THE BIOLOGY OF DENDRITIC CELLS IN THE CONTEXT OF AUTOIMMUNITY

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by
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ABSTRACT

Systemic lupus erythematosus (SLE) is a complex autoimmune disease that affects at least five million people worldwide. An increased expression of type I interferon (IFN) regulated genes is a hallmark of SLE, but the precise etiology of SLE initiation and flares is poorly understood. Because plasmacytoid dendritic cells (pDCs) are the primary type I IFN producers, their role in SLE has long been suspected, with murine pDC depletion models successfully delaying the progression of murine lupus-like disease. However, the mechanism behind how exactly how pDCs contribute to lupus autoimmunity is unknown, contributing to the current dearth lack of disease modifying treatments; current treatments only succeed in suppressing symptoms, and do not halt disease progression. In this study, we take a multifactorial approach to understanding the biology of pDCs in the context of lupus autoimmunity.

Although the exact etiology of lupus is unknown, infections are an important environmental trigger for lupus flares, and are a major cause of morbidity and mortality. Medically, bacterial biofilms are important for the establishment of chronic and persistent infections. Bacteria are embedded into their biofilms, allowing them to dramatically increase their ability to withstand physical insults and tolerate host defenses. To strengthen the extracellular matrix of their biofilms, bacteria produce amyloids, complex proteins with a conserved beta sheet structure. Our group has reported that *Salmonella* and *E. coli* biofilms contain the functional amyloid protein curli contained in *Salmonella and E. coli* biofilms form complexes with eukaryotic and bacterial DNA. Systemic administration of these curli/DNA complexes purified from biofilms accelerated autoimmunity in lupus-prone mice, and triggered production of autoantibodies in wildtype mice. Infections with curli-expressing *Salmonella* or *E. coli* gave the same results, implicating curli in lupus pathogenesis. We present here for the first time that bacterial amyloid curli/DNA complexes activate pDCs, the aforementioned primary producer of type I IFNs. Additionally, we show that curli/DNA complexes are capable of inducing expression of interferon-stimulated genes (ISGs) including ISG-encoded CXCL10 and robust production of IFNα. In addition, curli/DNA complexes are capable of inducing *in vivo* activation of DCs in lupus-prone and wildtype mice. Curli/DNA complex activation of *in vitro* DCs is partially hindered in the absence of toll-like receptor (TLR) 9, but is not inhibited in the absence of TLR2, indicating that signaling through the DNA-recognizing TLR9 is a possible mechanism for how bacteria amyloid is being recognized and activating pDCs. Interestingly, depletion of pDCs immediately prior and during curli injections delays in the development of autoantibodies, strongly implicating pDCs in the ability of bacterial amyloid curli/DNA complexes to stimulate autoimmunity. Our results identify bacterial amyloid curli/DNA complexes as a novel activator of pDCs and suggest that curli/DNA complexes may accelerate lupus by directly activating pDCs and their powerful type I IFN activity. Our results also provide evidence for a direct role for pDCs in bacterial amyloid-stimulated autoimmunity and highlight the important mechanistic role of TLR9. These results support a pathogenic role for bacterial infections in SLE and suggest that the activation of pDCs by curli/DNA complexes is one of the mechanisms mediating such pathogenicity.

Type I IFNs play a central role in immunology, orchestrating immunomodulatory responses to shape both the innate and adaptive immune response. The transcription of ISGs is canonically activated by the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway. Engagement of the IFN α/β receptor (IFNAR) activates JAK1 and tyrosine kinase 2 (TYK2), resulting in the tyrosine phosphorylation, dimerization, and translocation to the nucleus of STAT1 and STAT2 in complex with IRF9. Since some type I IFN responses were shown to require only STAT1, our previous studies showed that STAT2 is constitutively expressed in murine DCs and is required to signal IFN-dependent responses. It is unknown, however, whether a functional STAT2 in endogenous DCs is necessary for the development of cross-presentation responses, critical for the induction of antibody responses because of the DC ability to directly stimulate follicular T helper cells, which promotes the classic antigen-specific antibody response by providing help to B cells. In this study, we investigated the unique DC requirements of STAT2 with a conditional STAT2 knockout (KO) mouse that has a deletion in only CD11c+ cells, a surface marker expressed by most dendritic cells. We report for the first time that the STAT2 function in DCs is essential for cross-presentation to CD8+ T cells and stimulation of an Ag-specific cytotoxic T lymphocyte (CTL) response, while it is dispensable to elicit an antigen-specific antibody response. These findings indicate that STAT2 is crucial for specific DC responses.

Finally, our studies led us to explore pDCs as a therapeutic target that would be aimed at the root of SLE pathology. Changes in fatty acid metabolism are essential for pDC activation and response to type I IFNs, and our data confirms that inhibition of this metabolic reprogramming significantly impairs DC activation at doses that do not affect cell viability. We investigated two inhibitors of fatty acid metabolism, an inhibitor of oxidative phosphorlation (OXPHOS) and a dual inhibitor of glycolysis and OXPHOS. Etomoxir inhibits the enzyme of faty acid oxidation (FAO) carnitine palmitoyltransferase I, and 5-tetradecyloxy-2-furoic acid (TOFA) inhibits the fatty acid synthesis enzyme acetyl-CoA carboxylase. We also chose to test pyruvate analog ethyl pyruvate, which our group previously showed to inhibit glycolysis and OXPHOS and it has been shown by others to possess anti-inflammatory properties in various cell types *in vitro* and also in many animal models of disease such as severe sepsis and ischemia-reperfusion injury. We also investigated the effect of metformin on DC activation, which activates AMPK and inhibits oxidative phosphorylation by blocking the mitochondrial respiratory chain complex I. *In vitro*, our results demonstrate that the inhibition of FAO affects that upregulation of DC surface costimulatory molecules. Our results show that *in vivo* modulation of metabolic pathways necessary for DC activation may postpone the development of autoantibodies in lupus-prone mice, showing for the first time that the inhibition of DC metabolism may have significant therapeutic benefits in SLE.

Together, the findings in this thesis identify key mechanistic players in dendritic cell biology. Indeed, they provide an explanation for how DCs may contribute to autoimmunity by recognizing the bacterial amyloid from bacterial biofilms. Additionally, we discovered that STAT2, the signaling protein downstream of IFNAR, is essential for the ability of DCs to mount CTL responses, elucidating a mechanistic aspect of IFN signaling in DCs, while it is not necessary for antibody production, suggesting the lack of involvement of the response of DCs to type I IFNs in humoral autoimmunity. Finally, our findings identify a possible novel therapeutic target, by demonstrating how metabolic modulation of pDCs and other DC subsets may prevent their production of inflammatory cytokines without affecting cell viability. Overall, these findings help expand our knowledge on the complex intricacies of dendritic cell biology and autoimmunity.

To Danny, without whose fun presence this thesis would have been finished in half the time.

To Pippa, who is a better person than all of us even though she is a dog.

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"Knowledge is in the end based on acknowledgment." – Ludwig Wittgenstein, *Über Gewissheit (On Certainty)*, 1969

Knowledge has always been the heart of the philosophical method, and as such, the dangers of certainty in science inspired me to include this quote, one of a remarkable set of 676 aphorisms jotted down by Ludwig Wittgenstein in the years just prior to his death. Possibly one of the most important lessons I learned during this PhD thesis is that the human will to certitude is incontrovertibly fallibilist, and that the uncertainty about certainty is something to embrace.

I will admit that the poetics of this aphorism is unpalatably lessened by its usage here to belabor a substandard-at-best philosophical wordplay*, but in the interest of time, space, and modesty, I digress . . .

First and foremost, I would like to thank my advisor, Dr. Stefania Gallucci, for taking me under her wing during the last couple of years. Her unwavering support, patience, and perseverance throughout my studies has helped me to develop into a critical thinker, a teacher, a writer, and a scientist. Dr. Gallucci has granted me so many opportunities to ask my own questions and to take on projects, giving me a wonderfully well-rounded PhD training in this short amount of time. She has set me on my way to a lifetime of academic medicine, and I look forward to chatting about science many years from now.

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* David Foster Wallace's essay, "Authority and American Usage," has inspired me to take special consideration of syntax, grammar, and rhetoric, and more importantly, taught me through shame by my failure to recognize right away why the term "Acknowledgement" was on the page of literary abhorrence in the essay's inset in "Consider the Lobster." Hint: spelling.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
ANA	Anti-nuclear antibody
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
B6	C57Bl/6 wildtype mice
B cell	Bursa-derived lymphocyte
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CFSE	5- (and 6-) carboxyfluorescein diacetate succinimidyl ester
CpG	Cytosine guanine dinucleotide
CLEC4	C-type lectin domain family 4
CTL	Cytotoxic T lymphocyte
CXCL	Chemokine C-X-C motif ligand
DC	Dendritic cell
cDC	Conventional dendritic cell
pDC	Plasmacytoid dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ds	Double-stranded (as dsDNA)
eDNA	Extracellular DNA

DT	Diphtheria toxin
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
EP	Ethyl pyruvate
EPS	Extracellular polymeric substances
Eto	Etomoxir
FA	Fatty acid
FACS	Fluorescence-activated cell sorting
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLT3-L	*Fms*-related tyrosine kinase 3 ligand
FVD	Fixable viability dye
g	Gram
GM-CSF	Granulocyte-macrophage colony stimulating factor
h	Hour
IFA	Incomplete Freund’s adjuvant
Ig	Immunoglobulin (also IgA, IgG, etc.)
IFN	Interferon
IFNAR	Interferon receptor alpha receptor
IL	Interleukin
IMDM	Iscove’s modified Dulbecco’s medium
i.m.	Intramuscular

i.p.	Intraperitoneal
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
i.v.	Intravenous
JAK	Janus kinase
KO	Knockout
cKO	Conditional knockout
L	Liter
LPS	Lipopolysaccharide
2-ME	2-mercaptoethanol
Met	Metformin
MFI	Mean Fluorescence Intensity
mg	Milligram
min	Minute
ml	Milliliter
mRNA	Messenger RNA
μg	Microgram
μl	Microliter
NET	Neutrophil extracellular traps
ng	Nanogram
OD	Optical density
OVA	Ovalbumin
OXPHOS	Oxidative phosphorylation

p	Probability
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PRR	Pattern recognition receptor
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
T cell	Thymus-derived lymphocyte
Th	T-helper
TOFA	5-tetradecyloxy-2-furoic acid
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TYK	Tyrosine kinase
UTI	Urinary tract infection

CHAPTER 1

INTRODUCTION AND BACKGROUND

Review of the Literature

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the production of autoantibodies, i.e. antinuclear antibodies (ANAs), and an increased expression of type-I interferon (IFN) regulated genes, termed the IFN Signature.[1-7] The Lupus Foundation of America estimates that at least five million people worldwide have a form of SLE.[8] Genome-wide association studies (GWAS) have established that lupus is a polygenic disorders in which complex gene interactions predispose the individual to disease development.[9,10]

"Lupus" is cognate with the Latin *lupus*, originally adopted to describe skin lesions that resembled the bit of a wolf.[11] In the mid-1800s, the term "lupus érythémateux" was first used by Pierre Louis Alpheé Cazenave, a French dermatologist who practiced medicine at the Hôpital Saint-Louis in Paris.[12] Due in part to Cazenave's observations, cutaneous lupus lesions were recognized to be associated with significant internal conditions, including nephritis, serositis, arthritis, and neurologic disease, eventually leading to Edmund L. Dubois to develop the concept of lupus as a spectrum of disease in 1964.[13,14] An important advance in lupus disease research arose from the identification of antinuclear antibodies (ANAs) in 1957 by Friou, after which the association of specific autoantibodies to specific clinical manifestations was discovered by a number of investigators.[15] The use of antimalarial therapy for lupus was initiated in 1894 by Payne with the use of quinine to treat cutaneous lesions.[16] Synthetic antimalarials became the mainstay of therapies by the 1950s,[14,16-18] and remained so until immunosuppressants, including systemic corticosteroids, came into use during the mid 20th century.[12] Since then, caring for patients with lupus has only proved to be a challenge, and in the last 60 years, only a single drug, belimumab, has been approved for SLE treatment.[19] Although many categories of immunosuppressive drugs are used off-label in SLE patients, including steroid-sparing medications such as azathioprine and methotrexate, and inhibitors of DNA synthesis such as cyclophosphamide and mycophenolate mofetil, chosen depending on clinical manifestations and concurrent comorbidities.[20,21] Although challenges arise from disease heterogeneity, this severe paucity of adequate therapeutics for lupus remains inexcusable. While some difficulties can be attributed to methodological foibles, it is becoming evident that a better understanding of underlying pathologies down to the details at the cellular level could help overcome barriers and address limitations in current SLE treatments.

Clinical Manifestations

Nearly every organ can be affected by the protean clinical manifestations of SLE, however, the presentation from patient to patient can vary dramatically. Patients usually present with a mixture of constitutional complaints, with the most common patterns including serologic, hematologic, skin, musculoskeletal, renal, or central nervous system manifestations. Whatever pattern of clinical presentation that dominates during the early years of SLE pathogenesis tends to prevail throughout the disease course for that patient. In addition to the clinical course being highly variable, SLE is also characterized by periods of remissions and relapses. Additionally, lupus is characterized by a striking 9:1 female to male ratio of disease incidence,[22] but the molecular mechanisms behind the female predominance are still under research.[23-26]

The clinical manifestations of lupus can be categorized broadly by organ involvement (Table 1). To create a better system to document the wide variety of symptoms a patient may experience, classification criteria have been developed for SLE. Previously, the American Rheumatism Association (now the American College of Rheumatology, ACR) developed criteria established by cluster analysis.[27] However, limitations are apparent especially due to the criteria being established by data primarily from Caucasian patients, despite African American race being an increased risk factor for developing lupus. In 2012, a revised classification criterion was developed by the Systemic Lupus International Collaborating Clinics (SLICC) to address weakness of the older ACR classification criteria, achieving a sensitivity of 97% (versus 83% with the ACR classification).[28] The SLICC criteria grades the patient as having SLE if four of the criteria, including at least one clinical and one immunologic, are satisfied, or if the patient has biopsy-proven nephritis with ANAs (Table 2).

A number of comorbidities related to SLE disease or therapy are also common in lupus patients. These include immunodeficiencies, fibromyalgia, osteonecrosis, osteoporosis, thyroid disease, and infection. Seriously infectious complications develop in about half of SLE patients, with a large majority of infections (80%) attributable to pathogenic bacteria. The seriousness and commonality of infection in the SLE disease course and the importance of environmental factors in the manifestation of disease has led to research efforts, including ones covered in this thesis, to explore the clinical and scientific significance behind this finding.

Table 1. Clinical Manifestations of SLE[29-42]

Constitutional Symptoms	• Fatigue (80-100% of patients), fever (>50%), and weight loss are present in most patients at some point • Myalgia • Infection is a major cause of morbidity
Arthritis & Arthralgias	• Arthritis (65-70%), tends to be polyarticular and symmetrical. Usually nonerosive
Skin & Mucosal Involvement	• Acute cutaneous lupus erythema (ACLE) commonly known as the "butterfly rash" presenting as erythema in a malar distribution • Discoid lesions • Photosensitivity • Oral and/or nasal ulcers, usually painless (in contrast to herpetic chancres) • Nonscarring alopecia
Vascular Disease	• Raynaud phenomenon (up to 50%) • Vasculitis (11-36%), most common type is cutaneous small vessel vasculitis manifesting as palpable purpura and petechiae • Thromboembolic disease (especially in the context of antiphospholipid antibodies) • Arterial (11%) and venous (5%) thrombotic events
Renal Involvement	• 50% of patients • Several forms of glomerulonephritis can occur, with presentations varying from asymptomatic hematuria to nephrotic syndrome
Gastrointestinal Involvement	• Up to 40%, majority caused by infections or adverse medication reaction • Lupus hepatitis, rare
Pulmonary Involvement	• Pleuritis • Pneumonitis • Pulmonary hypertension • Interstitial lung disease

	• Alveolar hemorrhage
Cardiac Disease	• Most commonly manifesting as pericarditis (affection 25% of patients), also cardiac disease associated with SLE can also involve the myocardium, valves, arteries, and cardiac conduction system. • Verrucous endocarditis • Heart block in neonatal lupus
Neuropsychiatric Involvement	• Cognitive dysfunction • Delirium • Psychosis, depression, anxiety, mania • Seizures • Peripheral neuropathies • Focal neurologic problems may suggest arterial thromboemboli
Ophthalmologic Involvement	• Retinal vasculopathy (cotton wool spots) • Keratoconjunctivitis sicca • Optic neuropathy • Choroidopathy • Scleritis, episcleritis • Anterior uveitis
Hematologic Abnormalities	• Anemia of chronic disease • Leukopenia (50%) • Thrombocytopenia (usually mild)
Lymphadenopathy & Splenomegaly	• Lymphadenopathy usually involves the cervical, axillary, and inguinal nodes • Splenomegaly especially observed in patients with active disease

Table 2. SLICC Diagnostic Criteria[43]

Clinical Criteria	***Immunological Criteria***
Acute cutaneous lupus	ANA above laboratory reference range
Chronic cutaneous lupus	Anti-dsDNA above laboratory reference range, or ELISA twice above laboratory reference range
Oral or nasal ulcers	Anti-Sm antibodies
Nonscarring alopecia	Antiphospholipid antibodies
Synovitis (2+ joints)	Low complement
Serositis	Direct Coombs test in the absence of hemolytic anemia
Renal	
Neurologic	
Hemolytic anemia	
Leukopenia or lymphopenia	
Thrombocytopenia	
SLE if either: 1. 4+ criteria (at least one clinical, one immunologic) 2. Biopsy-proven nephritis with ANA or anti-dsDNA antibodies The presence of criteria does not need to be concurrent. Criteria are cumulative.	

Review of Clinical Trials

The paucity of approved SLE therapeutics serves to reflect the challenges in developing effective treatments for the diverse manifestations and complex mechanics behind the pathology. Despite only one drug, belimumab, earning approval for SLE treatment in the last 60 years, SLE clinical trials have served as landmarks in subsequent advances, providing information to learn from regarding both disease pathology and trial design.

Dehydroepiandrosterone (DHEA), a weak androgen, was found in lower levels in SLE patients compared to healthy controls.[44] DHEA has modest immune modulatory effects,[45] and lower circulating levels correlated with increased disease activity, guiding randomized control trials (RCTs) investigating DHEA (and the DHEA formulation prasterone) supplementation in SLE treatment. Initially, the RCTs showed no improvement with treatment compared to placebo.[46,47] While response rates did not appear different between randomized groups, retrospective data analysis showed that different groups of patients with similar disease activity considered separately may differ in their response rate, and that patients with inactive SLE were very likely to achieve the primary endpoint with placebo.[48] Protocols of two large phase III RCTs were amended to include active SLE into the inclusion criteria, and subsequent to this change, the primary endpoint was met with a significantly larger percent of the treatment group as compared to the placebo group.[48,49] FDA approval was not granted, however, because of the change in protocol. These RCTs suggest that DHEA may reduce SLE disease activity in women with moderately active SLE, but FDA approval was not granted due to the change in protocol acting as a barrier to understanding the true impact of DHEA treatment. These studies highlight the need for appropriate patient selection and inclusion and exclusion criteria.

Rituximab, a CD20 directed monoclonal antibody (mAb) was also a promising SLE treatment, aiming to improve disease activity by B-cell depletion.[50] A large RCT randomizing 257 patients with active SLE found that the primary endpoint of a larger proportion of rituximab versus placebo arm patients achieving “complete clinical response” was not achieved, despite a clear mechanistic reasoning behind the drug, and earlier uncontrolled studies demonstrating promising improvements. Detailed study of the patients did find that B-cell depletion was achieved in 89.5% of patients treatment with rituximab, and there were very significant decreases in pathogenic anti-dsDNA antibodies and increases in complement levels compared to placebo.[51] However, both the treatment and control arms had similar rates of disease improvement. In this RCT, background immunosuppressants were allowed to be continued during the study, and a strong prednisone burst with a prolonged taper was initiated at study entry. Failure to find a significant difference in the endpoint despite obvious differences in clinically relevant biologic measures suggests that initial glucocorticoid burst may have induced similar disease control in both arms, and clouded the ability of researchers to detect differences in the treatment arm.[52] Additionally, the handling of background therapy must be taken into consideration.

Another therapeutic targeting a specific cell type is abatacept, a CTLA-4 fusion protein targeting CD80/86 on the surface of antigen presenting cells (APCs).[53,54] Abatacept’s mechanism of action was promising, and in this RCT, the degree of disease activity, background therapy, and initial glucocorticoid dose to manage the flare was carefully considered. The primary endpoint of fewer flares, however, was not met, as there were similar rates of new flares in both the treatment and placebo arms.[55] In further analysis after the trial, it was discovered that patients with primarily polyarthritis had a pronounced response with significant improvements and fewer flares when treated with abatacept, suggesting that organ system involvement should be considered for inclusion criteria as well.[52]

As research into lupus pathology progressed, the role of type I IFN in disease progression became increasingly evident .[56] Therefore, anifrolumab, a type I IFN receptor antagonist, was an extremely promising new therapeutic, and results from the RCT were highly anticipated. An initial phase II RCT showed promise with a decrease in the IFN signature, confirming biologic activity.[57] Interestingly, a benefit was only noted in patients with an increased IFN gene signature. Nonetheless, two subsequent phase III RCTs enrolled both high IFN signature patients and low IFN signature patients. In August of 2018, AstraZeneca and MedImmune announced that one of the trials failed to meet the primary endpoint. Results will not be released until the conclusion of the second RCT.

After learning important lessons from failed trials, phase III RCTs were carefully designed for belimumab, a promising mAb inhibiting autoreactive B cells through binding BAFF/BLyS.[58,59] Only seropositive patients were enrolled, and background glucocorticoids were strictly controlled. At 10mg/kg, the primary efficacy endpoint was met, with fewer severe flares and clinically meaningful improvements.[60,61] Belimumab was approved in 2011 for the treatment of seropositive SLE patients with active disease. Follow-up studies provided reassuring evidence that belimumab has some modest benefit, but it is clear that it doesn’t induce a rapid clinical benefit.[52] Additionally, few African

American patients were included, and there is very little known about its effect on patients with renal, heart, lung, or CNS disease, subgroups of patients who would benefit from new therapies.[62] The many unanswered questions and lackluster clinical benefit emphasizes the benefits of a well-designed trial, and the continued need for additional lupus therapeutics research. Despite seemingly disappointing results from the clinical trials reviewed above, some drugs—most notably rituximab—are beginning to be successfully used off-label in SLE patients. This may herald new clinical trials to test the medications in different ways, possibly resurrecting them in the new future.

Murine Models of Lupus

The age long adage, "Of Mice and Men," while most customarily attributed to John Steinbeck's novella, is actually taken from the Robert Burns poem, "To A Mouse." Despite a mouse's careful plans and hard work to prepare for the winter cold, her efforts are in vain as the farmer accidently destroys her house with his plow,

> "The best laid schemes of mice and men
> Go often askew,
> And leave us nothing but grief and pain,
> For promised joy!"

Yet, in this poem, Burns reminds the reader, that the mouse is still blessed, compared to men; only the present touches the mouse, while men look to the past in tedium and the future in fear.

While many concerns and criticisms arise from how murine and human immune systems differ, murine models have proven to be invaluable to the understanding of the underlying mechanisms of lupus pathology. While some aspects of scientific research will go askew despite how much we plan, we can look to the past and anticipate the future. Although there are limitations, the importance of murine lupus models should not be marginalized, as they have made significant contributions to our understanding of this disease in humans.

Spontaneous Models of Lupus

As an autoimmune disease of multifactorial etiology, murine models of SLE that accurately reflect human disease have been a challenge to develop. The first recognized spontaneous murine model of SLE was the NZB/NZWF1 (NZBW) mouse, developed from the New Zealand Black (NZB) mouse crossed with the New Zealand White (NZW) mouse.[63,64] NZBW mice produce anti-dsDNA and ANAs, and develop immune complex glomerulonephritis and vasculitis, consistent with important aspects of human lupus disease. Additionally, the NZBW model reflects human disease with a female predominance and the development of splenomegaly and hypergammaglobulinemia. However, the long disease incubation time (disease manifesting at around six months of age) and a 50% mortality rate for female mice at 9 months of age and male mice at 15 months of age are major drawbacks.[65] Nonetheless, the similarities between the lupus-like disease in the NZBW mouse model and lupus disease in humans makes this mouse model appealing.

Through mating pairs of NZBW mice over multiple generations, the related NZM strains were developed, originally in response to the increased maintenance needed to crossbreed and maintain two separate mouse strains (NZB, NZW). Through this process, the NZM2410 was developed and found to display distinct phenotypes that allowed for the identification of the genomic positions of three loci that are very strongly associated with lupus susceptibility.[66] All three loci, *Sle1, Sle2,* and *Sle3,* are recessive, and located on chromosomes 1, 4, and 7, respectively.[67] B6 mice with *Sle1* develop IgG ANAs, B6 mice with *Sle2* display polyclonal B cell hyperactivity, and B6 mice with *Sle3* develop lupus nephritis.[68] The triple congenic Sle1,2,3 mice were developed through backcrossing NZM2410 mice to wildtype C57BL/6 mice, eliminating the limitation of NZBW mice not having a strict wildtype control. These mice therefore express all susceptibility loci on a B6 background. Similar to in human disease, Sle1,2,3 mice produce an IFN Signature, with a profound increase in the production of proinflammatory cytokines including IL-6 and TNF in response to TLR ligands, which drives T cell hyperactivity and disease progression in the mice.[69,70] The advantages of the Sle1,2,3 has led it to be a widely used spontaneous murine model of lupus.

Other spontaneous models include the MRL/lpr mouse, in which the lpr gene is a mutation in the Fas receptor, resulting in a defect of apoptosis.[71] The onset of disease is rapid and includes multiple manifestations of disease, but unlike in the majority of human cases of SLE, MRL/lpr lupus disease is not driven by IFNa.[72-74] Another spontaneous model is the BXSB/Yaa model of lupus, with a translocation of the Yaa genetic locus from the X chromosome onto the Y chromosome. This results in the duplication and subsequent overexpression of TLR7 in male mice and was important in demonstrating the critical role TLR7 plays in increasing type I IFNs and their role in SLE disease.[75] Since this model of lupus disease includes glomerulonephritis and it only occurs in male mice,[76] it is a good model to study lupus in males, an under-investigated disease that deserves more research attention. Although SLE is less frequent in men, it manifests equally, if not more severely, than what is seen in women, especially in the context of lupus nephritis.[77]

Induced Models of Lupus

While spontaneous models of lupus have given rise to great insight into the genetics behind lupus, environmental triggers play a huge role in initiating disease and flares in humans, leading to the development of induced models of SLE. Pristane is an extensively used induced model dependent on overproduction of type I IFNs, similar to most human patients with SLE.[78,79] Pristane, an isoprenoid alkane hydrocarbon oil, induces lupus-like disease with autoantibodies and immune complex glomerulonephritis in appropriate strains (Balb/c but not B6). Although the mechanism remains to be defined, pristine has a cytotoxic effect that is thought to induce apoptosis, providing a sufficient amount of autoantigen substrate for autoimmunity similar to SLE to develop.[80]

The TLR ligands resiquimod (R848) and imiquimod applied to the ears of mice is also an induced model of lupus, causing lupus-like autoantibody production, splenomegaly, and immune complex glomerulonephritis to develop within 2 to 4 weeks.[81] Resiquimod and imiquimod modulate the immune response through the activation of TLR7 and TLR8, leading to the production of proinflammatory cytokines. These mice also experience elevated type I IFN activity, and this model supports the findings that increased TLR7 activity can contribute to the development of systemic autoimmunity in lupus.

Transgenic Models of Lupus

Transgenic editing to introduce immunoglobulin heavy and light chain genes that code for autoantibodies was one of the first uses of transgenic technology. The manipulation of a gene has led to the ability for researchers to better understand the mechanisms and genes important for autoimmunity. By editing mice so all germinal center B cells express the transgenic VH3H9u heavy chain,[82,83] B cell tolerance and germinal center checkpoints can be studied in detail, which other researchers in the Gallucci lab are utilizing to understand the tolerance breakdown in lupus.[66,84]

Transgenic editing can also be used for receptor-mediated targeted cell ablation. The importance of the type I IFN signature highlights the potential large role of pDCs, which led us to utilize C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J mice to study the specific effect of plasmacytoid dendritic cells. These mice have a simian diphtheria toxin (DT) receptor under transcription control of the human C-type lectin domain family 4 (CLEC4) promoter, very specific to the plasmacytoid dendritic cell.[85] Therefore, injection of small amounts of DT leads to the sensitive and targeted conditional cell ablation of pDCs in this model. We utilize this model in Chapter 3 to study the specific role of pDCs in the development of autoimmunity in a bacterial amyloid induced lupus model.

Infection in Autoimmunity

While the biological mechanisms leading to the development of SLE are poorly understood, it is clear that both genetic and environmental factors contribute to the etiopathogenesis. [86-91] SLE disease in humans shows a clear genetic predisposition, but a concordance of about 25% in monozygous twins[92,93] clearly indicate the importance of environmental triggers in the development of disease. Because autoimmunity is due to the breakdown of tolerance, where the immune system attacks self-tissues as if they were foreign, infections and pathogens have been identified as possible candidates that contribute to the breakdown of self-tolerance.

Infections in SLE Patients

Interestingly, the incidence rate of infections in SLE patients is significantly higher than both the general population and the population of patients suffering from other common autoimmune diseases.[94] Specifically, of the 10.8 per 100 person-years incidence rate of infections in SLE patients, 96% of these infections are bacterial.[95] This is significant because enteric bacteria are more prone to disseminate into dangerous systemic infections in SLE patients, and may contribute to disease flares, manifesting as periodic increases in disease severity.[96-98] Despite the overwhelming evidence that infections are one of the leading causes of lupus morbidity and mortality, the exact mechanism of how bacterial infections contribute is unknown. Additionally, increasing evidence is showing that infections may accelerate lupus disease in humans, emphasizing the importance of vaccination in these patients.[99]

Infection-Induced Autoimmunity in Mice

Notably, previously discussed induced models of murine lupus mimic aspects of immune activation or cell death that can naturally be instigated by infection, leading to an increased interest in pathogens as an environmental trigger in lupus. This has led to models of murine lupus-like disease induced by infection. The earliest autoantibodies detected in lupus are against the RNA binding autoantigen Ro60.[100,101] Anti-Ro antibodies are pathogenic in lupus, and are best known for leading to cardiac conduction defects and cutaneous lesions due to their trans-placental spread in neonatal lupus erythematosus.[102,103] The spontaneous development of anti-Ro60 antibodies can be induced in germ-free wild-type C57Bl/6 mice when monocolonized by a common gut commensal that produces a Ro60 ortholog.[104] Within 3-5 months of monocolonization, sera are positive for anti-human Ro60 IgG. This spontaneous production of antibodies was equivalent to mice that were monocolonized by the same strain but had induced barrier inflammation and dysfunction from treatment with oral 0.1% imiquimod or 1-2% dextran sulfate sodium salt. Monocolonization with a different gut commensal does not result in the production of anti-human Ro60 IgG antibodies. Together, this model suggests that there is selective cross-reactivity between a Ro60 ortholog from commensal bacteria and human Ro60, further emphasizing how infection may play a role in triggering autoimmunity in lupus.

Another infection-induced model of lupus includes the administration of the bacterial amyloid curli, a component of biofilms produced by enteric bacteria that commonly cause infections in lupus patients, including *Escherichia coli* and *Salmonella spps.*[105] The role of biofilms as inducers of lupus is further in this thesis; here it is important to introduce the concept that components of biofilms have begun to be used to develop induced models of autoimmunity. The amyloid protein curli irreversibly binds with bacterial DNA during the biofilm formation processes, which in turn also accelerated amyloid polymerization.[106,107] These highly immunogenic complexes activate immune cells, including dendritic cells, and induce the production of the SLE type I IFN hallmark through powerfully stimulating TLR2 and TLR9.[107-109] Infection of lupus-prone mice with curli-producing bacteria triggered profoundly higher autoantibody titers compared to infection with the same strain of bacteria with a mutation rendering them unable to produce functional curli amyloid.[110] Additionally, the systemic administration of the curli/DNA complexes alone triggered autoantibody production in both lupus-prone and wild-type mice, suggesting that biofilm-producing enteric infections may be an important environmental trigger in SLE.

Similar to that of human patients, susceptibility to infection is also seen in murine lupus. MRL/lpr, BXSB, and particularly NZB mice are susceptible to *Trypanosoma cruzi* infection.[111] Trypanosomes are able to modulate the host immune response by using host antibodies to ligate Fc receptors and enter phagocytes.[112] In congruence, a greater susceptibility to *T. cruzi* infection in these lupus-prone mice correlates with higher levels of anti-*T. cruzi* antibodies.[111]

Studies exploring the role of gut microbiota on disease progression in lupus-prone mice further corroborated the importance of bacteria and infection on lupus development.[113] Consistent with findings that germ-free conditions do not influence disease outcome,[114] treating lupus-prone mice with antibiotics from the time of weaning also does not impact disease activity. However, when antibiotic treatment initiation was delayed until after disease onset, SLE autoimmunity was attenuated.[115] Lupus progression was thought to be attenuated by targeting Clostridial strains (i.e. *Lachnospiraceae)* found to be increased in both lupus mice and feces of human SLE patients while allowing for beneficial commensals found in healthy individuals (i.e. *Lactobacillus* spp.) to thrive. Treatment with vancomycin, which targets Gram-positive bacteria, thus, sparing Lactobacilli, also reduced translocation of LPS across the intestinal barrier, further suggesting that microbial translocation from barrier dysfunction may be an environmental trigger in SLE.[115-117]

Nucleic Acids as Autoantigens

Nucleic acids and the proteins that bind to nucleic acids are the main autoantigens (autoAgs) in systemic lupus erythematosus (SLE).[118] The main pattern recognition receptors (PRRs) that have been found to be involved in the pathogenesis of lupus are toll-like receptors (TLR) 7 and 9, which respectively recognize dsRNA and DNA rich in hypomethylated CpGs.[119,120] TLR7 and TLR9 are localized within endosomes, suggesting that their ligands are coming from the extracellular compartment, prompting the question of the origin of the nucleic acids being detected. There is abundant evidence—mostly in murine models of lupus—that genetic defects in cell death and clearance of dead cells (efferocytosis) lead to release of lupus autoAgs, the combination of which can trigger autoimmunity in the right genetic background.[84,121-123] Many of these defects, however, are not found in human SLE patients, indicating the need to search for alternative causes of release of nucleic acids in the extracellular compartment.

Infections in general are well known to expose the immune system to a variety of nucleic acid material, and infectious pathogens are thought to play a role in the development of autoimmune disease, contributing to abnormal immune responses through molecular mimicry, epitope spreading, and bystander activation.[124] While there are multiple theories in which infection by a pathogen is thought to lead to autoimmunity, a common thread that ties the many mechanisms together is the infections leading to exposure of the immune systems to nucleic acids the host otherwise would not be exposed to.[125] In addition to extracellular nucleic acids from pathogens during infection, host nucleic acids also exist in the extracellular compartment. Although circulating extracellular nucleic acids can be found in healthy individuals and was first described in 1948,[126] their role was largely ignored until 1967, when high levels of free DNA was found in SLE patients.[127] Since then, research has demonstrated that host-derived nucleic acids are also immunostimulatory, inducing IFN production through both TLR-dependent and independent pathways[128-130]. This release of host mammalian nucleic acids can be induced through cell death and neutrophil extracellular trap (NET) extrusion during infection.[131,132] Taken together, there is significant evidence to suggest that infections play a role in triggering self-reactive immune responses.

NETosis and Autoantigens

In addition to being the first cells attracted to both infected and sterile wounded tissues to clear microbes and debris by phagocytosis, neutrophils also release neutrophil extracellular traps, or NETs.[133,134] Neutrophils undergo NETosis, an active antimicrobial lytic cell-death mechanism, to release NETs.[134] Composed of nucleic acids, histones, and anti-microbial proteins, NETs are a host microbicidal mechanism that also act as a physical barrier to prevent further spread of microbes.[135] However, with the nature of NETosis characterized by the extrusion of self-nucleic acids, NETs have been increasingly implicated in autoimmune disorders.[136,137] Autoimmune disorders are defined by inappropriate immune responses against self-structures of the body, and NETosis exposes normally cryptic nucleic acids and other auto-epitopes that are normally not released into extracellular areas. These antigens can be picked up and presented to immunocompetent cells which could promote synthesis of autoantibodies[138,139]. This connection between NETosis and autoimmunity has been supported by numerous clinical observations, as increased NET formation is found in rheumatoid arthritis, antiphospholipid syndrome, antineutrophil cytoplasmic antibody-associated small vessel vasculitis, and other autoimmune disorders.[138,140-143] SLE is also associated with increased NET formation, and escalating disease activity and organ damage in SLE is associated with augmented NETosis.[142,144-146] An investigation of 98 lupus patients showed that levels of anti-curli/DNA antibodies correlated with persistent bacteriuria, and with disease flares and that curli/DNA complexes can stimulate NETosis.[147]

Infections and Cell Death in the Context of Autoimmunity

Because DNA is a major autoantigen in SLE, studies have attempted to determine whether an excess of circulating DNA may distinguish SLE patients from healthy subjects. As a whole, no differences were noted in SLE patients when compared to healthy individuals, except for SLE patients with vasculitis. SLE patients with vasculitis had higher levels of circulating DNA, suggesting that tissue damage affecting endothelia may result in the release of extracellular DNA at the site of damage.[148] This is corroborated with studies showing very high levels of plasma DNA in patients who recently underwent major surgery or experienced traumatic bodily injury, and together, suggests that cell death is the source of the extracellular DNA.[149-151] The concept was replicated in mice, when an injection of necrotic cells induces a rapid increase of plasma DNA levels.[151]

Cell death is a natural and necessary process, and efficient recognition and clearance of products is important to avoid eliciting an immune response. Whether occurring by the programmed and regulated apoptosis or the inflammatory necrosis, the accumulation of cell debris from inefficient clearance leads to the breakdown of self-tolerance.[84,152] Originally, it was hypothesized that defects in efferocytosis could be an underlying cause of lupus. Mice with deficiencies in receptors necessary for efferocytosis demonstrate the development of an SLE-like disease including splenomegaly and glomerulonephritis and generate high levels of hallmark antinuclear antibodies.[153] Mutations in BAI, MerTK, MFG-E8, and TIM-4 receptors involved in efferocytosis have all resulted in SLE-like disease in mice,[154-156] but there is a dearth lack of evidence of these mutations in human SLE patients, suggesting that the simple lack of clearance of apoptotic cells is not a key player in the development of lupus antinuclear antibodies.

Although apoptosis is most broadly recognized, a pathway of programmed cell death that is stimulated by microbial infections was more recently described and termed pyroptosis. Pyroptosis is a programmed process of cell death that is canonically dependent on the protease caspase 1, making this process inherently inflammatory. When caspase 1 is activated, plasma-membrane pores are rapidly formed, allowing for osmotic lysis and the release of inflammatory cytokines and cell contents, in contrast to the noninflammatory apoptosis. When LPS is recognized by caspase 4 or 5 in humans (caspase 11 in mice), caspase 1-independent pyroptosis is initiated. Both types of pyroptosis leads to the release of potent inducers of inflammasome activation and inflammation. Additionally, both nuclear and mitochondrial DNA are release by pyroptotic cells.[157-160] Another inflammatory protein released by pyroptotic cells is high-mobility group box 1 (HMGB1), a nuclear DNA binding protein ubiquitously expressed in eukaryotic cells.[161,162] Circulating anti-HMGB1 antibodies are present in SLE patients and increased extracellular expression of HMGB1 is found in cutaneous lupus lesions.[163,164] *In vitro*, HMGB1, when complexed with DNA, can stimulate TLR9 and subsequent production of type I IFN by dendritic cells.[165] Additionally, these immune complexes activate B cells via RAGE, supporting the role of HMBG1 in promoting the formation of autoreactive B cells.[165] Other cytokines released by pyroptosis include the caspase 1-dependent IL-1b and IL-18, both thought to play a part in promoting autoimmune disease.[166-168] Moreover, evidence from many studies are reporting increased pyroptosis in both human and murine SLE contributing to lupus manifestations including nephritis. Microarray analysis of kidney tissue from SLE patients reveal an increase of inflammasome-associated transcripts,[169] and low serum levels IL-1 receptor antagonist in SLE patients suffering from renal flares suggest a pathogenic role for IL-1 in lupus nephritis.[170] Additionally, polymorphisms in the IL-18 gene have been linked to SLE,[171,172] and these polymorphisms were found to lead to heightened expression of IL-18 and development of kidney disease.[173,174] These findings were further supported with the detection of heightened levels of sera and urine IL-18 in SLE patients, especially those with active lupus nephritis.[175,176] Together, these findings strongly suggest that pyroptosis may play an important pathogenic role in SLE.

Molecular Mimicry

The role of infection in autoimmunity has been highlighted by the ability of an infectious agent to initiate and/or exacerbate disease. Mechanisms of infection-induced autoimmunity include:

1. Epitope spreading, where a T cell response to a specific self-peptide can diversify through priming of T cells specific to other self-peptides. This was

first confirmed in a murine EAE model, where 40 days after immunization with MBP and 31 days after confirmation of a response directed towards a specific epitope, T cell responses developed to other MBP epitopes were detected. Interestingly, increasing evidence is showing that B cell epitope spreading can result in autoimmunity.[177] More specifically, endocytic processing and somatic hypermutation are molecular mechanisms that assist in driving epitope spreading, and such B cell epitope spreading can result in autoimmunity.

2. Pathogen superantigens, where superantigens can bind to a large variety of MHC class II molecules and can activate T cells through their TCR-b variable domain. Therefore, very large numbers of T cells are activated regardless of MHC/peptide specificity. The ability of activated cells to stimulate polyclonal B cells especially raises the possibility of superantigens in the precipitation of autoimmunity.[178] An example of pathogen superantigen induced murine autoimmune disease is the *Mycoplasma arthritidis* superantigen, which causes severe arthritis.

3. Molecular mimicry, where microbial peptides have a similar sequence to self-peptides, activating autoreactive B cells and T cells. A murine myocarditis model induced with peptides from *Chlamydia* utilizes the similarity of the 60kDa cysteine-rich outer membrane protein of the bacteria to a 30 amino acid peptide from the cardiac myosin heavy chain of mice, producing severe inflammatory heart disease.

In SLE, molecular mimicry between infectious agents is another theory behind the mechanism of how SLE is triggered, where infection in a susceptible genetic background may lead to the breakdown of self-tolerance.[179] This leads to the production of cross-reactive antibodies that have the ability to recognize self Ags, a hallmark of SLE disease. SLE has been linked to several bacterial and viral pathogens showing molecular mimicry between the pathogen and SLE-related antigens.

Epstein-Barr virus (EBV) has been linked to many autoimmune diseases, with SLE among one of them.[180] In mice, vaccination with an EB nuclear antigen (EBNA) 1 resulted in the development of SLE-associated anti-Sm and anti-dsDNA autoantibodies, giving evidence of antigenic cross-reactivity.[181] Additionally, immunization with EBNA-1 or EBNA-1-derived peptides induced SLE-like autoimmunity in rabbits, including renal insufficiency.[182] Of note, there is clinical evidence that EBV response is different in SLE patients, given that the EBV viral load is consistently 10-100-fold higher in SLE patients. This appears to be due to both impaired humoral and adaptive responses. SLE patients produce more antibodies to EBV, but the antibodies have a greater epitope diversity than compared to normal, and SLE patients have an increased CD4+ T cell response but a decreased CD8+ T cell response to EBV, suggesting difficulty in controlling latent EBV infection.[183-186] Antigenic cross-reactivity has also been described with the Coxsackie virus 2B protein with the binding site of the Ro60 autoantigen, and the parvovirus B19 VP1 protein with human cytokeratin.[187,188] Together, this suggests that cross-reactivity between viruses and SLE-associated self-antigens may implicate viral infection as a causative agent of SLE in the susceptible genetic background.

Notably, the development of antinuclear antibodies, arguably the hallmark of SLE, has been linked with bacterial infections in both humans and mice. Sera from human SLE patients have shown anti-dsDNA antibodies with similarity to peptides from burkholderia bacteria, and the relationship was substantiated when purified anti-dsDNA antibodies were shown to react with *Burkholderia fungorum* bacterial lysates.[189] A common anti-dsDNA idiotype in humans were also found in high amounts in patients infected with *Klebsiella pneumoniae*.[190] The interaction of anti-dsDNA antibodies to bacteria was also found in mice, where anti-dsDNA antibodies produced by lupus-prone mice reacted with endogenous murine flora.[180]

Additionally, candidate antigens for the pathogenic Th cells that allow for the expansion of autoreactive B cells include those with sequences that resemble both microbial proteomes and self-proteins.[179,191] The role for Th cells is well established in SLE, and autoreactive B cells have been shown to present variable region-derived idiotype peptides on their MHC class II molecules to ideotype-specific T helper cells.[192-195] Systemic autoimmune disease can be established in mice with prolonged idiotype-driven T helper cell and B cell collaboration.[179,196,197] Further studies in a lupus-prone mouse model demonstrate the idiotype-specific T helper cells support proliferation of anti-dsDNA B cells, with the mice developing clonal expansion of their B cell population.[198-200] In end-stage disease resembling characteristics of human SLE disease including nephritis and vasculitis, 40% of the B cell population consisted of anti-dsDNA B cells, further supporting the role of idiotype-specific T cells in triggering SLE.[201] Interestingly, an analysis of the seemingly dissimilar specificities of the T helper cells from lupus-prone mice showed a high rate of matches with microbial proteomes.[179] Additionally, these T helper cells also developed responses towards related sequences that resembled self-histones, suggesting that there is molecular mimicry between microbial peptides, idiotypes, and self-proteins.[179]

Bacterial Biofilms

Biofilms, a term coined by Bill Costerton in 1978 to describe a sessile, attached community of microbial cells embedded in microbe-produced extracellular matrix, was first described by Anton Von Leeuwenhoek—the inventor of the microscope—in the 17th century.[202,203]

Biofilm Structure

Since then, biofilms have been defined as an aggregation of microbial cells that are firmly attached or enclosed in an extracellular matrix produced by the microbes themselves.[204] Biofilms have become of public health interest through the recognition of their role in a number of infectious disease processes, including common infections such as urinary tract infections (UTI), otitis media, periodontitis, and a broad spectrum of indwelling medical devices.[205,206] While the primary matrix material in biofilms is extracellular polymeric substances (EPS), more than 40% of bacteria produce amyloids, proteins with a conserved beta sheet structure, which become a major structural component of the biofilm.[109,207] The primary protein component found in enteric biofilms is the amyloid curli.[208,209] The bacterial amyloid curli has been found in various enteric infections, including UTI, sepsis, and gastroenteritis.

Biofilms and Chronic Infections

Of note, most pathogenic—as well as many commensal—bacteria live and thrive in structured "biofilm" communities.[210] Consisting of polymeric conglomerations formed by the bacteria themselves, biofilms allow for adherence and resistance to foreign insults including the host immune system and antibiotics.[210,211] Biofilm formation *in vivo* protects the community from clearance, and robust biofilm formation can be positively associated with failure of antibiotic treatment.[212] In congruence, chronic infections including chronic middle ear infections, periodontal disease, chronic urinary tract infections (UTIs), and chronic joint infections can all be attributed to bacterial biofilms.[202,203,213-216] The permanence of the biofilm can be demonstrated by indwelling medical device infections,[217] where the implant needs to be removed in the case of infection, as it is so far impossible to clear the biofilm otherwise.[205] The tolerance to conventional antimicrobials and host defense that the biofilm endows upon its associated bacteria puts forth a major issue that needs to be addressed when thinking about chronic infections. Understanding of the role played by biofilms in infections and how it relates to autoimmunity will provide novel treatment approaches for lupus.

Curli and Lupus

The idea that DNA complexing with a protein antigen can induce SLE-like disease is not new, but more recent studies have shown that curli fibers are bound very tightly to extracellular DNA (eDNA) in biofilms.[110] Interestingly, the Tükel laboratory recently showed that curli, a bacterial amyloid produced in biofilms formed by enteric bacteria, forms tight complexes with DNA. These curli/DNA complexes are very immunogenic, and precipitate the production of ANAs, the hallmark of SLE disease when injected systemically in lupus-prone mice. Additionally, the curli/DNA complexes were found to be powerful stimulators of both the innate adaptive immune systems, and triggered autoantibody production when injected wild-type mice.[107] In congruence, infection with either the virulent *Salmonella Typhimurium* or the commensal *Escherichia coli* that express curli induced the production of high autoantibodies titers in lupus-prone NZBW mice.[107] Lupus-prone mice exposed to mutant bacteria that cannot produce curli or biofilms still developed autoantibodies, albeit at a much lower level when compared to those infected with bacteria that could produce curli, indicating that exposure to curli amyloid or infection with bacteria that can make biofilms induces the development of autoantibodies in susceptible mice.[110]

The mechanism of how curli elicits an autoimmune response can be explained by the ability of the amyloid to complex securely with DNA. The immunogenic curli/DNA complexes stimulate the immune system by binding to TLR2 with the beta-sheet structure of curli, allowing for internalization, after which the DNA portion of the complex binds to the endosomal TLR9. This results in the production of type I IFNs and subsequent production of autoantibodies. The autoantibody production response to curli/DNA complexes was attenuated in the absence of TLR2 or TLR9, suggesting that both TLR2 and TLR9 are necessary to shape the autoimmune response.[108,109] The findings that an amyloid component of bacterial biofilms forms complexes with DNA and can potently activate a type I IFN immune response further supports the link between bacterial infections and SLE disease, and highlights the important role that biofilms may play in progressing the generation of autoantibodies to nucleic acid.

Dendritic Cells

Dendritic cells (DCs) were first discovered by Ralph Steinman and Zanvil Cohn in 1973 at the Rockefeller University.[218] Steinman and Cohn recognized this unique population of cells by their tree-like processes and unusual movements leading Steinman to be the first to show that dendritic cells present Ag and stimulate T cells.[219] Although normally comprising 1% of cells in the spleen, by 1979, Steinman had achieved enriching this small DC population with a high degree of purity, allowing for functional studies on the cells to begin in laboratories worldwide.[220] Subsequent studies uncovered the powerful characteristics of DCs, finding that they are the only cells able to activate both naïve and memory T cells, with an equivalent capacity to activate and provide survival signals to B cells.[221-225] Ralph Steinman was awarded the Nobel prize for these discoveries.[220]

Dendritic Cell Subsets

DCs, with their powerful APC abilities, play a critical role in immune surveillance through their recognition of invading pathogens and cell damage via a variety of pattern recognition receptors (PPRs) and their ability to shape the immune response by antigen presentation and cytokine production. DCs also play an important role in preserving immunologic tolerance, as DCs can also tolerize lymphocytes reactive for self-antigens.[226,227] Further studies uncovered that there are a number of distinct DC subsets, each with characteristic functions and surface markers. Each subset's unique gene transcriptome, tissue localization, function, and surface expression of molecules define each population.

Conventional dendritic cells

Conventional DCs (cDCs), previously known as myeloid DCs (mDCs), compose the major subset of DCs, and reside in most tissues in the body. cDCs are the major bridge between the innate and adaptive immune systems, with strong antigen presenting properties.[228] cDCs are capable of phagocytosis, and foreign bodies are internalized via receptor-mediated endocytosis.[229] Transcripts unique to this subset include fms-like tyrosine kinase 3 (Flt3), stem cell factor tyrosine kinase receptor (c-kit), C-C chemokine receptor type 7 (CCR-7), and zinc finger transcription factor (zbtb46). cDCs are a heterogenous population of cells, but because research in this thesis project is in the context of the lupus autoimmune condition, the cDCs we refer to will be the Flt3-L-derived cells that are a model of cDCs resident in peripheral tissue including kidney, lymph nodes, and spleen. These cDCs express $CD11c^+$ $CD11b^+$ $MHCII^+$ and are $CD103^-$ $CD8^-$.

$CD8\alpha^+$ dendritic cells

Their ability to capture, process, and present antigens (exogenous and endogenous) coupled with their ability to migrate from tissue to secondary lymphoid organs labels cDCs as APCs, but only DCs expressing CD103 or CD8α and not CD11b surface markers are capable of cross-presenting antigen.[230,231] This subset of cDCs responsible for cross-presentation will be referred to from hereinafter as CD8α+ DCs. Their powerful capacity to activate both naïve and memory T cells makes the purpose of their existence clear. While other APCs are able to cross-present antigens to CD8+ T cells, CD8α+ DCs are the major *in vivo* cell responsible for cross-presentation.[232] Further studies investigating DC ability to cross-present ovalbumin (OVA) to CD8+ OT-I T cells show that the CD8α+ DCs are essential for cross-presentation of antigens, and are indispensable for the differentiation of effector CD8+ T cells in inflammatory conditions.[231]

Plasmacytoid dendritic cells

Plasmacytoid DCs (pDCs) are a unique and rare subtype of DCs that performs less antigen presentation, but instead, produce 10-100 times more type I IFN that any other cell type, labeling them the major producer of type I IFN in the body despite their rarity in number. Type I IFNs play a central role in cancer biology and tumor immunology by inhibiting tumor growth and inducing tumor cell death, by acting as danger signals during viral infections, and by orchestrating immunomodulatory responses to shape both the innate and adaptive immune response, and will be covered in more depth in the next section of this chapter.[233-236] Importantly, pDCs are thought to be the major contributor to the high levels of type I IFN causing the IFN Signature in most patients with SLE.[2,237,238] In congruence, TLR7/8 and TLR9, key nucleotide sensing proteins that are the main inducers of pDC activation, are exclusively expressed by pDCs in humans. While they are only widely expressed by murine pDCs, TLR7/8 and TLR9 are still the major inducers of pDCs in mice.[239-241]

Dendritic Cells in Autoimmunity

In contrast to their importance in the establishment and maintenance of self-tolerance, DCs also seem to be able to be powerful inducers of autoimmunity, supported by the finding of accumulated DCs in secondary lymphoid organs and affected tissues in autoimmune disease.[242-245] Although the causative mechanism is still unclear, DCs with a defect in apoptosis develop a lupus-like autoimmune disease and priming active DCs with self-antigen induces and accelerates autoimmunity.[243,246] When DCs are removed from some autoimmune strains of mice, the DCs continue to show abnormalities in culture, suggesting an intrinsic defect in the DCs that are leading to their overactivation.[69] In contrast, DCs in other autoimmune mice only exhibited abnormalities when *in vivo* or isolated *ex vivo*, suggesting that some DC dysfunctions instead may be a consequence of an autoimmune environment.[247,248] Nonetheless, in SLE patients, the increased type I IFN is strongly attributed to DC function due to the ability of DCs to produce so much more type I IFN than other cells. This is supported by DCs from sle1,2,3 cultured in isolation still expressing an IFN signature, indicating that DCs are an independent source of these proinflammatory signals. Because polymorphisms in genes within or related to the signaling pathway of type I IFNs have been associated with a higher risk of developing lupus, the overexpression of type I IFN in lupus patients may be a combination of exogenous stimulation of DCs and genetic predisposition.[4,5,69]

Type I Interferons

First discovered in 1957 by Alick Isaacs and Jean Lindenmann, interferons were identified as a secreted protein specially produced by virally-infected cells that prevented both infected and non-infected cells to produce viral replication-inhibiting proteins[249]. This action of the secreted protein to interfere with infection contributed to its name. Initially thought to be one single protein, subsequent research revealed a large family of proteins, differentiated primarily through their amino acid sequences. Despite a relatively similar sequence between the many protein family members, type I interferons were identified as the primary defense against viral infections, due to the large number of nucleic acid sensors that can trigger the production of these proteins, the history and details of which are covered in the following reviews.[250-252] Of the type I IFN proteins, 13 IFNα and IFNβ are the most important members from an immunologic perspective.[253] Interestingly, soon after their discovery, the idea that type I IFNs may play a pathogenic role in SLE disease was raised by Steinberg et al in his description of the acceleration of autoimmune disease in a murine lupus model following the administrator of an inducer of type I IFN.[254] In this seminal paper, it was reported that the induced production of type I IFN in young lupus-prone mice *in vivo* prior to evident pathology accelerated the production of autoantibodies and precipitated subsequent tissue damage.[254] This hypothesis of the role of type I IFN in human lupus pathology was more recently confirmed in 1979 with the discovery of increased type I IFN activity in patients with autoimmune diseases[255] and confirmed to be mainly in SLE.[256] Later, the key role that type I IFN plays in SLE pathogenesis was supported with the critical findings that pDCs secrete a remarkable amount of type I IFN[257], that this secreted type I IFN can activate immature cDCs[258], and that cDC activation in response to excess type I IFN may result in a break in peripheral tolerance.[227,259,260].

Major functions of type I IFNs include the induction of cell-intrinsic antiviral responses and the activation of adaptive antigen-specific B and T cell responses, all of which are regulated by interferon-stimulated genes (ISGs). A plethora of microarray and RNA-Seq analyses have shown the extent of the IFN response, with studies showing the modulation of specific ISGs upon different viral infections.

Type I Interferon Signaling

The transcription of ISGs is canonically activated by the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway.[261-263] Engagement of IFN α/β receptor (IFNAR) activates JAK1 and tyrosine kinase 2 (TYK2), resulting in the tyrosine phosphorylation, dimerization, and translocation to the nucleus of STAT1 and STAT2.[264-266]

A deeper understanding of the multiple and complex type I IFN signaling and feedback mechanisms have increasingly labeled type I IFNs as a double-edged-sword.[267,268] While type I IFNs are generally considered beneficial in cancer immunology, ongoing type I IFN signaling has been implicated as a key driver of immune dysfunction in some malignancies, with newer data implicating type I IFNs play a negative role through promoting immunosuppression.[269,270] Therefore, discovering the differences of how type I IFNs signals are transduced and their effects on specific cell types is crucial in understanding type I IFNs. *In vitro,* type I IFNs activate DCs while *in vivo,* type I IFNs act as an adjuvant.[271] They can be secreted as an amplifier of innate immunity, and are able to promote cross-priming of CD8+ T cells and isotype switching in activated B cells.[272]

IFNAR Deficiency in Dendritic Cells

IFN-Is are well known to directly enhance dendritic cell (DC) functions, and IFN-I-induced activation of DCs is essential for spontaneous antitumor immunity.[273,274] IFN-Is produced by CD11c+ DCs has been detected in tumor-draining lymph nodes prior to antigen-specific CTL responses.[275,276] A lack of IFNAR function in DCs results in defects in their ability to activate and produce IFN-I-dependent cytokines, and importantly, cross-priming and tumor rejection is severely impaired in IFNAR KO mice.[273,277-279] The unique requirement of IFN-Is by DCs for this process is emphasized by normal tumor rejection in mice lacking IFNAR1 in just granulocytes or macrophages.[273]

Dendritic Cell Metabolism

Given the limited efficacy of available therapies, a full dissection of the aberrant operations of key immune players in SLE is needed to understand the processes that govern pathogenesis, in order to design interventional treatments that could cure this disease, rather than just mitigate symptoms. As we begin to more intimately link metabolism with cell activation state, metabolic manipulation of key players in SLE is emerging as a promising new target. The immune system is composed of cells that are relatively quiescent in their steady state, but have the ability to rapidly respond to perturbations such as infection and inflammation.[280] It is known that metabolic changes occur in cells in response to many factors, including nutrients, oxygen levels, and/or cytokine signaling.[281] This mounting of a response typically requires a change in expression of many genes to allow for the acquisition of new functions, such as production of cytokines, ability to migrate, and cell division, and such changes in gene expression necessitate changes in energy pathways.[282] Specific to SLE, mitochondrial metabolism supports the chronic activation of CD4+ T cells, and these metabolic processes are elevated in CD4+ T cells in lupus-prone mice.[283,284]

Metabolism of Resting DCs

Resting DCs face few anabolic demands, as the catabolism of proteins and triacylglycerols can fuel ATP production through their production of amino acids and fatty acids being utilized for oxidative phosphorylation (OXPHOS). Resting DCs belonging to different subsets utilize primarily OXPHOS, but also consume some glucose, but it is unclear whether the glucose consumption is used for another pathway other than OXPHOS.[285]

Metabolism of Activated DCs

DCs show an increase in glucose consumption following TLR agonist stimulation. Because the ontogeny and function of cDCs and pDCs are distinct, they differ metabolically. Early in the metabolic changes in response to activation, cDCs increase activity through the glycolysis pathway, often within minutes of activation. In contrast, pDCs undergo a metabolic switch to upregulate oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) in response to TLR activation.[285,286] The increased FAO and OXPHOS exhibited by pDCs is critical for activation. Interestingly, this metabolic change is not accompanied by an increase aerobic glycolysis, in contrast to cDCs, which respond to TLR agonists by a large and rapid increase in aerobic glycolysis.[286] Given the importance of DCs in lupus and the stark differences of metabolic requirements in response to activation between DC subsets, approaches that take advantage of this difference can alter downstream effects of specific cells types and may allow for better therapeutics that provide a powerful but targeted response.

Metabolic Modulators

Using metabolic modulators is an attractive therapeutic option, as these drugs would benefit patients without causing the severe immunosuppression resulting from drugs traditionally used in SLE.[287,288] Studies have shown that metabolic modulation of immune cells does not affect normal immune response, confirming the safety of this potential therapeutic approach.[283,289] The safety of metabolic modulators has been evaluated in several diseases.[290-292] T cells from SLE patients and lupus-prone mice have a higher energy metabolism, and normalization of elevated glycolysis and oxidative phosphorylation reduced production of pro-inflammatory interferon-γ, while restoring secretion of interleukin-2, important for tolerance and immunity, suggesting suppression of undesirable immune over-activation while preserving function.[283] *Thus, metabolic modulators have more advantages over traditional therapy*. Moreover, the benefits of success from this project are not limited to SLE patients, as metabolic modulation could apply to other autoimmune diseases with neutrophils playing a central role, including autoimmune vasculitides and blistering skin diseases.[142]

Specific Aims

The immunologic details of lupus autoimmunity are often staggeringly complex. Accordingly, this project investigates a key cell type, dendritic cells, from three different approaches. First, we investigate the role that dendritic cells play in bacterial amyloid induced autoimmunity because of the significance of infections in lupus patients. Secondly, we investigate the importance of STAT2, a signal transducer downstream from the IFNα receptor, providing details on the mechanism of type I IFN signaling, an increase of which is seen in the vast majority of lupus patients. Finally, we investigate the possibility of modulating the metabolism of dendritic cells, exploring a novel targeted therapeutic approach to remedy the paucity of lupus therapeutics currently on available for patients.

Aim 1

Investigate the bacterial amyloid curli/DNA complexes as a novel activator of pDCs.

- Do curli/DNA complexes induce an IFN signature in pDCs *in vitro,* and how does curli/DNA complex-induced activation of pDCs differ from CpG activation?
- Do curli/DNA complexes activate pDCs *in vivo,* and does this contribute to the development of autoantibodies?

Aim 2

Investigate the role of STAT2 signaling pathway involvement in DC activation.

- Does the absence of type I IFN signaling molecule STAT2 in cDCs and pDCs impair their function?

Aim 3

Investigate the importance of metabolic modulation in DC activation.

- What are the effects of inhibiting metabolic pathways necessary for pDC activation *in vitro?*

CHAPTER 2

LUPUS PATHOGENESIS: THE DENDRITIC CELL RESPONSE TO CURLI AMYLOID

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the formation of autoantibodies. Twin studies reveal some genetic component to the development of disease, but infections are considered a major environmental trigger. Bacterial infections in particular are associated with lupus flares and are a major cause of morbidity and mortality in lupus patients. The underlying mechanism, however, remains unclear. The collaboration between the Gallucci lab and the Tükel lab has discovered that the curli bacterial amyloid complexes with bacterial DNA and can accelerate autoimmunity in lupus-prone mice.[107] Human studies also show that curli/DNA complexes induce immune responses in SLE patients.[147,293] Our lab has previously shown that curli/DNA complexes are sufficient in activating inflammatory conventional dendritic cells (cDCs), as generated *in vitro* in the presence of GM-CSF and *in vivo* in the spleen, and that curli fibers are a major contributor to the immune response to bacterial biofilms from curli producing pathogens.[107] The studies in this chapter focus on cDCs, the important professional antigen presenting cell type, and pDCs, with their capacity to produce vast amounts of type I IFNs and their implication in both the induction and maintenance of tolerance, as both DC subsets are generated *in vitro* in the presence of FLT3-L. In this chapter, we show how curli activates cDCs and pDCs, which have long been postulated to play central roles in SLE pathogenesis.[6,23,248,274,294-301]

Materials and Methods

Mice

C57BL/6 (B6) wildtype nonautoimmune mice were bred and maintained in our colony. Breeding pairs were originally purchased from Jackson Laboratory. A C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J (CLEC4-DTR) transgenic male mouse was purchased from Jackson Laboratory for breeding with B6 female mice purchased from Jackson Laboratory. Resulting CLEC4-DTR mice were bred hemizygously and maintained in our colony. Both male and female mice were used. Mice were between 8 and 16 weeks of age for most experiments.

Mice were housed in the Laboratory Animal Resources Unit Temple University School of Medicine of Philadelphia, an AALAC accredited facility. The University maintains an Institutional Animal Care and Use Committee (IACUC), the members of which are appointed by the President of the University. Animals are housed and cared for in accordance with established National Institutes of Health (NIH) guidelines for the humane use of animals in laboratory research. The colonies are routinely screened for the presence of common murine viruses. Separate rooms are available for animal isolation and for procedures involving use of biohazardous materials and infectious agents.

The mice are caged and housed according to NIH guidelines for humane care of laboratory animals. All injections were well tolerated by the mice, but we carefully monitor for sign of local pain or inflammation. If necessary, we were ready to use local analgesic to limit pain, although it was never necessary in these experiments. All injected substrates do not induce pain or distress to the mice.

Of necessity, the mice were humanely sacrificed to permit analysis of the cellular immune response or to avoid suffering. The mice were sacrificed with CO_2 in a chamber with a fill rate of 10-30% per minute and monitored for a minimum of 1 minute after respiration ceases. Cervical dislocation immediately followed to ensure fatality. This method is consistent with the most recent recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. After death was been confirmed, the blood, bone marrow, and/or spleen were harvested to provide a source of serum and cells for culture and/or analysis.

Bone marrow from TLR2 knockout mice were used as a generous gift from the Çagla Tükel lab at the Lewis Katz School of Medicine at Temple University. Bone marrow from TLR9 knockout mice were used as a generous gift from the Edward Behrens lab at the Children's Hospital of Pennsylvania at the University of Pennsylvania Perelman School of Medicine.

Bone Marrow-Derived Dendritic Cells

Bone marrow derived pDCs and cDCs were generated as per the laboratory's published protocols.[69,110,302-305] See Appendix for detailed method. Briefly, bone marrow precursors were flushed from femurs and tibias of mice and differentiated into pDCs and cDCs in the presence of FLT3-L in complete RPMI medium (10% FBS, L-glutamine, penicillin/streptomycin, 2-mercaptoethanol) for 7 days.

In Vitro Dendritic Cell Studies

DCs generated as described above were sorted using the EasySep PE positive selection kit with a PE-B220 antibody to separate out the pDCs from the cDCs. Purity of cells averaged at least 95%. Sorted cells were plated in complete RPMI medium with FLT3-L overnight, then stimulated the next morning. Curli/DNA complexes were a kind gift from the Tükel lab and were purified from *Salmonella Typhimurium msbB,* a mutant that expresses a Lipid A that does not bind TLR3, using previous protocols reported.[107,306] Curli/DNA complexes were used to stimulate DCs in increasing concentrations. TLR ligands used were LPS 100ng/ml (Sigma Aldrich) or CpGA 5ug/ml (ODN 2336 synthesized by IDT Biotechnologies). Supernatant was collected at 6 hours post-stimulation for measurement of IL-6 and TNFα production. Supernatant was collected at 24 hours post-stimulation for measurement of IL-6, IL-10, IL-12, and CXCL10 production. Cells were harvested at 24 hours post-stimulation by gentle retro-pipetting for analysis by flow cytometry.

In Vivo Dendritic Cell Studies

Purified curli fibers were inject intraperitoneally (i.p.) at 50ug/mouse in 100μl PBS vehicle. 24 to 72 hours later, mice were sacrificed for study. Spleens were harvest and incubated in plain medium with collagenase type IV and DNase I for 45 min at 37°C. Single cell suspensions were produced using a 100 μm filter. Red blood cells (RBCs) were lysed using ammonium-chloride-potassium (ACK) lysing buffer. Cells were subsequently stained and analyzed by flow cytometry as described below.

Cytokine ELISAs

IL-6, IL-10, IL-12, and TNFα were measured in supernatants were collected from DC cultures post-TLR stimulation for the measurement of IL-6, IL-10, IL-12, and TNFα using the BD Pharmingen ELISA kits and CXCL10 was measured using the R&D kit, according to the manufacturer's protocol. Optical densities were measured at 650 nm and 405 nm and results analyzed with SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA).

In Vivo Acceleration of Lupus Autoimmunity by Curli/DNA Complexes

8 to 10 weeks old female and male B6 and CLEC4-DTR mice were injected i.p. with 50ug/mouse of curli/DNA complexes in 100μl PBS vehicle thrice a week for 5 weeks. Mice were bled before the first injection and then weekly. 50 μl of blood or less were collected per week. Mice were sacrificed at the end of the treatment and spleens were analyzed for immune lineage and activation markers.

pDC Depletion in C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J Mice

C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J mice were purchased from Jackson Laboratories (Stock No: 014176) and bred hemizygously. Mice 8-16 weeks of age were injected with 100ng of DT i.p. or two days prior to the start of the experiment, then three times a week to maintain depletion. See Appendix for genotyping and injection schedule.

Autoantibody ELISAs

Blood samples were collected from mice via the tail vein weekly (50 μl of blood or less collected per week). Sera were isolated from the whole blood and analyzed for autoantibodies to dsDNA and chromatin as per established protocol.[307] Briefly, 96-well PVC plates were coated with calf thymus dsDNA or chicken chromatin, blocked with borate-buffered saline (BBS) containing 3% bovine serum albumin (BSA) and 1% Tween 80 (BBT), then coated with serum samples diluted in BBT at 1:250. Sera from aged Sle1,2,3 mice and from young B6 mice were used as positive and negative controls, respectively, in each assay. Avidin-alkaline phosphatase conjugate (Sigma) was used at a diluted 1:8000 concentration, followed by alkaline phosphatase pNPP substrate (Sigma). Plates were read at 650 nm and 405 nm and results analyzed with SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA). See Appendix for details.

Flow Cytometry

Splenocytes after processing and cultured DCs following harvest were incubated with rat anti-mouse CD16/CD32 mAb (clone 2.4G2) for 15 min on ice to block FcγRs. Cells were then stained for 30 min in the dark on ice using specific antibodies for surface lineage markers and costimulatory molecules. See Appendix for details on antibodies used. See Table 3 for antibody combinations used to identify specific cell types. Cells were also analyzed by flow cytometry for cell viability using 7-AAD or Fixable Viability Dye eFluor 780 (Thermo Fisher). Cells stained for viability using 7-AAD were analyzed live, 1 min after the addition of 7-AAD. Cells stained for viability using Fixable Viability Dye eFluor 780 were fixed in 2% paraformaldehyde in PBS with 1% BSA. All cells were analyzed on a FACSCanto flow cytometer (BD Bioscience) with FlowJo software (Tree Star, Ashland, OR, USA).

Table 3. Distinguishing Surface Markers used in Flow Cytometry Analysis

Cell Type	Distinguishing Surface Markers
cDC	B220- CD11b+ CD11c+ CD19- BDCA1- CD8α- Activation: CD80, CD86, CD40, MHCII
pDC	B220+ CD11b- CD11c+ CD19- BDCA1+ CD8α- Activation: CD80, CD86, CD40, MHCII
CD8α+ DC	B220- CD11b- CD11c+ CD19- BDCA1- CD8α+ Activation: CD80, CD86, CD40
B cell	B220+ CD11b- CD11c- CD19+ Activation: CD80, CD86, CD40
CD4+ T cell	CD3+ CD4+ CD8- Activation: CD69
CD8+ T cell	CD3+ CD4- CD8+ Activation: CD69

Statistical Analysis

Prism 8 (GraphPad software, San Diego, CA, USA) was used for data analysis. Means and standard error of means (Mean ± SEM) were calculated by averaging results from independent experiments. Statistical significance was determined using unpaired two-tailed Student's t-test for comparison between two groups. One-way ANOVA and Two-way ANOVA were used for multiple comparisons followed by the Bonferroni and Sidak multiple comparisons post-hoc correction tests respectively. *p*-values marked in the figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ were considered significant.

Results

Bacterial Amyloid Curli Activates FLT3-L-Derived Conventional Dendritic Cells

The Gallucci lab has previously shown that bacterial curli amyloid induced proinflammatory cytokine production by bone marrow-derived conventional dendritic cells (BMDCs) from B6 mice, derived using GM-CSF as previously described[69,107,303,304]. We utilized FLT3-L derived DCs because these DCs possess characteristics that more closely resemble lymphoid organ resident DCs, while GM-CSF derived BMDCs more closely resemble inflammatory DCs found *in vivo*.[308] We purified pDCs from cDCs by positive selection for B220+ cells. After an overnight rest, we stimulated both single populations of cDCs and pDCs with a dose titration of curli/DNA complexes, ranging from 10 μg/ml to 10 ng/ml. Curli/DNA complexes were purified by the biofilms generated in vitro by Salmonella *Typhimurium msbB*. This is a mutant of this strain of Salmonella that expresses a Lipid A incapable of binding TLR4, therefore eliminating the confounding stimulatory properties of LPS that might contaminate the curli preparation.[107,209] We also stimulated cDCs with 100 ng/ml of LPS as positive control, because cDCs are known to express TLR4 and respond to this concentration of LPS with optimal expression of costimulatory molecules, cytokines and develop the ability to efficiently stimulate T cells.[271,302] Moreover, we used a second positive control, CpGA ODN2336, which has been shown to stimulate both murine cDCs and pDCs, as well as human pDCs[309,310] and provide the advantage of being able to stimulate both cDCs and pDCs with the same compound and therefore compare the extent of activation.

We found that curli/DNA complexes induced in cDCs a strong upregulation of the costimulatory molecules CD86 and CD40 by FLT3-L-derived cDCs in response to curli, two different surface costimulatory molecules upregulated when DCs are activated.[311,312] Curli/DNA complexes were able to induce a robust upregulation of CD86 and CD40 by cDCs in a dose dependent manner, where upregulation in response to curli/DNA complexes involved up to 80% of the cDCs and was equivalent in strength to the cDC response to known TLR4 ligand LPS and TLR9 ligand CpGA ODN 2336, even at doses of curli/DNA complexes as low as 25 ng/ml, much lower than the other TLR ligands (Figure 1).

These results are the first evidence that curli/DNA complexes activate a model of sentinel cDCs *in vitro*.

Bacterial Amyloid Curli is a Novel Activator of Plasmacytoid Dendritic Cells

Because of the importance of type I IFN in lupus disease, and the forefront role pDCs play in type I IFN production, we next asked whether curli/DNA complexes can activate pDCs *in vitro*. pDC upregulation of CD86 in response to the highest dose of curli/DNA complexes was about 75% of the pDC response to TLR9 ligand CpGA, a known strong activator of pDCs. Even at the lowest doses of curli/DNA complexes, pDC upregulation of CD86 was higher than the pDC response to TLR4 ligand LPS, which we used as a negative control to confirm cell purity and rule out ligand promiscuity, as data regarding murine pDCs expression of TLR4 receptor is contradictory. Humans and non-human primate pDCs do not express TLR4. Some research shows that mice pDCs express high levels of TLR4, but isolated murine pDCs do not consistently respond to LPS stimulation, supporting the idea that murine pDCs do not express or express very little TLR4.

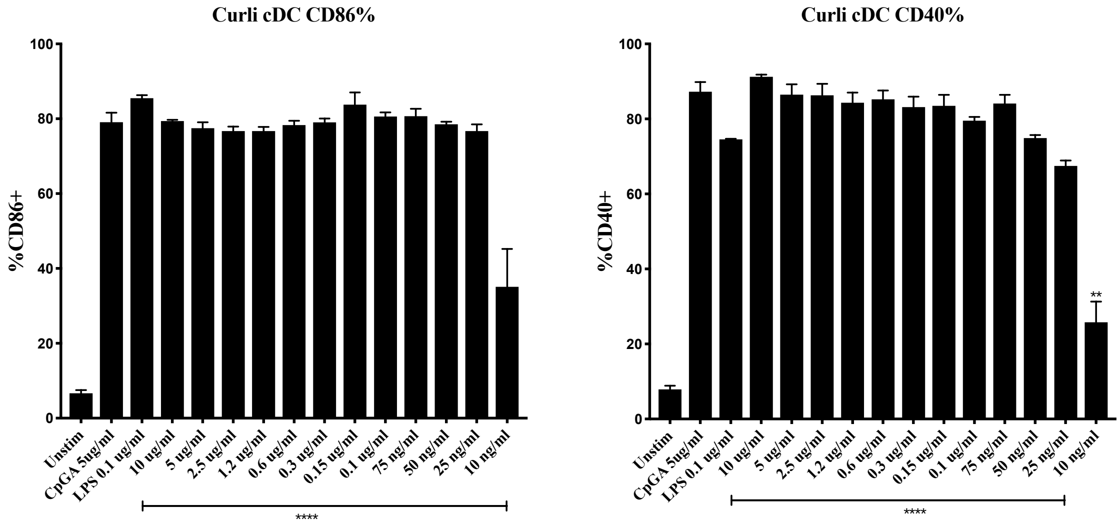

Figure 1. Curli/DNA complexes induce upregulation of CD86 and CD40 in FLT3-L-derived conventional dendritic cells

FLT3-L derived cDCs generated from bone marrow precursors of C57BL/6 mice and sorted after 7 days, were stimulated for 24 h with a dose titration of purified curli/DNA complexes, isolated from Salmonella Typhimurium msbB biofilm. Unstim = untreated cells in the same medium. TLR4 ligand LPS and TLR9 ligand CpGA ODN 2336 were used as positive controls. Cells were stained for lineage and activation markers, analyzed with a Flow Cytometer, and gated as alive (FVD negative) and CD11c+. cDCs were gated for B220- CD11c+ CD11b+. Results are shown as the mean ± SEM from 6 independent experiments, each with an N of 2 or 3. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ were considered significant.

pDCs demonstrate a more robust upregulation of CD40 in response to curli amyloid compared to its upregulation of CD86, since up to 50-60% of the pDCs upregulated CD40, compared to 25-35% of pDCs upregulating CD86. Upregulation of CD40 at high (10 μg/ml) doses of curli/DNA complexes reach levels comparable to the pDC response to CpGA. Even at lower doses (0.1 μg/ml) of curli/DNA complexes, pDCs upregulate surface CD40 expression more strongly than in response to LPS. The sensitivity of pDCs to curli/DNA complexes appeared to be similar or lesser than the one of cDCs, by judging the doses able to induce CD86 (25 ng/ml) and CD40 (75 ng/ml) respectively.

These results are the first evidence that curli/DNA complexes activate a model of pDCs *in vitro*.

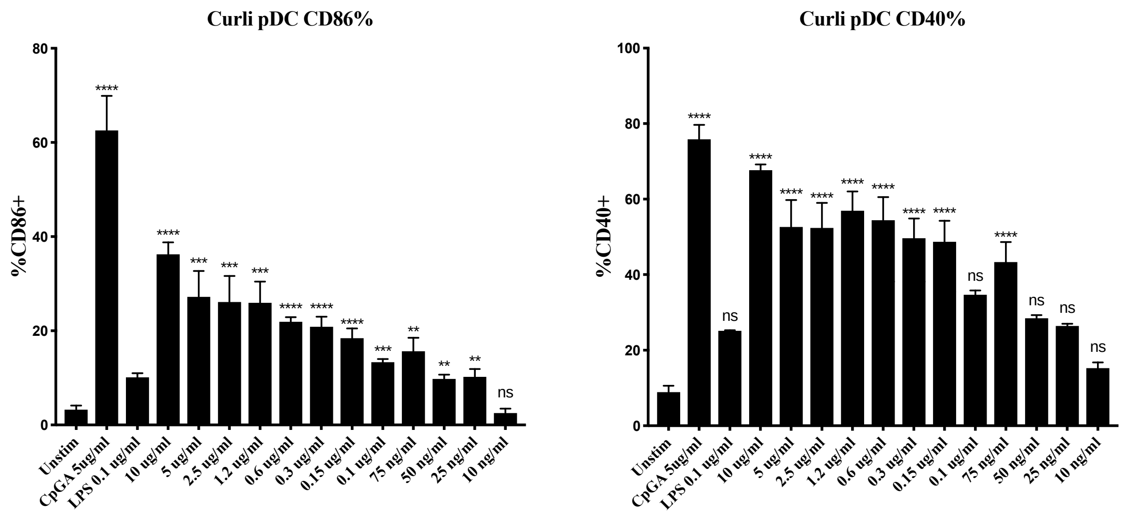

Figure 2. Curli amyloid is a novel activator of plasmacytoid dendritic cells Upregulation of surface costimulatory molecules CD86 and CD40 by FLT3-L-derived pDCs generated from bone marrow precursors of C57BL/6 mice. DC cultures were sorted after 7 days in FLT3-L supplemented complete medium and stimulated for 24 h with TLR4 ligand LPS, TLR9 ligand CpGA ODN 2336, or purified curli isolated from *Salmonella* biofilm. Unstim = untreated cells in the same medium. pDCs were gated for B220+ CD11c+ CD11b-. Results are shown as the mean ± SEM from 6 independent experiments, each with an n of 2 or 3. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ were considered significant.

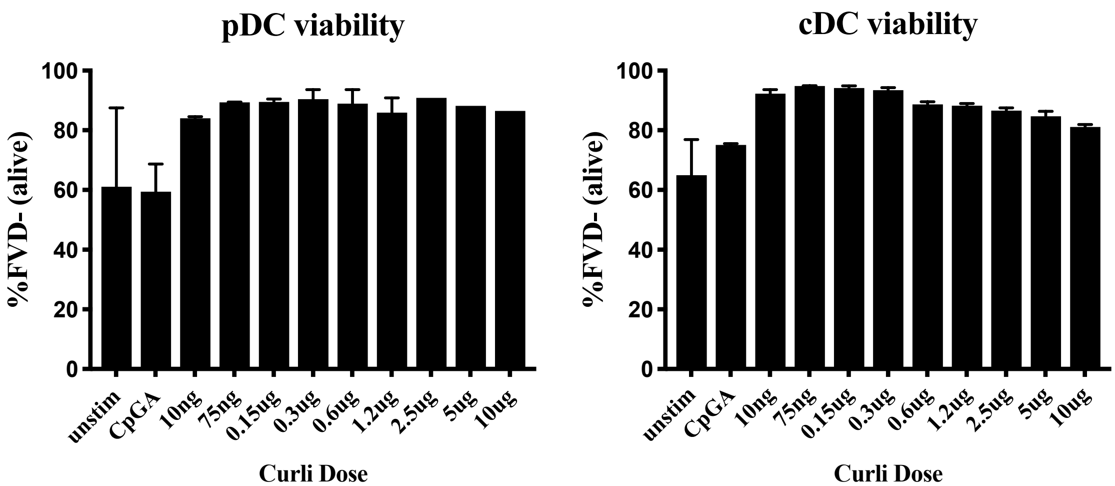

Figure 3. Curli amyloid does not affect viability of dendritic cells
Fixable Viability Dye 780 staining of FLT3-L derived pDCs and cDCs generated from bone marrow precursors of C57BL/6 mice reveal that curli amyloid does not affect viability *in vitro*. FVD stains dead or dying cells. Data shows the percent of FVD- cells, which are alive. Results are shown as the mean ± SEM from 4 independent experiments, each with an n of 2. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ were considered significant.

Curli/DNA Complexes Do Not Affect Dendritic Cell Viability

Gating of each cell type is detailed in Table 3. All analyzed cells were also stained with a viability dye and gated for cell viability to reduce the chance of nonspecific staining due to cell death. In Figure 3, we show that treatment with amyloid curli/DNA complexes does not affect cell death significantly, neither in pDCs nor in cDCs, suggesting that curli/DNA activate DCs without affecting their viability.

Curli/DNA Complexes Induce Dendritic Cell Production of Cytokines

Next, we wanted to determine whether the DC response to amyloid curli/DNA complexes is proinflammatory. We measured proinflammatory cytokines in the supernatants of pDCs and cDC stimulated as described in the previous two paragraphs. In particular, we analyzed TNFα after 6 hours of stimulation (Figure 4), and cytokines IL-6, IL-10, and IL-12 after 24 hours of stimulation with curli/DNA complexes, CpGA and LPS (Figure 5). Both FLT3-L-derived cDCs and pDCs stimulated with curli/DNA complexes produced significant amounts of TNFα after 6 hours, and the production of TNFα in response to curli/DNA complexes by cDCs was almost twice as much as the TNFα produced in response to TLR9 ligand CpGA, suggesting that curli/DNA is a strong activator of TNFα, one of the most important pro-inflammatory cytokines.

Robust IL-6 and IL-12 production was also detected in both DC subsets at 24 hours, suggesting that amyloid curli/DNA complexes induce a strong and sustained activation of pDCs and cDCs, and induces a robust production of proinflammatory cytokines *in vitro*. In the case of these cytokines, LPS did not induce any IL-12 and low amounts of IL-6 in pDCs, confirming the purity of the pDC purification and suggesting that the modest up-regulation observed in the expression of CD40 may be independent of TLR triggering.

The comparison of the cytokine response shown in Figure 5 and the upregulation of costimulatory molecules shown in Figure 1-2 suggests that pDCs and cDCs have similar sensitivities to curli/DNA, with a threshold of 25 ng/ml.

Additionally, curli/DNA complexes did induce IL-10 production by both pDCs or cDCs at higher doses. IL-10 is a cytokine with pleotropic effects and is capable of inhibiting synthesis of proinflammatory cytokines.

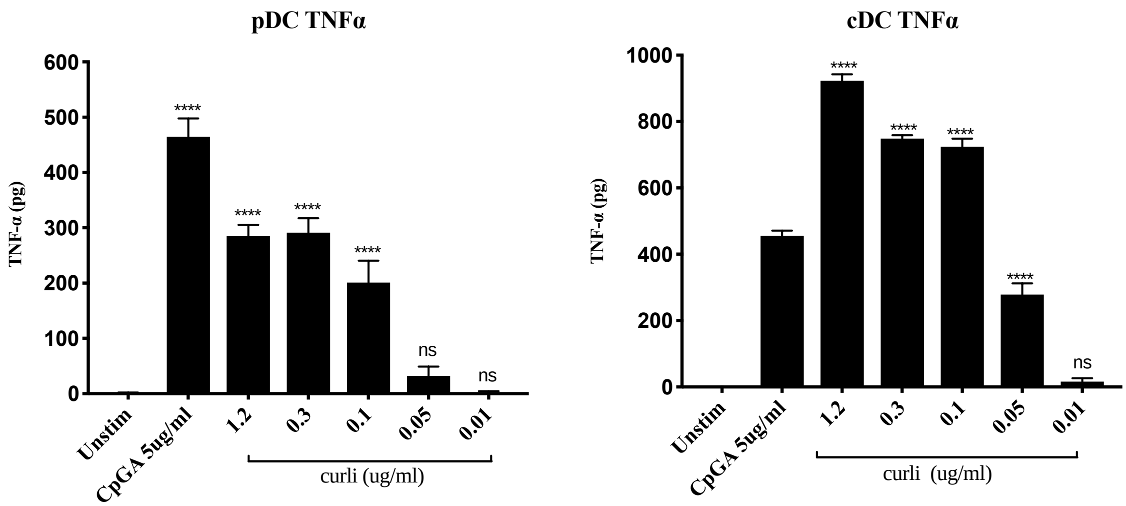

Figure 4. Curli induces a rapid and robust production of TNFα

Proinflammatory cytokine TNFα production by FLT3-L derived pDCs and cDCs generated from bone marrow precursors of C57BL/6 mice after treatment with TLR7 ligand, curli, or untreated complete medium control. Supernatant was harvested for ELISA 6 hours after stimulation Results are shown as the mean ± SEM from four independent experiments, each with biological duplicates, for a total N=8. Each biological duplicate was evaluated as an average of technical duplicates or triplicates. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 were considered significant.

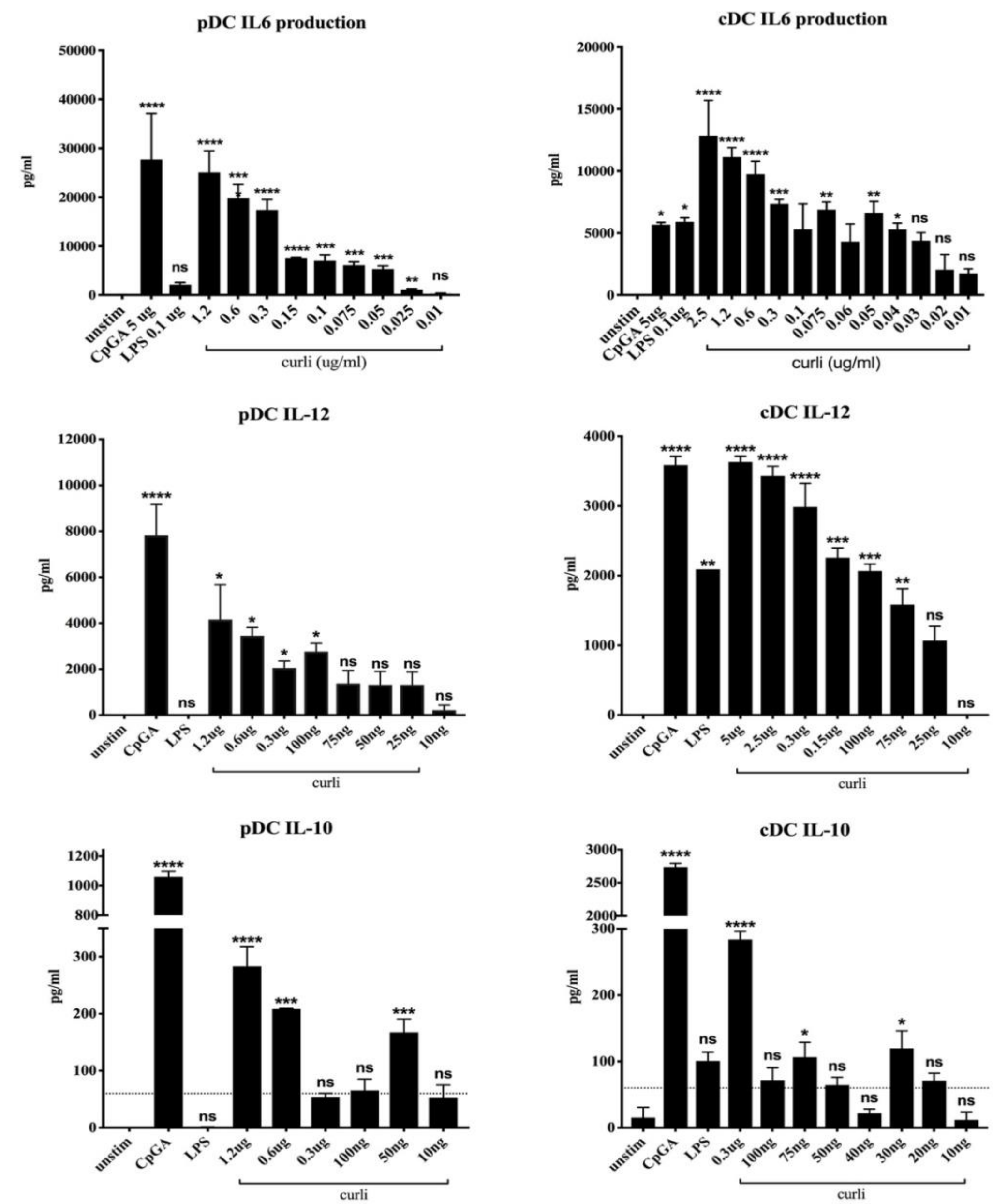

Figure 5. Curli induces production of proinflammatory cytokines IL-6 and IL-12 and the anti-inflammatory cytokine IL-10.

Curli amyloid induces production of proinflammatory cytokines IL-6 and IL-12 by FLT3-L derived pDCs and cDCs generated from bone marrow precursors of C57BL/6 mice but does not induce significant production of IL-10. Supernatant was harvested for ELISA 24 hours after stimulation. Results are shown as the mean ± SEM from four independent experiments, for a total N=8. Each biological duplicate was evaluated as an average of technical duplicates or triplicates. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ were considered significant. Dotted line signals threshold of detection.

Curli/DNA Complexes Induce a Robust Production of ISG-Encoded CXCL10

Now that we have determined that curli/DNA complexes activate FLT3-L-derived cDCs and identified amyloid curli/DNA complexes as a novel activator of pDCs, we wanted to evaluate whether amyloid curli/DNA complexes could induce the production of cytokines under IFN control. Our previous studies have shown that curli/DNA complexes stimulate the production of type I IFNs, and that the compounds can accelerate autoimmunity in lupus-prone mice. Because of the key role pDCs play in the global production of type I IFN, we aimed to ask whether curli induces the production of CXCL10, an interferon inducible cytokine often used as a measure of type I IFN transcriptional activity. Using sorted FLT3-L-derived pDCs and cDCs from B6 mice, we treated the cells with varying doses of curli/DNA complexes, TLR ligand positive control, or plain medium as a negative control. Supernatant was harvested for ELISA measurement of CXCL10 production 24 hours after stimulation. We found that curli/DNA complexes induce a very robust production of CXCL10 by both cDCs and pDCs (Figure 6). Of interest, pDC produced higher levels of CXCL10 in response to curli/DNA complexes, amounts which were significantly higher than pDC production of CXCL10 in response to TLR9 ligand CpGA, a very strong positive control for pDC activation, further signifying the strong activation curli/DNA complexes induced pDCs. Curli/DNA complexes also induced a strong cDC production of CXCL10, although the amounts of CXCL10 were less than what pDCs produced in response to the same doses of curli/DNA complexes.

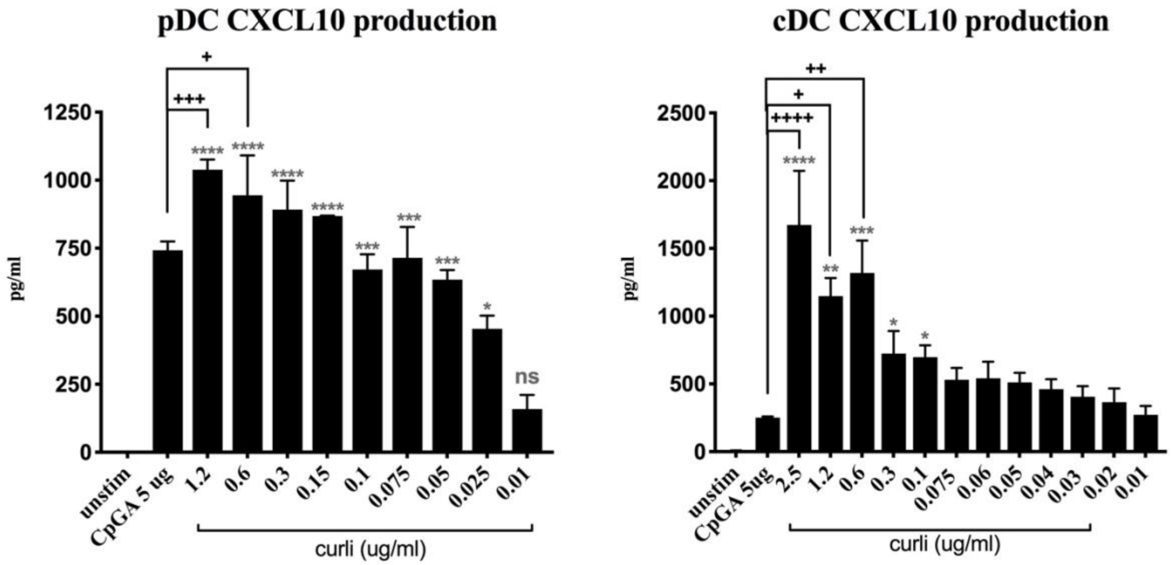

Figure 6. Curli induces robust production of interferon encoded cytokine CXCL10

Curli amyloid induces production of CXCL10, a cytokine under interferon transcriptional control, by FLT3-L derived pDCs and cDCs generated from bone marrow precursors of C57BL/6 mice. Supernatant was harvested for ELISA 24 hours after stimulation. Results are shown as the mean ± SEM from 6 independent experiments, each with an n of 2 or 3. Each biological duplicate was evaluated with technical duplicates or triplicates. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 were considered significant. Significance against CpGA sample is marked in the figures as + p<0.05, ++ p<0.01, +++ p<0.001 and ++++ p<0.0001.

Curli/DNA Complexes Induce Production of Interferon α by pDCs, an Important Cell Type in Lupus Pathogenesis

After confirming that amyloid curli/DNA complexes induce the production of CXCL10, a cytokine under interferon transcriptional control, we wanted to directly confirm that curli amyloid induced type I IFN production by DCs. We followed the FLT3-L-derived DC protocol described above and sorted treated pDCs, generated as described above with 5ug/ml of amyloid curli/DNA complexes. The protein transport inhibitor Monensin (GolgiStop) was added to the medium at hour 18, 6 hours prior to harvest for flow cytometry. 24 hours after stimulation, cells were harvested and permeabilized for intracellular staining with an anti-IFNα antibody. Technical duplicates were stained for surface markers to confirm purity. Figure 7 shows that treatment with curli amyloid induces significant IFNα production, captured intracellularly, compared to the unstimulated pDCs. The mean fluorescence intensity (MFI) of the curli treated pDCs is about 30% higher than the MFI of the CpGA treated pDCs, suggesting that IFNα production induced by curli amyloid is significantly higher than the IFNα production induced by TLR9 ligand CpGA. A surface stain only control shows that the positivity in the intracellular stain channel is not due to auto-fluorescence or spill-over from other channels. It is worthy to note that in vitro cultured pDCs express constitutively a conspicuous amount of IFNα, much higher than other types of cells, a possible reaction to the in vitro conditions.

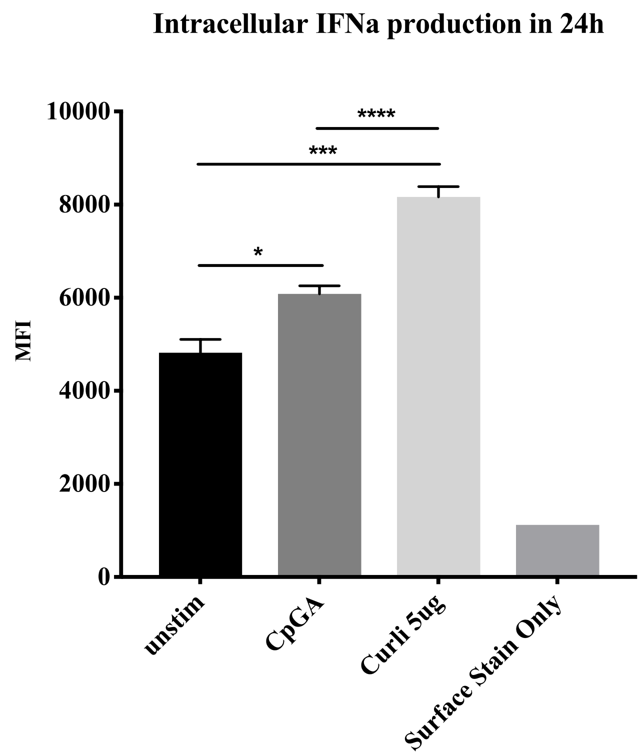

Figure 7. Curli induces IFNα production by pDCs

Curli amyloid induces production of IFNα by pDCs, the primary producer of type I IFNs, making it a cell of great interest in the study of lupus pathogenesis. Sorted FLT3-L-derived pDCs were treated with 5ug/ml of curli. A protein transport inhibitor containing monensin was added to the media 18 hours after stimulation, and cells were harvest 24 hours after stimulation for analysis by flow cytometry. Cells were permeabilized for staining with an intracellular IFNα antibody. Technical duplicates were stained for surface markers to confirm purity and to rule out fluorescence spill-over from other channels. Results are shown as the mean ± SEM from 2 independent experiment each with an n of 2 or 3, representative of 3 independent experiments. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ were considered significant.

Injection with Curli Activates All Dendritic Cell Subtypes In Vivo

After establishing that curli amyloid activates pDCs and cDCs *in vitro*, resulting in a robust production of IFNα, IFN-regulated cytokines, and other proinflammatory cytokines, we next asked whether this effect is seen *in vivo.* To test the activation state of DCs *in vivo* after exposure to curli amyloid, we injected B6 mice intravenously (i.v.) with curli. We chose the dose of 50 μg of curli/DNA complexes per mouse, as per previous experiments which showed that intraperitoneal (i.p.) injections of 50 μg of curli three times a week can induce the production of autoantibodies in wildtype nonautoimmune mice in as early as two weeks. Because we wanted to test the acute activation state of DCs soon after exposure to curli/DNA complexes, mice were sacrificed 24 hours after injection. Although 50 μg of curli/DNA complexes injected i.p. can induce autoantibodies in as soon as two weeks, we chose to inject i.v. to measure the acute activation state of DCs in the short time point of only 24 hours. Spleens were harvested, and processed with collagenase type IV and DNase I, to free DCs from the extracellular matrix of the spleen, then subjected to red blood cell lysis to obtain a clean single cell suspension. The single cell suspension was stained for surface markers as per Table 3.

There was no mouse mortality observed due to the i.v. injection of curli. Absolute spleen counts did not change, which is expected in the short 24-hour time point. However, we did see significant upregulation of surface costimulatory markers CD86 and CD40 on three different subsets of DCs (Figure 8). cDCs, the primary antigen presenting DC subset, pDCs, the primary type I IFN producer, and CD8α+ DCs, the primary cross-presenting cell all upregulated surface costimulatory markers, suggesting that curli rapidly induces a robust and global activation of multiple DC subsets *in vivo*.

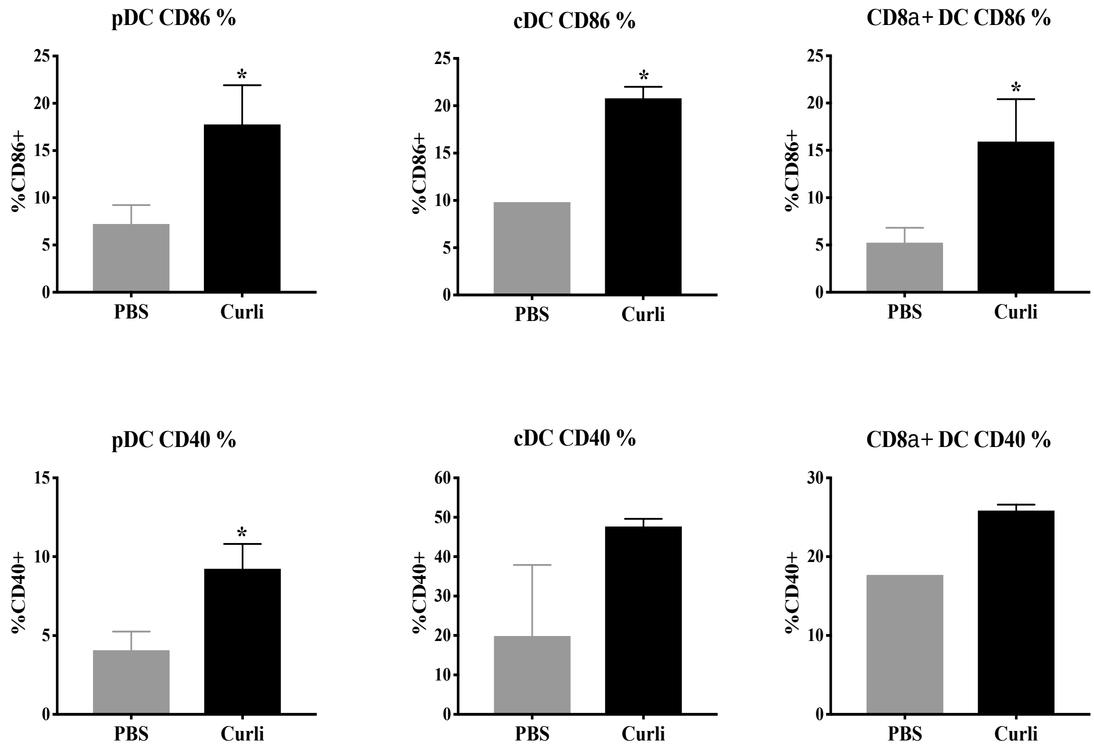

Figure 8. Curli amyloid activates multiple dendritic cell subsets *in vivo*
To test whether curli activation of DCs seen *in vitro* with FLT3-L-derived cells also is seen *in vivo*, we injected B6 mice with 50 μg of curli i.v. Mice were sacrificed 24 hours after injection, and spleens were processed for flow cytometry analysis. Three different subsets of DCs, cDCs, pDCs, and CD8α+ DCs, all showed upregulation of surface costimulatory markers CD86 and CD40 in response to curli. Results are shown as the mean ± SEM. n=2-4, representative of 3 independent experiments. Significance was calculated using One Way ANOVA and post-hoc Bonferroni against the unstimulated sample and p-values marked in the figures as * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 were considered significant.

Depletion of Plasmacytoid Dendritic Cells Potentially Delays the Production of Curli-Induced Autoantibody Production

With the discovery that bacterial amyloid curli is a novel activator of pDCs, and that curli injection induces the activation of multiple DC subsets *in vivo*, we next sought to investigate how important this dynamic is for the curli amyloid induced autoantibody production previously discovered by our lab. Because of the prevalence of the IFN Signature in the majority of lupus patients despite the heterogeneity of disease manifestations, and because of the pDC capacity to produce 10-100 times more type I IFN than any other cell types previous reports sought to test the role of pDCs in the development of spontaneous lupus, depleting in vivo pDCs in lupus prone mice, either through a depleting antibody or through genetic manipulation. These studies showed that pDCs are indeed required in the development of spontaneous lupus. Similarly, we decided to utilize a unique transgenic pDC depletion model, the C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J mouse. The mice were originally purchased from Jackson Laboratories and subsequently bred hemizygously and maintained in our laboratory with regular genotyping. The transgene in this mouse is the simian diphtheria toxin (DT) receptor under transcriptional control of the human C-type lectin domain family 4 (CLEC4) promoter, which is very specific to pDCs.[85] Just one molecule of DT can kill a pDC in this mouse, but mice in general are not very susceptible to the effects of DT usually seen in humans. The LD50 of DT for mice is 0.3 μg/g, so for a 30g adult mouse, this equates to 9 μg, making the 100ng injection quite a small amount. Using this method, we achieved a sensitive and targeted conditional cell ablation of pDCs in this model. After three days of injections of 100 ng per mouse of DT, mice remained healthy with no change in spleen cell counts. When staining the spleen for a variety of DC surface markers, we determined that the small but distinct subset of pDCs was completely ablated, without affecting any other cell type. Figure 9 shows the presence of the pDC subset in C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J (CLEC4-DTR) mice injected with PBS, and the absence of the pDC subset in CLEC4-DTR mice (designated as Tg for transgenic) injected with DT. B6 wildtype mice injected with DT showed no changes in the pDC subset or otherwise.

After confirming depletion of the pDC subset, we injected a larger cohort of WT control and CLEC4-DTR mice with 100μl of PBS with or without DT three times a week and 50 μg/ml of curli/DNA complexes thrice a week as we previously reported.[107] We collected blood to analyze the sera for ANAs weekly. Figure 10 shows that after the depletion of pDCs may delay the production of anti-dsDNA antibody induced by amyloid curli/DNA complexes during the first 2 weeks, while the levels reach a maximum response which is the same with and without pDCs depletion, after 4 weeks of exposure to curli/DNA complexes. Our lab has previously shown that injection of WT mice with bacterial amyloid curli or infection with curli-producing bacteria can induce the production of autoantibodies. Our data showing that depleting pDCs may delay the onset of anti-dsDNA antibody productions suggests that pDCs may play an important role in curli induced autoantibody production, possibly elucidating part of the mechanism behind bacterial infections and how they are a major cause of morbidity and mortality in lupus patients.

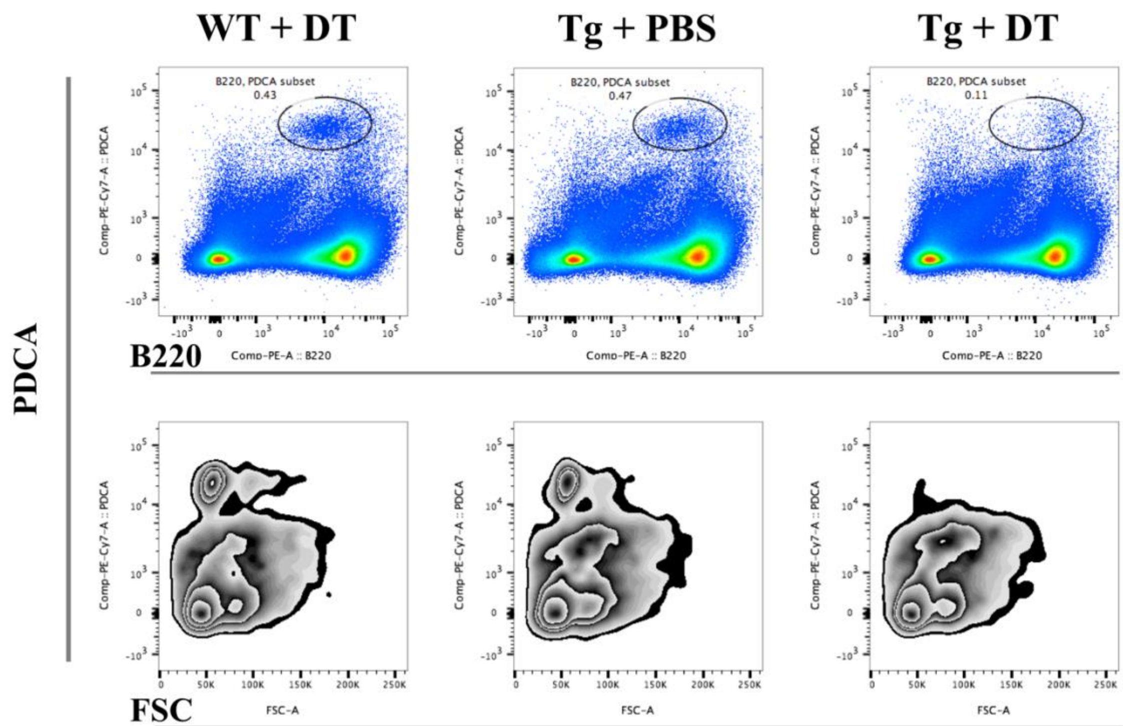

Figure 9. Diphtheria toxin produces a sensitive and targeted deletion of the pDC subset in CLEC4 transgenic mice

C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J mice have a simian DT receptor under transcriptional control of the CLEC4 promoter, specific to pDCs, allowing for a targeted deletion of the pDC subset with the injection of small amounts of DT. WT mice injected with DT and transgenic (Tg) mice injected with PBS vehicle all retain their pDC subset, gated here with B220 and PDCA. Tg mice injected i.p. for 3 days of 100ng of DT per mouse per day show a specific ablation of the pDC subset. Images are representative of depletion checks for all experiments involving pDC depletion. Splenocytes in the upper row were gated for appropriate forward scatter and side scatter, singlet cells, and viability (FVD-), while in the lower row they were further gated for B220+ CD11c+ CD11b- lineage markers specific for pDCs.

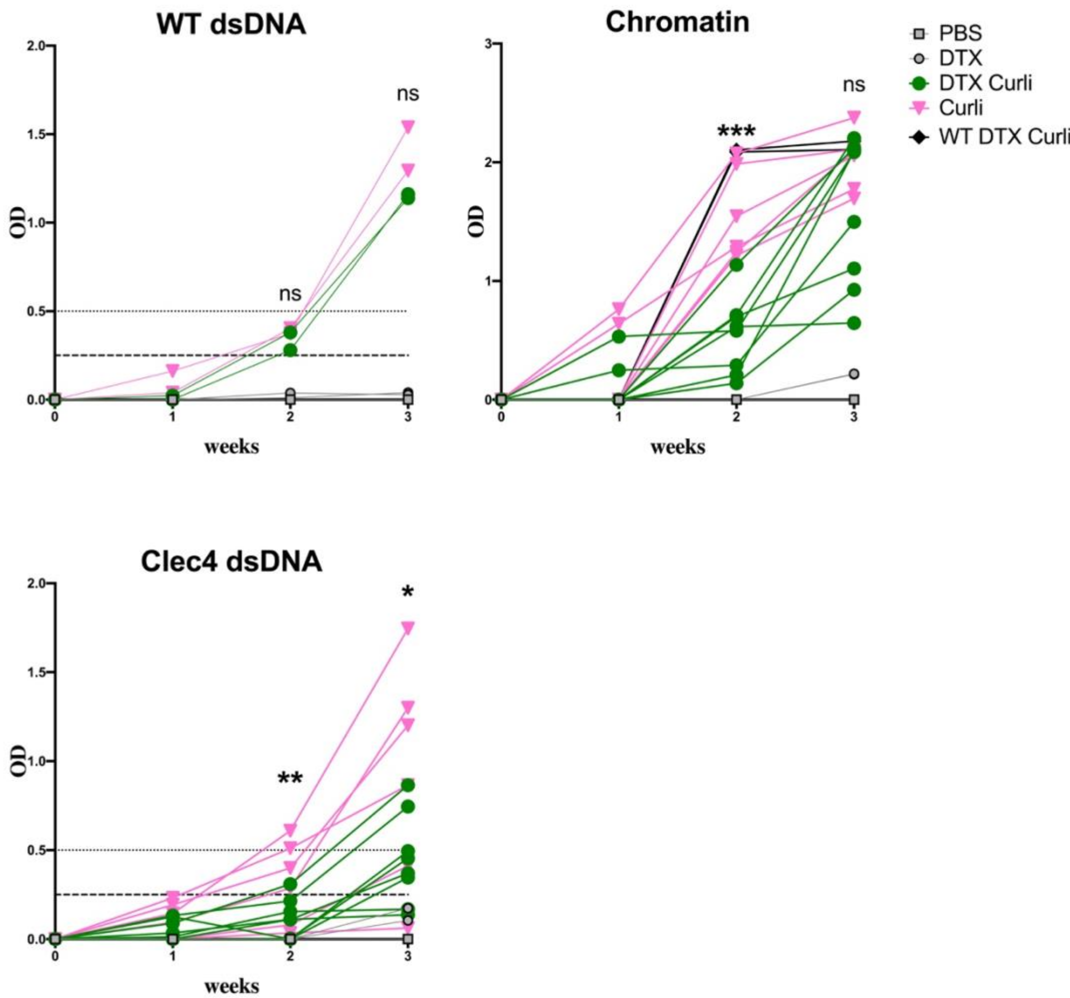

Figure 10. Depletion of pDCs delays the onset of autoantibody production induced by bacterial amyloid curli production

Our lab has previously shown that injecting WT mice with bacterial amyloid curli can induce autoantibody production. We show here that by depleting pDCs in a transgenic mouse on a B6 background, the production of anti-dsDNA autoantibody is slightly delayed. This suggests that pDCs may play a central role in the initiation of bacterial amyloid induced autoantibody production. Each line here represents an individual mouse. N ranges from 2 for the controls, to 5 per group for the mice injected with curli or DT and curli.

TLR2 is not essential for the DC response to curli

Now that we have shown the important role that DCs, and especially pDCs due to their capacity to produce type I IFN, are important in the bacterial amyloid promotion of autoimmunity, we wanted to better understand how pDCs recognize curli. Because DCs are the sentinels of the immune system through their recognition of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), and because our lab has previously shown that DCs from lupus prone mice overexpress type I IFN in response to TLR stimulation,[69] we wanted to investigate which specific TLR ligands are used by DCs to recognize curli. Previous studies by collaborating labs have shown that TLR1/2 and TLR9 may play an important role in the host ability to mediate an immune response to curli amyloid. Therefore, we wanted to investigate whether either of these two TLRs are essential for the curli activation of DCs. As mentioned in detail in the materials and methods section of this chapter, we obtained bone marrow from TLR2 global knockout (KO) mice, a generous gift from the Çagla Tükel lab at Temple University School of Medicine.

Bone marrow from TLR2 KO mice were processed as previously discussed. Briefly, cells were differentiated in FLT3-L supplemented complete RPMI medium for 7 days, then sorted into pDC and cDC subsets. Sorted cells were left to rest overnight, then stimulated with TLR ligand positive controls or curli at varying doses. Both pDCs and cDCs from TLR2 KO mice show the ability to upregulate surface costimulatory molecules upon stimulation with curli/DNA complexes as normal, statistically insignificantly different from the upregulation of surface costimulatory molecules in response to curli by WT mice (Figure 11). This suggests that TLR2 is not essential for the DC upregulation of surface costimulatory molecules in response to curli. Both CD86 and CD40 was upregulated by both pDCs and cDCs in the absence of functional TLR2. In congruence, TLR2 is also not essential for the production of CXCL10, a cytokine under transcriptional control of type I IFN (Figure 12). Production of CXCL10 by TLR2-KO pDCs was not significantly higher than production of CXCL10 by WT pDCs. Pam3C, a known TLR2 ligand, was used at 200 μM as a positive control to ensure TLR2 activity in WT DCs, and the absence of TLR2 activity in TLR2 KO DCs.

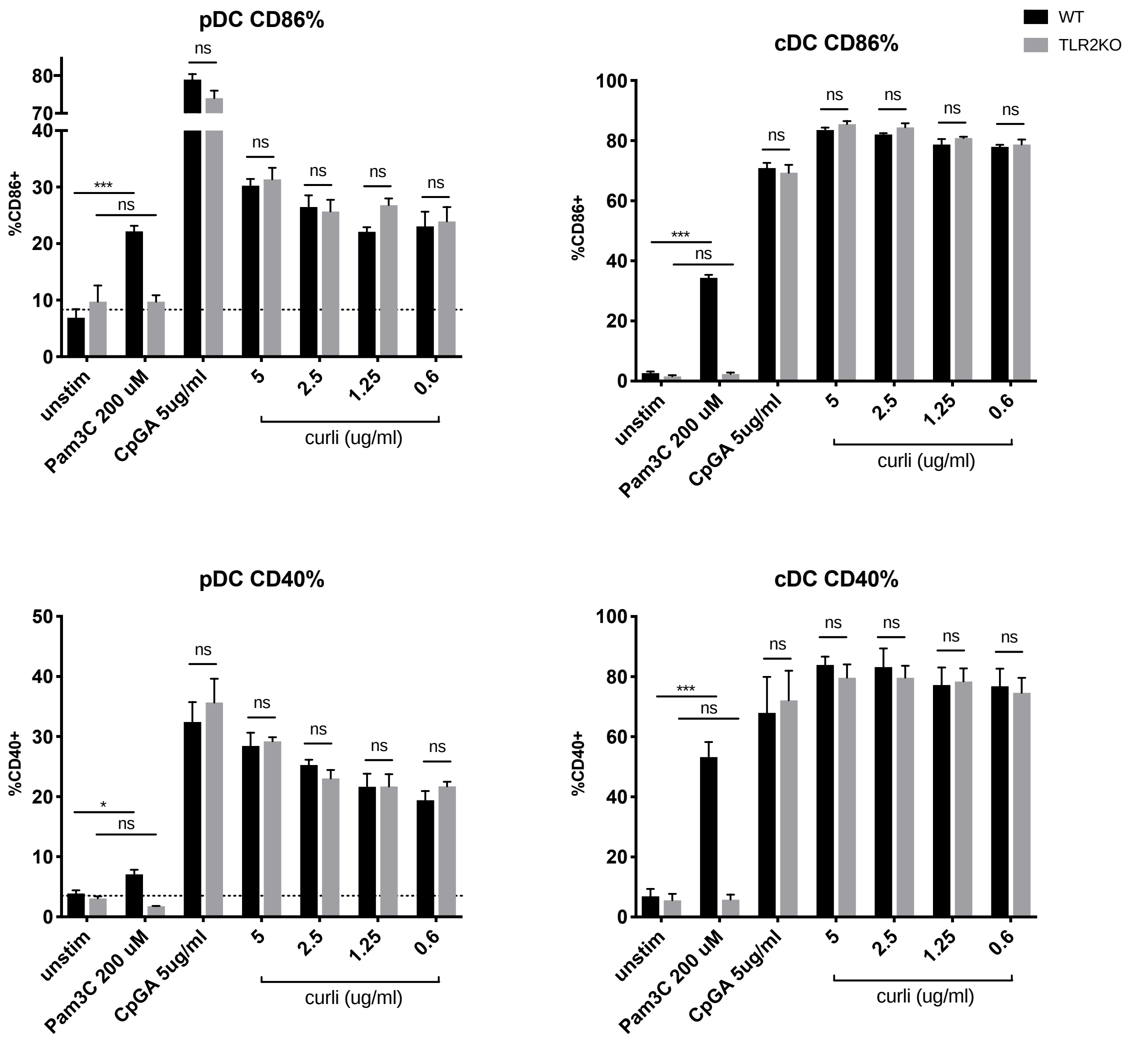

Figure 11. TLR2 is not essential for the upregulation of surface costimulatory molecules in response to curli

FLT3-L-derived pDCs and cDCs from TLR2 KO mice were stimulated with varying doses of curli, CpGA, or known TLR2 ligand Pam3C. Both WT and TLR2 KO DCs activated surface costimulatory molecules CD86 and CD40 to the same degree, suggesting that TLR2 is not essential for the upregulation of surface costimulatory molecules in the DC response to curli. Results are shown as the mean ± SEM. n=4, representative of 3 independent experiments.

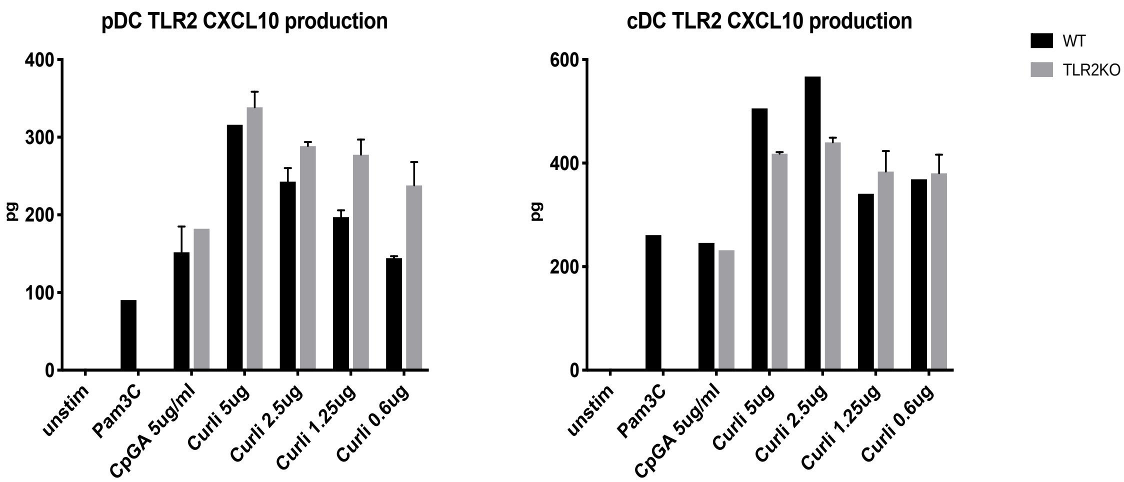

Figure 12. TLR2 is not essential for the production of type I IFN regulated cytokine CXCL10

FLT3-L-derived pDCs and cDCs from TLR2 KO mice were stimulated with varying doses of curli, CpGA, or known TLR2 ligand Pam3C. Both WT and TLR2 KO DCs produced similar amounts of CXCL10, suggesting that TLR2 is not essential for the DC production of type I IFN regulated cytokine CXCL10 in response to curli. Results are shown as the mean ± SEM. n=4, representative of 3 independent experiments.

TLR9 plays a role in the CXCL10 production in response to curli

Because TLR9 has also been implicated in mediating the immune response to curli,[109] we wanted to see whether functional TLR9 was necessary for the DC response to curli. As mentioned in detail in the materials and methods section of this chapter, we obtained bone marrow from TLR9 KO mice, a generous gift from the Behrens lab at The Children's Hospital of Pennsylvania.

Bone marrow from TLR9 KO mice were processed as previously discussed. Briefly, cells were differentiated in FLT3-L supplemented complete RPMI medium for 7 days, then sorted into pDC and cDC subsets. Sorted cells were left to rest overnight, then stimulated with TLR ligand positive controls or curli at varying doses. Both pDCs and cDCs from TLR9 KO mice show the ability to upregulate surface costimulatory molecules as normal, statistically insignificantly different from the upregulation of surface costimulatory molecules in response to curli by WT mice (Figure 13). This suggests that TLR9 is not essential for the DC upregulation of surface costimulatory molecules in response to curli. Both CD86 and CD40 was upregulated by both pDCs and cDCs in the absence of functional TLR9. In contrast, the DCs from TLR9 KO were significantly impaired in their ability to produce CXCL10, compared to DCs from WT mice with functional TLR9. Although the expression of CD86 in cDCs is statistically different between WT and TKR9-KO cells, the up-regulation of this marker is so high (>60% of positivity against <10 in unstimulated cDCs) that we conclude that most of the stimulation of CD86 upon curli/DNA is TLR9 independent. This suggests that TLR9 is not essential for the DC upregulation of surface costimulatory molecules in response to curli. In contrast, the DCs from TLR9 KO were significantly impaired in their ability to produce CXCL10, compared to DCs from WT mice with functional TLR9. This suggests that, although TLR9 may not be essential for the DC upregulation of surface costimulatory molecules in response to amyloid curli/DNA complexes, it is essential for the production of CXCL10, a cytokine under transcriptional control of type I IFN (Figure 14). CpGA, a known TLR9 ligand, was used at 5 μg/ml as a positive control to ensure TLR9 activity in WT DCs, and the absence of TLR9 activity in TLR2 KO DCs.

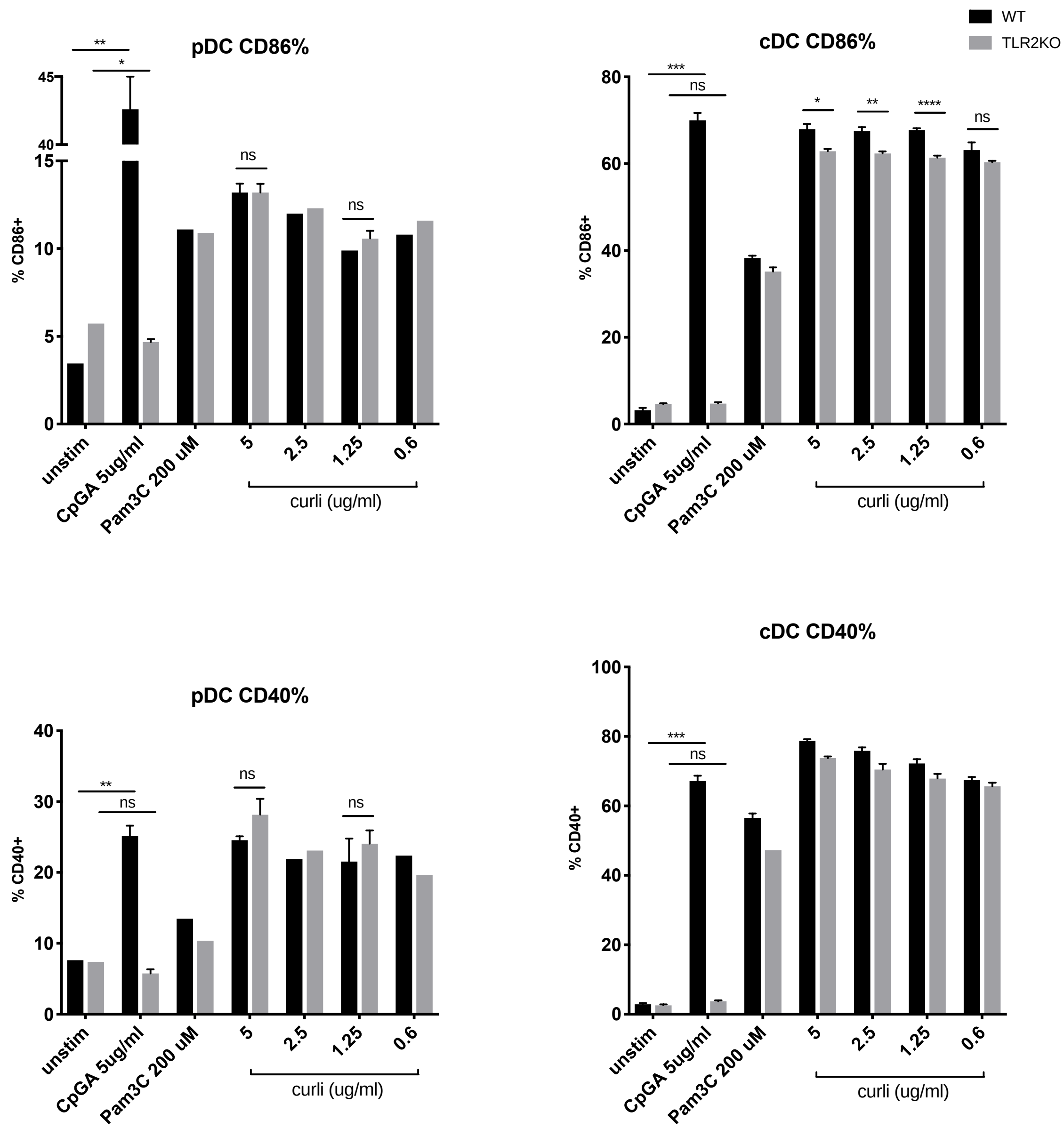

Figure 13. TLR9 is not essential for the upregulation of surface costimulatory molecules in response to curli

FLT3-L derived pDCs and cDCs from TLR9 KO mice were stimulated with varying doses of curli or known TLR9 ligand CpGA. Both WT and TLR9 KO DCs activated surface costimulatory molecules CD86 and CD40 to the same degree, suggesting that TLR9 is not essential for the upregulation of surface costimulatory molecules in the DC response to curli. Results are shown as the mean ± SEM. n=4, representative of 3 independent experiments

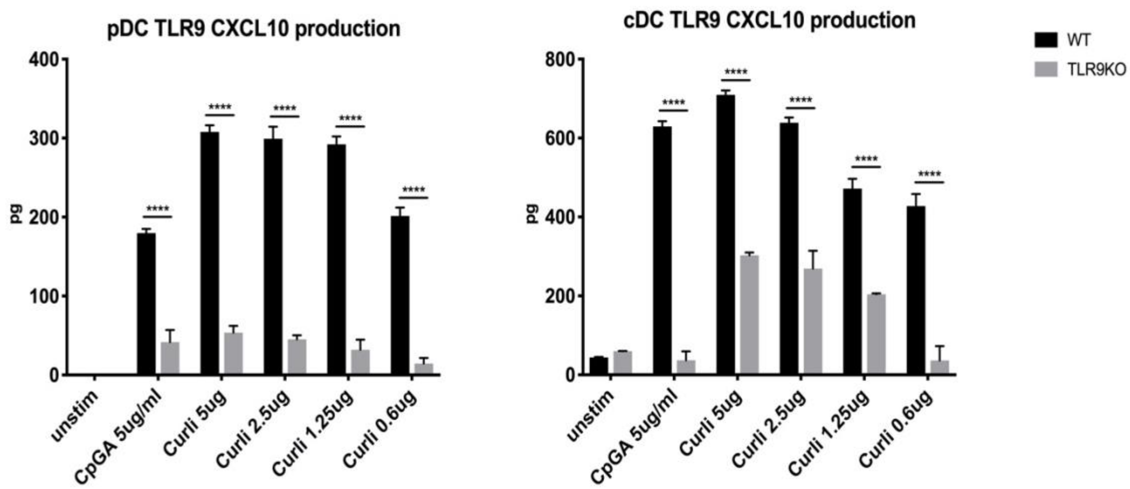

Figure 14. TLR9 is essential for the production of type I IFN regulated cytokine CXCL10

FLT3-L-derived pDCs and cDCs from TLR9 KO mice were stimulated with varying doses of curli or known TLR9 ligand CpGA. While treating DCs from WT mice with curli elicited a robust production of CXCL10, DCs from TLR9 KO mice were significantly impaired in their production of CXCL10, suggesting that TLR9 is essential for the DC production of type I IFN regulated cytokine CXCL10 in response to curli. Results are shown as the mean ± SEM. n=4, representative of 3 independent experiments.

Discussion

Systemic lupus erythematosus, for the last couple centuries has proven to be a challenging disease to treat, supported by its convoluted and complex pathophysiology behind each disease manifestation. The identification of DCs, and pDCs specifically because of their remarkable ability to produce type I IFNs, has led researchers in the direction to study how these sentinels of the immune system may contribute to lupus disease. As lupus as a disease became better defined, it became evident that the role of infections in autoimmunity is something important to explore, and the ability of DCs to recognize PAMPs and DAMPs to shape the immune response further implicated DCs as an important cell type to study. The Gallucci Dendritic Cell Biology Lab, in collaboration with the Tükel lab, discovered that curli, an amyloid produced by bacteria implicated in the infections commonly found in lupus patients, is a PAMP that can stimulate the immune system, and the inflammatory dendritic cells in particular. They also discovered that curli/DNA composites are strong inducers of type I IFNs and can remarkably accelerate autoimmunity in lupus prone mice.

These findings set the base for this thesis project, where we discovered the ability of amyloid curli/DNA to directly activate multiple subsets of DCs both *in vitro* and *in vivo*, identifying curli/DNA as a novel stimulator of these cells. We discovered that curli/DNA induces the upregulation of surface costimulatory molecules and proinflammatory cytokine production in both cDCs, the primary APC, and pDCs, the primary type I IFN producer. Importantly, we discovered that amyloid curli/DNA also induces a very strong type I IFN response, as evidenced by the identification of intracellular IFNα in pDCs, and the stronger production of type I IFN regulated cytokine CXCL10. Type I IFN responses induced by curli/DNA were significantly stronger than that induced by the TLR9 ligand CpGA, the so far best stimulator of pDCs. These results further highlight the important stimulatory effects of curli/DNA on pDCs.

Our results also show that the depletion of pDCs may delay the onset of amyloid curli/DNA-induced autoantibody production in wildtype mice, highlighting the role of pDCs in how curli/DNA can trigger autoimmunity even in non-autoimmune mice. This finding raises the question of why autoimmunity is not more common in the human population, which normally is exposed to a wide variety of bacteria and infections expressing curli/DNA complexes. Coupled with conflicting studies showing the lupus-prone mice in germ-free conditions develop SLE-like disease, this suggests that genetics do play an important part in disease manifestation.[114] To account for both of these findings, it is thought that genetic predispositions for immune dysfunction in susceptible humans and mice may increase the host exposure to nucleic acid material during infections, while in nonautoimmune-prone humans and mice, infections do not normally result in the release and exposure of nucleic acid material.[110] Alternatively, or in combination, it has been proposed that genetic predispositions can lead to weaker mucosal barrier and therefore higher leakage and systemic exposure to bacterial products that can activate the immune system. This is supported by reports of high levels of circulating endotoxin and more frequent bacteremia from infection in SLE patients.[313-315] We propose that in genetically predisposed individuals, curli/DNA complexes stimulate the immune system, and pDCs in particular, once they gained systemic access during chronic clinical and subclinical infections.

In conclusion, this study highlights the role of bacterial amyloid curli in the development of lupus-like autoimmunity and identifies curli amyloid as a novel activator of pDCs, the primary type I IFN producer heavily implicated in the IFN Signature seen in the vast majority of human lupus patients. Our findings provide a possible explanation for the mechanism behind how curli amyloid can induce a type I IFN response, and how pDCs are important in the development of autoantibodies in response to bacterial amyloid (Figure 15).

Future Directions

Future directions for this aim include utilizing a genetic pDC-specific knockout that would allow for the longer-term exploration of any deficits or lack thereof in response to amyloid curli-DNA complexes, due to the relatively short-lived effect of diphtheria toxin-induced selective pDC depletion. Although the transgenic diphtheria toxin model is more accurate and thorough than the traditional antibody depletion models, the otherwise healthy B6 wildtype background allows for the mouse to develop antibodies against the diphtheria toxin after a couple weeks of thrice-weekly injections. Diphtheria toxin is well known to have an adjuvant effect and is used to our advantage in human vaccines. Thus, the development of neutralizing diphtheria toxin antibodies rendering this depletion model ineffective is not surprising. A pDC gene-specific genetic that could be used is the C57BL/6J-Pdc^{em1Smoc} mouse, generated by CRISPR/Cas knockout of the exon 3 region of the pDC, although this strain is not well studied. A pDC KO mouse could also be back-crossed onto a lupus-prone background, such as the triple congenic Sle1,2,3 mouse also on a B6 background, to see if the lack of pDCs would affect the spontaneous development of lupus disease.

Additionally, this aim could be expanded through exploring changes in the transcriptome that occur due to the depletion of pDCs and its role in delaying the early production of autoantibodies in response to amyloid curli/DNA complexes. In either a short-term diphtheria-toxin induced depletion or a long term pDC KO model, the transcriptome and/or type I IFN gene products could be monitored to see what changes occur during the amyloid curli/DNA complex-induced antibody production. Through careful monitoring, gene product changes and the development of specific aspects of the interferon signature could be charted in a mouse model, which lacks the variability seen in human patients. In this way, the candidate genes that pDCs upregulate that results in the induction of the production of autoantibodies can be identified, highlighting new possible targets and bringing new insights into lupus pathogenesis that could really propel bench-to-bedside research occurring in the clinical setting.

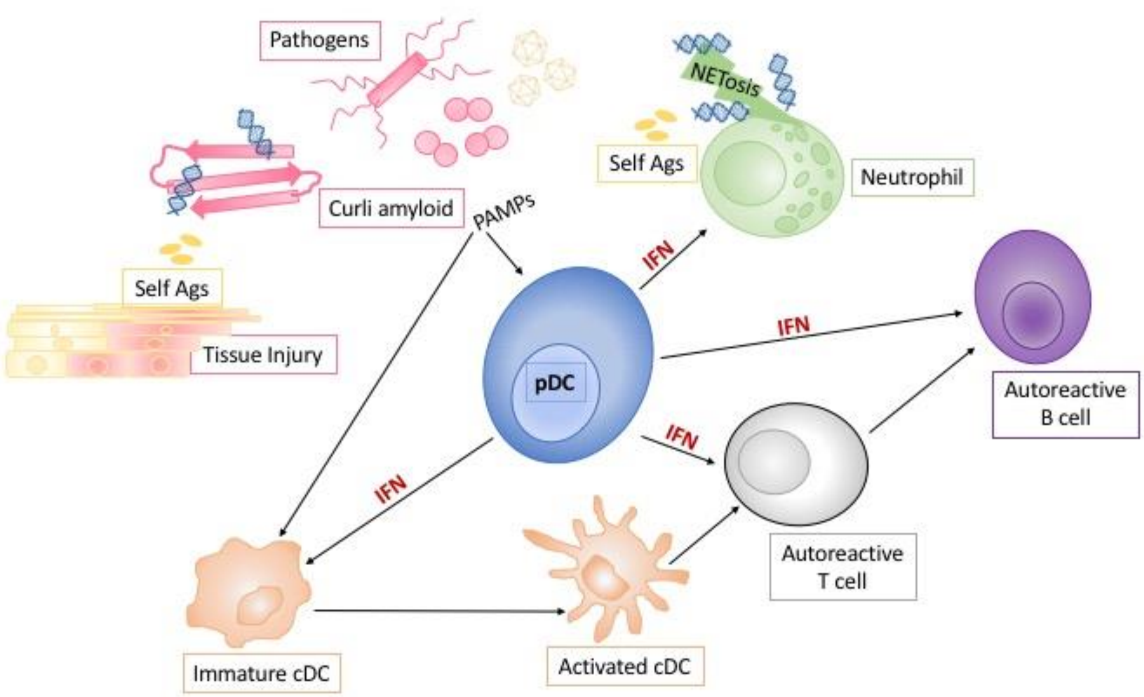

Figure 15. Working model

The production of type I IFN by pDCs is a known mechanism of pathogenesis in SLE, and the bacterial amyloid curli/DNA complex is a novel activator of pDCs, acting as a PAMP. The curli/DNA complexes activate pDCs at least partially via TLR9, inducing type I IFN and proinflammatory cytokine production. The bacterial amyloid curli/DNA complex also activates cDCs and promotes the release of inflammatory cytokines. Type I IFN produced by pDCs is capable of promoting T cell activation, B cell autoantibody production, cDC activation and maturation, and release of neutrophil NETs containing nucleic acid material and self antigens. Activated cDCs are capable of releasing proinflammatory cytokines and priming T cells specific for self antigens (a process known to be facilitated by type I IFN).

CHAPTER 3

DENDRITIC CELL SIGNALING: THE ROLE OF IFNAR DOWNSTREAM SIGNALING MOLECULE STAT2

Introduction

Because some IFN-I responses are STAT1-independent,[236,279] our previous studies led us to show that STAT2 is constitutively expressed in murine DCs and is required for the upregulation of costimulatory molecules and ISGs in response to various toll-like receptor (TLR) ligands, establishing that the primary signal transducer molecules of IFN-Is in cDCs are the canonical STAT1-STAT2 heterodimers.[236,279,316] We also reported that the lack of a functional STAT2 molecule in cDCs impaired the cross-presentation of tumor antigens by bone marrow-derived cDCs to CD8+ T cells *in vitro*, using a tumor antigen and a non-tumor antigen models, underscoring the importance of STAT2 function in DCs in antitumor and general immunity.[278,302] Additionally, we demonstrated that priming WT mice with *Stat2-/-* bone marrow-derived cDCs pulsed with OVA protein resulted in a decreased induction of specific cytolytic activity towards target OVA-pulsed splenocytes, compared to mice immunized with WT cDCs pulsed with OVA, suggesting that STAT2 in cDCs is necessary to induce cross-presentation *in vivo*.[302] However, it is unknown whether a functional STAT2 in endogenous DCs is necessary for the development of cross-presentation responses, and whether the necessary function of STAT2 is specific to discrete subsets of DCs.

Materials and Methods

Mice

In this study, we investigated the unique DC requirements for STAT2 by utilizing a conditional STAT2 knockout mouse that has a deletion of the STAT2 gene only in CD11c+ cells. Because most dendritic cells express CD11c+, this conditional knockout allowed us to investigate the specific role that STAT2 plays in IFN-I directed responses by DCs. Stat2$^{fl/fl}$; CD11c Cre^{+} mice on a C57BL/6 background used in these studies were gifted by the Gamero Lab at Temple University, to be used in collaborative investigations. C57BL/6 mice were used as WT, full STAT2-KO mice and parental Stat2$^{fl/fl}$ mice and CD11c Cre^{+} mice were used as controls.

Immunization with Chicken Egg Ovalbumin

Naïve mice were immunized by intramuscular (i.m.) injection with 50μg of chicken egg ovalbumin (OVA) in Complete Freund's Adjuvant (CFA, Sigma) and boosted with 100μg of OVA in Incomplete Freund's Adjuvant (IFA, Sigma) two weeks later. The blood was collected prior to immunization with OVA antigen in CFA, 2 weeks after the initial immunization, before the booster, and 2 weeks after the booster immunization. The blood samples were taken from the tail vein, allowed to coagulate at room temperature, then centrifuged to isolate the serum. Isolated serum was centrifuged again, and serum from the second centrifugation was collected and immediately frozen.

Anti-OVA antibody ELISA

After sera from all time points were collected, the frozen serum samples were thawed and analyzed in the same ELISA.[317] 96-well vinyl assay plates (Corning) were coated with 100ul/well of 30ug/ml of OVA protein in BBS overnight at 4°C. The plates were washed with BBS, blocked with 3% bovine serum albumin (BSA, Gemini) in borate buffered saline (BBS) for 2-3 hr at 37°C, and washed again with BBS. Thawed serum was diluted 1:500 in blocking buffer (BBT) containing borate BBS, 3% BSA, and 1% Tween 80. 100ul of diluted serum or mouse anti-OVA antibody standard (Clone OVA-14, Sigma) was added to each well, and samples were incubated at 4°C overnight. The plates were then washed with BBS and coated with alkaline phosphatase conjugated antibodies. The plates were rocked for 1 hr at room temperature, then washed with BBS. 100ul/well of 1mg/ml of p-nitrophenyl phosphate (PNPP, Sigma) substrate was added, and the plates were rocked at room temperature. The optical densities were measured at 405nm with wavelength correction at 650nm after 90 minutes. Results were expressed as optical density units (O.D.). See Appendix for injection schedule.

In Vivo CTL killing Assay

Mice that had been immunized with OVA antigen and had blood collected for antibody production assays were boosted a second time with 50μg of OVA in IFA two weeks after the first booster immunization. Seven days later, splenocytes from naïve WT C57BL/6 mice were divided into two equal number populations that were pulsed or not with 1uM of OVA SIINFEKL peptide (Invitrogen) for 1 hr at 37°C in RPMI media (Fisher) supplemented with fetal bovine serum (FBS, Gemini). The pulsed target splenocytes were stained with low-endotoxin phosphate buffered serum (PBS) containing 0.1% BSA and 5uM CFSE ($CFSE^{hi}$ positive cells), and unpulsed splenocytes were stained with PBS containing 0.1% BSA and 0.5uM CFSE ($CFSE^{low}$ positive cells). Pulsed and unpulsed splenocytes were combined in equal numbers at a final density of $5x10^6$ cells/200ul PBS. The immunized mice and control naïve mice were injected intravenously (i.v.) via tail vein with 200ul PBS with or without $5x10^6$ of 1:1 mixed CFSE labeled target splenocytes. The mice were sacrificed after 24 hrs, and their spleens were collected for flow cytometry analysis of $CFSE^{hi}$ and $CFSE^{lo}$ positive target cells. The percent of specific lysis was calculated as $1-[r_{naive}/r_{immunized}]x100$, where $r=\%CFSE^{lo}$ cells / $\%CFSE^{hi}$ cells (27233962). See Appendix for injection schedule.

In Vivo Stimulation of Dendritic Cells

The mice were injected i.v. in the tail vein with 30μg of the TLR7 ligand R848 (Invivogen). The mice were sacrificed after 24 hrs. The spleens were collected and digested in collagenase type IV (Worthington) and DNAse from bovine pancreas type II (Sigma) for 45 min at 37°C in neat IMDM media to free APCs from the splenic extracellular matrix and filtered through a 100um filter. The harvested single-cell suspension was pelleted by centrifugation and resuspended in 2mL of ammonium-chloride-potassium red blood cell lysing buffer (VWR) for 5min at room temperature. The reaction was stopped by adding IMDM media containing 10% FBS (Gemini). The cells were pelleted by centrifugation and resuspended in buffer for staining for flow cytometry analysis.

Bone marrow-derived cell cultures

Femurs were harvested from mice and bone marrow precursors were flushed from the femurs into a single-cell suspension. To generate macrophages, bone marrow cells were cultured in complete DMEM medium (Corning) supplemented with 10% FBS (Gemini), 1% penicillin/streptomycin (Fisher), 1% L-glutamine (Corning), 0.1% 2-mercaptoethanol (Life Technologies), and 10ng/ml of recombinant mouse macrophage colony-stimulating factor (M-CSF 315-02, Peprotech). To generate conventional dendritic cells (24489947), bone marrow cells were cultured in complete IMDM medium (Corning) supplemented with 10% FBS (Gemini), 1% penicillin/streptomycin (Fisher), 1% L-glutamine (Corning), 0.1% 2-mercaptoethanol (Life Technologies), and 1% of the supernatant of a cell line secreting murine granulocyte-macrophage colony-stimulating factor (GM-CSF). Fresh medium was added at day 2, and half of the medium was replaced at day 5. On day 6, cells were gently harvested, washed with PBS, dry-pelleted, and frozen on dry ice for later analysis by Western blot.

Flow cytometry

Single cells suspension of splenocytes, obtained as described in the previous paragraph, was suspended in cold PBS containing 1% BSA and incubated with rat anti-mouse CD16/CD32 (clone 2.4G2) mAb for 10 min on ice to block the FcγR. The cells were then stained for 30 min on ice with antibodies specific for surface markers (B220 clone RA3-6B2, CD11c clone N418, CD11b cloneM1/70, CD3 clone 17A2, CD4 clone RM4-5, and/or CD8α clone 53-6.7) and activation markers (CD86 clone GL1, CD40 clone MR1). Cells were analyzed on a FACSCanto cytometer (BD Biosciences). Data analysis was performed using FlowJo software. Statistical analysis was performed using GraphPad Prism7.

Statistical Analysis

Prism 8 (GraphPad software, San Diego, CA, USA) was used for data analysis. Means and standard error of means (Mean ± SEM) were calculated by averaging results from independent experiments. Statistical significance was determined using unpaired two-tailed Student's t-test for comparison between two groups. One-way ANOVA and Two-way ANOVA were used for multiple comparisons followed by the Bonferroni and Sidak multiple comparisons post-hoc correction tests respectively. P-values marked in the figures as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ were considered significant.

Results

Conditional Deletion of STAT2 in CD11c+ Dendritic Cells

By collaborating with the Gamero Lab at the Lewis Katz School of Medicine at Temple University, we were able to study the unique DC requirements for STAT2 by generating a conditional STAT2 knockout mouse that has a deletion of the STAT2 gene only in CD11c+ cells. Using Cre-Lox site-specific recombinase technology, the Gamero lab was able to carry out the deletion of STAT2 specifically in CD11c+ cells (Figure 15). Because most dendritic cells express CD11c+, this conditional knockout allowed us to investigate the specific role that STAT2 plays in IFN-I directed responses by DCs. Western blotting of various cell types and organs confirms the specific KO of STAT2 (Figure 16).

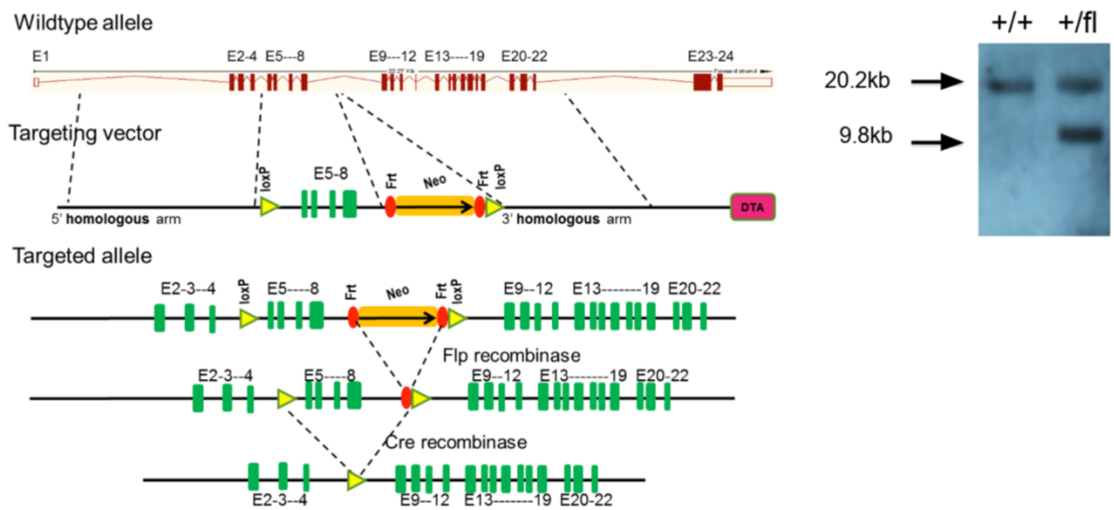

Figure 16. Targeting strategy for the specific deletion of STAT2 in CD11c+ cells

Cre-Lox site-specific recombinase deletion of STAT2 in CD11c+ Cells. Figure and data courtesy of the Gamero lab.

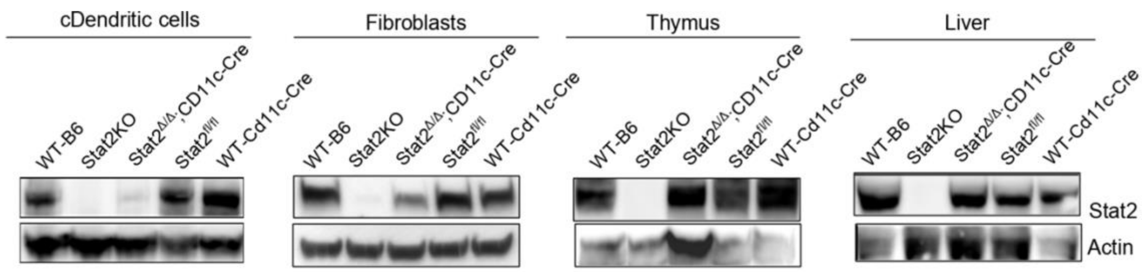

Figure 16. Confirmation of the absence of STAT2 protein in the correct cells
Western blotting demonstrates the specific KO of STAT2 in CD11c+ cells. Cell lysates from bone marrow-derived cDCs, generated in culture in presence of GM-CSF, embryonic fibroblasts, thymus and liver of WT, full Stat2-KO, the conditional Stat2 floxed in CD11c+Cre, and the parental mice Stat2 flox and CD11c+ Cre mice. Western Blotting courtesy of the Gamero lab.

The Production of Anti-OVA Antibodies Requires STAT2 but is not Dependent on STAT2 Function in CD11c+ Cells

To study the role of STAT2 in promoting a humoral response and to determine whether STAT2 function in CD11c$^+$ cells is necessary for the murine capacity to produce antibodies to a specific protein antigen, we utilized chicken egg ovalbumin (OVA), a T cell dependent antigen for which the immune response has been well characterized.[318,319] We immunized naïve CD11c-Cre$^+$ mice, Stat2$^{fl/fl}$ mice, CD11c-Cre$^+$-Stat2$^{\Delta/\Delta}$ mice, full Stat2-KO, mice and WT mice with OVA protein in CFA and boosted at week 2 with OVA in IFA. Blood was collected prior to immunization, prior to the booster (week 2), and 2 weeks after booster immunization (week 4). We measured the levels of anti-OVA antibodies in the serum using an enzyme-linked immunosorbent assay (ELISA) specific for anti-OVA IgG and we discovered that the antigen-specific antibody production is not dependent on STAT2 function in CD11c$^+$ cells, since the CD11c-Cre$^+$-Stat2$^{\Delta/\Delta}$ mice produced similar levels of anti-OVA IgG as the WT and the CD11c-Cre$^+$ and Stat2$^{fl/fl}$ control littermate mice (Figure 17). No difference was observed after the first immunization and the booster, indicating that both primary and secondary immune responses do not require a functional STAT2 in DCs. In contrast, the full Stat2 knockout mice were unable to produce anti-OVA serum IgG antibodies after the first immunization and showed very low levels of OVA-specific antibodies, about 8 times lower than the WT mice ($p<0.0001$) after the booster (Figure 17). These results indicate that STAT2 in cells other than CD11c$^+$ DCs is required for a normal humoral response.

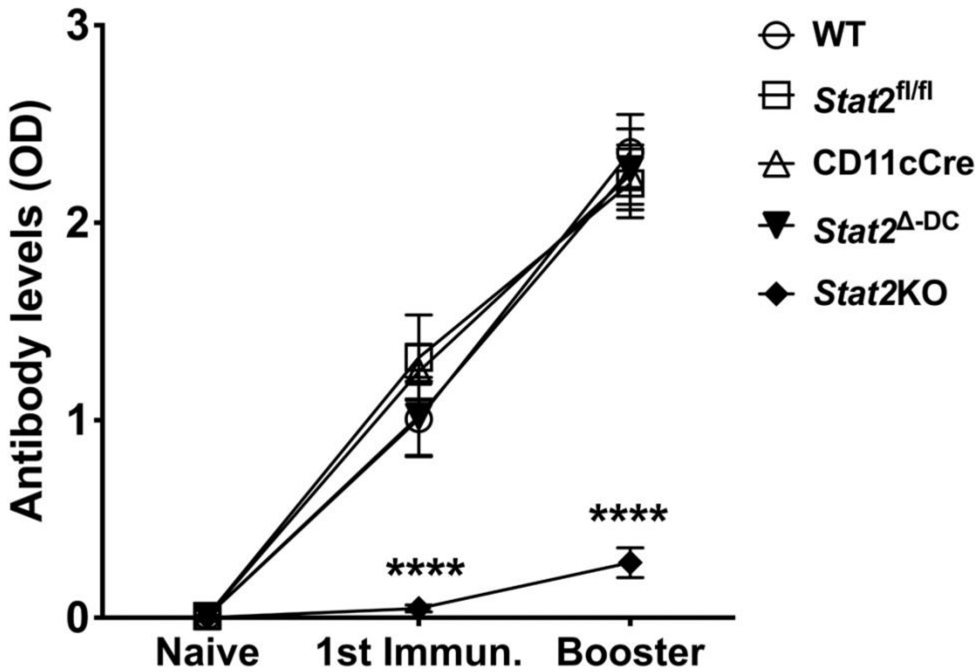

Figure 17. STAT2 function is necessary for the production of anti-OVA antibodies but is not dependent on STAT2 function in CD11c+ cells

WT mice, mice carrying the flox Stat2 gene, mice carrying the Cre under the CD11c promoter, mice with the deletion of Stat2 in CD11c+ cells, and full Stat2-KO mice were immunized with 50 μg of OVA in Complete Freund's Adjuvant (CFA). Two weeks later, mice were boosted with 100 μg of OVA in incomplete Freund's Adjuvant (IFA). Serum samples from each mouse were collected at baseline prior to immunization, two weeks after initial immunization, before the booster, and two weeks after booster immunization. The anti-OVA antibody levels were measured by ELISA and expressed as a mean optical density (OD). Data are shown as averages of 7 to 14 mice per group, tested in three separate experiments. Statistical significance was analyzed using one-way ANOVA followed by the Dunn's multiple comparisons test against WT and is shown by the symbol **** $p<0.0001$. ns, not significant.

Induction of CTL Killing Response Requires STAT2 in DCs In Vivo

Cross-presentation by DCs is a very important cellular mechanism in host defense and it has been shown to be pivotal to elicit a cytotoxic T cell lymphocyte (CTL) killing response in $CD8^+$ T cells against antigens not expressed by DCs but acquired from the environment.[232,320] $CD8\alpha^+$ DCs were shown to be the main DC subset responsible for performing this type of presentation.[321,322] To test whether cross-presentation by endogenous DCs requires STAT2, we measured CTL killing of OVA-specific CFSE labeled target cells *in vivo* by flow cytometry. We used the mice previously immunized with OVA to measure the humoral response, namely CD11c-Cre^+ mice, $Stat2^{fl/fl}$ mice, CD11c-Cre^+-$Stat2^{\Delta/\Delta}$ mice, full Stat2-KO mice, and WT mice. We administered to them a second booster immunization of OVA protein. Seven days later, we performed the CTL killing in vivo assay by injecting the mice with target splenocytes containing a mix of OVA-pulsed and unpulsed splenocytes easily discernible by a differential CFSE staining intensity. After 24 hrs, we analyzed spleens for the presence of the injected target cells, and we calculated the specific lysis of OVA positive targets as the ratio of $CFSE^{high}$ OVA-pulsed cells and $CFSE^{low}$ unpulsed cells. WT, Stat2 WT CD11c-cre^+, and $Stat2^{fl/fl}$ CD11c-cre^- mice, which are all functionally WT, mounted an efficient CTL killing response to the OVA-labeled target cells (Figure 18). The functionally WT mice achieved an average specific lysis of 75-80%, compared to naïve unimmunized mice, which represented the specific lysis of 0%. Interestingly, the $Stat2^{\Delta/\Delta}$ CD11c-cre^+ and global Stat2 knockout mice were both significantly impaired in their ability to mount a CTL killing response, with the conditional STAT2 knockout mice averaging a specific lysis of 22.5%, and the complete STAT2 knockout mice averaging 14.5% (Figure 18). Our results show that the WT mice displayed strong specific cytolytic activity as expected. The results that the full STAT2 knockout mice are significantly impaired in their ability to mount a CTL response *in vivo* (*p*<0.0001) indicate the pivotal role for STAT2 and the I-IFN response in the process of cross-presentation and stimulation of CTL killing *in vivo*. Our results reveal that STAT2 in DCs is specifically required for efficient cross-priming of CTL response *in vivo*, and that the absence of STAT2 function in CD11c+ DCs significantly impairs the CTL killing response (p<0.0001) (Figure 18), indicating that intrinsic DC responses to type I IFNs are necessary to efficiently cross-present to CD8 T cells and induce killing responses.

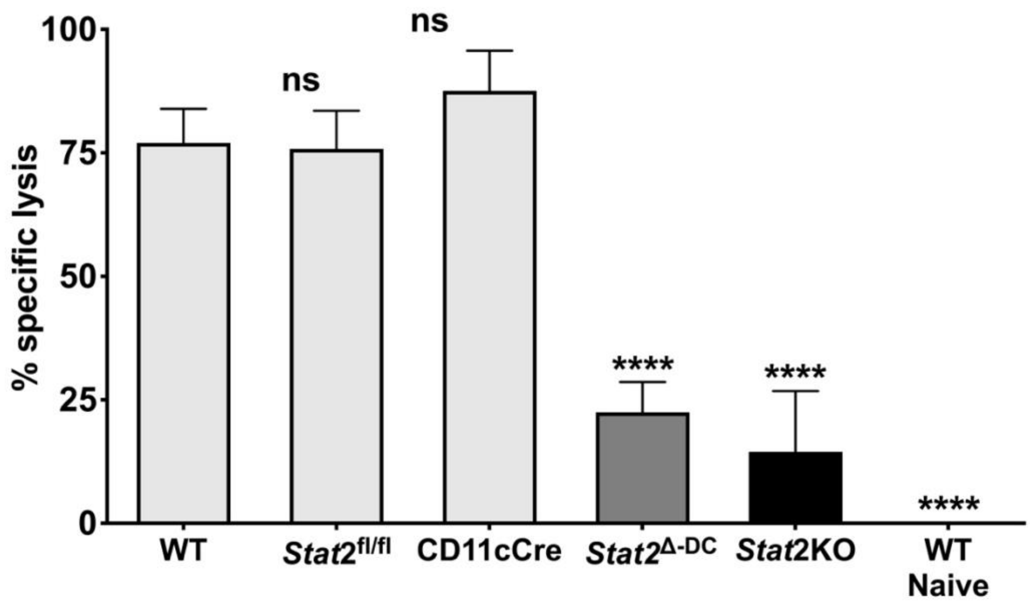

Figure 18. STAT2 function in DCs is necessary for mounting a CTL killing response in vivo
Two weeks after the booster immunization, mice were boosted a second time with 50 µg of OVA in IFA. Seven days later, CTL killing assay was performed by injecting mice with splenocytes pulsed with or without OVA peptide and recognizable as labeled with a high or low concentration of CFSE. Mice were sacrificed after 24 hours and their spleens analyzed by flow cytometry. The percentage of specific lysis was compared with the naive mice, calculated from a ratio of $CFSE^{high}$ and $CFSE^{low}$ cells. Data are shown as averages of 4 to 14 mice per group, tested in two experiments. Error bars indicate the standard error of means (SEM). Statistical significance was analyzed using one-way ANOVA followed by the Dunn's multiple comparisons test against WT and is shown by the symbol **** $p<0.0001$. ns, not significant.

STAT2 Function is Necessary for the Activation of DCs In Vivo

Since our results show that STAT2 function in CD11c+ cells are critical in mounting a cross-presentation response, we decided to determine whether DCs require STAT2 to activate and up-regulate costimulatory molecules *in vivo* and which subsets are affected. We tested the response to the TLR7/8 ligand R848 as example of DC activation. The intracellular TLR7 and TLR8 mediate the recognition of ssRNA by DCs and plays a major role in the ability of these cells to recognize foreign bodies and produce an immune response.[323] Additionally, TLR7 enables cross-presentation by multiple DC subsets, and results in a vigorous production of type I IFN and IL-12,[324,325] which have been demonstrated to be required for the cross-priming of $CD8^+$ T cells.[326-328] To assess the importance of STAT2 in the ability of DCs to activate in response to R848, we injected mice with 50μg of R848 or PBS vehicle. Mice were sacrificed after 24 hrs, and splenocyte suspensions were stained for antibodies specific for mouse DCs (B220, CD11c, CD11b, CD8α) and DC activation markers (CD86, CD40). We evaluated the conventional DCs ($B220^-$ $CD11c^+$ $CD11b^+$ cDCs), the plasmacytoid DCs ($B220^+$ $CD11c^{Int}$ $CD11b^-$ pDCs), and the $CD8\alpha^+$ DCs ($CD3^-$ $CD8\alpha^+$ $CD11c^+$ $B220^-$), the latter are important for the cross-priming of $CD8^+$ T cells. We analyzed the total numbers of splenocytes and the frequencies of cDCs, pDCs and $CD8^+$ DCs and found that the deletion of STAT2, both in the whole mouse and specifically under the CD11c protomer, does not affect the total cellularity of the spleen and the frequency of DC subsets, baseline and in response to TLR stimulation *in vivo* (Figure 19). All three DC subsets in WT or functionally WT mice demonstrated an upregulation of the surface costimulatory markers CD86 and CD40 in response to R848, as expected (stimulated WT mice are shown as normalized to 1).[302,328] In contrast, all three

DC subsets in the global STAT2 knockout mice failed to upregulate surface costimulatory makers ($p<0.0001$), indicating that STAT2 is required for *in vivo* DC responses to TLR7 (Figure 20). The upregulation of the costimulatory molecules in the $CD11c^+$ conditional STAT2 knockout mice was impaired in the cDC subsets, with the most pronounced impairment in the $CD8\alpha^+$ DC subset, while pDCs show a trend to a lesser upregulation that did not reach statistical significance. (Figure 20). Our results show that the function of STAT2 in $CD11c^+$ DCs is important for DC response to a systemic TLR7/8 agonist, while does not affect their presence in the spleen.

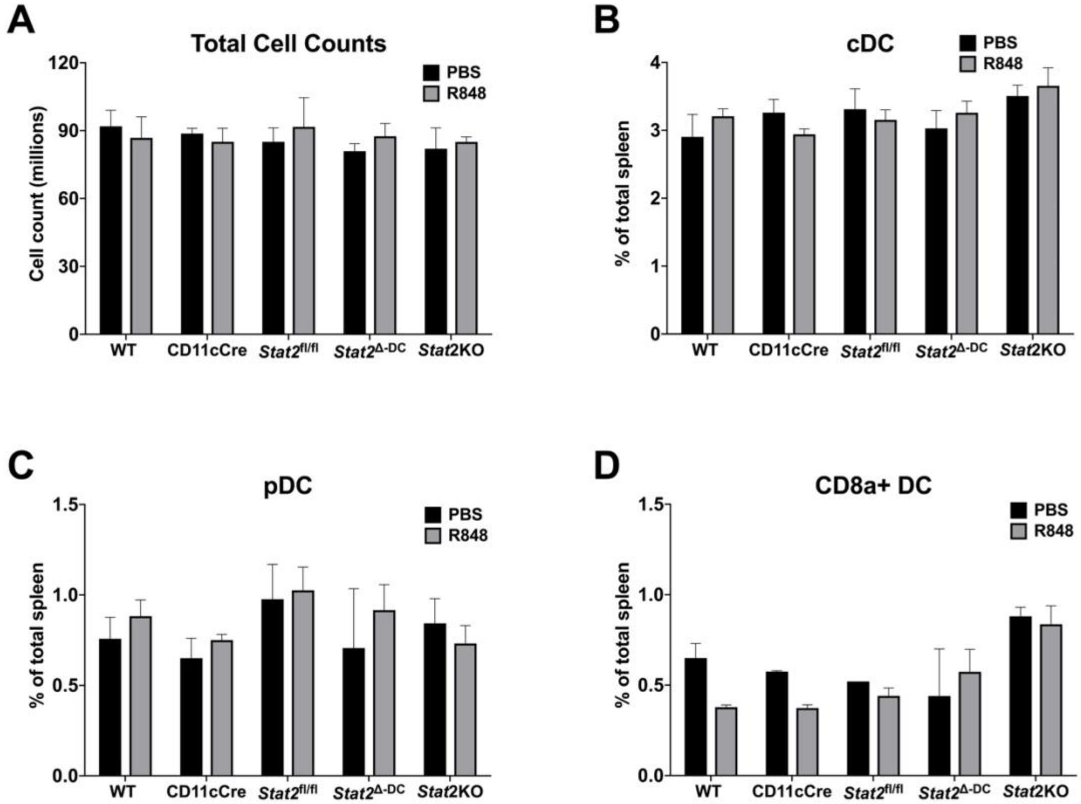

Figure 19. STAT2 deletion does not affect the frequency of DC subsets at baseline and in response to TLR stimulation *in vivo*

WT mice, mice carrying the flox Stat2 gene, mice carrying the Cre under the CD11c promoter, mice with the deletion of Stat2 in CD11c+ cells, and full Stat2-KO mice were injected i.v. with 30 μg of TLR7 ligand R848 or PBS vehicle. 24 hours later, splenocytes were stained for surface lineage and analyzed by flow cytometry. Results show the absolute numbers of splenocytes and the frequency of pDCs, cDCs, and CD8α+ cDCs. 2-8 mice per group. Error bars indicate the standard error of means (SEM). No statistical significance differences were found between groups constitutively and upon TLR stimulation using one-way ANOVA.

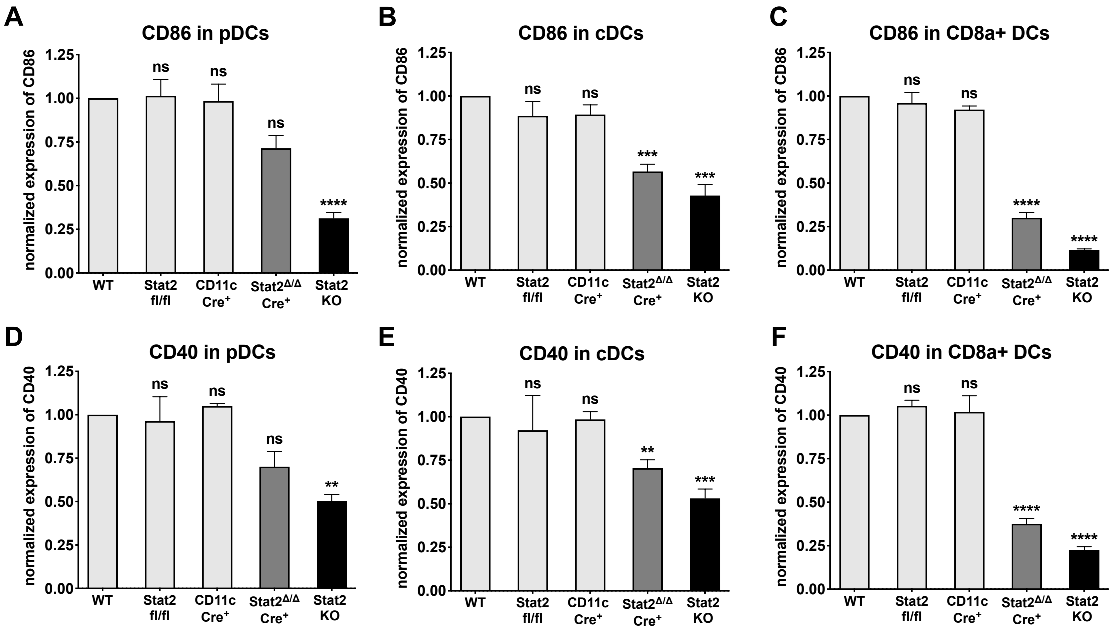

Figure 20. Effects of STAT2 function on the ability of DC subsets to respond to TLR stimulation *in vivo*

WT mice, including B6 and CD11c Cre+ mice, mice with the deletion of Stat2 in CD11c+ cells, and full Stat2-KO mice ice were injected i.v. with 30 μg of TLR7 ligand R848 or PBS. 24 hours later, splenocytes were stained for surface lineage and activation markers and analyzed by flow cytometry. Expression of the activation markers CD86 (**A, B, C)** and CD40 (**D, E, F**) was analyzed in pDCs (**A, D**), cDCs (**B, E**), and CD8α+ cDCs (**C, F**). Results are shown normalized to the WT mice. 3-4 mice per group. Error bars indicate the standard error of means (SEM). Statistical significance was analyzed using one-way ANOVA followed by the Dunn's multiple comparisons test against WT and is shown by the symbol * p<0.05, ** p< 0.01, *** p<0.001 **** p<0.0001, ns, not significant.

Discussion

It is known that IFN-Is strongly enhance humoral immunity, and studies show that the response to IFN-I is required for the development of antibody responses through demonstrating that full IFNAR knockout (KO) mice are unable to produce antibodies to a specific antigen following immunization.[329] That study further emphasized the role of DCs in the ability of IFN-I to induce isotype switching by showing that enhanced production of IgG2a antibodies could be induced when DCs were the only cell type able to respond to IFN-I *in vivo*.[329] In contrast, IFN-I given directly to activated B cells did not promote IgG2a production. Although these clearly support the importance of DCs in IFN-I enhanced antibody production, it was unknown whether the response of DCs to IFN-Is is required to stimulate humoral responses and whether it depends on a functional STAT2.

In this study, we investigated the unique DC requirements for STAT2 by generating a conditional Stat2 knockout mouse that has a deletion of the Stat2 gene only in CD11c+ cells. Because most dendritic cells express CD11c+, this conditional knockout allowed us to investigate the specific role that STAT2 plays in IFN-I directed responses by DCs. Using this Stat2$^{fl/fl}$; CD11c Cre^{+} mouse, we report for the first time that STAT2 function in DCs is not necessary for the development of an antigen-specific humoral response. In contrast, we present the novel finding that full Stat-2 KO mice are deficient in developing antigen-specific IgGs, indicating that STAT2 in cells other than CD11c+ DCs is required for the induction of humoral responses. Since STAT2 is very specific of the signaling pathway downstream of type I IFNs, these results also suggest that the response to type I IFNs in cells other than CD11c+ DCs is required for humoral responses. Since it was shown that DCs require the ability to respond to type I IFNs to prime humoral immune responses[329], the present results suggest that other cells can substitute cDCs to induce antibody responses. In the context of a lupus-oriented research, this finding suggests that STAT2 could be a therapeutic target to suppress auto-antibody production by using specific inhibitors that need to have access to STAT2 in the general immune cells, while specific inhibition of STAT2 in cDCs would not be sufficient.

We show here that STAT2 function in DCs is essential for cross-presentation to CD8+ T cells and stimulation of an Ag-specific CTL response. Loss of STAT2 specifically in DCs in mice also impaired the antitumorigenic effects of IFN-I, suggesting that the antitumorigenic effects of IFN-I depends largely on STAT2 signaling in DCs. These findings indicate that STAT2 is crucial for the DC response to type I IFN.

By virtue of their ability to deliver tumor antigens for cross presentation and their capacity to shape the immune response through cytokine production and immune stimulation, DCs are also recognized to be central regulators in cancer immunity, supported by a large body of literature suggesting that the transfer of the tumor associated antigen to DCs and the subsequent cross-presentation of the antigen by the DCs to CD8+ T cells to be crucial for the induction of antitumor immunity.[330-332] Although a role for host type I IFNs in cancer immunosurveillance has been recognized, the exact mechanism is unclear.

Type I IFNs produced by CD11c+ DCs has been detected in tumor-draining lymph nodes prior to antigen-specific CTL responses.[275,276] A lack of IFNAR function in DCs results in defects in their ability to activate and produce type I IFN-dependent cytokines, and importantly, cross-priming and tumor rejection is severely impaired in IFNAR KO mice.[273,277-279] The unique requirement of type I IFNs by DCs for this process is emphasized by normal tumor rejection in mice lacking IFNAR1 in just granulocytes or macrophages.[273]

We also reported that the lack of a functional STAT2 molecule in cDCs impaired the cross-presentation of tumor antigens by bone marrow-derived cDCs to CD8+ T cells *in vitro*, using a tumor antigen and a non-tumor antigen models, underscoring the importance of STAT2 function in DCs in antitumor and general immunity.[278,302] Additionally, we demonstrated that priming WT mice with *Stat2-/-* bone marrow-derived cDCs pulsed with OVA protein resulted in a decreased induction of specific cytolytic activity towards target OVA-pulsed splenocytes, compared to mice immunized with WT cDCs pulsed with OVA, suggesting that STAT2 in cDCs is sufficient to induce cross-presentation *in vivo*.[302] However, was is unknown whether a functional STAT2 in endogenous DCs is necessary for the development of cross-presentation responses, and whether the necessary function of STAT2 is specific to discrete subsets of DCs. In studies performed by our collaborator and the creator of the STAT2 conditional KO mouse, Dr. Ana Gamero, it was discovered that mice with STAT conditionally knocked out in CD11c+ cells failed to reduced tumor size in a B16 tumor model in response to type I IFN administration. The lack of tumor immunity seen in STAT2 CD11c conditional KO mice was the same as the lack of tumor immunity seen in STAT2 global KO mice. Both are in stark contrast to the ability of WT mice to reduce tumor size in response to type I IFN administration. This impairment of antitumorigenic effects suggests that the antitumorigenic effects of type I IFN depends largely on STAT2 signaling in DCs. These findings support the findings in this thesis showing that STAT2 is crucial for the DC response to type I IFN, and also demonstrate that this IFN signaling molecule plays a pervasive role in tumor immunity. The Gallucci and Gamero labs have previously reported that STAT2 is not necessary for DCs development[302] and we have confirmed here that the STAT2 deficiency conditional in DCs similarly does not alter the number and frequency of the main DC subsets normally present in the spleen. Therefore, the deficient cross-priming of CD8+ T cells cannot be explained by an absence of DC subsets in charge of cross-presenting to T cells, but rather to their inability to properly activate, as suggested by our results that cDCs and CD8+ cDCs were deficient in up-regulating costimulatory molecules upon *in vivo* exposure to TLR7 ligand R848. The fact that pDCs show a partial upregulation of CD86 and CD40 upon R848 can be explained by an inefficient knocking down of Stat2 in these cells caused by the low levels of CD11c expressed in these cells, that might have induced insufficient levels of Cre expression to knock down Stat2, as it has been suggested by others in other systems.

In conclusion, together with the results that our lab previously reported in 2016,[302] in which it was shown the WT DCs injected in WT mice were capable of inducing antigen-specific CTL killing while STAT2-KO DCs did not, the present results that mice deficient in STAT2 specifically in DCs are incapable to mount a CTL killing response, indicate a pivotal role for cDCs in the induction of this immune response and the requirement of STAT2 in the cDCs for their stimulatory function.

Future Directions

Our research delineates part of the downstream signaling of an important pathway in DC activation. Of relevance, we show that STAT2 function in CD11c+ DCs is essential for the cross-presentation of tumor antigens that allow for a CTL killing response in response to IFN. In light of recent research highlighting the role of cross-presentation by DCs as a key part of what makes tumors “hot” and responsive to checkpoint inhibitors, our research in STAT2 and IFN signaling could be expanded to explore whether IFN therapy could be a useful adjuvant in cancer therapy. The failure of “cold” tumors to respond to checkpoint inhibitors was originally thought to be due to a lack of T cells infiltrating the tumor, but the Vonderheide group has proposed that the defect is truly due to a priming deficit by DCs, explaining why these tumors do not have an abundance of T cells but the phenotype can be turned “hot” (and response) by the addition of CD40 as an adjuvant.[333-335] Our data provides information on how STAT2 function in DCs is important in the IFN pathway in the cross-presentation of tumor antigen, and a future direction this could take is the exploration of how critical IFN signaling is to the ability of DCs to cross-present antigen. While we showed that type I IFN signaling is required to stimulate cross-priming and trigger anti-tumor immunity, increasing evidence is showing that chronic activation of this pathway may mediate resistance to certain cancer treatments, suggesting that the plasticity and dynamisms of type I IFN signaling in cancer should be further studied.

Interestingly, findings could also be explored in the context of SLE, due to known chronic type I IFN signaling. Cross-priming abilities or deficiencies have not been studied in SLE. An association between SLE and an increased risk of blood, GI, and lung cancers is well established, but SLE does not have an effect on the development of most cancers and is associated with a decreased risk of other cancers (prostate, cutaneous melanoma).[336] A further direction this aim could take would be to explore how chronic type I IFN signaling effects the cross-presentation of tumor antigens in patients with and without SLE, which could give clues into the plasticity of type I IFN signaling and how an autoimmune background can affect it. Further exploring type I IFN and cross presentation in SLE patients could also provide insight into long-term type I IFN therapy in cancer. Identifying a helpful role could possibly provide a reason to administer type I IFN in conjunction with CD40 to address a priming deficit in “cold” tumors unresponsive to checkpoint inhibitors.

CHAPTER 4

THERAPEUTIC STRATEGIES: MODULATING DENDRITIC CELL METABOLISM

Introduction

Dendritic cells (DCs) comprise a small percentage of the total population of immune cells, but their powerful capacity to produce pro- and anti-inflammatory cytokines and present antigens to T cells grants them the ability to shape an immune response.[225,337,338] While this is beneficial in a healthy immune system, the central role that DCs play has implicated overactive DCs in the pathogenesis of many autoimmune diseases.[298,339-341] Of these, plasmacytoid dendritic cells (pDCs), the primary producer of the family of cytokines type I IFNs, has been implicated in systemic lupus erythematosus (SLE),[247,301] a complex systemic autoimmune disease in which the majority of patients show increased expression of type I IFN-regulated genes.[3,237,342,343] This immune dysregulation is seen in up to 80% of SLE patients and is termed the Interferon Signature.[6,237] While pDCs are major producers of IFNα, conventional DCs (cDCs) produce mostly IFNβ, together with many other pro-inflammatory cytokines such as IL-6, and DC subsets have been found abnormally activated in lupus autoimmunity.[69,248,344]

The Lupus Foundation of America estimates that at least five million people worldwide—including 1.5 million Americans—have a form of lupus.[8] However, the precise etiology of SLE initiation and flares are poorly understood, thus targeted therapies for this disease do not currently exist. Current treatments in SLE include antimalarials and systemic corticosteroids, which induce general immunosuppression and manage symptoms but do not often halt disease progression.[345] Recent clinical trials inhibiting IFNα have also failed, and it is evident that a full dissection of the aberrant operations of key immune players in SLE is needed to understand the processes that govern pathogenesis, in order to design interventional treatments that could affect disease progression.[346] As we begin to link metabolism with cell activation state, metabolic manipulation of key players in SLE is emerging as a promising new target.[347]

The immune system is comprised of cells that are relatively quiescent in the steady state, but rapidly respond to perturbations.[280] Changes in energy pathways are necessary to mount an immune response.[282] Recent studies have shown that changes in the way in which immune cells produce their energy is important for their functions[281,348] and is a putative therapeutic target in autoimmunity.[283] In particular, fatty acid (FA) metabolism has been proposed to be essential for pDC activation and response to type I IFNs.[286] Specifically, type I IFNs have been shown to promote both fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS). In Toll-like receptor 9 (TLR9) agonist CpGA-stimulated pDCs, the enhanced FAO due to autocrine type I IFN signaling was found to be critical for pDC activation. Inhibition of this metabolic reprogramming significantly impaired full pDC activation while having no effect on cell viability.[286] The role of FAO and FAS in cDCs, which can be generated in culture from murine bone marrow precursors in presence of FLT3-L, was not investigated. On the contrary, bone marrow-derived cDCs generated in culture in presence of GM-CSF and considered a murine model of pro-inflammatory DCs (iDCs), have been shown to upregulate glycolysis and shutdown oxidative phosphorylation upon stimulation, and require mostly glycolysis to sustain activation and their Ag presentation.[305,349] In this study, we investigated the effects of the pharmacological blockade of FAO and fatty acid synthesis (FAS) to manipulate pDC and cDC activation. This may be a safer and more efficient approach in autoimmune diseases mediated by high expression of type I IFNs, as targeting pDC and cDCs output may spare IFNα generation in other cell types. Specific to SLE, mitochondrial metabolism supports the chronic activation of CD4+ T cells, and these metabolic processes are elevated in CD4+ T cells in lupus-prone mice.[283,284] The mitochondrial electron transport chain (ETC) complex I inhibitor Metformin and the glycolysis inhibitor 2-deoxy-D-glucose (2DG) significantly reverse T cell activation in lupus-prone mice and SLE patients.[283] While these results implicate T cell metabolism in SLE pathogenesis, detailed studies of SLE patients and murine models have identified type I IFN and expression of the IFN Signature as a chief pathogenic mediator, leading us to direct our study target towards DCs.[237,350] DCs are commonly subdivided into cDCs, best characterized by their specialization in antigen presentation, and pDCs, best characterized by their unique ability to produce large amounts of type I IFN.[6,294] High serum levels of type I IFN are observed in more than 70% of SLE patients, in association with the most serious complications of SLE.[4-6] The pathogenic role of type I IFN in SLE has led to clinical trials attempting to block the response to IFNα,[351] with results that suggest beneficial effects but do not achieve the expected clinical success. Since murine models of SLE show that depletion of the major producers of type I IFNs, the pDCs, stop onset and progression of autoimmunity,[296,297] we propose it is important to suppress pDC activities that include but also go beyond the production of type I IFNs.

New results suggest a lesser known role for cDCs in SLE, as producers of type I IFNβ with alterations in function and phenotype.[2,300] Because we isolate DC subtypes in our experiments, we will evaluate both cDCs and pDCs. We propose to study the metabolism of pDCs and cDCs in lupus-prone mice and SLE patients. The discovery that lupus DCs have increased energy metabolism would be highly significant because it would highlight a novel pathogenic mechanism. We will test several metabolic modulators to determine the most efficacious block of DC activation and production of the IFN Signature. These experiments will provide novel knowledge of DC biology in SLE. Moreover, they are pre-clinical studies to support metabolic modulation as novel therapeutic strategies in SLE. Using metabolic modulators is an attractive therapeutic option, as these drugs would benefit patients without causing the severe immunosuppression resulting from drugs traditionally used in SLE.[287,288] The safety of metabolic modulators has been evaluated in several diseases.[290-292] The benefits of success from this project are not limited to SLE patients, as metabolic modulation could apply to other autoimmune diseases where DCs and Type I IFNs are pathogenic.[2,238,352,353]

Among the current pharmacologic tools used to manipulate cell metabolism, we investigated metformin, the most commonly prescribed medication for type II diabetes known to have an effect on oxidative phosphorylation. Additionally, we explored the effects of etomoxir, an inhibitor of carnitine palmitoyltransferase-1 (CPT-1), a key enzyme in FAO. Additionally, we investigated the effects of 5-tetradecyloxy-2-furoic acid (TOFA), an inhibitor of acetyl-CoA carboxylase-α, targeting the rate-limiting enzyme in long-chain fatty acid synthesis. Our findings explore how fatty acid metabolism may be essential for DC activation and production of proinflammatory cytokines. We show that modulating FA metabolism by etomoxir and TOFA suggest these drugs as novel therapeutics for affecting DCs.

Metformin

1,2-dimethylbiguanide, more commonly referred to as metformin, is a biguanide derivative that serves as the anti-hyperglycemic drug-of-choice in the oral treatment of type II diabetes.[354,355] First introduced clinically in the 1950s, metformin was thought to restore the response to insulin through targeting insulin directly, effectively reducing the risk of hypoglycemia that is of concern in other oral diabetic medications.[356] Further research revealed that metformin inhibits gluconeogenesis by the liver, therefore inhibiting endogenous glucose production. This finding led to the discovery that metformin also activates the metabolic regulator adenosine monophosphate kinase (AMPK), which increases insulin signaling and glucose transport into muscle tissues.[357,358] However, it was later discovered that metformin has no effect on MAPK phosphorylation by liver kinase B1 (LKB1), suggesting that metformin cannot directly activate AMPK.[359] This contradicting data was mitigated by the finding that metformin imposes and mild but specific blockade of the mitochondrial respiratory chain complex I in multiple cell types and tissues.[360-363]

This further understanding of how metformin functions has led to the discovery of how metformin has other benefits in addition to treating type II diabetes. Because metformin-regulated lipogenesis trough AMPK leads to a decrease in fatty acid synthesis (FAS), metformin has been found to be beneficial in treating fatty liver disease.[364] Consistent with these findings that metformin inhibits some pathways in lipogenesis, it also possesses anti-obesity effects, and weight loss is a relatively common side effect in diabetic patients undergoing treatment with metformin.[358,365,366]

These findings led to the study of metformin in more detail in other diseases.

Because metformin has also been shown to prevent HMGB1 release in LPS-treated cells, effectively preventing lethality of endotoxemic mice,[357] metformin became of interest in the field of immunology. More recently, metformin was found to alter the metabolic profile of T cells from lupus-prone mice but not nonautoimmune wildtype mice.[283] T cells from lupus-prone mice are characterized by increased mitochondrial metabolism, and the metformin was found to inhibit mitochondrial respiration, effectively restoring IL-2 production and reducing IFNγ levels, both more consistent with healthy T cell function. While metformin treatment *in vivo* did not affect disease progression in lupus-prone mice, a combination of metformin with 2-deoxyglucose, an inhibitor of glycolysis, reversed murine lupus, possibly through the restoration of T cell metabolism.[283,367]

The large role that DCs play in lupus naturally led to the study of the effect of metformin in DCs. An early study showed that short-term treatment of DCs *in vitro* could reduce expression of surface costimulatory molecules and inhibit cross-presentation.[368] Further studies in the Gallucci lab discovered that metformin does not affect maturation and viability of inflammatory GM-CSF bone marrow-derived DCs, establishing the safety of this drug for this cell type. However, we also discovered that metformin affects MHC expression in LPS-stimulated cDCs, and also causes a partial reduction in nitro oxide production. However, nothing is known about the effect of metformin on other subsets of DCs.

Ethyl Pyruvate

The aliphatic ester ethyl pyruvate (EP) is best known as a derivative of pyruvate. In *in vitro* studies and in animal models of sepsis and ischemia-reperfusion injury, EP has demonstrated a high safety profile and anti-inflammatory properties at doses that are clinically relevant. While these findings show promise for EP in disease settings displaying an excess of inflammation, the effect of EP on DCs is unclear.

An aliphatic ester, EP exists in a keto-enol tautomerism state of chemical equilibrium.[369] Prior to the studies in our lab, only one report existed in the literature assessing effects of EP on DC responses,[370] finding that EP inhibits HMGB1, and enhances DC expression of surface costimulatory molecules in response to thermal injury.

Etomoxir

A small-molecule, etomoxir inhibits FAO through the irreversible inhibition of carnitine palmitoyl-transferase 1a (CPT1a).[371] Studies in DCs have shown that etomoxir inhibits an increase in basal OCR usually seen in TLR agonist-stimulated pDCs.[286] Because CPT1a is the critical transporter of long chain fatty acids into mitochondria for oxidation, inhibition of the transporter by etomoxir would allow us to study whether FAO is essential for FLT3-L-derived pDC and cDC activation in response to TLR agonists.

TOFA

5-tetradecyloxy-2-furoic acid, more concisely referred to as TOFA, is an inhibitor of the enzyme acetyl-CoA carboxylase-α, the rate-limiting enzyme in fatty acid synthesis (FAS).[372] Dendritic cell dysfunction in cancer has been associated with lipid accumulation,[373] and administration of TOFA has been found to normalize DC functional activity. Because the normalization of T cell metabolism has been shown to have a beneficial effect in lupus-prone mice,[367] we wanted to investigate whether modulating DC fatty acid metabolism by TOFA could suppress activation, suggesting a possible metabolic target in the context of overactivated DCs in SLE.

Materials and Methods

Mice

C57BL/6 (B6) wildtype nonautoimmune mice were bred and maintained in our colony. Breeding pairs were originally purchased from Jackson Laboratory. Lupus-prone NZBW/F1 mice were purchased from Jackson Laboratory. Mice were between 8 and 16 weeks of age for most experiments.

Mice were housed in the Laboratory Animal Resources Unit Temple University School of Medicine of Philadelphia, an AALAC accredited facility. The University maintains an Institutional Animal Care and Use Committee (IACUC), the members of which are appointed by the President of the University. Animals are housed and cared for in accordance with established National Institutes of Health (NIH) guidelines for the humane use of animals in laboratory research. The colonies are routinely screened for the presence of common murine viruses. Separate rooms are available for animal isolation and for procedures involving use of biohazardous materials and infectious agents.

The mice are caged and housed according to NIH guidelines for humane care of laboratory animals. All injections were well tolerated by the mice, but we will carefully monitor for sign of local pain or inflammation. If necessary, we will use local analgesic to limit pain. All injected substrates not induce pain or distress to the mice.

Of necessity, the mice were humanely sacrificed to permit analysis of the cellular immune response or to avoid suffering. The mice were sacrificed with CO_2 in a chamber with a fill rate of 10-30% per minute and monitored for a minimum of 1 minute after respiration ceases. Cervical dislocation immediately followed to ensure fatality. This method is consistent with the most recent recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. After death was been confirmed, the blood, bone marrow, and/or spleen were harvested to provide a source of serum and cells for culture and/or analysis.

Bone Marrow-Derived Dendritic Cells

Bone marrow derived pDCs and cDCs were generated as per the laboratory's published protocols.[69,110,302-305] See Appendix for detailed method. Briefly, bone marrow precursors were flushed from femurs and tibias of mice and differentiated into pDCs and cDCs in the presence of FLT3-L in complete RPMI medium (10% FBS, L-glutamine, penicillin/streptomycin, 2-mercaptoethanol) for 7 days.

In Vitro Dendritic Cell Studies

Bone marrow derived pDCs and cDCs were generated as per the laboratory's published protocols.[69,110,302-305] Briefly, bone marrow precursors were flushed from femurs and tibias of mice and differentiated into pDCs and cDCs in the presence of complete RPMI medium (Thermo Fisher), containing 10% FBS (Gemini Bio Products), L-glutamine (VWR), penicillin/streptomycin (VWR), 2-mercaptoethanol (Gibco, Thermo Fisher), and enriched with 15% FLT3-L conditioned medium from a FLT3-L-producing cell line, in 24 well plates for 7 days. DCs were then sorted using the EasySep PE positive selection kit (StemCell Technologies) with a PE-B220 antibody (StemCell Technologies) to separate out the pDCs from the cDCs. Purity of cells averaged at least 90%. Similar experiments were repeated using pDCs and cDCs that were sorted by Flow Cytometer sorting, reaching a purity >98%. Sorted cells were plated in complete RPMI medium with FLT3-L overnight, then stimulated the next morning. TLR9 ligands CpGA 5ug/ml (ODN 2336 synthesized by IDT Biotechnologies) was used to stimulate both pDCs and cDCs. R-(+)-Etomoxir and TOFA were both purchased from Cayman Chemical and used at varying doses, adding them 1 hour before the CpG-A stimulation. Supernatants were collected at 24 hours post-stimulation for measurement of IL-6 production. Cells were harvested at 24 hours post-stimulation by gentle retro-pipetting before to be stained with specific antibodies for analysis by flow cytometry.

In vivo EP injection and spleen and lymph node cell staining

C57BL/6 mice were injected i.p. with 80mg/kg of EP in 200ul PBS (vehicle) one hour before the injection of 30 μg/mouse of TLR7 ligand R848 in 200ul PBS (vehicle). EP was further administered 4, 8 and 20h after R848 stimulation. Spleens and mesenteric lymph nodes (mLN) were harvested 24h post-R848 stimulation and single cell suspensions were prepared using DNase I (Sigma-Aldrich) and collagenase IV (Worthington Biochemical Corporation) as well as ACK red blood cell lysis buffer for spleens (VWR). Cell viability was assessed by either staining with fixable viability dye eFluor780 (eBioscience) or 7-AAD. Cells were then incubated with purified rat anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2) prior to the addition of fluorochrome-conjugated antibodies against DC surface markers: rat anti-mouse CD11c (N418), CD86 (GL-1), PDCA-1 (eBio927), Ly-6C (HK1.4), CD11b (M1/70) and B220 (RA3-6B2). Cells not stained with 7-AAD were fixed with 1% paraformaldehyde. Samples were acquired on a FACSCanto Cytometer then analyzed with FlowJo.

Cytokine ELISAs

IL-6 was measured in the supernatants of pDC cultures using the BD Pharmingen ELISA kits, according to the manufacturer's protocol. Optical densities were measured at 650 nm and 405 nm and results analyzed with SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA).

Flow Cytometry

Following harvesting, pDCs and cDCs were incubated with rat anti-mouse CD16/CD32 mAb (Biolegend) for 15 min on ice to block FcγRs. Cells were then stained for 30 min in the dark on ice using specific antibodies for surface identification markers CD11c (eBioscience), CD11b (BD Bioscience), and B220 (StemCell Technologies), and costimulatory marker CD86 (BD Bioscience). Cells were analyzed for cell viability using Fixable Viability Dye eFluor 780 (Thermo Fisher). Cells were fixed in 2% paraformaldehyde (Thomas Scientific) in PBS (Fisher) with 1% BSA (Gemini Bio Products). All cells were analyzed on a FACSCanto flow cytometer (BD Bioscience) with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical Analysis

Prism 8 (GraphPad software, San Diego, CA, USA) was used for data analysis. Means and standard error of means (Mean ± SEM) were calculated by averaging results from independent experiments. Statistical significance was determined using unpaired two-tailed Student's t-test for comparison between two groups. One-way ANOVA and multiple comparisons post-hoc correction test was used when appropriate. P-values marked in the figures as * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$ were considered significant.

Results

Metformin Inhibits Activation of Plasmacytoid But Not Conventional Dendritic Cells

As sentinels of the immune system, DCs are relatively quiescent at the steady state, but are able to very rapidly respond to perturbations. To determine the role of fatty acid metabolism in dendritic cell activation, we grew bone marrow precursors in presence of FLT3-L, a well establish protocol that elicits a mixed population of pDCs and cDCs. After sorting with magnetic beads, we obtained single subsets of pDCs and cDCs, with a purity of at least 90% (Figure 21). Similar experiments were repeated using pDCs and cDCs that were sorted by Flow Cytometer sorting, reaching a purity >98%.

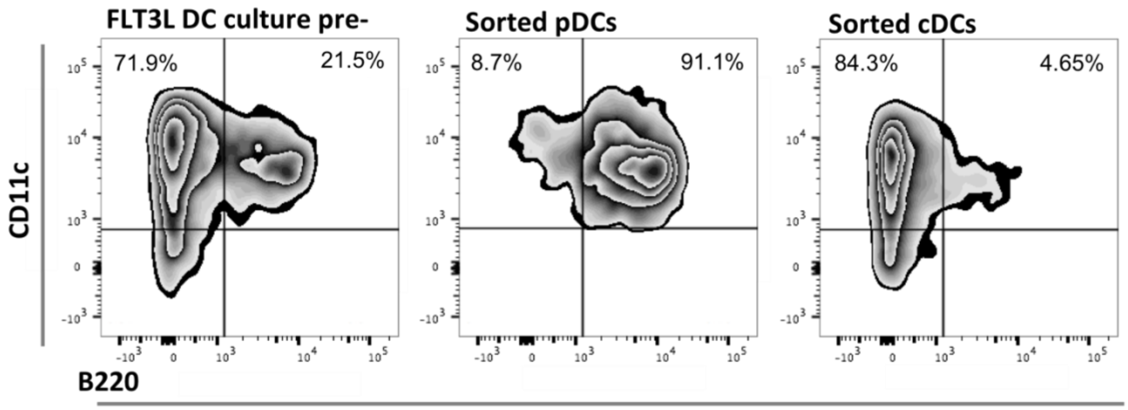

Figure 21. Sorting FLT3-L DC culture by positive selection of pDCs using surface expression of B220.
pDCs and cDCs were generated *in vitro* from bone marrow precursor cells harvested from the femur and tibia of WT mice in FLT3-L enriched-medium. After 7 days in culture, cells were gently harvested, and pDCs were positively selected for surface expression of B220, a CD45 isoform expressed by pDCs but not cDCs. The positively selected cohort of cells for all experiments displayed a purity of at least 90%. Flow diagrams are representative of purity checks for all experiments.

After an over-night rest, we treated pDCs and cDCs with a dose titration of metformin. After 1 hour, we stimulated both DC subsets with TLR9 ligand CpGA 2336, an oligonucleotide that is able to stimulate both subsets of murine DCs, or TLR4 ligand LPS, which we expect to activate cDCs. While human pDCs do not express TLR4, murine pDCs express very little TLR4. We found that metformin suppressed pDC activation by CpGA as measured by the expression of surface co-stimulatory molecules CD86 and CD40. LPS resulted in very little activation of pDCs, and metformin had no effect. In contrast, metformin had no effect on cDC activation in response to either CpGA or LPS (Figure 22).

Metformin Affects Viability of Resting Dendritic Cells

Of note, metformin did not have a negative effect on viability of either pDCs or cDCs that were activated with a TLR agonist after treatment with metformin. In contrast, metformin did cause significant cell death as measured by FVD positivity when cells were not activated (Figure 23). This suggests that metformin treatment of resting cells is affecting the OXPHOS metabolism exhibited by baseline DCs enough to cause significant cell death. However, when activated by TLR agonists, our results confirm that DCs upregulate different metabolic pathways. cDCs have been shown to strongly upregulated glycolysis,[286] which explains how metformin treatment of resting cDCs causes cell death, but when activated, the shift to glycolysis is enough to keep cDCs alive. Concurrently, metformin, by affecting OXPHOS, does not have an effect on activated cDC metabolism, and cDCs activated normally in response to TLR agonists. In contrast, pDCs upregulate OXPHOS significantly in response to TLR agonists.[286] Our data suggests that the upregulated OXPHOS activity is sufficient to keep the pDCs alive, but the metformin inhibition is able to suppress pDC activation, demonstrating the importance of OXPHOS in the ability of pDCs to activate in response to TLR agonists.

In summary, our results show that metformin affects DC OXPHOS metabolism, which does not have an effect on TLR ligand-activated DC viability but does cause cell death in resting DCs. Our results also show the different metabolism exhibited by different DC subsets and show how pDCs primarily upregulate OXPHOS and cDCs primarily upregulate glycolysis in response to TLR agonists. This data suggests that metformin, as an OXPHOS target, can suppress pDC activation without affecting cDC activation.

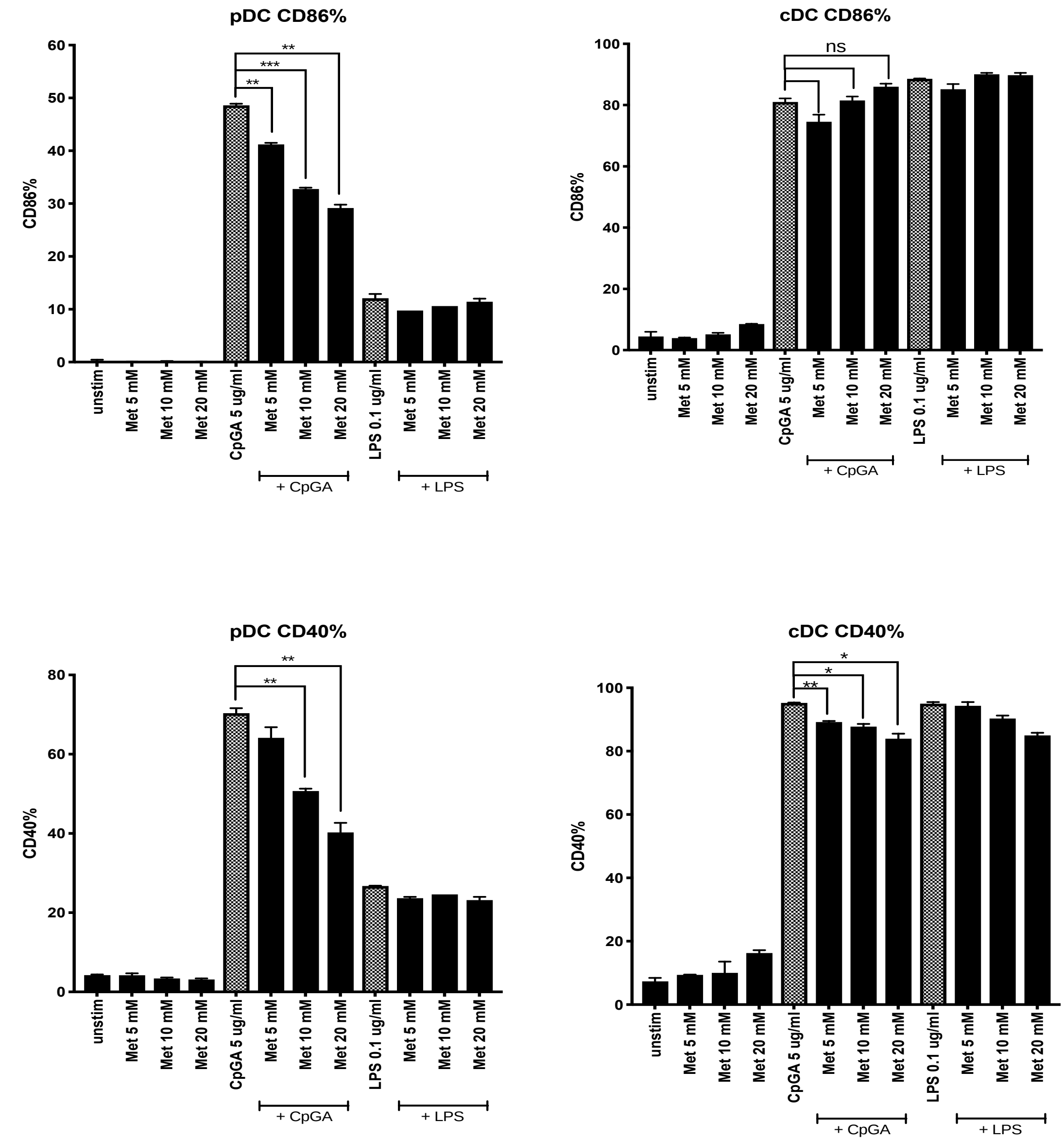

Figure 22. Metformin inhibits activation of plasmacytoid but not conventional dendritic cells

Purified pDCs and cDCs were treated for 1h with metformin, then stimulated with TLR9 agonist CpGA 5μg/ml or TLR4 agonist LPS (0.1μg/ml). Activation was measured after 24h by CD86 expression via flow cytometry. pDCs were gated for B220+CD11c+CD11b-. cDCs were gated for B220-CD11c+CD11b+. Results are shown as the mean ± SEM, from 3 independent experiments.

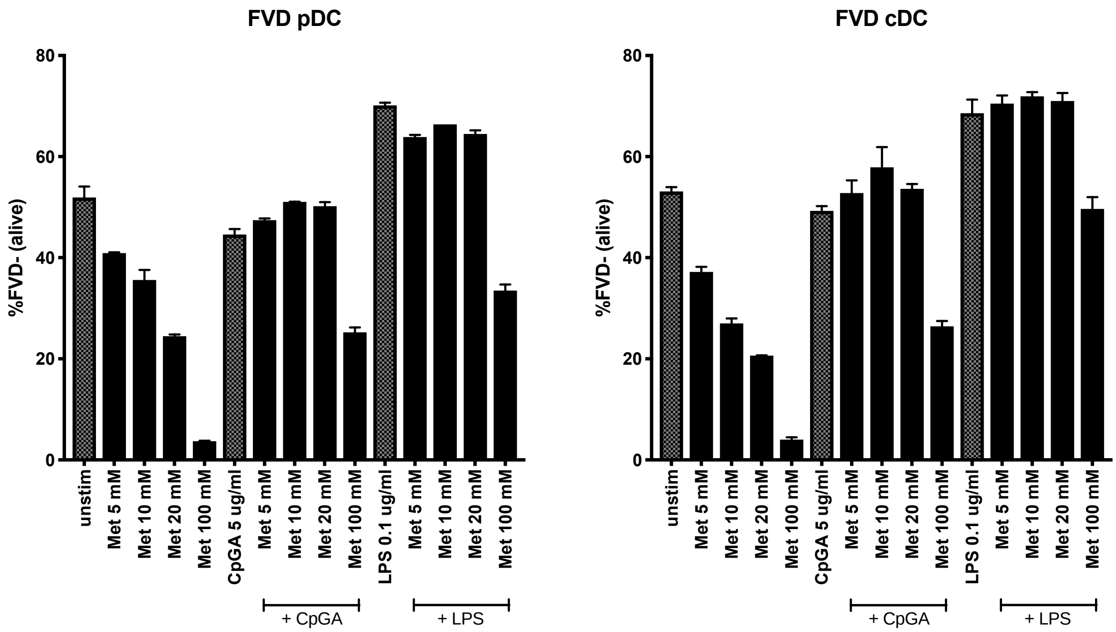

Figure 23. Metformin affects viability of resting dendritic cells

Purified pDCs and cDCs were treated for 1h with metformin, then stimulated with TLR9 agonist CpGA 5μg/ml or TLR4 agonist LPS (0.1μg/ml). Viability was measured. Data shows the percent of FVD- cells, which are alive. Results are shown as the mean ± SEM from 4 independent experiments. Results are shown as the mean ± SEM, from 3 independent experiments.

Inhibition of fatty acid oxidation by etomoxir suppresses DC up-regulation of costimulatory molecules

In separate sets of experiments, after an over-night rest, we treated pDCs and cDCs with a dose titration of etomoxir, an inhibitor of the key fatty acid oxidation (FAO) enzyme carnitine palmitoyltransferase I (CPT1). We chose this dose range following previous literature in DCs and T cells.[286,292,374] After 1 hour, we stimulated both DC subsets with TLR9 ligand CpG-A 2336, an oligonucleotide that is able to stimulate both subsets of murine DCs (Figure 24). We found that etomoxir suppressed both pDC and cDC activation as measured by the expression of surface co-stimulatory molecule CD86 (Figure 24a and b). Etomoxir suppressed TLR-induced CD86 up-regulation of both DC subsets in a dose-dependent manner, and pDCs were sensitive to inhibition at lower doses than cDCs (100μM vs 200μM). In both DC subsets, etomoxir did not affect cell viability, neither in resting state or upon stimulation (Figure 24c), suggesting that suppressing FAO inhibits the metabolic switching needed for activation, without inducing cell death in resting nor stimulated DCs.

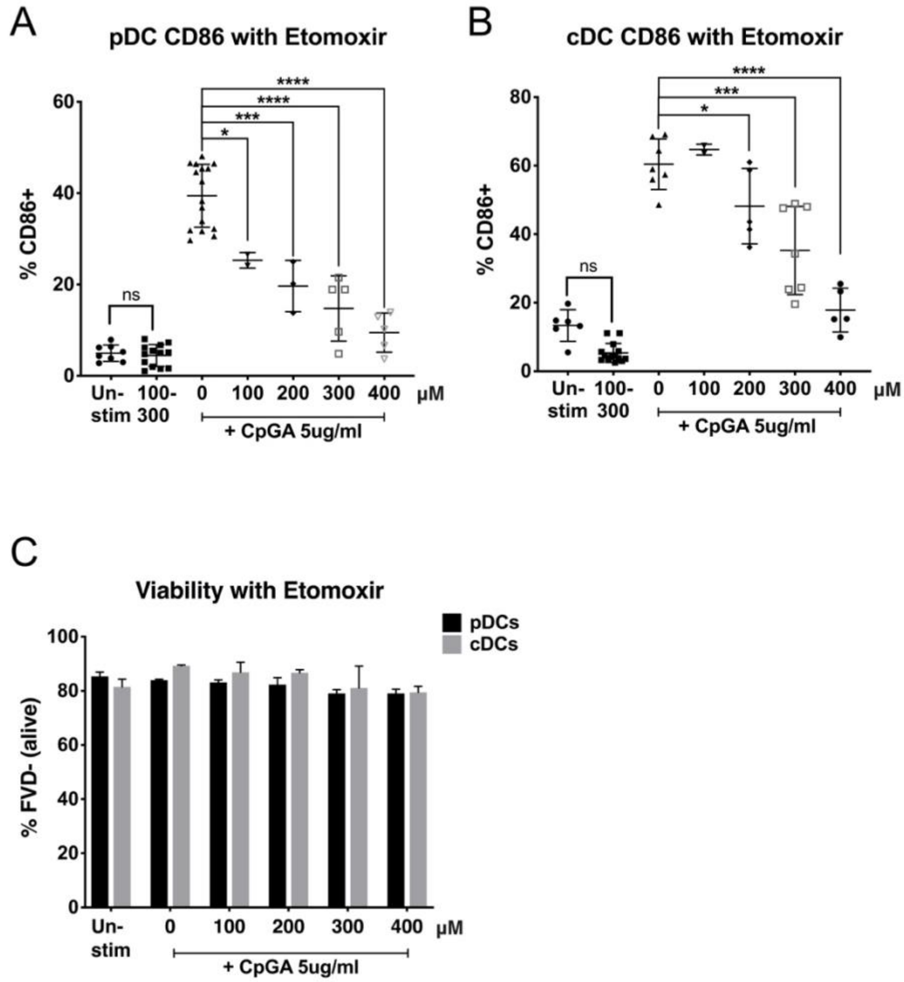

Figure 24. Inhibition of fatty acid oxidation by etomoxir suppresses DC upregulation of CD86

Purified pDCs and cDCs were treated for 1h with CPT1 inhibitor etomoxir (doses reported in μM), then stimulated with TLR9 agonist CpGA 5μg/ml. Activation was measured after 24h by CD86 expression via flow cytometry. pDCs were gated for B220+CD11c+CD11b-. cDCs were gated for B220-CD11c+CD11b+. Viability of cells (C) is expressed as % of cells negative for the Fixable Viability Dye and was not affected at effective doses of etomoxir. Results are shown as the mean ± SEM, from 5 independent experiments.

Inhibition of fatty acid synthesis by TOFA suppresses DC activation

Next, we investigated whether inhibiting fatty acid synthesis (FAS), which may also contribute to FAO in DCs, would also affect activation. We treated purified pDCs and cDCs with a dose titration of 5-tetradecyloxy-2-furoic acid, or TOFA, an inhibitor of acetyl-CoA carboxylase-α, using doses previous tested in immune cells.[286,373] We found that TOFA did not affect cell viability (Figure 25c), neither in resting state nor upon stimulation, but inhibited the upregulation of surface co-stimulatory molecule CD86 induced by CpG-A in both DC subsets at the same dose of 100μM (Figures 25a and 23b). As for etomoxir, these findings suggest that TOFA is effective in inhibiting the metabolic switch needed for DCs to activate without shutting down the metabolic pathways that are keeping them alive.

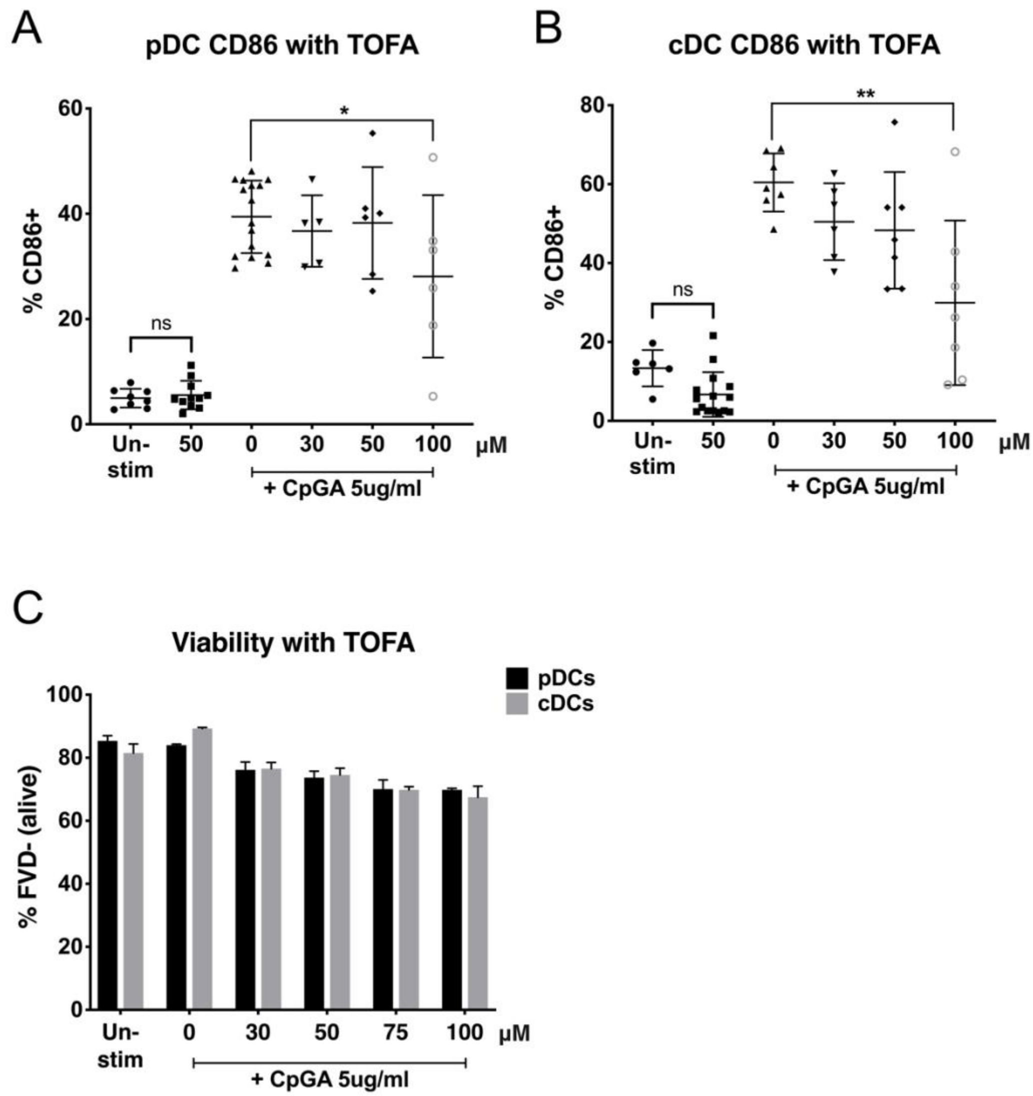

Figure 25. Inhibition of fatty acid synthesis by TOFA suppresses DC upregulation of CD86

Purified pDCs (A) and cDCs (B) were treated for 1h with TOFA (doses expressed in μM), an inhibitor of acetyl-CoA carboxylase-α, targeting the rate-limiting enzyme in long-chain fatty acid synthesis, then stimulated with TLR9 agonist CpGA 5μg/ml. Activation was measured after 24h by CD86 expression via flow cytometry. pDCs were gated for B220+CD11c+CD11b-. cDCs were gated for B220-CD11c+CD11b+. Viability of cells (C) is expressed as % of cells negative for the Fixable Viability Dye and was not affected at effective doses of TOFA. Results are shown as the mean ± SEM, from 5 independent experiments.

Inhibition of fatty acid metabolism suppresses DC production of proinflammatory cytokine IL-6

While surface costimulatory molecules are important for T cell stimulation by DCs, we next investigated whether inhibiting FA metabolism affects the production of proinflammatory cytokines by DCs. Notably, IL-6 production is found to be higher in lupus patients and has been shown to reflect disease activity.[70,300,375,376] Testing the supernatants of pDCs and cDCs treated with etomoxir to inhibit FAO and TOFA to inhibit FAS, as described above, we found that the inhibition of FA metabolism is sufficient to suppress the production of proinflammatory cytokine interleukin (IL)-6 by pDCs (Figure 26a and 26b), and by cDCs (Figure 26c and 26d). This finding suggests that etomoxir and TOFA are able to suppress a proinflammatory response, while still allowing both pDCs and cDCs to maintain baseline metabolism, preserving their viability.

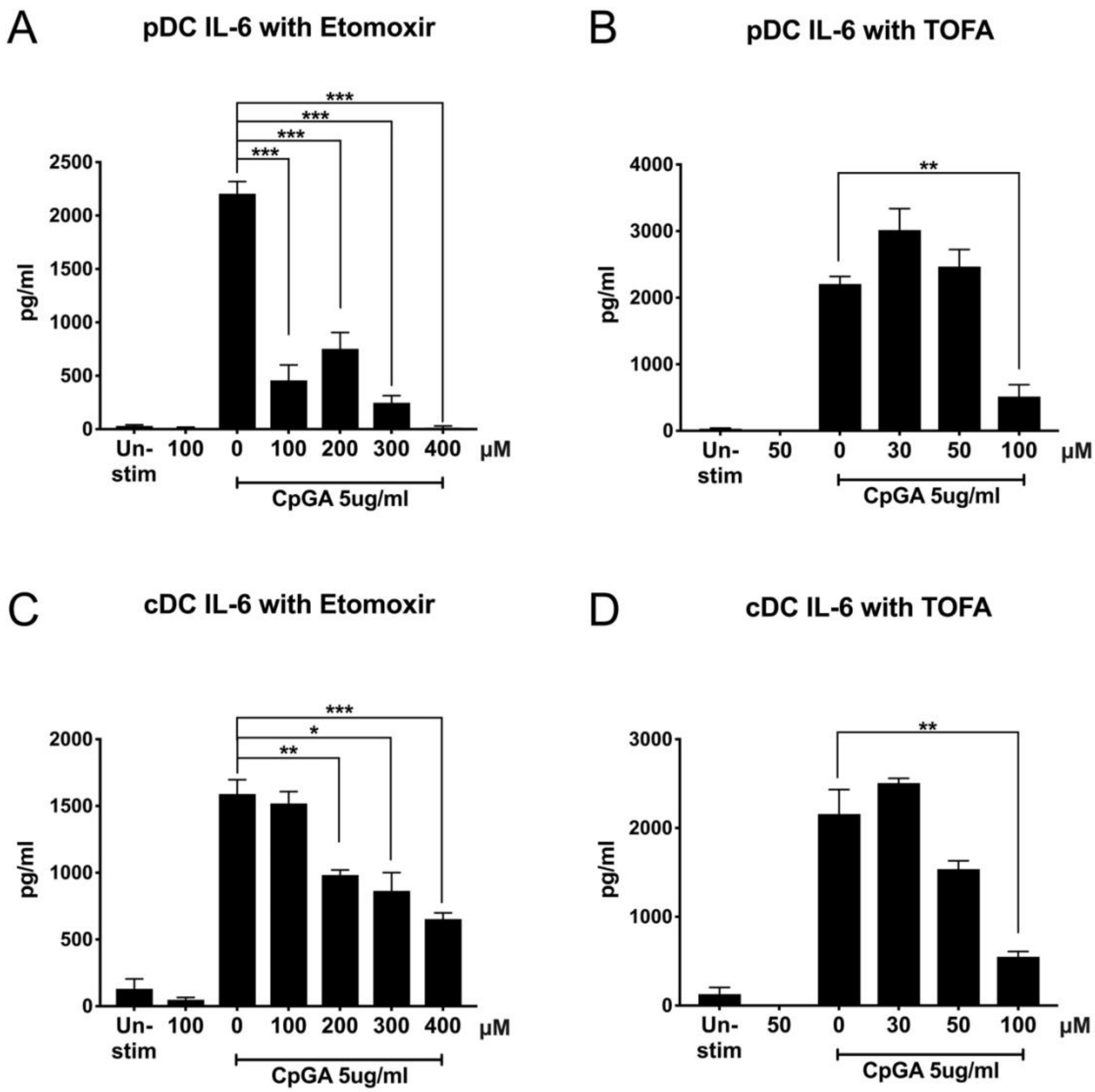

Figure 26. Metabolic modulation of fatty acid metabolism suppresses proinflammatory IL-6 production.

Purified pDCs and cDCs were treated for 1h with CPT1 inhibitor etomoxir (μM) or ACC inhibitor TOFA(μM), then stimulated with TLR9 agonist CpGA 5μg/ml. Supernatant was collected after 24h and analyzed for IL-6 production using an ELISA kit. Results are shown as the mean ± SEM, from 2 independent experiments.

Ethyl Pyruvate Inhibits Activation of Dendritic Cells

Next, we treated pDCs and cDCs with a dose titration of ethyl pyruvate, that we have previously shown to inhibit glycolysis and OXPHOS in GM-CSF-cDCs.[305] After 1 hour, we stimulated both DC subsets with TLR9 ligand CpG-A 2336, an oligonucleotide that is able to stimulate both subsets of murine DCs (Figure 27). We found that ethyl pyruvate suppressed both pDC and cDC activation as measured by the expression of surface co-stimulatory molecule CD86. Ethyl pyruvate suppressed TLR9-induced CD86 up-regulation of both DC subsets in a dose-dependent manner.

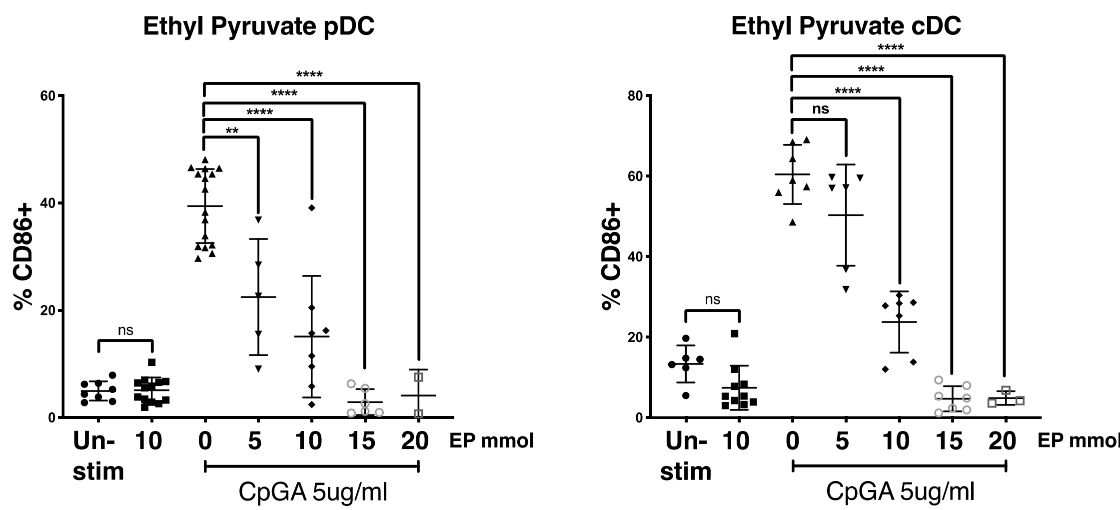

Figure 27. Ethyl pyruvate inhibits upregulation of CD86 in pDCs and cDCs
Purified pDCs and cDCs were treated for 1h with pyruvate analog ethyl pyruvate, then stimulated with TLR9 agonist CpGA 5μg/ml. Activation was measured after 24h by CD86 expression via flow cytometry. pDCs were gated for B220+CD11c+CD11b-. cDCs were gated for B220-CD11c+CD11b+. Results are shown as the mean ± SEM, from 5 independent experiments.

Ethyl Pyruvate Inhibits Activation of Conventional Dendritic Cells Stimulated by TLR Ligands In Vivo

To test whether the suppressive effects of EP on DC activation *in vitro* were also observed *in vivo*, we harvested spleens and mesenteric lymph nodes (mLN), 24h after B6 mice were injected with the TLR7 ligand R848 while receiving 80 mg/kg EP 1h before as well as 4, 8 and 20h after R848. We chose the TLR7 ligand R848 because it is a potent murine cDC activator, which, unlike LPS, was not reported to induce DC death. We stained the cells *ex vivo* for surface lineage markers recognizing conventional DCs (cDCs) and inflammatory monocytes (iMo), two subsets of innate immune cells that are represented *in vitro* by the GM-CSF-DCs[23]. We found that the stimulation with R848 induced a decrease in splenic cDCs, which was unexpected because R848 was not previously associated with cell death, and an increase in iMo in mLN, while EP did not change the frequency of either innate subset both in R848-treated cells as compared to unstimulated (Figure 28A and 28C). Moreover, we assessed cell activation status by analyzing the expression of surface CD86 and found that EP significantly decreased the percent of R848-induced CD86-positive cDCs (Figure 28B). In addition, EP was able to reduce R848-induced up-regulation of CD86 on iMo from mLN (Figure 27D). These results were not due to cell death as no change in absolute cell numbers was detected (data not shown), confirming once again that EP does not induce cell death. This data indicates that EP is able to directly affect cDCs *in vivo* by decreasing their activation without compromising cell viability, a promising finding for therapeutic purposes.

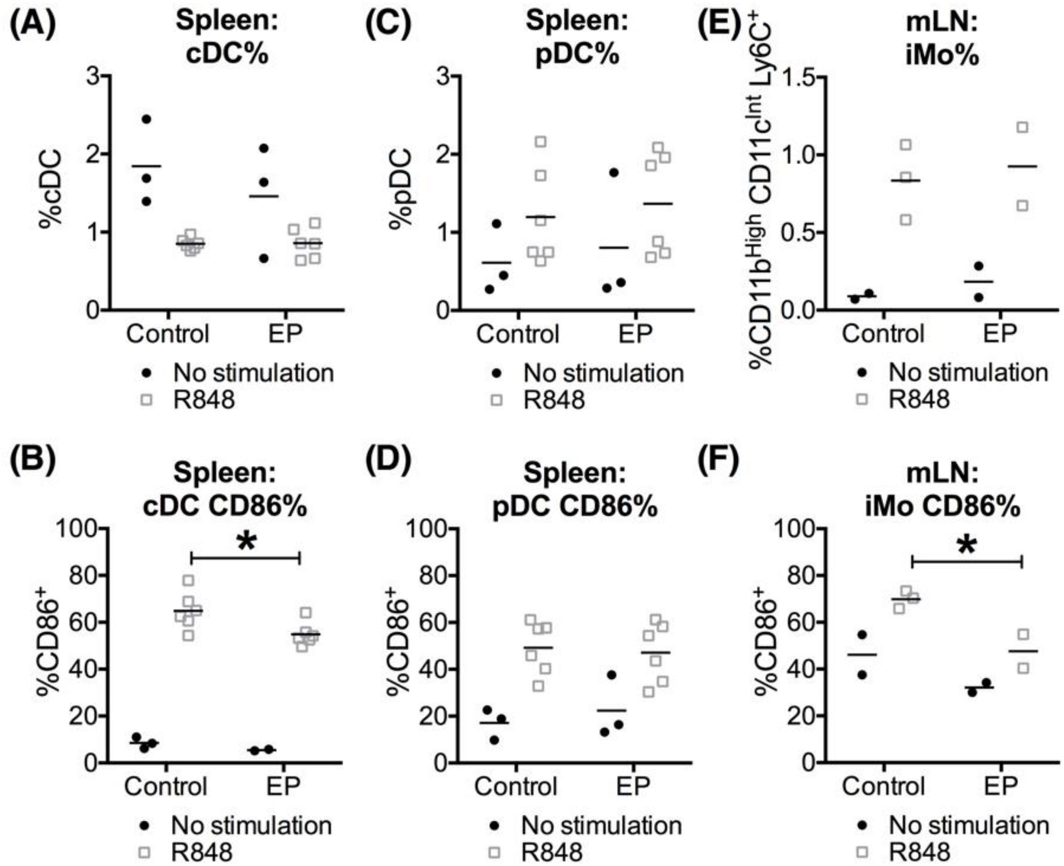

Figure 28. EP decreases the in vivo TLR-induced cDC activation without affecting their survival

C57BL/6 mice were injected i.p. with 80 mg/kg of EP in PBS (vehicle) one hour before the injection of 30 μg/mouse of TLR7 ligand R848 in PBS (vehicle). EP was further administered again 4, 8 and 20 hours after R848 stimulation. (A, C, E) Spleens and mesenteric lymph nodes (mLN) were stained for surface lineage markers to recognize cDCs ($CD11c^{Hi}$, $CD11b^{Int}$), pDCs ($CD11c^{Int}$ $B220^{pos}$ $PDCA^{pos}$) and inflammatory monocytes (iMo, $CD11c^{Int}$, $CD11b^{Hi}$ and $Ly6C^{pos}$) and (B, D, F) the costimulatory molecule CD86 24 hours after the TLR stimulation. Results are expressed as mean and single results of 2 independent experiments (n=3-6 for spleens and n=2-3 for mLN). Data was analyzed using the two-way ANOVA and the Sidak correction for multiple comparisons test. P-values $p<0.05$ were considered of statistical significance. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Discussion

The immune system is comprised of cells that are relatively quiescent in the steady state, but rapidly respond to perturbations.[280] DCs act as the bridge between the innate and adaptive immune systems and, through presentation to T cells and production of cytokines, they direct the immune response.[377] Changes in energy pathways are necessary to mount an immune response.[282] Indeed, both innate cells and T lymphocytes rely on the process of mitochondrial oxidative phosphorylation to obtain energy when they are quiescent, while they increase their metabolic capacity and shift to glycolysis and fatty acid metabolism upon activation.[280] DCs are a diverse population comprising many subsets, with different roles and functions, and this diversity is associated with different metabolic requirements.

Our findings confirm that a shift to FA metabolism is important for TLR-induced activation of pDCs, as previously reported[281,286]. Moreover, we show for the first time that FLT3-L-derived cDCs also activate with a similar requirement for FAO and FAS. These results suggest that cDCs have different energy requirements than iDCs, known to shut down oxidative phosphorylation after activation, and therefore it may be possible to therapeutically target one subset and not the other. By using FAO inhibitor etomoxir and FAS inhibitor TOFA, we suppressed the TLR-induced upregulation of surface costimulatory molecule CD86, which is important for DCs to interact with T cells to mount an adaptive immune response. Additionally, the overexpression of CD86 in DCs from lupus patients suggests that manipulating CD86 could be a clinically relevant response.[378] Furthermore, etomoxir and TOFA suppressed the TLR-induced production of IL-6, a proinflammatory cytokine found to be produced in excess by stimulated DCs from lupus patients.[379,380] and proposed to be an accurate biomarker of SLE disease activity. [376] The feasibility of inhibiting the upregulation of these markers of pDC and cDC activation with FAO and FAS inhibitors suggests a therapeutic potential for these drugs in lupus.

Moreover, we have recently reported that iDCs from lupus-prone mice have a higher energy metabolism than WT iDCs,[23] and it will be important to determine whether also cDCs and pDCs from lupus-prone mice have higher metabolism, and in particular higher FAO and FAS, the interdependence between FA metabolism and the lupus IFN Signature, and whether FAO and FAS inhibition may normalize DC abnormalities in lupus.

Pharmacologic inhibition of FAO with etomoxir or FAS with TOFA has been reported inhibiting proliferation and sensitizing to cell death human tumor cells, like leukemia cells[381] and glioblastoma cells.[371] Therefore, we analyzed a possible effect of these drugs on DC survival. We used doses of etomoxir and TOFA that were suggested by the literature and found in both cases a similar sensitivity to levels of 100-200μM, which were shown feasible in murine in vivo studies.[292,382-384] The results that both drugs inhibit metabolic reprogramming to a degree that suppresses DC activation without affecting cell viability, further suggest that these metabolic shifts may be an exploitable target for novel therapies.

Interestingly, *in vitro* treatment of DCs with ethyl pyruvate did result in the suppression of both pDC and cDC activation as measured by upregulation of surface costimulatory molecules. However, the suppressive effect of ethyl pyruvate was only seen in cDCs. Due to limits in the ability to measure specific cell type metabolism *in vivo*, most studies performed on DC metabolism have been done *in vitro*. Our studies provide important insight into how metabolic modulators may work differently in DCs *in vitro* vs *in vivo.* However, the recapitulation of our findings that ethyl pyruvate does suppress cDC activation *in vivo* is an important finding highlighting the need for additional research into the metabolic changes of pDCs *in vivo*, and that the effects of metabolic modulation of cDCs using ethyl pyruvate is reproducible *in vivo*.

Future Directions

Studies of metabolic modulation of a systemic system are not complete without addressing the metabolome. Our *in vitro* findings are promising, and the *in vivo* administration of these drugs may be successful due to the fewer number of mitochondria naturally in DCs compared to other cell types, suggesting that a systemic administration of these metabolic modulators (many affecting mitochondria-tangential pathways) could be effective at a lower dose by having a larger effect on DCs, but not affecting other important cell types (i.e. cardiac muscle) that have many more mitochondria. *In vivo* studies of these metabolic inhibitors and an analysis of the metabolome of either the entire organism or specific lupus-relevant organs (i.e. kidney) could provide insight as to why the metabolic modulator works (or doesn't work) *in vivo*, where the system is much more complicated than the administration of the drug to a specific culture-differentiated cell type *in vitro*.

Additionally, this aim could be expanded further through the exploration of these drugs in cutaneous lupus. This would address the difficulties of systemic administration and disease that hampers the development of these therapies. Because the pathogenesis of cutaneous lupus is well known to be dependent on pDC activation[385] and exacerbated by UV damage[386] (and subsequent release of nucleic acid material), and pDCs are well established to be recruited to cutaneous lupus lesions[387,388], it would be exciting to explore whether the topical application of such metabolic modulators subsequent to sun exposure could result in the amelioration of disease through the prevention of pDC activation. This could be performed easily in murine models, but the systemic safety profile of the metabolic modulators in humans suggests that the topical application could be very safe, and a viable therapeutic modality to explore in cutaneous lupus. Although this would not cure systemic lupus, it could provide relief to a very common and very painful aspect of lupus disease.

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APPENDICES

Appendix A. Flow Cytometry Antibodies

Table 4. Antibodies for Flow Cytometry

Target	Color	Clone	Company
B220	PE	RA-6B2	BD
B220	APC	RA-6B2	BD
B220	APC-Cy7	RA-6B2	BD
CD3	FITC	17A2	BD
CD4	PE	RM4-5	BD
CD4	PE-Cy7	RM4-5	BD
CD8	FITC	53-6.7	BD
CD8	PE	53-6.7	BD
CD8	PerCP	53-6.7	Biolegend
CD8	APC	53-6.7	BD
CD11b	PE-Cy7	M1/70	BD
CD11c	APC	N418	eBio
CD40	FITC	HM40-3	BD
CD40	PE	3/23	BD
CD40L	PE	MR1	Pharmingen
CD69	FITC	H1.2F3	BD
CD69	PE	H1.2F3	BD
CD80	FITC	16-10A1	BD

CD80	PE	16-10A1	BD
CD80	APC	16-10A1	eBio
CD86	FITC	GL1	eBio
CD86	PE	GL1	eBio
CD86	APC	GL1	eBio
FVD	eFluor 780	n/a	Thermo Fisher
PDCA-1 (CD317)	PE-Cy7	Ebio927	eBio
SiglecH	FITC	Ebio440c	eBio

Appendix B. Protocols

Bone Marrow Culture and Sorting Protocol

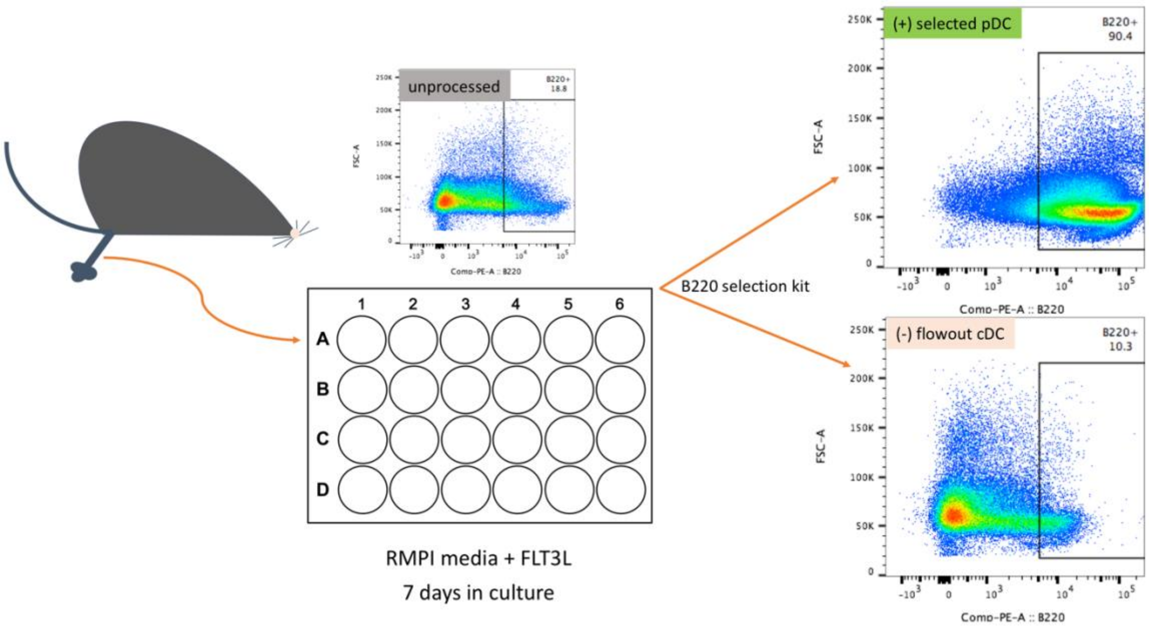

Figure 29. Sorting of FLT3-L DC culture

Materials

FLT3-L DC Media

- RPMI medium 500 ml
- Fetal Bovine Serum 50 ml
- 2-mercaptoethanol 500 µl
- Pen/Strep (100U/ml) 5 ml
- L-glutamine (100x) 5 ml
- FLT3-L supernatant 5%

Bone Marrow Harvest

- 1x sterile PBS
- 70% Ethanol
- 50 ml tubes
- 100 µm cell strainer
- 6 well plate or small petri-dishes
- 1cc syringe
- 27-gauge needles
- Sterile gauze
- 24 well plate

Day 0

- Sacrifice mice with CO2, cervical dislocate
- Remove skin from leg area
- Remove femur at hip joint
- Transfer the bones to small petri dish (or well of a 6 well plate)
- Clean muscle off bones
- Dislocate femur from tibia
- Rinse bones in fresh media
- Add bones to 70% ethanol for 1 min
- Rinse bones in PBS
- Rinse bones in fresh media
- Gently cut off distal and proximal ends of each bone
- Use syringe to flush out bone into a 100µm filter over a 50 ml conical tube using fresh media until marrow space is clear
- Centrifuge 10 min 4C 1600 RPM
- Resuspend cells in fresh media
- Count cells
- Centrifuge 10 min 4C 1600 RPM
- 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
- Plate in 24-well plate at 2x10^6 cells/well with 1 ml fresh media

Day 2

- Add 1 ml fresh media per well

Day 5

- Carefully remove 1 ml media from each well
- Add 1 ml fresh media per well

Day 7

- Gently harvest cells by retropipetting. No need to scrape
- Centrifuge 10 min 4C 1600 RPM
- Resuspend and count cells
- Centrifuge 10 min 4C 1600 RPM
- Resuspend in sorting buffer
- Cells ready to sort using StemCell Technologies EasySep sorting kit

Autoantibody ELISA Protocol

Materials

- Poly-L-Lysine – 0.01% in PBS (1:10 dilution). 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
- 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)
- Avidin-alkaline phosphatase conjugate (Sigma, cat. No. A7294)
- Alkaline phosphatase pNPP liquid substrate
- Flat-bottom 96-well PVC plate

Experimental Protocol

Day 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)
- Wash three times with distilled water or BBS
    - This can be stored for up to 1 week before use at RT
- Dilute dsDNA and chromatin in BBS
    - dsDNA (calf thymus DNA)
        - 2.5µg/mL BBS
        - Our stock is 4.2µg/µl, so for 10ml add 6µl
    - Chromatin (chicken chromatin)
        - 3µg/mL BBS
        - Our stock is 1µg/µl, so for 10ml add 30µl
- Coat each well with 100µl of above dsDNA/Chromatin solution. Seal the plate with parafilm and keep at 4^{o}C overnight.

Day 2

- Wash plate 3x with 200µl BBS.
- Add 200µl of BBT to each well and block for 2hrs at RT by gentle rocking.
- Make 1:250 dilution of MRL/lpr control serum and test samples in BBT in eppendorf tubes.
- Wash plate 5x with BBS.
- 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.
- Add 100µl of diluted test serum to the respective wells.
- Seal the plate with parafilm and keep it at 4°C overnight.

Day 3

- Wash plate 5x with BBS.
- Add 100µl of 1:5000 dilution of biotinylated goat anti-mouse IgG in BBT to each well. Incubate for 2 hrs at RT.
- Wash plate 5x with BBS.
- Add 100µl of 1:8000 dilution of avidin-alkaline phosphate conjugate in BBT to each well. Incubate for 2 hrs at RT.
- Wash plate 5x with 200µl BBS
- 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.)
- Incubate at 37°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.

C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J Genotyping Protocol

Table 5. CLEC4-DTR Genotyping Primers

Primer	**5’ -> 3’**	**Type**
11638	GCC CCC GGG CAG CAC AGC CAC TGC CGG TCC	Transgene forward
11639	GGC CAA GCG CTT GGG CAC TGT TCC CTC CCT	Transgene reverse
OIMR8744	CAA ATG TTG CTT GTC TGG TG	Internal positive control forward
OIMR8745	GTC AGT CGA GTG CAC AGT TT	Internal positive control reverse

Table 6. CLEC4-DTR Genotyping Master Mix

Master Mix					
# of Samples	**30**				
	Concentrations			MM for 1 Sample	
ddH2O	Up to final Volume			12.4	µL
Kapa 2G HS buffer (1.5mM)	1.3	µL		5	µL
MgCl2 (25mM)	2.6	mM		1	µL
dNTP KAPA	0.26	mM		0.5	µL
11638	0.5	µM		1.25	µL
11639	0.5	µM		1.25	µL
oIMR8744	0.5	µM		1.25	µL
oIMR8745	0.5	µM		1.25	µL
Glycerol	6.5	%		-	µL
Kapa 2G HS taq polymerase	0.3	U/µL		0.1	µL
DNA - added last into individual PCR tube	1	µL		1	µL
				12.6	µL
			Total volume per well	25	µL

Follow Jackson Laboratory Cycling Steps for Tg(CLEC4c-HBEGF)
Will see internal positive control at ~ 200bp, Transgene at ~ 432bp, example gel below:

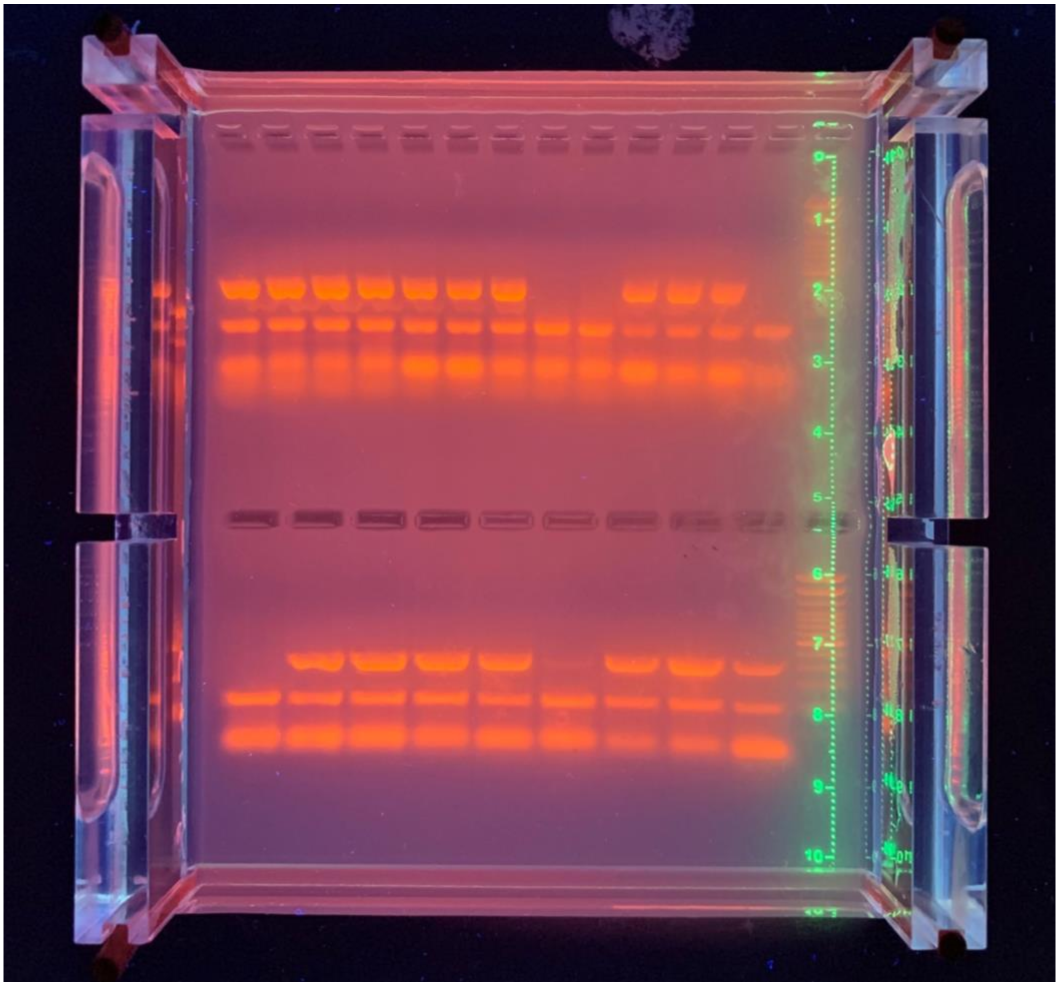

Figure 30. CLEC4-DTR genotyping gel

pDC Depletion Protocol

- Inject 100ng of DTX per mouse on days -2, -1, and 0. Start experiment on day 0
- Inject 100ng of DTX per mouse 3 times per week (Mon, Wed, Fri)

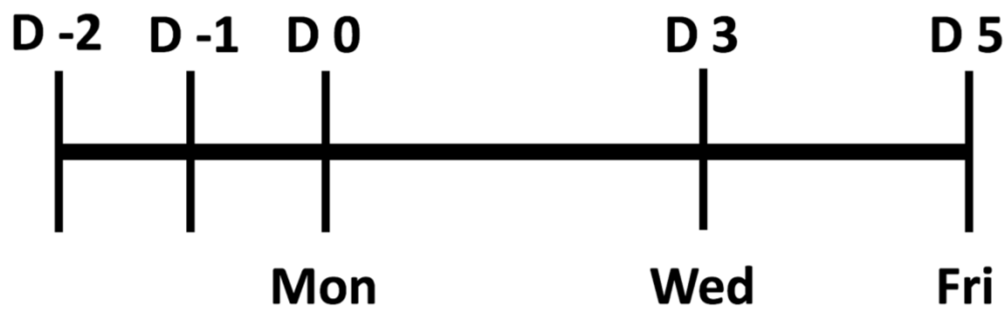

Figure 31. pDC depletion injection schedule

CTL Killing Protocol

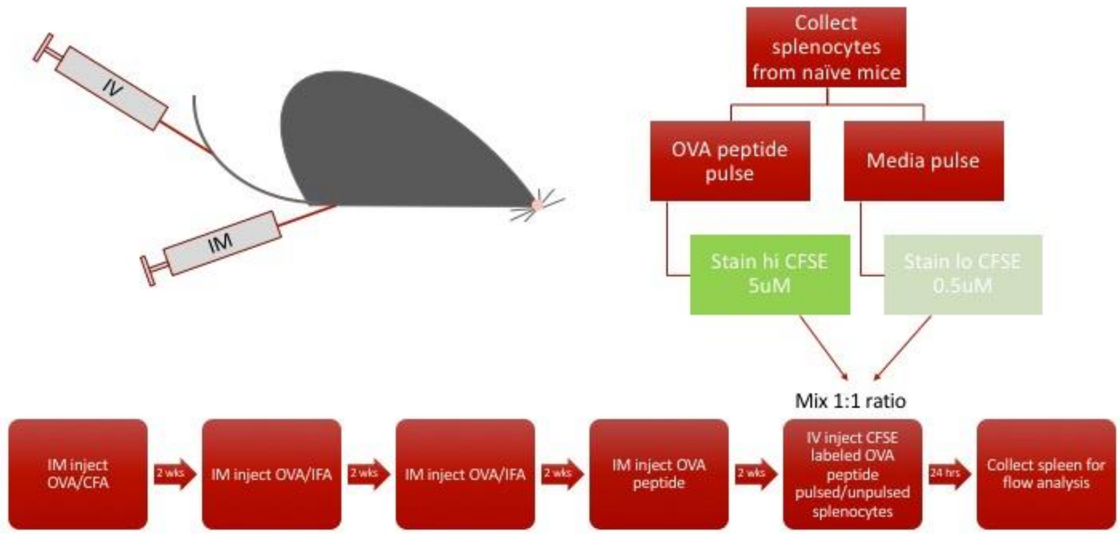

Figure 32. OVA antibody and CTL killing injection schedule

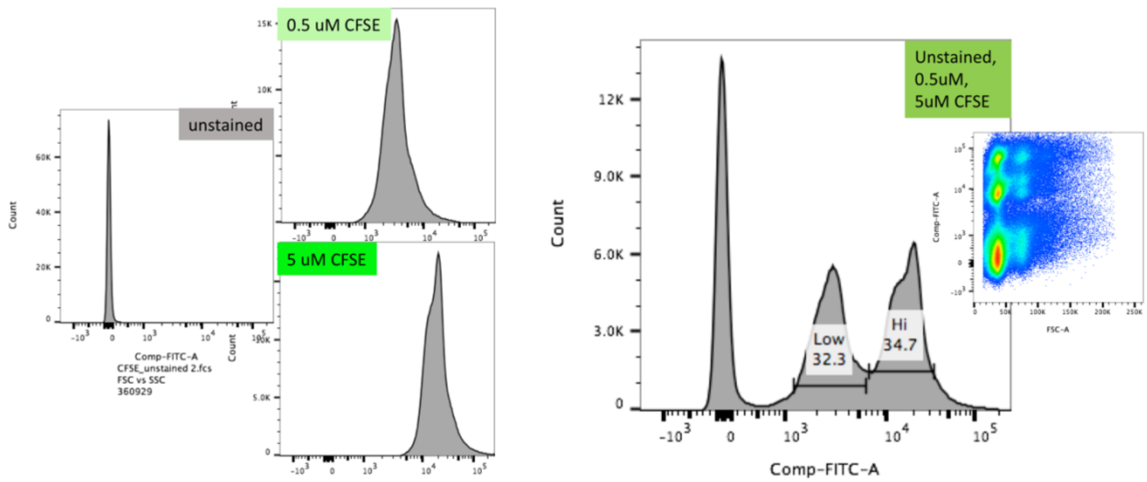

Figure 33. CTL killing CFSE staining

- After immunizing mice, harvest B6 splenocytes from naïve mice
- Count cells and split into 2 equal aliquots
- Pulse one aliquot with OVA peptide for 1 hour
- Leave second aliquot in fresh media
- Stain unpulsed aliquot with 0.5 μM CFSE for 10 min
- Stain OVA peptide pulsed aliquot with 5 μM CFSE for 10 min
- Rinse cells well
- Count cells
- Add 1:1 ratio of splenocytes into syringe
- Inject 100μl i.v.
- Sacrifice mice after 24 hours