *Il10* mRNA levels were elevated in Sle1,2,3-derived DCs. IL-10 is known to be elevated in the serum of lupus patients with active disease and first degree relatives of lupus patients are more likely to have elevated serum IL-10 levels. Here we show that elevated IL-10 levels can also be seen in BMDCs from lupus-prone mice, suggesting that dysregulation of cytokine signaling in the myeloid compartment may contribute to IL-10 dysregulation in SLE patients.

Crosstalk between Type I IFN and IL-10

IL-10 and type I IFN are genetically related. Indeed, the type III IFNs share genetic and structural similarities with type I IFNs, and use the IL-10R2 chain for signaling. A number of lines of evidence have shown that type I IFNs can induce IL-10 production. Because type I IFNs are known to be elevated in SLE patients and in Sle1,2,3 mice we hypothesized that the elevated IL-10 production in Sle1,2,3 DCs was due to elevated type I IFN feedback. First we found that we could mimic the pattern seen with Sle1,2,3 TLR-induced IL-10 overproduction in B6 DCs by pretreating with type I IFNs, suggesting that type I IFNs alone are enough to dysregulated IL-10. However this did not directly implicate IFN feedback in the elevated IL-10 found in Sle1,2,3 DCs. To determine if IFN was responsible for IL-10 overproduction in Sle1,2,3 DCs we used a blocking antibody to IFNAR1. Blockade of IFN signaling led to a significant reduction of IL-10 production in Sle1,2,3 DCs, suggesting that excessive IFN feedback is responsible for IL-10 dysregulation in SLE.

A number of mechanisms have been described for IFN-induced IL-10 production including activation of GSK3-β[376], IRF1/STAT3,[377] and Erk pathways.[378] We explored Erk activation because it has been suggested that Erk is abnormally active in B cell of lupus-prone mice.[315] We found that Sle-derived DCs had higher levels of phospho-Erk after simulation with LPS, suggesting that MAP kinase pathways in DCs from lupus prone mice are more sensitive to TLR stimulation. We also found that Erk activation was required for IL-10 overproduction from Sle-derived DCs and that IFNAR blockade lowered Erk activation, suggesting IFN feedback contributes to Erk activation and overproduction of IL-10.

Summary

Our work suggests that overproduction of IL-10 in SLE is the result of excessive type I IFN feedback. Figure 29 shows a schematic for the hypothesized role of IFN and IL-10 crosstalk in SLE. DCs recognize danger signals using pattern recognition receptors. Type I IFNs are overexpressed basally and overproduced in response to danger signals. Autocrine type I IFN mediates signaling though IFN receptors, thereby amplifying IL-10 transcription. IL-10 dampens innate immune responses, but may also gain pro-inflammatory functions as a result of the IFN signature. Increased IL-10 may also contribute to increased DC cell death, B cell proliferation, and autoantibody production (Figure 29).

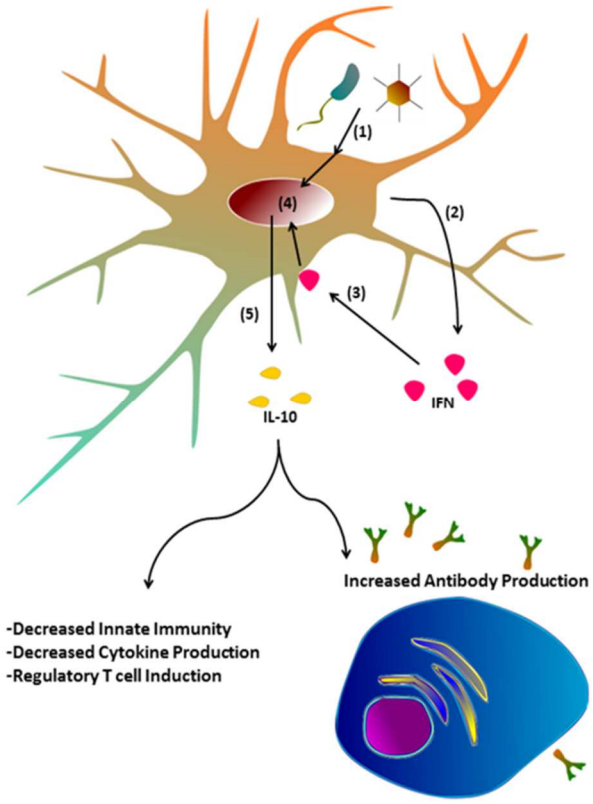

Figure 29: Proposed Mechanism of IL-10 production in Sle1,2,3 DCs. (1) DCs recognize danger signals using pattern recognition receptors. (2) Type I IFNs are overproduced in response to danger signals. (3) Autocrine IFN mediates signaling though IFN receptors. (4) Signaling from both pattern recognition receptors and IFN receptors induces IL-10 transcription. (5) The resulting IL-10 dampens responses of the innate immune system, but also gains pro-inflammatory functions as a result of the IFN Signature. Increased IL-10 also contributes to increased DC cell death. We show that these pathways are aberrantly activated in Sle1,2,3 DCs.

Results

Glomerular ErbB2 is Overexpressed in Diseased NZB/W-F1 Mice

Renal disease remains a major cause of morbidity and mortality in lupus. Our collaborators Drs. Kate Sullivan and Patricia Costa Reis (University of Pennsylvania) have recently observed that kidneys from lupus patients with nephritis have increased glomerular expression of HER2 (ErbB2) (unpublished observations). Similar results were found in lupus-prone NZM2410 mice. To determine if Type I IFN could increase ErbB2 expression in NZB/W-F1 mice we treated mice with a single dose of an IFN-expressing adenovirus to accelerate disease onset and compared kidney histology to young pre-diseased mice. Kidneys from IFN-accelerated, disease-positive NZB/W-F1 mice had higher levels of ErbB2 expression compared to healthy NZB/W-F1 mice, including in the glomerulus (Figure 30). Because ErbB2 is a growth factor receptor best known for its involvement in the proliferation of some cancers, we hypothesized that the expression of ErbB2 may be contributing to the maladaptive proliferative and fibrotic processes found in lupus nephritis.

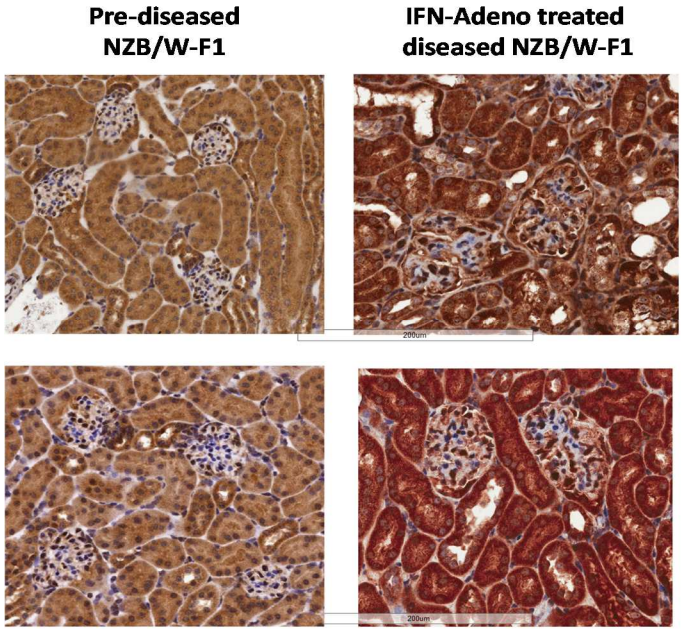

Figure 30: Glomerular ErbB2 is overexpressed in diseased NZB/W-F1 mice
Kidneys from pre-diseased and IFN-accelerated diseased NZB/W-F1 mice were sectioned and stained by immunohistochemistry for ErbB2. Brown indicates ErbB2 positivity. Two representative pictures are shown from each group.

Study Design

To explore the importance of ErbB2 in the development and progression of lupus nephritis we used an accelerated model of murine lupus in which lupus-prone NZBW-F1 are i.v. injected with a single dose of an IFNα-expressing adenovirus (Figure 31). We sought to treat these mice with an ErbB1/ErbB2 dual kinase inhibitor, lapatinib. Temple University biostatistician Dr. Huaqing Zhao was consulted for power calculations during study design. We divided thirty mice into three groups of 10 mice each: 1) vehicle, 2) prevention, and 3) treatment; and i.v. injected all mice with a single dose of IFNα-expressing adenovirus at 10 weeks of age to accelerate disease. We treated vehicle group mice with vehicle by oral gavage twice a day from the start of the trail; prevention group mice with lapatinib (75mg/Kg) in vehicle by oral gavage twice daily, 6 days a week, from the start of the trial; and treatment group mice with lapatinib in vehicle by oral gavage after twice confirmed proteinuria or after 50% of all treatment group mice developed proteinuria.

Lapatinib is an oral small molecule 4-anilinoquinazole derivative that inhibits both EGFR and HER2 (ErbB2) by reversibly binding the intracellular ATP-binding domain kinase, leading to lower activation of downstream mediators including Erk MAP kinase and Akt.[379] Oral administration of lapatinib enters the systemic circulation and a 75mg/Kg dose is within range for effective tissue perfusion up to 16 hours in mice.[380] Many pre-clinical investigations using lapatinib have been in cancer models, showing efficacy against HER2-positive tumors.[381] In human trials of lapatinib the most common adverse effects were diarrhea and rash.[382] Severe adverse effects include hepatic,[383] cardiac,[384] and gastrointestinal toxicities (reviewed in [385]). Renal toxicity has not been regularly described with the use of lapatinib, although clinical studies often exclude those with pre-existing impaired kidney function.[386] Additionally, lapatinib has a modest benefit in patients with renal cell carcinoma,[387] suggesting that lapatinib does not worsen renal disease in this context. This work led us to believe lapatinib would be a good candidate for the treatment of lupus nephritis.

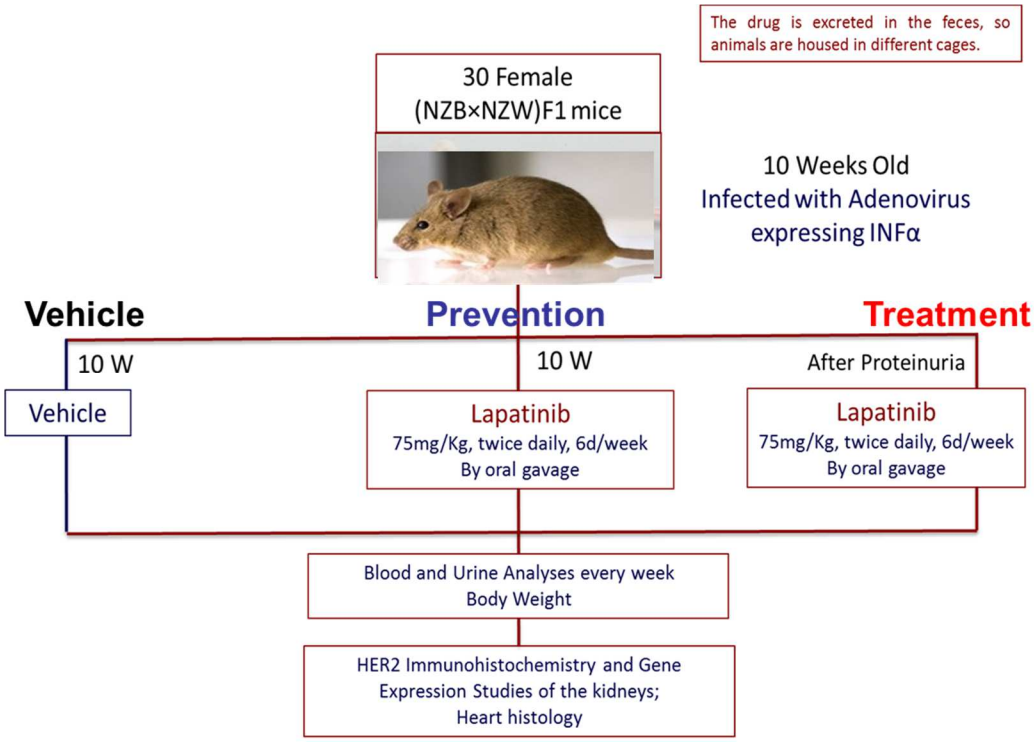

Figure 31: Lapatinib in murine lupus study design
Thirty 10 week old NZB/W-F1 mice were randomly separated into three groups of 10 mice each. All mice were treated with a single dose of an IFN-α-expressing adenovirus via tail i.v. injection in order to accelerate disease onset. "Vehicle" group mice received oral gavage of empty drug vehicle from the start of the experiment. "Prevention" group mice received oral gavage of lapatinib in vehicle twice daily, 6 days a week at 75mg/kg from the start of the experiment. "Treatment" group mice received oral gavage of lapatinib in vehicle twice daily, 6 days a week at 75mg/kg after the onset of proteinuria.

Mouse Weight

Mice were weighed once per week. Blood was collected by tail vein bleed once every two weeks and urine was collected once or twice per week. Although individual mice would gain a significant amount of weight as the result of kidney disease (visible as ascites in Figure 39) the average weight of all three groups stayed relatively consistent (Figure 32).

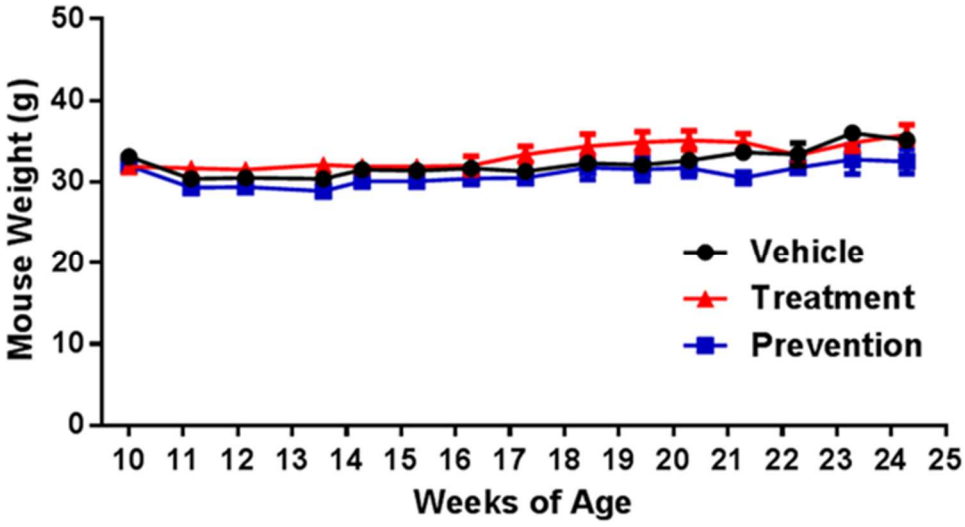

Figure 32: Weight of lapatinib-treated murine lupus
Mice were weighed once every week. The average and SEM are shown for each group.

Autoantibodies in Lapatinib-treated Murine Lupus

Blood was collected every two weeks and assessed for autoantibodies to dsDNA and chromatin by direct ELSIA. Mice from all three groups developed autoantibodies to dsDNA and chromatin. Autoantibody titers remained elevated despite treatment with lapatinib, although autoantibodies in the prevention group were significantly lower (Figure 33A). After death or euthanasia, spleens were weighed. Spleens from both lapatinib-treated groups were significantly smaller compared to vehicle-treated mice (Figure 33B). These results suggest that lapatinib may inhibit immune cell functions and limit the development of autoantibodies in lupus-prone mice. We confirmed ELISA results using ANA. Figure 34 shows representative ANAs from all three groups. The majority of mice from vehicle and treatment groups were strongly positive, showing either nuclear or perinuclear staining. However, mice from the prevention group did not stain as strongly, supporting our previous ELISA results and suggesting lapatinib administration lowers autoantibody titers in IFN-accelerated murine lupus.

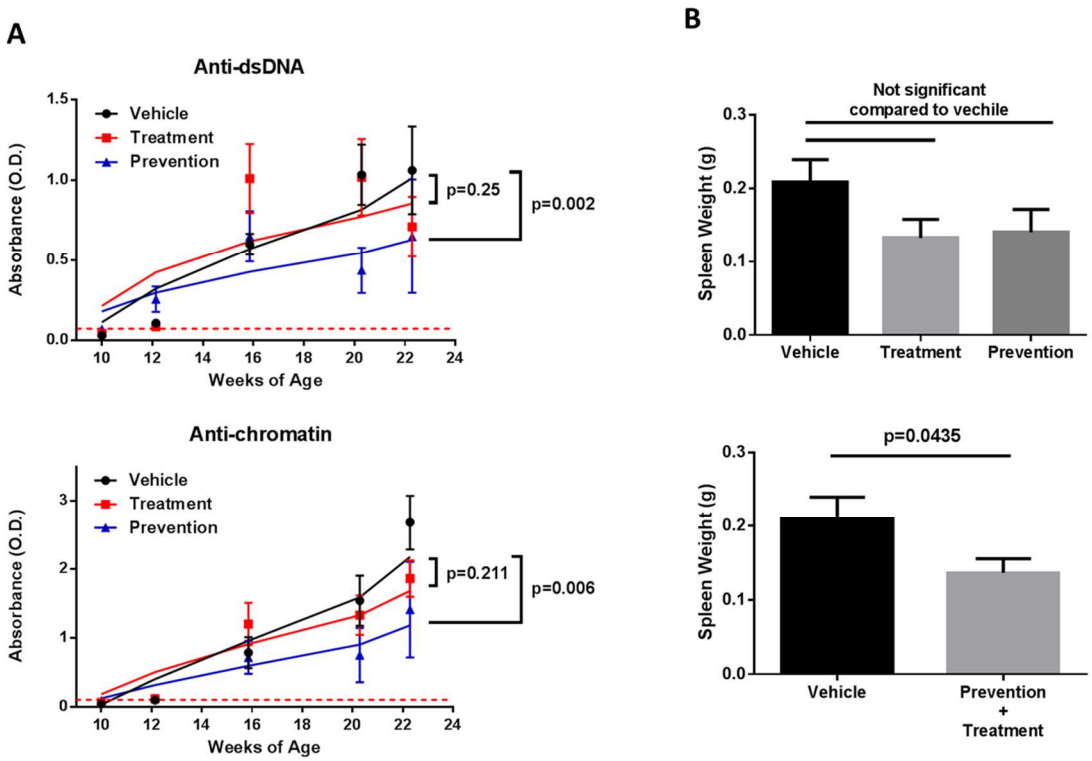

Figure 33: Autoantibodies in lapatinib-treated murine lupus
Mice were bled periodically via tail bleed over the course of the trial. Anti-dsDNA and anti-chromatin autoantibodies were measured by direct ELISA. The red dotted line represents the cutoff for positivity, calculated as two standard deviations above the B6 average (negative control). n=10 mice per group. Error bars are SEM. Two-way ANOVA for repeated measures was used to calculate p values.

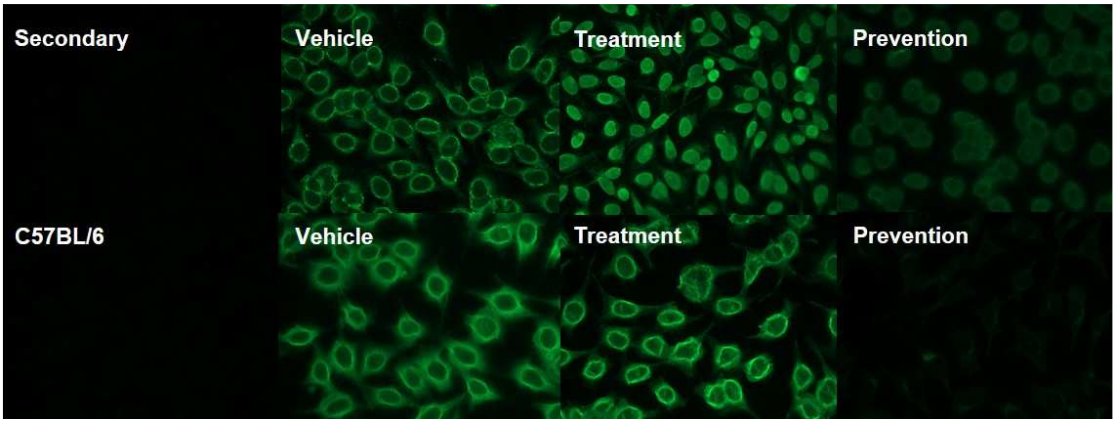

Figure 34: Representative ANA
Serum was collected from mice every two weeks and at the termination of the study. The final bleed (closest to the time of death) from each mouse was analyzed by ANA using the Hep2 cell line. Two representative pictures from each group are shown.

Kidney Function in Lapatinib-treated Murine Lupus

Because lapatinib was able to lower autoantibody levels in murine lupus we predicted that lapatinib may also improve kidney function. Mice were followed for approximately four months after the initiation of the study. Blood urea nitrogen (BUN) and urine protein were used as indicators of kidney function. All experimental animals had elevated BUNs compared to B6 controls but lapatinib administration did not affect BUN levels significantly (Figure 35). Contrary to our expectations, proteinuria increased earlier in the prevention group and showed sustained elevation throughout the study (Figure 36A). Urine sediment from mice with proteinuria was cellular and contained both granular and waxy casts, indicative of renal inflammation and glomerular disease (Figure 36B). So although lapatinib decreased autoantibody production, it failed to improve renal disease and worsened kidney function in mice receiving drug as a preventative measure.

Survival in Lapatinib-treated Murine Lupus

Figure 37 shows the survival curves for all three groups. The black arrow indicates the beginning of the study at which 10 week old NZBW-F1 mice were injected i.v. with IFNα-expressing adenovirus and treatments begun for the vehicle and prevention groups. The red arrow indicates when 50% of all treatment group mice became proteinuria positive and all treatment group mice began treatment. Mortality in the prevention group was significantly accelerated compared to the vehicle control group, suggesting that lapatinib exacerbated disease in these mice. The treatment group did not seem to benefit from lapatinib after the onset of proteinuria and the experiment was ended when it became apparent that treatment group animals where not protected. The treatment group survival curve was not significantly different from the vehicle group at the time the trial was ended (Figure 37).

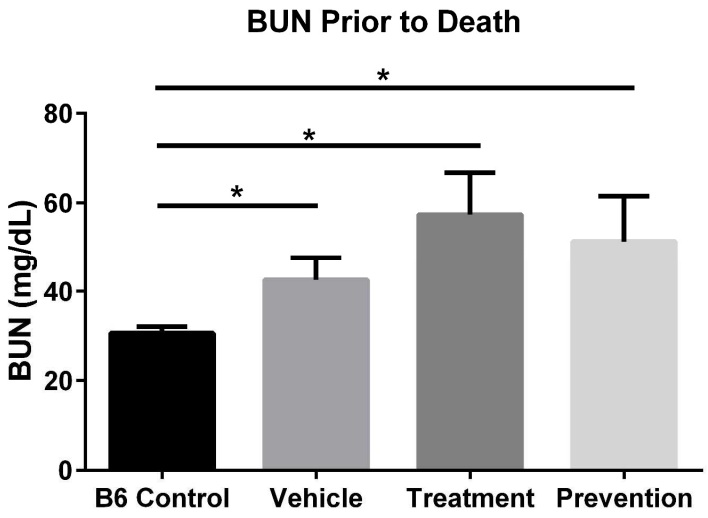

Figure 35: Blood urea nitrogen prior to death
Serum was collected from mice every two weeks and at the termination of the study. The final bleed (closest to the time of death) from each mouse was analyzed for blood urea nitrogen (BUN).

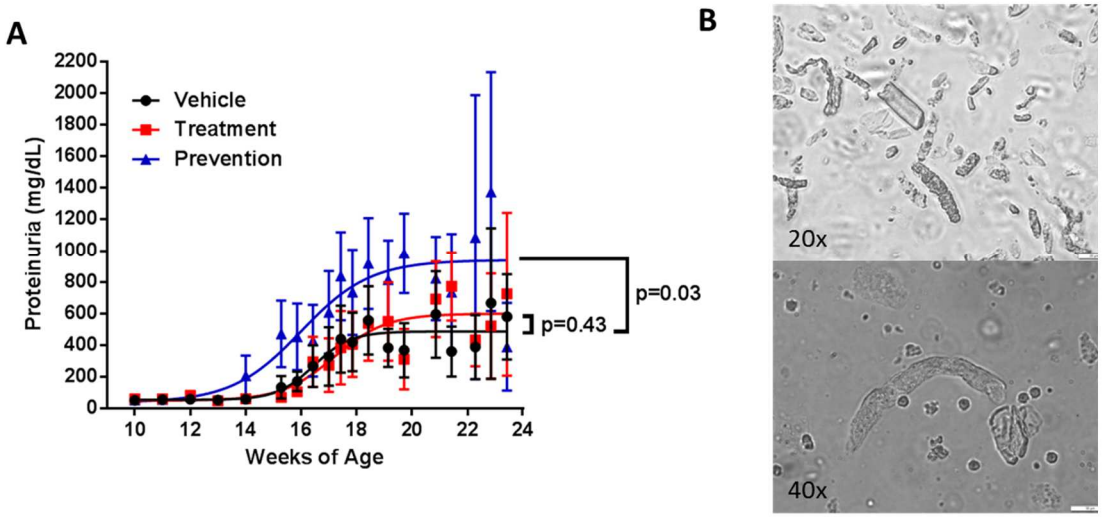

Figure 36: Proteinuria in lapatinib-treated murine lupus
NZBW-F1 mice were divided into three groups (10 mice per group) and treated as described in the methods section. A) Proteinuria during the course of the trial. n=10 mice per group. Error bars are SEM. Two-way ANOVA for repeated measures was used to calculate p values. B) Examples of urine sediment from proteinuria-positive mice. Sediments were cellular and contained both granular and waxy casts.

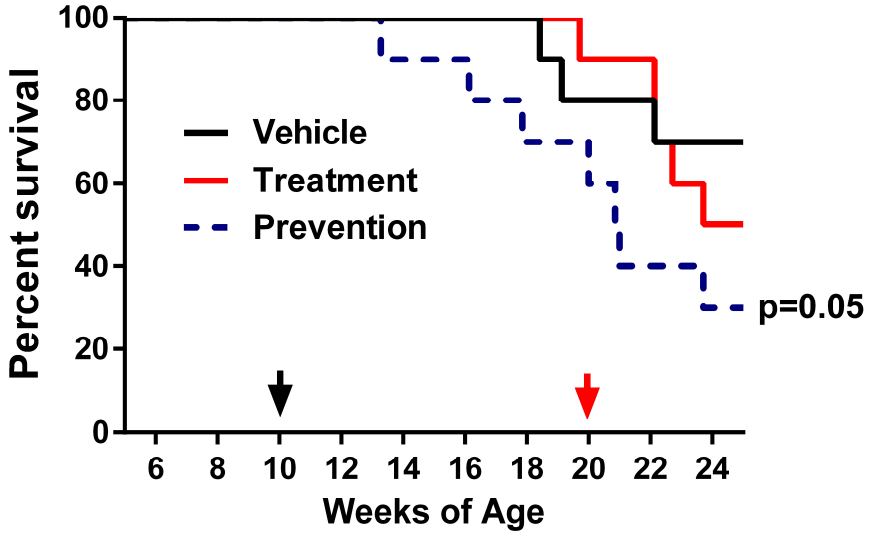

Figure 37: Survival in lapatinib-treated murine lupus
NZBW-F1 mice were divided into three groups (10 mice per group). All mice were i.v. injected with a single dose of IFNα-expressing adeno virus at 10 weeks of age (black arrow) to accelerate disease. Vehicle group mice were treated with vehicle by oral gavage from the start of the trail (black arrow). Prevention group mice were treated with lapatinib in vehicle by oral gavage from the start of the trial. Treatment group mice were treated with lapatinib in vehicle by oral gavage after twice confirmed proteinuria (red bars) or after 50% of the Treatment group mice developed proteinuria. Log-rank test showed a significant difference in the prevention group.

Primary Endpoints in Lapatinib-treated Murine Lupus

We chose survival and proteinuria greater than 300mg/dL as the two primary endpoints at the beginning of the trial. Figure 38 shows the percent of mice in each group meeting either of these two endpoints and confirms that lapatinib worsened disease progression in prevention group mice.

Physical Findings

Mice that became sick showed classic signs of murine lupus and renal insufficiency. After the onset of proteinuria, mice eventually became bloated with excess fluid (ascites and anasarca) and fur became ruffled (Figure 39A). In some mice digits became pale, likely the result of anemia. When possible, we euthanized mice before disease became severe, although some mice were found dead. Kidneys from sick mice were universally pale, indicative of renal damage (Figure 39B). Kidneys from some mice found dead had necrotic poles, possibly indicating circulatory failure as the cause of death (Figure 39C).

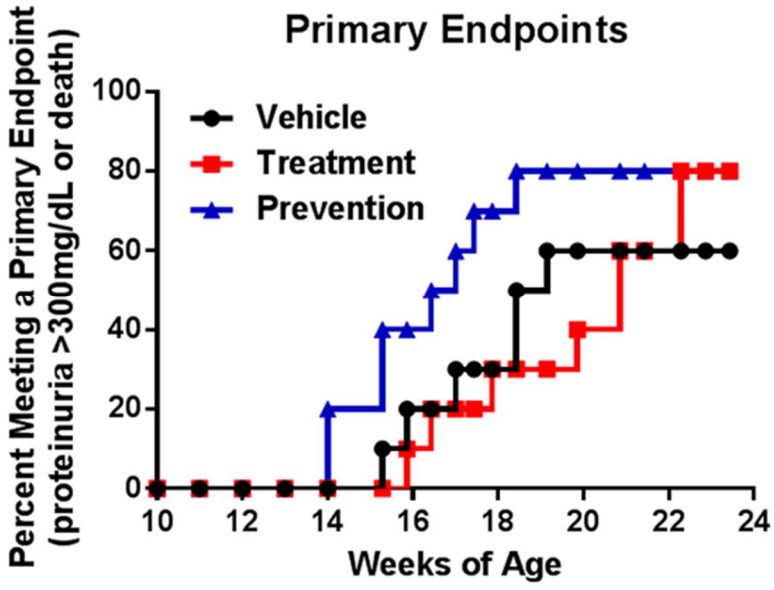

Figure 38: Primary endpoints in lapatinib-treated murine lupus
Primary endpoints were defined as proteinuria >300mg/dL or death.

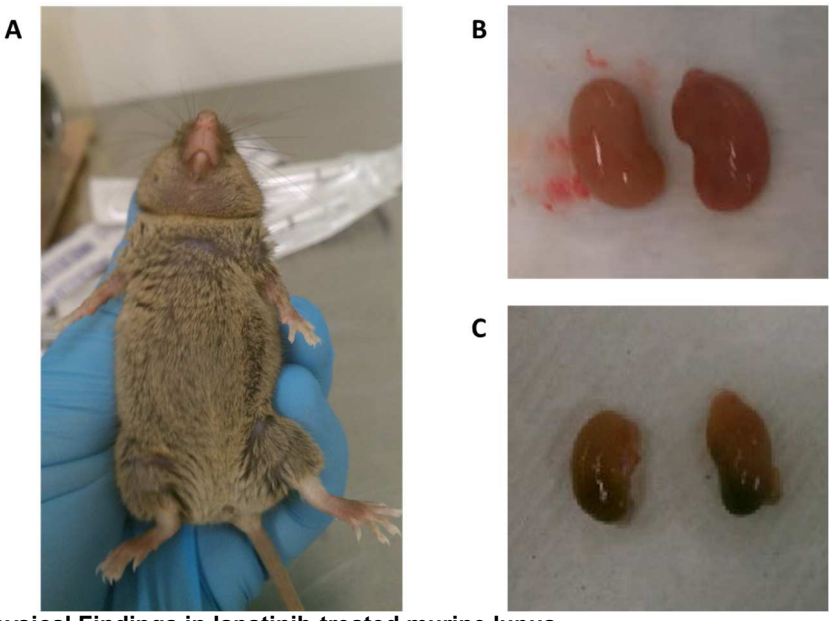

Figure 39: Physical Findings in lapatinib-treated murine lupus
A) As kidney disease progresses, mice become bloated (anasarca). Fur becomes ruffled and digits may become pale. B) Left and right kidneys from a euthanized mouse. Kidneys have become pale from renal disease. C) Left and right kidneys with necrotic poles from a mouse found dead.

Microscopy

Kidneys were harvested from euthanized mice for microscopic analysis. Frozen sections were used to probe for glomerular immune complex deposition as indicated by IgG and complement C3. Experimental mice stained positive for glomerular IgG and C3. Mice in the vehicle and treatment group showed robust staining for both IgG and C3 whereas mice in the prevention group had less intense staining (Figure 40). This observation is consistent with our data showing that lapatinib use in the prevention group decreases autoantibody titers.

Formalin fixed kidney sections were stained by H&E and scored by a blind third party. All experimental animals showed evidence of renal damage, however mice in the prevention group tended to have higher scores in for glomerular crescents and interstitial infiltrate (Figure 41). These results clearly show that lapatinib is not useful in the treatment of lupus nephritis and suggests that lapatinib may worsen renal disease in SLE.

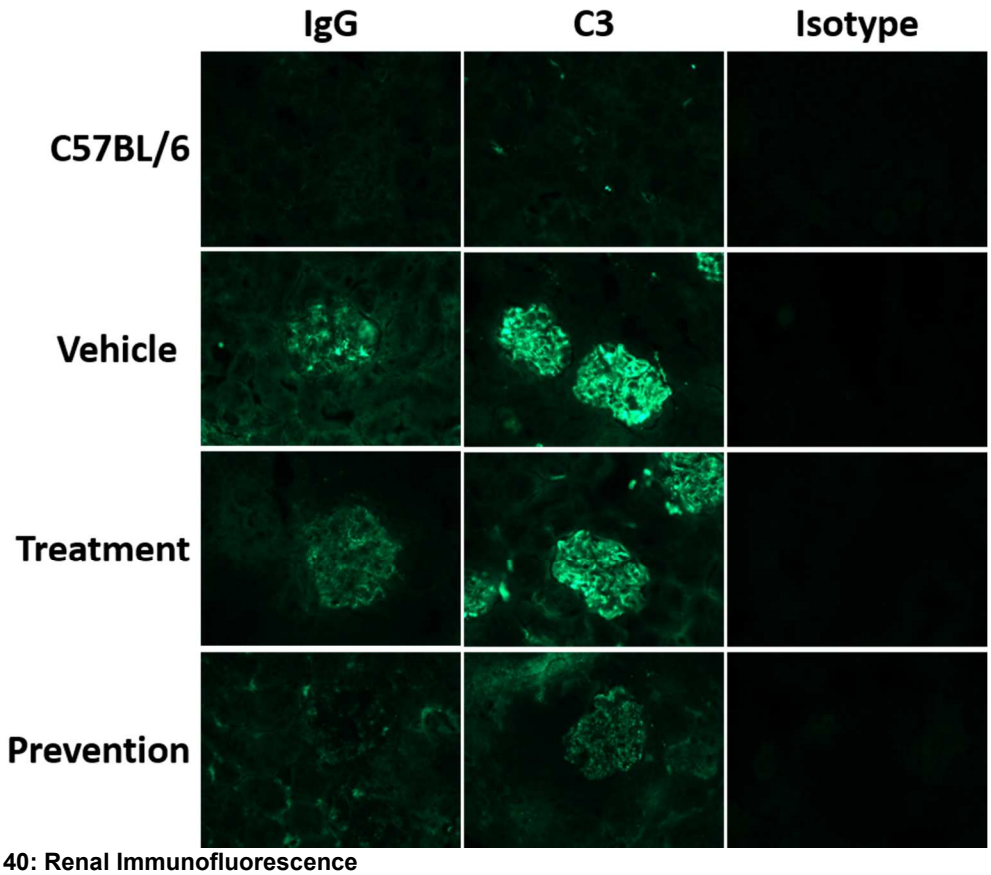

Figure 40: Renal Immunofluorescence
Kidneys were collected after euthanasia. Frozen sections were probed for IgG and C3. Pictures are representative for each group.

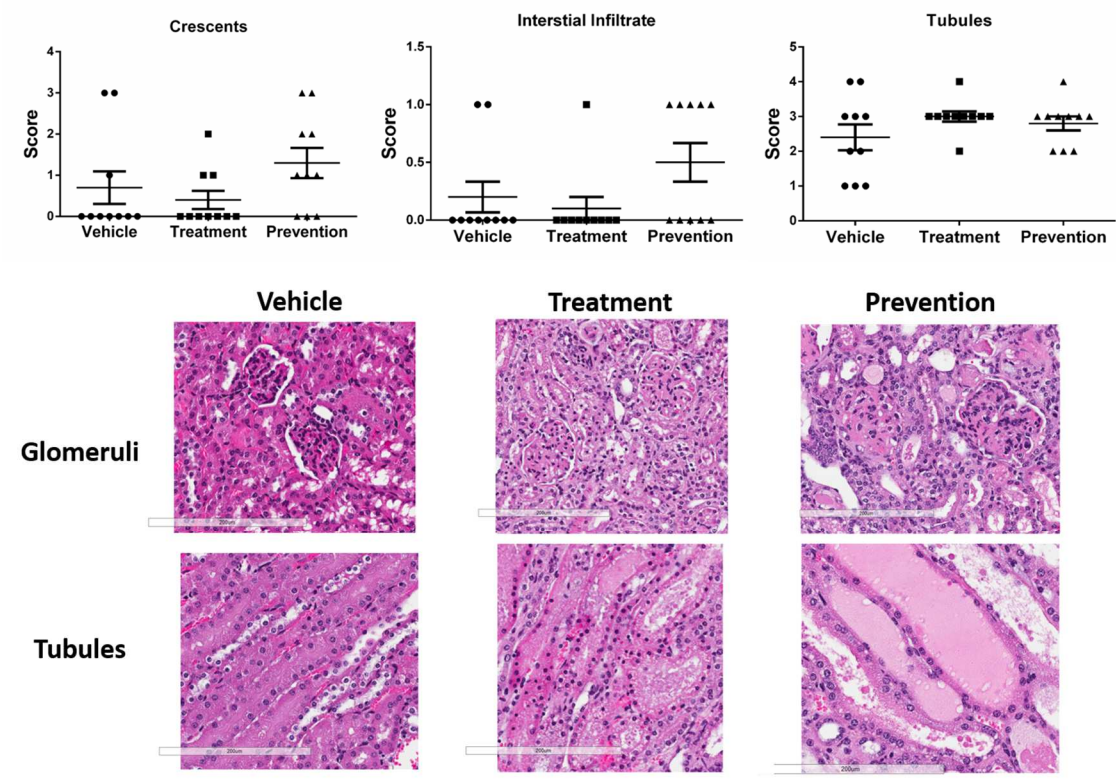

Figure 41: Renal histology of lapatinib treated mice
Kidneys were collected after euthanasia. Formalin fixed sections were stained and scored. Scoring was blinded and conducted by a third party. Pictures are representative for each group.

Fibrotic Markers are Increased in Lapatinib Treated Kidneys

We next analyzed kidney samples by western blot. Mice that were found dead were excluded. Blots were probed for type I collagen and α-smooth muscle actin (α-SMA) as markers for tissue fibrosis. Deposition of type I collagen is indicative of matrix production and scaring, while α-SMA is indicative of mesangial and fibroblast activation (reviewed in [388,389]). Consistent with worsened renal function, mice that received lapatinib had elevated fibrotic markers compared to vehicle controls (Figure 42). These results support the notion that lapatinib administration worsens renal disease in IFN-accelerated murine lupus.

Lapatinib Prevents Renal Cell Tissue Repair In Vitro

Although lapatinib seems to lower autoantibody titers, renal disease is worse in prevention group mice. These results suggest that lapatinib may in fact impair tissue repair processes in the kidney, as opposed to preventing maladaptive proliferation as hypothesized. To test if lapatinib inhibits tissue repair *in vitro* we grew primary renal cells from B6 kidneys until confluent. Cells were treated with lapatinib and/or IFNα 30 minutes prior to scratching with a 1000μl pipette tip. Twenty four hours later scratches in the renal cell culture were assessed for growth back into the scratched area (Figure 43). After 24 hours, untreated and IFN-treated renal cells grew back into the scratched area. In contrast, renal cells treated with lapatinib showed an impaired ability to grow into the scratched area, suggesting that EGFR-family signaling is required for tissue repair in the kidney (Figure 43).

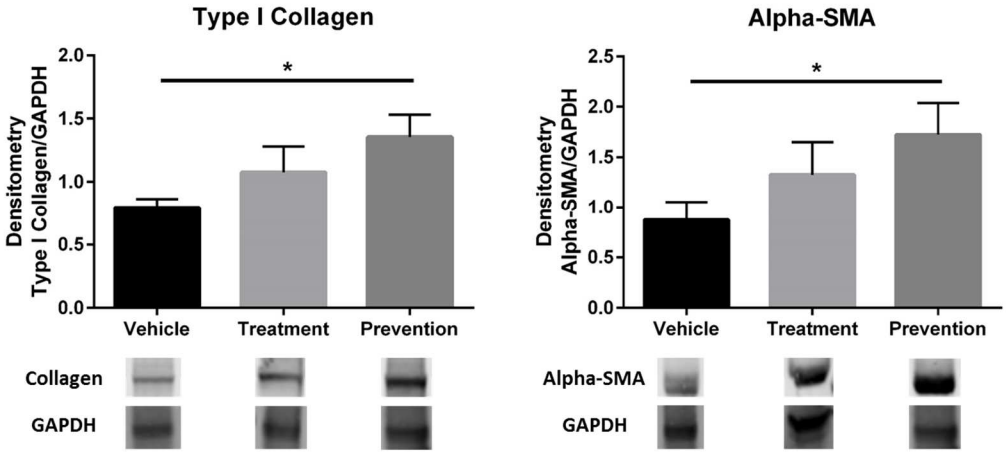

Figure 42: Fibrotic markers are increased in lapatinib treated kidneys
Kidneys were collected from mice and processed for western blot analysis. Blots were probed for type I collagen and α-smooth muscle actin as markers for fibrosis. Mice that were found dead were excluded. Representative bands are shown. Densitometry was used for semi-quantitative analysis. Student T-test was used to compare groups (* $p<0.05$).

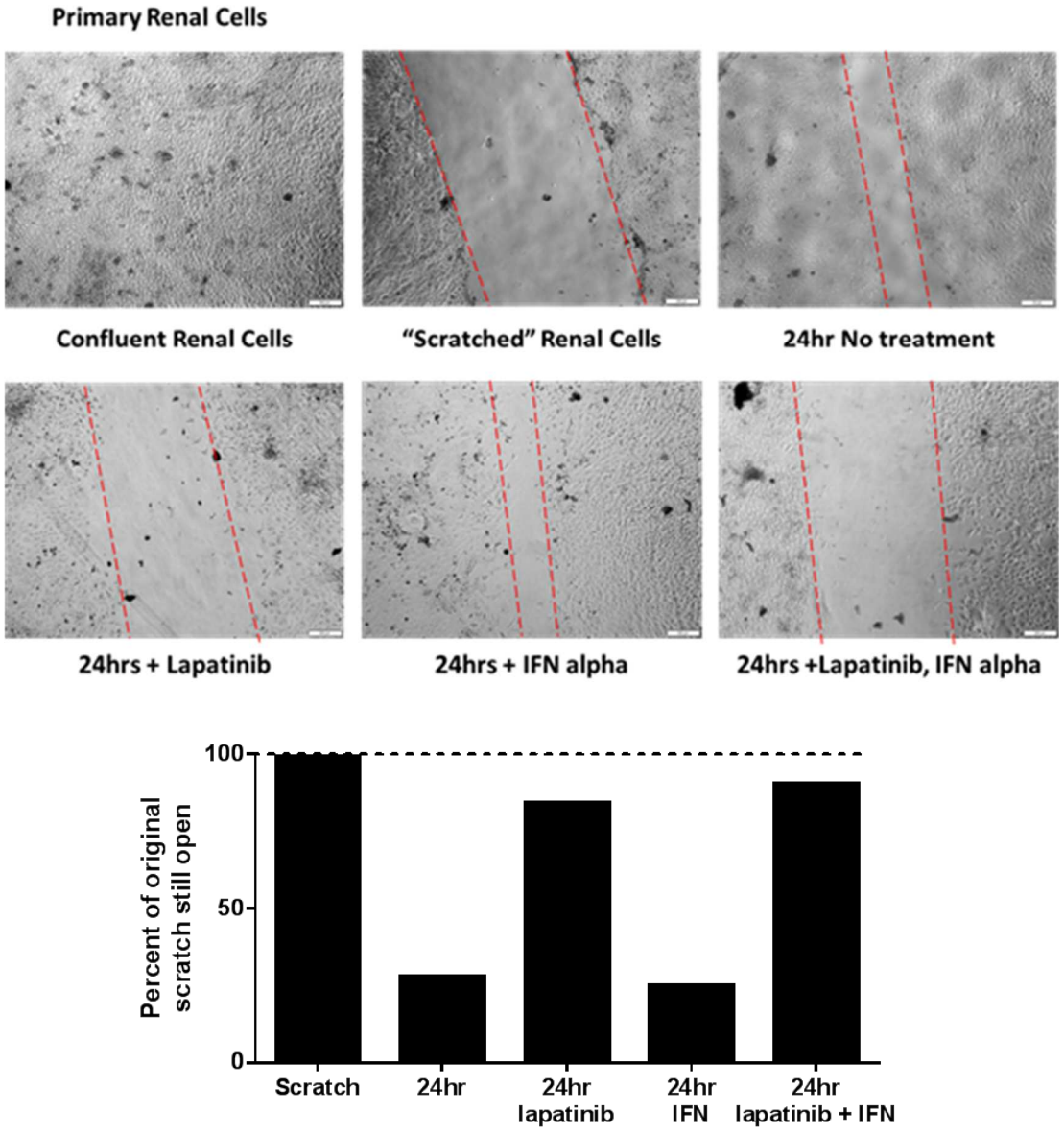

Figure 43: Lapatinib prevents renal cell tissue repair *in vitro*
Primary renal cells were cultured from B6 mice as described in the materials section. Cells were treated with Lapatinib and/or IFN-α 30 minutes prior to scratching. A scratch was made in the renal cell monolayer with a 1000μl pipette tip. Pictures were taken at the time of scratching and 24 hours later. Pictures are representative of three independent experiments.

Discussion

General Conclusions

In this investigation we explored the role of EGFR-family receptors in the pathogenesis of lupus nephritis. We built on the observation that the kidneys of both SLE patients and lupus-prone mice showed significant up-regulation of HER2 (ErbB2) compared to controls. Because of their potential to stimulate growth and proliferation we hypothesized that EGFR-family receptors might contribute to maladaptive tissue repair processes, leading to the proliferative glomerulonephritis characteristic of lupus. This hypothesis was supported by previous work suggesting that EGFR-family receptors promote immune-mediated kidney disease (see chapter 1). We therefore designed an investigation to test the safety and efficacy of lapatinib, an EGFR-ErbB2 dual kinase inhibitor, in an accelerated model of murine lupus.

To model severe lupus nephritis we used lupus-prone NZBxW/F1 mice and accelerated natural disease progression with a single dose of an IFN-expressing adenovirus. Unfortunately, we found that lapatinib did not improve renal disease in this mouse model. Indeed administration of lapatinib before the onset of overt kidney disease (the prevention group) worsened proteinuria and accelerated mortality. Interestingly mice receiving lapatinib from the beginning of the trial (the prevention group) had significantly lower autoantibody levels compared to the control group, suggesting that lapatinib might have an effect on the B cell compartment. Despite lowered autoantibody levels, lapatinib administration seemed to worsen renal function – likely in part due to the inhibition of cell growth and repair in the kidney (Figure 43).

Reasons for Lapatinib Treatment Failure

In comparing our results to those found in the literature, we found a number of differences that may have contributed to the “failure” of lapatinib to treat murine lupus nephritis. The first consideration is with the use of lapatinib itself. Unlike other EGFR-family inhibitors that have been used in murine models of kidney disease, lapatinib is a dual kinase inhibitor – blocking both EGFR and ErbB2. By blocking both receptors we may have obstructed any protective redundancy within the signaling pathway that would otherwise promote tissue repair. Indeed,

although EGFR-family receptors might promote fibrosis, they also mediate a number of anti-inflammatory and tissue repair processes. The EGFR ligand amphiregulin, for example, is known to orchestrate tissue repair (reviewed in [390]) and has recently been shown to support regulatory T cell functions.[343] In our investigation lapatinib may have overcompensated, hindering the ability of the kidney to heal itself.

A second consideration is the mouse model. The NZBxW/F1 mouse is a well-studied model of spontaneous polygenic lupus. However, accelerating the disease using IFN-expressing adenovirus may alter the pathophysiology of this otherwise chronic disease (reviewed in [50]). IFN overexpression may make the nephritis in this model more acute and therefore make EGFR-family signaling more important in tissue repair. In other words, the IFN acceleration may have been too strong a stimulus to study EGFR-family inhibition in lupus nephritis. So while our results argue against the hypothesis that EGFR-family receptors promote lupus nephritis, they by no means rule it out. Future work will be required to determine if other drugs targeting this pathway might be useful in spontaneous lupus.

Lapatinib in Human Use

Our results raise another important issue. Lapatinib is approved for human use as a therapy for EGFR-positive cancers, including advanced breast cancers. During clinical trials lapatinib was found to cause significant liver damage.[391] Warnings therefore exist for the use of lapatinib in patients with hepatic insufficiency. However, very little lapatinib is excreted by the kidneys and clinical trials have not explored the use of lapatinib in renal disease – leaving the safety of lapatinib in renal disease unknown. Renal failure has been reported in patients receiving lapatinib, but these patients often have multi-organ damage and it is unclear if lapatinib contributed to failure.[392] Lapatinib has also been studied in patients with advanced renal cell carcinoma. Lapatinib had a modest benefit in renal cell carcinoma patients, suggesting that lapatinib did not worsen renal disease in this context.[387] In this investigation we found that lapatinib worsened kidney disease in a murine model of lupus nephritis, suggesting that lapatinib may hasten the progression of chronic kidney disease. The incidence of cancer in lupus patients

is generally similar to that of the general population – although lupus patients may have a higher risk of developing some lymphomas and a slightly decreased risk of breast cancer. To our knowledge there are no studies that specifically investigate the safety of lapatinib or other EGFR inhibitors in lupus patients with cancer. Our results suggest that the use of lapatinib in lupus patients with EGFR-responsive cancers should be done carefully.

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APPENDIX A: BONE MARROW CULTURE

Bone Marrow Culture Protocol

PURPOSE
To grow bone marrow derived dendritic cells for *in vitro* experiments.

BACKGROUND
DCs are isolated from other cell types in the bone marrow. Physical disruption will lead to production of type I interferons, so we plate them in the wells that the experiments will be done in. IL-4 can be used (at 500 µl added to DC media) depending on the DC physiology you want.

MATERIALS

DC Media

- Iscove's modified IMDM - 500 ml – Cat. 10-016-CV (Cellgro)
- Fetal Bovine Serum - 50 ml – Cat. 100-106 (Gemi bioproducts)
- 2-mercaptoethanol (optional) - 500 µl – Cat. 21985-023 (Gibco)
- Pen/Strep (100U/ml) - 5 ml – Cat. 15140-122 (Invitrogen)
- L-glutamine (100x) - 5 ml – Cat. 25030-081
- Gentamicin (50mg/ml) - 500 µl – Cat. 15750-060 (Invitrogen)
- Recomb. GM-CSF (from working stock) - 500 µl – Cat. 554586 (Pharmingen)

- 1x sterile PBS
- Ethanol
- 50 ml tubes – Cat. 227270
- 100 micron cell strainer – Cat. 352360 (BD bioscience)
- 6 well plate or small petri-dishes
- 3 ml syringe and 18 or 21 guage needle
- Sterile gauze
- 24 well plate

MACS buffer

- 1x sterile PBS - 500 ml
- FBS - 5 ml
- 0.5 M EDTA - 5 ml
- Sterile filter and let the vacuum fun for 10 minutes (degassing to avoid air bubbles or clogging in the MACS comumns)

Other

- LS columns - Cat. 130-052-201 (Miltenyi)
- Thy 1.2 microbeads - Cat. 130-049-101 (Miltenyi)
- MHC II microbeads - Cat. 130-052-401 (Miltenyi)

EXPERIMENTAL PROTOCOL

Day 1

- De-pant and remove legs, move to hood in conical filled.
- Transfer the bones to small petri dish (or well of a 6 well plate)
- Clean muscle off bones.
- Add 2 ml of 70% ethanol to a small petri dish and add cleaned bones for 1 minute (only).
- Take a clean gauze and dry the bones for 1 minute. Put the bones into a new petri dish with fresh IF-10.
- Use scalpel to cut off the top of the bone.
- Use syringe to flush out bone into a 50 ml conical tube using 5 ml media.
- Repeat the last step from both ends of the bone until the bone is cleaned out.
- Usually takes about 25-30 ml to flush one set of bones.
- Take another clean tube and place the 100 micron mesh inside. Pipet the cell suspension with a 25 ml pipet and pass it through the mesh.
- Count cells
- Centrifuge the suspension at 1600 rpm for 10 min at 4 deg. C
- From a normal mouse about 6-8 weeks of age, about 60-90 million bone marrow precursors can be obtained.
- Resuspend the cell pellet in DC media at desired concentration.

Time Table

- Usually start culture on Wednesday (Day 0)
- Add 1 ml DC media on Friday evening (Day 2)
- Replace 1 ml DC media on Monday morning (Day5) – carefully take off 1 ml of media and add 1 ml of fresh media to each well. Use pipet if necessary, but be careful to remove only about 1 ml from each well)
- The cells are ready to use by Tuesday and could be used until Friday for any assay.
- Typically over 70% will be $CD11c^+$ cells by flow analysis. With IL-4 included in the media the percentage is considerably higher.

Autoantibody ELISA

Purpose
To measure autoantibodies to dsDNA and chromatin in mouse or human serum

Background
Lupus prone mice – including MRL/lpr, NZB/W-F1, and Sle1,2,3 – develop high titers of autoantibodies against dsDNA and chromatin. Sle1,2,3 females develop autoantibodies around 12 to 16 weeks of age. Nephritis does not set in until 4 to 6 months of age.

Materials
Poly-L-Lysine (Sigma cat# P8920-100mL) – 0.01% in PBS (1:10 dil).
For full plate, 9mL PBS + Poly-L-Lysine

BBS Wash Buffer (4x) – 17.5g NaCl + 2.5g H_3BO_3 + 38.1g Na. Borate.$10H_20$
In 1000mL ddH_20. pH 8.4
1x BBS – 250mL of above solution + 750mL ddH_20

BBT (Blocking Buffer) – BBS + 3% BSA + 1% Tween 80
1.5g BSA and 500µl Tween 80 in 50 mL BBS

BBST (Sample and Antibody dilution buffer) – BBS + 0.5% BSA + 0.4% Tween 80
0.25g BSA and 200µl Tween 80 in 50mL BBS

Biotinylated goat anti-mouse IgG (Jackson ImmunoRes cat. No. 115-065-071)
1:5000 dilution in BBT (or BBST)

Avidin-alkaline phosphatase conjugate (Sigma, cat. No. A7294)
1:8000 dilution in BBT (or BBST)

Alkaline phosphatase pNPP liquid substrate

Flat-bottom 96-well PVC plate

Experimental Protocol
Day-1

1) For dsDNA, pretreat plates with 100ul/well of 0.01% poly-L-lysine for 1 hr at room temperature (this is not required for chromatin)
2) Wash three times with distilled water or BBS
   a) This can be stored for up to 1 week before use at RT
3) Dilute dsDNA and chromatin in BBS
   a) dsDNA (calf thymus DNA)
      i) 2.5µg/mL BBS
      ii) Our stock is 4.2µg/µl, so for 10ml add 6µl
   b) Chromatin (chicken chromatin)
      i) 3µg/mL BBS
      ii) Our stock is 1µg/µl, so for 10ml add 30µl
4) Coat each well with 100µl of above dsDNA/Chromatin solution. Seal the plate with parafilm and keep at 4°C overnight.

Day-2

1) Wash plate 3x with 200µl BBS.
2) Add 200µl of BBT to each well and block for 2hrs at RT by gentle rocking.
3) Make 1:250 dilution of MRL/lpr control serum and test samples in BBT in eppendorf tubes.
4) Wash plate 5x with BBS.
5) Add 200µl of 1:250 diluted control to first two wells and to the rest of the wells at 100µl of BBT. Now perform two-fold serial dilutions up to 1:512,000 on the plate itself.
6) Add 100µl of diluted test serum to the respective wells.
7) Seal the plate with parafilm and keep it at 4^{o}C overnight.

Day-3

1) Wash plate 5x with BBS.
2) Add 100µl of 1:5000 dilution of biotinylated goat anti-mouse IgG in BBT to each well. Incubate for 2 hrs at RT.
3) Wash plate 5x with BBS.
4) Add 100µl of 1:8000 dilution of avidin-alkaline phosphate conjugate in BBT to each well. Incubate for 2 hrs at RT.
5) Wash plate 5x with 200µl BBS
6) Add 100µl of pNPP alkaline phosphatase substrate solution to each well. (You will see some crystal formation in the bottle. It will help if you warm it at RT before adding, but even if there is some crystal left, it won't interfere with your reading.)
7) Incubate at 37^{o}C and read the optical densities at time points 1hr, 4hr, and 20hrs at 650nm and 405nm using ELISA plate reader. Subtract the reading obtained at 650nm from that obtained at 405nm.

Anti-Curli IgG ELISA

Purpose

To measure antibodies to curli in mouse or human serum.

Background

Adapted from ELISA used to measure autoantibodies:

- Cohen P, Maldonado M. Animal models for SLE. Current protocols in immunology / edited by John E. Coligan ... [et al.]. 2003;Chapter 15.

Materials

BBS Wash Buffer (4x) – 17.5g NaCl + 2.5g H_3BO_3 + 38.1g Na.Borate.10H_20

- In 1000mL ddH_20. pH 8.4

1x BBS – 250mL of above solution + 750mL ddH_20

BBT (Blocking Buffer) – BBS + 3% BSA + 1% Tween 80

- 1.5g BSA and 500µl Tween 80 in 50 mL BBS

Biotinylated goat anti-mouse IgG Fc (Jackson ImmunoRes cat. No. 115-065-071)

- 1:5000 dilution in BBT
- Other biotinylated secondary antibodies can be used to measure other isotypes.

Avidin-alkaline phosphatase conjugate (Sigma, Cat. No. A7294)

- 1:8000 dilution in BBT

Alkaline phosphatase pNPP liquid substrate (Sigma, Cat. No. P7998–100ML)

Flat-bottom 96-well PVC plate (Any PVC plate should do: BD Falcon, REF 353912)

Experimental Protocol

Day-1

5) Dilute curli in BBS (3µg/mL)
6) Coat each well with 100µl of above curli solution. Seal the plate with parafilm and keep at 4°C overnight.

Day-2

8) Wash plate 3x with BBS.
9) Add 200µl of BBT to each well and block for 2hrs at RT by gentle rocking.
10) Make a 1:250 dilution in BBT in eppendorf tubes for each sample (or other ratio determined empirically).
11) Wash plate 5x with BBS.
12) Add 100µl of diluted test serum to the respective wells.
13) Seal the plate with parafilm and keep it at 4°C overnight (or for 4 hrs).

Day-3

8) Wash plate 5x with BBS.
9) Add 100µl of 1:5000 dilution of biotinylated goat anti-mouse IgG in BBT to each well.
10) Incubate for 2 hrs at RT.
11) Wash plate 5x with BBS.
12) Add 100µl of 1:8000 dilution of avidin-alkaline phosphate conjugate in BBT to each well.
13) Incubate for 2 hrs at RT.
14) Wash plate 5x with BBS
15) Add 100µl of pNPP alkaline phosphatase substrate solution to each well. (You will see some crystal formation in the bottle. It will help if you warm it at RT before adding, but even if there is some crystal left, it won't interfere with your reading.)
16) Incubate at room temperature and read the optical densities using ELISA plate reader at 650nm and 405nm at time points 20 min, 1hr, and 2 hrs (or other time point determined empirically). Subtract the reading obtained at 650nm from that obtained at 405nm.

APPENDIX D: B7H1-GM HYBRIDOMA CELL LINE

B7H1-GM Hybridoma Cell Line for GM-CSF Conditioned Media

Purpose

Recombinant GM-CSF is extremely expensive. Growing B7H1-GM hybridoma cell line that produces mGM-CSF will help save money. Supernatant containing GM-CSF can be used for growing bone marrow derived dendritic cells (DCs).

Background

The cells come from a collaborator at Penn. They are fibroblast hybridomas. Cells were frozen in 10% DMSO. I don't know much about the cells themselves and I had trouble finding helpful literature. GM-CSF production requires adding Hygromycin B, but we don't want Hygromycin B in the final supernatant. So cells must be grown with Hygromycin B first. Then, once enough cells are present, cells are cultured in media without Hygromycin B to produce final supernatant.

Materials

- Hygromycin B: Roche # 10 843 555 001
    - Stock = 1g in PBS (20ml). 50 mg/ml, sterile
    - Use final concentration 1.2 mg/ml
- IF-10
    - Icove's media (IMDM) plus 10% FBS, 1% P/S, and 1% Q, and 50 mM 2-ME.
- T-75 flasks

Protocol Notes

- The protocol given to us (PDF from collaborator) uses RPMI. But we usually grow DCs in Icove's media (IMDM), so I grow this cell line in IMDM. They grow well in IMDM.

Experimental Protocol

Day 1

1. Quickly thaw cells and dilute in IF-10
2. Centrifuge for 5 minutes
3. Resuspend the pellet in IF-10 and then add into 3 wells of a six well plate.
    a. The protocol from the collaborator says to do a dilution and plate the supernatant left over from the centrifugation. I'm not sure this is necessary. Could probably plate resuspended cells directly into a T-75 flask.

Day 2

1. Aspirate off all the media to remove dead cells.
2. Wash the cells to remove remaining DMSO and add fresh media.
    - If the cells look good (adherent and relatively confluent), you may add media with 1.2 mg/ml Hygromycin B. But I usually let them grow a bit more and add Hygromycin when I plate them in flasks.

Following Days

1. Culture cells in IF-10 with Hygromycin B (1.2 mg/ml).
    a. I usually do 20 ml in each T-75 flask and let them grow to confluence.
    b. For splitting use 1% Trypsin (according to the given protocol, but I've used the 5% Trypsin more commonly available).
    c. Freeze down some healthy cells in IF-10 + 10% DMSO
2. Split the cells in IF-10 with Hygromycin B until you have enough flasks for the volume you'd like.
3. For final splitting, withdraw the Hygromycin B, wash in IF-10 medium **without Hygromycin.**

4. Let the cells grow to confluence in the flask (without Hygromycin B) and then collect the supernatant.
    a. We have begun using multilayer flasks for the final splitting. These flasks have 3 or 5 layers of cells within the same volume. This will help produce a more concentrated supernatant.
5. Filter the supernatant, and freeze at -80
    a. For DC cultures, the amount of GM-CSF needs to be within a range (i.e. adding too much GM-CSF will cause the culture to act more like macrophages).
    b. We generally make a big batch of supernatant and then test it for the best concentration. To test it we grow DCs in different concentrations of supernatant and stimulate some wells with LPS (or other TLR ligand). We then measure cytokine production and analyze cells for DC markers CD11c, CD11b, and MHC class II by flow cytometry. We also have a GM-CSF ELISA to get an idea about the optimal concentration.

APPENDIX E: GENERAL URINALYSIS PROTOCOL

General Urinalysis Protocol

Purpose

To evaluate renal function in mice

Background

Adapted Urinalysis protocol from:

- Seavey M, Lu L, Stump K. Animal models of systemic lupus erythematosus (SLE) and ex vivo assay design for drug discovery. *Current protocols in pharmacology / editorial board, S.J. Enna (editor-in-chief) ... [et al.]*. 2011;Chapter 5:Unit 5.60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21935901.

Materials

- PBS
- From Seavey, et al: *Standard protein solution: prepared from normal mouse sera and used as a standard for mouse urinary protein assay by turbidity—prepare by collecting or purchasing normal mouse sera (Jackson Immunoresearch, cat. no. 015-000-120), generating a 1 mg/ml serum standard in PBS (based on manufacturer-reported values), then filter-sterilizing*
    - I've used BSA dissolved in PBS as a protein standard.
- Urine samples
- 3% sulfosalicylic acid (Ricca chemical company, cat. No. 8115-16) (can be ordered through fisher)
- BD Falcon 96-well flat bottom plate
- Can also use Siemens Uristix stick assay
    - Note: for what we use "multistix 5" works well (Siemens No 2309) Others may be more expensive.

Experimental Protocol

Urine Collection

- Hold the mouse in one hand, holding the scruff of the neck between the index and thumb and the tail with the little finger.
- Hold the mouse over an Eppendorf tube to collect the urine or over a clean plastic surface (for uristix).
- Gently massage the abdomen/bladder with the free hand, taking care to direct the urine into the tube or onto the clean surface.

Urinalysis

- If using uristix, touch the detection surface to the urine on the clean plastic. Read at the time directed by the sticks' instructions. Wipe surface clean before repeating with the next mouse.

- If using turbidity assay, keep samples on ice or freeze at -20 until ready.
  Adapted from Seavey, et al:
- Add 5, 10, 15, 20, 30, 40, and 50 µl of a 1 mg/ml standard protein solution in duplicate into two columns of the 96-well plate, designating two wells as blanks to control for background.
- Add sufficient PBS to individual wells to adjust the final volume to 50 µl.
- Microcentrifuge urine samples 3 min at maximum speed, 4◦C.

- Add 1 to 50µl of the urine supernatant in duplicate, then add PBS to adjust the volume to 50µl. (I usually do 5µl)
- Add 200 µl of 3% sulfosalicylic acid to the test columns.
- Allow the microplate to stand for 10 min at room temperature and read the plates using an ELISA reader with single beam at 450 nm.