The Role of Neutrophils in the Pathogenesis of Systemic Lupus Erythematosus

by

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BIOGRAPHICAL SKETCH

Anna Bird was born in Seattle, WA. She received her Bachelor of Science degree in Biochemistry from Hillsdale College in 2011. In 2011, she began pre-doctoral training in the Immunology, Microbiology and Virology graduate program at the University of Rochester, and received her Masters of Science degree in Immunology in 2014. She received an NIH T32 pre-doctoral fellowship from the Center for Musculoskeletal Research from 2015-2017. In 2015, she was awarded a Trainee Pilot Award from the Clinical and Translational Science Institute at the University of Rochester to investigate dysregulation in hematopoiesis in human lupus.

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ABSTRACT

Neutrophils are short-lived effector cells that participate in the innate phase of acute immune responses. However, recent literature shown that neutrophils can shift the balance between acceleration or suppression of an adaptive immune response, particularly in chronic inflammatory states such as malignancy, chronic viral infection, and—more recently described—in autoimmunity. Here, we investigated the contribution of neutrophils to adaptive immune dysregulation in systemic lupus erythematosus (SLE).

While neutrophils mediate tissue damage in SLE, it is unclear whether neutrophils propagate or restrain loss immunologic self-tolerance by the adaptive immune compartment. Neutrophils produce pro-inflammatory mediators in lupus including B-cell activating factor (BAFF), tumor necrosis factor-alpha (TNF α), as well as stimulating type I interferon production by plasmacytoid dendritic cells. However, several studies have suggested that neutrophils are able to suppress proliferation and effector function of adaptive immune cells via production of reactive oxygen species (ROS) and other immunosuppressive mediators. Drawing from this literature, we hypothesized that neutrophils within peripheral lymphoid tissues directly influence loss of self-tolerance by the adaptive immune compartment. Additionally, we hypothesized that neutrophil functional abnormalities observed in lupus patients have their origin in bone marrow pathology.

In order to examine whether neutrophils are predominantly protective or deleterious toward loss of self-tolerance in peripheral lymphoid tissues, we used the NZB/W murine model of lupus, and found that neutrophils accumulate in the secondary lymphoid tissue

with lupus progression and localize in close proximity to extra-follicular B and T lymphocytes. Additionally, we found that neutrophil depletion done in the early stages of lupus dramatically accelerates the progression of autoimmunity, including anti-dsDNA production, splenomegaly, and renal damage. These results indicated that, in early-stage lupus, neutrophils have the capacity to suppress the progression of auto-reactivity by the adaptive immune compartment.

To investigate which inflammatory mediators may underlie neutrophil protective effects observed during neutrophil depletion, we used RNA sequencing to characterize changes in the transcriptional program of splenic neutrophils at different stages of lupus in the NZB/W model. Over the course of lupus, we see significant changes in neutrophil transcripts encoding both pro- and anti-inflammatory and immunosuppressive mediators, including Cd274 [PD-L1] (inhibitor of T cell activation) and Csf1 [M-CSF], providing hypotheses as to which factors that may underlie changes in neutrophil effects on the adaptive immune system at different stages lupus.

Finally, we examined whether the aberrant phenotypic profile of neutrophils identified in the peripheral circulation in human lupus originates with bone marrow inflammation. We find an abnormal cytokine milieu in the bone marrow of a subset of lupus patients, including elevated G-CSF, GM-CSF, and TNF α . This inflammation is associated with abnormalities in the acquisition of neutrophil effector function within the bone marrow and corresponding functional defects in circulating neutrophils. These results implicate bone marrow inflammation as an underlying factor in the generation of functionally abnormal neutrophils in lupus.

CONTRIBUTORS AND FUNDING SOURCES

This work was completed under the supervision of Professor Jennifer Anolik (advisor) as well as members of the dissertation committee: Professor Deborah Fowell (Department of Microbiology and Immunology), Professor Minsoo Kim (Department of Microbiology and Immunology), Professor Richard Phipps (Department of Environmental Medicine), and Professor Steve Georas (Department of Environmental Medicine). The work described in this thesis was supported via generous funding from an NIH T32; A Clinical and Translational Science Institute (University of Rochester) Trainee Pilot Award (NIH Clinical and Translational Science Award, 5UL1TR000042); Lupus Foundation of America LIFELINE Grant, the Bertha and Louis Weinstein research fund, NIH grants P01-AI078907, R01-AI-077674, NIAMS Accelerated Medicines Partnership (1UH2AR067690).

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LIST OF ABBREVIATIONS

ANA	Anti-nuclear antibodies
ANOVA	Analysis of variance
APRIL	A proliferation-inducing ligand
ASC	Antibody secreting cell
BAFF	B-cell activating factor
BCR	B cell receptor
BHN	B-helper neutrophils
BM	Bone marrow
BMA	Bone marrow aspirate
cDNA	Complementary deoxyribonucleic acid
CDx	Cluster of differentiation x
DAMP	Danger-associated molecular pattern
DAVID	Database for Annotation Visualization and Integrated Discovery
DEG	Differentially expressed genes
dsDNA	Double-stranded deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
ELISPOT	Enzyme-linked immunospot assay
Ets1	Protein C-ets-1
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum

FC	Fold change
FDR	Benjamini-Hochberg False-Discovery Rate (adjusted p-value)
FOXP3	Forkhead box protein 3
GC	Germinal center
G-CSF	Granulocyte colony stimulating factor
GEO	Gene expression omnibus - a functional genomics database
GM-CSF	Granulocyte macrophage colony stimulating factor
gMDSC	Granulocytic myeloid-derived suppressor cells
GO	Gene ontology
Gr-1	Myeloid Differentiation Antigen (Ly6G/Ly6C)
GROx	Growth-regulated protein
HLA	Human leukocyte antigen
Hmgb1	High mobility group protein B1
IFN	Interferon
IFN-I	Type I interferon
IFN-II	Type II interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-x	Interleukin-x
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis
LDG	Low density granulocyte

Ly6x	Lymphocyte antigen 6 x
MDSC	Myeloid-derived suppressor cells
MeV	MultiExperiment Viewer
MIPx	Macrophage inflammatory protein x
MMPX	Matrix metalloprotease X
MOMA	Monoclonal anti-macrophage antibody
MPO	Myeloperoxidase
MSC	Mesenchymal stem cells
MZ	Marginal zone
NCBI	National Center for Biotechnology Information
NDS	Normal donkey serum
NET	Neutrophil extracellular trap
NETosis	Neutrophil extracellular trap formation
NF- κ B	Nuclear factor kappa B
NK cells	Natural killer cells
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	Nitric oxide
NZB/W	(New Zealand Black x New Zealand White)F1 hybrid mice
PAD4	Protein-arginine deiminase type-4
PB	Peripheral blood
PBS	Phosphate buffered saline
PCA	Principal components analysis

PCNA	Proliferating cell nuclear antigen
PD-L1	Programmed death ligand 1
PLO	Peripheral lymphoid organs
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear cell (mature neutrophil)
PNA	Peanut agglutinin
PRR	Pattern recognition receptor
Ptpn22	Tyrosine-protein phosphatase non-receptor type 22
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus erythematosus disease activity index
SLO	Secondary lymphoid organs
SN	Supernatant
Socs3	Suppressor of cytokine signaling 3
STATX	Signal transducer and activator of transcription X
STING	Stimulator of interferon genes protein
TCR	T cell receptor
TFH	T follicular helper cell
TGF β	Transforming growth factor-beta
TLRx	Toll-like receptor x
TNFAIP3	Tumor necrosis factor alpha-induced protein 3

TNF α	Tumor necrosis factor-alpha
Treg	Regulatory T cell
Trex1	Three-prime repair exonuclease 1
UV	Ultraviolet

CHAPTER 1 Introduction

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disorder with heterogeneous manifestations. SLE can affect almost any organ system, causing ongoing tissue damage and, in some cases, life-threatening organ dysfunction¹. SLE is defined based on classification criteria that include signs and symptoms such as photosensitive skin rash, alopecia, arthritis, serositis, neurologic disorder, renal disorder (including proteinuria or cellular casts) and laboratory abnormalities like anemia, leukopenia, low complement, and presence of disease-specific autoantibodies^{2, 3}. Although the presence of autoantibodies is essential for both pathogenesis and diagnosis of SLE, patients may exhibit a heterogeneous profile of auto-antibodies, with more than 180 different specificities identified among various human subjects⁴.

Auto-antibodies in lupus include IgM, IgA, and IgG isotypes⁵, and several autoantibodies with high prevalence, disease specificity, and diagnostic utility in SLE include anti-ssDNA, anti-dsDNA, anti-cardiolipin, anti-C1q, as well as anti-nuclear antibodies (ANA) that often target nuclear components such as ribonucleoprotein, SSA/Ro, SSB/La, Smith, and histone⁴. The elevated production of autoantibodies in SLE can lead to ongoing tissue damage, providing antigenic fuel to the adaptive immune system. This antigenic burden fuels further autoantibody production, completing a positive feedback loop that overcomes the mechanisms of immunologic self-tolerance.

Prevalence estimates for SLE in the United States vary due to the complexity of diagnosis, with approximately 1:1000 people diagnosed with suspected or definite SLE^{6, 7}. SLE disproportionately affects women (10:1), particularly women of childbearing age⁸. Individuals affected by SLE often experience significantly reduced quality of life, with many patients experiencing fatigue, fever, headaches, memory problems, and joint pain—symptoms that contribute to a 38% lower employment rate compared to healthy individuals^{9, 10, 11}. Although the SLE mortality rate has improved significantly in recent decades—with a five-year survival rate rising from 64-87% in the 1980s to better than 95% currently—medications used for SLE—including corticosteroids, anti-malarials, cyclosporin, B cell depletion, and BAFF blockade—are not always effective in cases of severe disease¹². These immunosuppressive medications often increase risk of infection and in the case of steroids, cause weight gain, osteoporosis, and in rare cases, steroid-induced psychosis¹³. The significant side effects of current therapies often lead to medication non-compliance in lupus patients, contributing to the elevated mortality in these individuals^{14, 15}. Overall, SLE patients have a significantly elevated risk of premature mortality, particularly increased risk of death due to cardiovascular disease (~3-fold), infection (~5-fold), and renal disease (~8-fold), compared to healthy individuals¹².

More targeted and efficacious therapies for SLE are still needed to improve survival and quality of life for those diagnosed with this disorder, but SLE remains a challenging disease to treat due to its heterogeneous and complex pathogenesis. Improving the therapeutic options for lupus patients will require a more complete understand of the factors

that contribute to tissue pathology, failure of self-tolerance mechanisms, and dysregulation of both innate and adaptive immune compartments in SLE.

1.2 Several physiologic processes contribute to the pathogenesis of SLE

The physiologic pathways that contribute to the pathogenesis of SLE are heterogeneous, and typically involve a complex genetic predisposition, hormone milieu, failure of self-tolerance mechanisms, aberrant exposure of the adaptive immune compartment of self-antigens, viral infections, tissue damage (e.g. UV light exposure), and certain medications (over 90 have been identified as risk factors for systemic autoimmunity)^{16, 17, 18}.

The genetic background that confers susceptibility to SLE is typically complex and heterogeneous, with a variety of gene variants with different degrees of penetrance. Although monogenic SLE is uncommon, instances are illustrative of the physiologic pathways that can contribute to systemic autoimmunity. Examples of monogenic susceptibility factors for SLE include defects in the complement cascade (e.g. loss of function mutations in C1q, C1r/s, C2, and C4), apoptosis (e.g. mutation in FAS or FASL), DNase enzymes (eg TREX1 mutations), cytosolic DNA sensing enzymes (e.g. gain of function mutations in STING), and genes that regulate B cell proliferation and TCR or BCR signaling (eg ETS1 and PTPN22)^{19, 20}. Defects in these pathways suggest that self-tolerance depends on both adequate clearance of self-antigen as well as the leukocyte responsiveness to alarmins and self-antigen. HLA haplotype is also highly predictive of susceptibility to SLE. For example, a SNP at rs2187668 within HLA-DR3 has been

associated with anti-dsDNA production¹⁹. Polygenic susceptibility factors also include genetic variants of interferon regulatory factors (IRF5, IRF7, and IRF8), transcription factors which regulate the production of type I interferon downstream of TLR engagement¹⁹. Other notable examples of risk variants include defects in STAT4, a mediator of cytokine signaling, as well as TNFAIP3, a regulator of NF- κ B signalling¹⁹. Thus, genetic risk variants for SLE are often associated with physiologic pathways involving dying cell clearance, apoptosis, antigen presentation, regulation of the activation thresholds for TCR and BCR signaling, leukocyte proliferation, and cytokine signaling, particularly IFN-I signaling.

An abnormal cytokine milieu is a prevalent feature of SLE, with elevated IFN-I, BAFF, IL-6, IL-10, IL-17, IL-21, TNF α , IFN γ , IL-1 β , and others often seen in the serum of SLE patients²¹. Although all of these cytokines have been demonstrated to contribute to lupus pathogenesis, cytokines of particular interest include BAFF and IFN α . Upregulation of interferon stimulated genes (ISGs) (i.e. the ‘type I interferon signature’) is observable in a majority of SLE patients, correlates with disease flare, and is reduced via glucocorticoid treatment²². The importance of IFN α in the etiology of SLE is demonstrated by the elevated risk of SLE in patients undergoing extended treatment with recombinant IFN α (historically a treatment for hepatitis C infection)^{23, 24, 25, 26}. The mechanism underlying elevated IFN-I is thought to be self-derived nucleic acid engagement of endosomal and cytoplasmic PRRs, particularly TLR7/8, TLR9, and STING²⁷. IFN-I is highly pleiotropic and contributes to SLE by mediating maturation of antigen presenting dendritic cells, differentiation of B cells to plasma cells, impairment of Treg function, augmenting cytotoxic activity in NK

cells and CD8+ T cells, as well as increasing production of BAFF²⁷. BAFF is an important mediator in the survival and proliferation of B cells, and carefully regulated BAFF levels in particular is critical in the maintenance of B cell anergy²⁸. Chronically high BAFF levels are observable in some lupus patients, and may allow the survival and maintenance of auto-reactive B cells in SLE²⁹. In murine models of lupus, BAFF deficiency ameliorates disease and BAFF-overexpression causes systemic autoimmunity^{30, 31, 32}.

The presence of elevated auto-antibodies is necessary but not sufficient to explain the development of SLE however. Auto-reactivity within the adaptive immune compartment is prevalent in humans, particularly in women over the age of 12, who exhibit ANA positivity (by HEp-2 staining) at a rate of approximately 18%³³. Only a small minority of these individuals develop the systemic autoimmune pathology and proliferation of auto-antibody specificities seen in SLE. Additionally, auto-antibodies have been shown to persist in SLE patients even during disease remission³⁴. A critical contributor to emergence of auto-reactivity and progression to fulminant SLE is an aberrant exposure of self-antigen to the adaptive immune compartment. This can occur in a number of ways, including through a defect in cell clearance, an overabundance of self-antigen due to tissue damage, as well as exposure of self-antigens that have been modified either post-translationally or via haptenization^{35, 36, 37}. The latter is a mechanism that may contribute to drug-induced lupus^{38, 39, 40}.

There are a wide array of endogenous molecules which are not exposed to immune cells under homeostatic conditions, but are immunostimulatory when released into the extracellular space and not cleared rapidly⁴¹. These include alarmins (endogenous danger-

associated molecular patterns) such as HMGB1, which is released during non-apoptotic cell death, ligates the RAGE receptor, and acts as a chemoattractant and activation factor for macrophages in SLE⁴². Calgranulins (S100 proteins) are pro-inflammatory mediators when released by myeloid cells into the extracellular space during inflammation states including lupus, and can contribute to both leukocyte activation through ligation of TLR4 as well as CD8+ T cell auto-reactivity⁴³. Certain self-molecules have inherently immunogenic properties, such as nuclear antigens with nucleic acid co-factors including RNP and histone, which, if endocytosed by B cells may ligate both an auto-reactive BCR as well as intracellular nucleic acid sensing TLR, providing a dual signal that drives B cell activation^{44, 45}. Lastly, self-nucleic acids, when inadequately cleared, can drive both auto-reactivity as well as IFN-I production by pDC when they are present within immune complexes, which allows combined ligation of Fc receptors and TLRs^{46, 47}.

In summary, SLE is a disorder involving failure of self-tolerance in the adaptive immune compartment, ongoing tissue damage, inflammation mediated by innate immunity, and the aberrant availability of self-antigen. Nearly all immune cell lineages have been implicated in the pathogenesis of lupus, although lupus research has classically focused on T and B lymphocytes as well as antigen presenting cells including macrophages and dendritic cells. Neutrophils have been characterized primarily as contributors to tissue damage in lupus, particularly nephritis⁴⁸. However, a growing body of literature now suggests that neutrophils contribute to the pathogenesis of lupus not only as mediators of tissue damage, but also as modulators of adaptive immune dysregulation in SLE. The work

in this thesis investigates the role of neutrophils in the dysregulation of the adaptive immune response in SLE.

1.3 The classical paradigm of neutrophil participation in inflammatory responses

Following injury or infection, neutrophils are recruited from blood circulation, the marginating pool, and—especially in severe inflammation—from the both immature and mature neutrophil populations in the bone marrow⁴⁹. Neutrophils migrate into an inflamed tissue through use of proteases such as MMP8 and MMP8 that allow neutrophil migration through the extracellular matrix and mediate tissue damage and remodeling⁵⁰. Resolution of a successful immune response involves prompt macrophage-mediated clearance of dying neutrophils and neutrophil debris, reverse transmigration of neutrophils out of a tissue and back into circulation, and conversion of neutrophil eicosanoids to pro-resolving lipoxins^{51, 52}. Effective clearance of apoptotic neutrophils by macrophages leads to the release of factors such as TGF β , which promotes tissue healing⁵³. Neutrophils also participate in resolution of inflammation in an active, coordinated fashion through local oxygen depletion⁵⁴, and release of proteases and decoy receptors such as DARK and D6, which limit further chemokine signaling, as well as release of find-me and eat-me signals such as annexin A1 and serpins^{53, 55, 56}, which limit leukocyte migration to the site of inflammation. However, in chronic inflammatory states such as lupus are marked by unsuccessful resolution of inflammation, although it is unknown whether neutrophil pro-

resolving mechanisms are defective in lupus or are overwhelmed by dysregulation in other immune cells subsets.

1.4 Neutrophil abnormalities contribute to disease pathogenesis in SLE

Historical models of neutrophil contributions to inflammation have described the neutrophil as a short-lived effector cell that participates only in the innate phase of the immune response. Indeed, neutrophils do migrate to sites of inflammation to clear invading pathogens before being cleared themselves. Because neutrophils are terminally differentiated and have a circulating half-life of around 5-12 hours in humans (8-10hrs in mice), it was thought until recently to be unlikely that neutrophils tailor their transcriptional and phenotypic program to different inflammatory states^{57, 58, 59}. Evidence has now accumulated that not only can neutrophils utilize tailored transcriptional and effector programs and that these programs affect both the onset and resolution of an immune response^{60, 61}. However, little is known about the effector program of neutrophils present in SLE, how this program changes over time, or how neutrophils affect the progression of auto-reactivity within the adaptive immune compartment.

There have been several findings in recent literature suggesting that neutrophils may have a significant impact on the progression of auto-reactivity and sterile adaptive immune responses in SLE. One possible mechanism by which neutrophils may exert influence on the adaptive immune system is via elevated cytokine production. Neutrophils isolated from the peripheral blood and bone marrow of lupus patients and characterized *ex vivo* show an elevated propensity for production of several pro-inflammatory cytokines compared to

healthy controls, including IFN γ , TNF α , IL-1 β , IFN α both spontaneously in culture or upon stimulation⁶². Lupus neutrophils also produce elevated amounts of BAFF, which was demonstrated to contribute to the progression of auto-reactivity in the B6.Fas^{lpr}/J/*Tnfrsf17*^{-/-} murine model of systemic autoimmunity^{63, 64, 65}. Because neutrophils are the most abundant leukocyte in humans, even moderate alterations in their cytokine production may have a significant impact on the systemic cytokine milieu in lupus. The cytokines produced by lupus neutrophils have been identified mainly as pro-inflammatory, there is currently a paucity of data quantitating neutrophil production of anti-inflammatory factors such as TGF β , IL-1RA, and pro-resolving lipid mediators, likely because of the persisting historical paradigm of the neutrophil as strictly pro-inflammatory.

An additional mechanism by which neutrophils may contribute to adaptive immune dysregulation in SLE is as a source of self-antigen. In 16-40% of SLE patients, neutrophil components are targeted by auto-antibodies, indicating their contribution to the antigenic burden in lupus^{66, 67, 68}. Neutrophil components have also been found in circulating immune complexes, which are a major mediator of both tissue damage (especially renal damage)^{69, 70}. The frequency of apoptotic neutrophils in peripheral blood is elevated in SLE, further suggesting inadequate clearance of neutrophil components^{69, 71, 72}. Neutrophils isolated from many lupus patients are also primed to undergo spontaneous neutrophil extracellular trap formation (NETosis), a pro-inflammatory form of cell death in which intracellular components, including nucleic acids, histone, and cytotoxic granular components are released into the extracellular space^{62, 73}. Neutrophil ETs contribute to vasculitis, skin, and renal pathology in lupus^{68, 74, 75}. Spontaneous NETosis by lupus neutrophils also contributes

to lupus pathogenesis by stimulating type I interferon production by pDC that encounter NET components within immune complexes^{69, 76, 77}. Lupus NETs have also been implicated in activation of caspase-1 and NLRP3 inflammasome activation in macrophages, thereby increasing IL-1 β and IL-18 production, which has been proposed as a mechanism of tissue damage^{69, 76, 77}.

Finally, neutrophils may contribute to adaptive immune dysregulation via direct interactions with and regulation of adaptive immune cells. Currently, little is known regarding the direct interactions of neutrophils with adaptive immune cells in SLE. Recent literature has begun to assess the idea that neutrophils influence the course of adaptive immune dysregulation in lupus, as has been demonstrated in other chronic inflammatory conditions, such as malignancy and chronic viral infection^{78, 79, 80, 81, 82}. In 2011, a publication by Puga et al. identified a unique subset of neutrophils—called B-helper neutrophils (BHN)—that have the capacity to enhance immunoglobulin production, class-switching, and even somatic hyper-mutation in human splenic MZ B cells, an effect primarily mediated by BHN secretion of BAFF, APRIL, and IL-21⁸³. Although the idea that neutrophils influence B cell Ig production was novel and controversial⁸⁴, the central findings of Puga et al. have been reproduced, supporting the model that neutrophils can regulate B cells responses within secondary lymphoid organs in both mice and humans^{85, 86, 87}. The Puga et al. (2011) study was conducted using tissue from humans in a variety of inflammatory responses including infection, but it is currently unknown whether BHN influence the progression of auto-reactivity in SLE.

Studies asking whether neutrophil influence adaptive immune dysregulation in SLE have thus far yielded conflicting results on whether neutrophils exert predominantly deleterious or protective on the progression of auto-reactivity. Evidence that neutrophils are deleterious comes from studies showing that inhibition NETosis using PAD4 inhibitors Cl-amidine and BB-Cl-amidine results in significantly ameliorated vascular and renal pathology, as well as a decrease in type I interferon signaling, although surprisingly NETosis inhibition also increases levels of serum anti-dsDNA^{74, 75, 77, 88}. Although cytotoxic NETs appear to mediate tissue damage and have been proposed as a source of self-antigen^{68, 89}, their effects on auto-reactivity are unclear. In particular, it is unclear whether the rise in serum anti-dsDNA with NETosis inhibition is due to increased formation of anti-dsDNA B cells, an increase in anti-dsDNA secretion by antibody secreting cells (ASC), or a decrease in available self-antigen for complexing with anti-dsDNA⁷⁵. Additional evidence for neutrophils' deleterious effects on adaptive immunity include the finding that granulocyte production of ROS has the capacity to suppress Treg differentiation *in vitro*⁹⁰. This finding is complicated by the lack of information about whether neutrophils do in fact interact with T cells (specifically naïve T cells) *in vivo*, and in the context which tissue and stage of disease. To address this knowledge gap, experiments in Chapter 2 provide an assessment of neutrophil-lymphocyte contacts and neutrophil localization within secondary lymphoid organs in murine lupus at different stages of disease progression in lupus.

1.5 Neutrophils restrain the progression of adaptive immune dysregulation in SLE

Although many studies examining the contribution of neutrophils to lupus pathogenesis have described neutrophils as pathogenic, evidence has accumulated that neutrophils have the capacity to suppress adaptive immune cell function, in many cases limiting the inflammatory process or promoting resolution. Also with the growing knowledge of diverse neutrophil effector programs, it is becoming clear that neutrophils help coordinate a wide array of immunologic processes. At local sites of infection or inflammation, neutrophils regulate an immune response via secretion of a wide array of cytokines, chemokines and lipid mediators⁹¹, recruiting other immune cells such as Th17 cells and monocytes^{92, 93}. In addition to promoting recruitment, neutrophils also transport antigen to secondary lymphoid organs, present antigen to T cells, polarize T helper cells, regulate T cell proliferation, and stimulate maturation of APC^{94, 95, 96, 97, 98, 99, 100, 101, 102, 103}. In chronic inflammatory conditions such as HIV infection, cancer, hepatitis B, and rheumatoid arthritis, neutrophil polarization toward granulocytic myeloid-derived suppressor cells (gMDSC) can tip the balance between adaptive immune activation and quiescence, primarily via MDSC regulation of lymphocyte effector function^{78, 79, 80, 81, 82}. In cancer models, gMDSC have primarily been described as suppressors of NK cell and T cell proliferation and effector function, often mediated by neutrophil production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)¹⁰⁴. Neutrophils with gMDSC-like function have recently been identified in both human and murine lupus, although it remains unclear whether gMDSC exert pathogenic or protective effects on adaptive

immune dysregulation in lupus or whether neutrophils in secondary lymphoid organs exert suppressive function^{90, 105, 106}.

Two studies have identified neutrophils with gMDSC-like function in SLE, in which the suppressive capacity of gMDSC appears to ameliorate the progression of auto-reactivity. Trigunaite et al. (2013) describe neutrophils present in both male and pre-pubescent female NZB/W mice, which are able to suppress B cell differentiation and Ig production *in vitro*. Ig production was completely rescued by addition of either a respiratory burst inhibitor N-acetyl-L-cysteine or by addition of iNOS inhibitor *N*_ω-L-arginine methyl ester hydrochloride, suggesting both ROS and NO are necessary for neutrophil suppressive function¹⁰⁵. The results of this study may be confounded by the rapid spontaneous apoptosis that neutrophils undergo when isolated *ex vivo* in combination with the ROS that is normally produced during neutrophil apoptosis independent of any disease in the donor animal^{107, 108, 109}. It is plausible that neutrophils present in SLO in lupus are not adequately cleared (or may be activated to produce ROS), but it is unknown whether SLO B cells encounter neutrophil ROS *in vivo* in SLE.

Further suggesting that neutrophil ROS may have protective effect on loss of self-tolerance in SLE, an *in vivo* study by Huang et al. (2015) found that neutrophil production of ROS is immunosuppressive in the context of a unique amyloid-induced lupus mouse model¹⁰⁶. Disease in this model is induced by injection of normal mice (i.e. BALB/c and C57BL/6) with a combination of human serum albumin amyloid in complex with DNA, which is thought to induce systemic autoimmunity via potent activation of the inflammasome with accompanying IL-1 β production. Lupus induction in this model causes

rapid neutrophil infiltration at the site of injection (the peritoneal cavity). Huang et al. demonstrated that when neutrophils were pre-depleted before amyloid injection or when amyloid was used in *NOX2*^{-/-} B6 mice (deficient in phagocyte NADPH oxidase), autoimmunity was significantly ameliorated. The mechanism of this protective effect shown to be neutrophil suppression IL-15 production by NK cells, thereby reducing systemic levels of IFN γ and thereby reducing autoantibody production¹⁰⁶. As in other studies, neutrophil suppressive effects on NK cells were found to be mediated by ROS production¹⁰⁶. As mentioned above, neutrophil ROS may not necessarily function in an immunosuppressive fashion, depending on the cell type that encounters ROS⁹⁰. Furthermore, evidence suggests that phagocyte (neutrophil, DC, macrophage) production of ROS in lupus is predominantly protective, as was demonstrated by knock-out of Nox2 (eliminating the function of phagocyte NADPH oxidase 2) in the MRL/lpr lupus model, which resulted in significantly augmented auto-reactivity¹¹⁰. Thus, although ROS has a vast array of effects, phagocyte production of ROS seems to restrain the progression of lupus.

Apart from ROS, neutrophils have a number of immunosuppressive tools in their effector repertoire. Neutrophil factors that may help restrain the progression of inflammation include TGF β , PD-L1, IL-1RA, Arginase-I, IL-10, IDO, iNOS, lipoxins, annexin A1, CCR5 (a chemokine scavenger)^{111, 112}, although little is known about whether these factors are altered in lupus or which might provide promising therapeutic targets or insight into lupus pathogenesis. Among these factors, PD-L1 is of particular interest, since it has a potent ability to regulate T cell responses to presented antigen and maintain

peripheral self-tolerance¹¹³. It was recently (2016) shown that circulating neutrophils in SLE express of PD-L1 and that degree of expression is positively correlated with disease activity¹¹⁴. Although the impact of neutrophil PD-L1 in lupus is currently unknown, neutrophil PD-L1 expression has been associated with suppression of an immune response in the context of HIV and malignancy^{115, 116, 117}, and may have similar immunosuppressive effects lupus as well.

The hypothesis that neutrophils are predominantly protective toward adaptive immune dysregulation supported by growing evidence, although it has yet to be determined in which contexts neutrophil deploy protective mediators, such as ROS and TGF β . The paradoxical results in current literature claiming that neutrophils exert both protective and deleterious functions may be resolved by considering neutrophil phenotype within different contexts. Neutrophils may both contribute or deter development of auto-reactivity depending on the murine model (or patient), stage/severity of disease, tissue localization, and the specific cell types that neutrophils interact with. Experiments discussed in Chapter 2 delineate the impact of these variables on neutrophil influence on adaptive immune dysregulation and loss of self-tolerance.

Neutrophils can tailor their effector program to various inflammatory contexts, including acquisition of immunosuppressive or pro-resolving roles, and we hypothesize that the comparison of lupus neutrophils to neutrophils in other inflammatory states such as infection shed light on the functional role of neutrophils in lupus. In Chapter 3, we use transcriptional profiling of splenic neutrophils isolated at 3 stages of lupus progression in order to a) characterize the changes in neutrophil phenotype over the course of lupus

progression b) conduct a comparison between lupus neutrophils at the 3 stages of disease with neutrophils in other inflammatory states, and c) generate hypotheses regarding the mechanisms by which neutrophils impact to loss of self-tolerance in both protective and regulatory capacities.

1.6 Bone marrow pathology in SLE

Despite observations that circulating neutrophils in SLE show functional abnormalities, including defects in phagocytic capacity and spontaneous NETosis¹¹⁸, the mechanisms underlying the generation of these functionally impaired neutrophils is unclear. We hypothesize that the generation of functionally abnormal neutrophils in lupus is attributable to inflammation within the bone marrow. Although bone marrow is the primary site of hematopoiesis and enforcement of central tolerance in developing B cells, although dysregulation of the bone marrow in lupus has been characterized in a very limited fashion. The extent to which marrow dysregulation influences lupus pathogenesis as a source of functionally abnormal leukocytes remains unclear. In chapter 4, we asked whether inflammation within the bone marrow is associated with functional defects in both developing and circulating neutrophils in SLE.

Lupus bone marrow pathology has been described in several contexts, most commonly a histological analysis of the tissue morphology, cellular proliferation, and overall tissue organization. Findings in various lupus patients are heterogeneous and include fibrosis, necrosis, hypocellularity, neoplastic outgrowth of myeloid cells (myelofibrosis), atypical localization of immature precursors, and an elevated proportion

of plasma B cells^{119, 120, 121}. Peripheral cytopenias such as anemia, thrombocytopenia, and leukopenia are common in SLE¹²² and are associated with bone marrow pathology, particularly hypo-cellularity and necrosis⁹⁹. Cell death in the marrow is likely a major cause of SLE cytopenias and associated risk of infection (a prevalent cause of mortality in SLE)^{123, 124}.

The mechanisms underlying defects in bone formation and hematopoiesis are unknown, although we hypothesize these defects are driven by ongoing inflammation within the marrow itself. Supporting this hypothesis, elevated IFN-I signaling and elevated levels of TNF α have both been identified in lupus marrow^{65, 125}. Both of these factors may underlie elevated cell death or defects in hematopoiesis^{65, 125}. Conversely, little is known regarding which cells may drive inflammation in the marrow, although both neutrophils and monocytes were implicated in one study as a source of TNF α (a mediator of marrow necrosis)^{125, 126}. Several studies have examined the cellular mechanisms that may underlie bone marrow pathology in lupus. Limited evidence has indicated defects in central tolerance in SLE¹⁰⁴, the frequency of developing B cell subsets is altered in SLE, alterations associated with elevated IFN-I signaling in the bone marrow⁶⁵. Also, mesenchymal stem cells (MSC) support hematopoiesis in the marrow, but in lupus, MSC show deficiency in production of cytokines known to support hematopoietic competence (e.g. IL-6, IL-7)¹²⁷, as well as an enhanced capacity to stimulate transitional and mature naïve B cell differentiation to plasma cells¹²⁸. Hematopoietic stem cells in lupus marrow exhibit elevated apoptosis and proliferative defects^{129, 130}, associated with defects in hematopoiesis, including for example, decreased frequency and impaired functional

maturation in NK cells¹³¹. Similar to NK cells, we hypothesize that defects in circulating, peripheral neutrophils (described in 1.5) originate with defects in granulopoiesis driven by bone marrow inflammation.

1.6 Neutrophils in bone marrow pathology in SLE

Evidence suggesting abnormal granulopoiesis in bone marrow comes from transcriptomic profiling of peripheral blood mononuclear cells (PBMC) from lupus patients as well as from total bone marrow aspirates^{22, 132}. Analyses of both tissues in SLE patients revealed elevated expression of an array of genes typically seen in immature granulocytes, such as transcripts encoding defensins, elastase, myeloperoxidase, and other factors that are highly expressed only during developmental acquisition of granular components during the early stages of granulopoiesis^{133, 134}. This granulopoiesis signature was positively correlated with SLEDAI¹³². Microscopy of peripheral blood neutrophils demonstrated that a subset of circulating neutrophils in SLE have an immature nuclear morphology, further suggesting premature egress from the marrow⁶². Although neutrophil left-shift is associated with elevated neutrophil consumption in various inflammatory states such as sepsis⁴⁹, it remains unclear whether a left-shift in lupus indicates a pathological development in granulopoiesis due to marrow-intrinsic tissue dysregulation or is simply a healthy compensatory response to increased neutrophil consumption in the periphery. Tangential evidence for granulopoietic dysregulation was provided by Singh et al. who discovered significantly increased genomic damage in circulating neutrophils in SLE compared to healthy controls, including loss of heterozygosity and microsatellite

instability¹³⁵. Because mature neutrophils are terminally differentiated, genomic abnormalities almost certainly arises during cell division during early granulopoiesis. The causes of neutrophil genomic damage are unclear, but may disrupt normal granulopoiesis as well as underlie the appearance of a unique subset of peripheral neutrophils called ‘low density granulocytes,’ which are both pathogenic and functionally impaired¹³⁵.

Low density granulocytes (LDG) were described by Denny et al. (2010), in which it was noted that ficoll-derived PBMC preparations from lupus blood commonly contain a significantly higher than normal frequency of granulocytes⁶². LDG exhibit a number of functional abnormalities, including impaired phagocytic capacity, elevated expression of IFN α , IFN γ , and TNF α , as well as exhibiting spontaneous NETosis *ex vivo*⁶². However, the mechanisms underlying the generation of these abnormal neutrophils is unclear, and based on their immature morphology, combined with the granulopoiesis transcriptomic signature found in lupus blood leads us to hypothesize that tissue dysregulation in the marrow contributes to the generation of the functionally abnormal and pathogenic neutrophils seen in lupus peripheral blood.

1.7 Summary of Hypotheses and Aims

Lupus is a complex and heterogeneous disease characterized by defects in both adaptive and innate immunity and is associated with substantial mortality and morbidity. Current treatments are broadly immunosuppressive and often ineffective. Moreover, there has been only 1 new treatment (Belimumab) approved for lupus in the past 50 years¹³⁶, further highlighting the need to better understand disease pathogenesis so that rational,

targeted therapies can be developed. A growing body of research indicates that adaptive immune dysregulation in SLE can be dramatically influenced by the innate immune system. This has been best exemplified by the role of pDCs as IFN-I producers in response to lupus autoantibody containing immune complexes and in turn driving activation of B and T cells and promoting loss of tolerance. More recently, the role of neutrophils in the disease has been heavily studied but with conflicting results. Much of the literature supports pro-inflammatory roles for neutrophils in SLE including production of inflammatory mediators and provision of autoantigen via enhanced NETosis. However, neutrophils also have inflammation resolving functions in other contexts, and a handful of recent literature suggests that neutrophils may also have regulatory roles in lupus. Our over-riding hypothesis is that the neutrophil effector function in lupus will vary depending on the inflammatory milieu, both during bone marrow development and peripheral activation, and the cellular context or predominant cell population with which neutrophils are interacting.

Aim 1 - Assess whether neutrophils exert predominantly protective or deleterious effects on loss of self-tolerance comparing early versus late stages of lupus in the NZB/W murine model.

Rationale: A more complete *in vivo* model is needed to reconcile the conflicting data pointing toward both pathogenic and regulatory effects on the adaptive immune compartment in SLE. Here we define the localization, changing cellular contacts, and transcriptional program of splenic neutrophils at several stages during the course of murine lupus and the effect of neutrophil depletion on disease.

Aim 2 – Characterize changes in the transcriptomic program of NZB/W neutrophils from peripheral lymphoid tissue at several stages in the progression of disease

Rationale: To further define how the neutrophil effector program changes over the course of lupus progression and gain insights into which immunologic mediators neutrophils produce at different stages of lupus, RNA sequencing was conducted at 3 disease time-points.

Aim 3 - Determine whether phenotypic and functional abnormalities in human lupus neutrophils arise during bone marrow development in the inflammatory milieu of the lupus marrow.

Rationale: Recent literature has suggested that neutrophils contribute to human SLE disease pathogenesis as mediators of tissue damage and as a source of cytotoxic NETs and inflammatory mediators including TNF α and IFN-I. We hypothesize that pathogenic and functionally abnormal neutrophils are generated as a product of dysregulated granulopoiesis within an inflammatory cytokine milieu in the bone marrow.

CHAPTER 2 Neutrophils slow disease progression in murine lupus via modulation of auto-reactive germinal centers

2.1 Abstract

Neutrophils are well-characterized as mediators of peripheral tissue damage in lupus, but it remains unclear whether they influence loss of self-tolerance by the adaptive immune compartment. Lupus neutrophils produce elevated levels of factors known to *fuel* autoantibody production, including interleukin-6 and B cell survival factors, but also reactive oxygen intermediates, which can suppress lymphocyte proliferation. In order to assess whether neutrophils directly influence the progression of auto-reactivity in secondary lymphoid organs (SLO), we characterized the localization and cell-cell contacts of splenic neutrophils at several stages in the progression of disease in the NZB/W murine model of lupus. Neutrophils accumulate in SLO over the course of lupus progression, preferentially localizing near T lymphocytes early in disease and B cells with advanced disease. RNA sequencing reveals that the splenic neutrophil transcriptional program changes significantly over the course of disease, with neutrophil expression of anti-inflammatory mediators peaking during early- and mid-stage disease, and evidence of neutrophil activation with advanced disease. To assess whether neutrophils exert predominantly protective or deleterious effects on loss of B cell self-tolerance *in vivo*, we depleted neutrophils at different stages of disease. Neutrophil depletion early in lupus resulted in a striking acceleration in the onset of renal disease, SLO germinal center formation, and auto-reactive plasma cell production. In contrast, neutrophil depletion with

more advanced disease did not alter SLE progression. These results demonstrate a surprising temporal and context dependent role for neutrophils in restraining auto-reactive B cell activation in lupus.

2.2 Introduction

A central feature of lupus pathogenesis is production of autoantibodies that target multiple organ systems, resulting in chronic inflammation and, in severe cases, life-threatening organ damage. However, the mechanisms underlying the dysregulated adaptive immune response and progression of auto-reactivity in lupus remain incompletely characterized. Although B and T cells are well known to play a key role in SLE disease pathogenesis¹³⁷, the contribution of innate immune cells to disease is increasingly recognized¹³⁸. Neutrophils help orchestrate the outcome of immune responses by tailoring their effector program to an acute inflammatory response during infection, immunosuppression during chronic inflammatory states such as malignancy, or resolution of inflammation to promote healing. In SLE, neutrophils are known to mediate tissue damage and act as a source of self-nucleic acids that drive aberrant plasmacytoid dendritic cell (pDC) activation^{62, 66, 139}. Neutrophil-specific components are targeted by autoantibodies in 10-20% of SLE patients, likely due to a high neutrophil turnover rate, impaired dying cell clearance, and elevated propensity for extracellular trap formation (NETosis)^{48, 62, 66, 67, 68, 71, 74, 88}. Because NETosis involves extravasation of nucleic acid and histone, this form of cell death may provide antigenic fuel for auto-reactive lymphocytes as well as interferon-producing pDC^{62, 139}. *In vivo* inhibition of NETosis with

peptidylarginine deiminase (PAD4) inhibitors in murine lupus models ameliorates vascular, dermal, and renal tissue pathology and reduces systemic IFN-I signaling^{74, 75}.

In contrast, recent literature has contended that neutrophils in lupus—similar to neutrophils in other inflammatory conditions such as malignancy and sepsis—may acquire an immunosuppressive phenotype and restrain development of auto-reactivity^{104, 140}. In an amyloid-induced model of systemic autoimmunity, neutrophil production of reactive oxygen species (ROS) mediates suppression of systemic IFN- γ levels and auto-antibody production¹⁰⁶. Additionally, *in vitro* co-culture of splenic neutrophils with B lymphocytes isolated from the NZB/W lupus model demonstrated that neutrophils can suppress B cell immunoglobulin production. Interestingly, neutrophil capacity for suppression of B cells was only apparent early in disease, suggesting that the neutrophil effector program may change with disease progression¹⁰⁵. Neutrophils may have different effects on adaptive immunity depending on the type of cell encounter. Thus, splenic neutrophils isolated in murine lupus were also able to suppress Treg differentiation *in vitro*, effects that should propagate autoimmunity^{90, 105, 141}. However, little is known about which immune cell types neutrophils interact with *in vivo*. It has been suggested that the predominant effect of neutrophil ROS in SLE may be protective, as was demonstrated by the finding of accelerated autoimmunity in MRL/*lpr* mice deficient in NADPH oxidase (NOX2, the enzyme responsible for oxidative burst in neutrophils)¹¹⁰. Aside from ROS, circulating neutrophils in SLE also produce elevated amounts of pro-inflammatory mediators including interleukin-6 (IL-6), tumor-necrosis factor-alpha (TNF α), B-cell activation-factor (BAFF), and a proliferation inducing ligand (APRIL), factors known to promote

adaptive immune dysregulation in lupus^{62, 65, 142, 143}. The cytokine profile of neutrophils in SLE remains incompletely characterized, particularly neutrophil production of anti-inflammatory factors such as TGF β and IL-1RA.

Thus, although recent literature highlights that neutrophils influence the course of autoimmune disease, a more complete *in vivo* model is needed to reconcile the conflicting data pointing toward both pathogenic and regulatory effects on the adaptive immune compartment. We hypothesize here that neutrophils have the capacity for either deleterious or protective influence on the development of auto-reactivity, dependent on variables including stage and severity of disease, tissue localization, cellular interactions, and differences in inflammatory pathways driving SLE. Here, we assess whether neutrophils contribute directly to loss of B cell self-tolerance within secondary lymphoid tissues using the NZB/W murine model of lupus. Specifically, we characterize the localization, changing cellular contacts, and transcriptional program of splenic neutrophils at several stages during the course of autoimmunity. We find that neutrophils are in close proximity to splenic T and B lymphocytes and that lymphocyte-neutrophil contacts in the spleen change over the course of disease. Transcriptome profiling of splenic neutrophils demonstrates that neutrophils display a regulatory transcriptional program that is apparent during the initiation of disease, but is lost with advanced disease. Consistent with this result, neutrophil depletion early in disease resulted in a striking acceleration in the development of B cell auto-reactivity and renal proteinuria, a result that was not apparent in advanced disease. Collectively, our findings demonstrate that the neutrophil effector program and the nature of neutrophil impact on autoimmunity are strongly dependent on the stage and

severity of disease, a critical consideration in the assessment of neutrophils as a potential therapeutic target in lupus.

2.3 Methods

Mice

Female (NZBxNZW)F₁ mice were purchased from The Jackson Laboratory and housed in the pathogen-free animal facility at the University of Rochester. All murine experiments were conducted in accordance with the policies established by the University of Rochester's University Committee of Animal Resources and the Institutional Animal Care and Use Committee.

Depletion of neutrophils with anti-Ly6G antibody

Female (NZBxNZW)F₁ mice were injected intraperitoneally with neutrophil-depleting antibody anti-Ly6G (clone 1A8) (BioXCell) or ChromPure rat whole IgG isotype control (Jackson ImmunoResearch) using 500µg antibody per mouse every 2 days over the entirety of the indicated depletion periods. Mice were sacrificed 24 hours following the last antibody injection. Flow cytometry was used to verify the efficacy of neutrophil depletion.

Quantitation of renal pathology and serum anti-dsDNA

Proteinuria was quantitated using urinalysis reagent sticks (Teco Diagnostics). At sacrifice, kidneys were formalin fixed for histological analysis. Kidney sections (4 µm) were stained with hematoxylin and eosin. Pathology was analyzed and scored in a blinded fashion (B.I.G.)¹⁴⁴. Briefly, the severity of glomerular, interstitial, and vascular lesions

was determined on a scale of 0 to 4+. Multiple sections at a minimum of two different levels were examined, with each section typically containing >50 glomeruli and >25 blood vessels. Blood was collected by submandibular bleed and serum anti-dsDNA IgG concentration was determined by ELISA (Alpha Diagnostic International).

Flow cytometric quantitation of leukocyte subsets

Bone marrow was isolated by flushing of murine femur and tibia with RPMI 1640. Splenocytes were isolated by mechanical disaggregation. Erythrocytes were lysed with ammonium chloride buffer and leukocytes stained for FACS analysis with an LSR II (Becton-Dickinson). Lymphocyte populations were identified as CD3⁺CD4⁺CD8⁻ (CD4⁺ T cells), CD3⁺CD4⁺CD8⁻CD25⁺FoxP3⁺ (Tregs), CD3⁺CD4⁺CD8⁻CXCR5^{hi}PD1⁺ICOS⁺ (T_{FH} cells), CD3⁻CD19⁺ (B cells), and CD19⁺CD95⁺Peanut-agglutinin⁺ (germinal center B cells). Myeloid cells were identified using B220⁺/CD4⁺/CD8⁺ cell exclusion and expression of CD11b⁺Gr-1^{hi}Ly6C^{int} (neutrophils) and CD11b⁺Gr-1^{lo-int}CD11c⁺F4/80^{hi} (monocytes/macrophages), which were further sub-gated into Ly6C^{lo}CX3CR1^{hi}SSC^{lo} (M2 Mφ) or Ly6C^{hi}CX3CR1^{lo}SSC^{lo} (M1 Mφ). Dead cells were excluded from all analyses using AQUA (Invitrogen). Antibodies used for flow cytometry: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-8.7), anti-CXCR5 (L138D7), anti-Foxp3 (150D), anti-CD25 (3C7), anti-PD1 (29F.1A12), anti-ICOS (15F9), anti-CD19 (6D5), anti-CD95 (SA367H8), anti-B220 (RA3-6B2), anti-Gr-1 (RB6-8C5), anti-Ly6C (HK1.4), anti-CX3CR1 (SA011F11).

Immunohistochemical characterization of secondary lymphoid organs (SLO)

Freshly isolated spleen was snap-frozen in OCT buffer and cut into 4 μ m sections for immunohistochemical staining. Sections were blocked (5% normal donkey serum/PBS), antibody-labeled, and mounted in Prolong Gold Antifade with DAPI (Life Technologies). Tissue sections were imaged with Zeiss Axioplan Microscope at 200x magnification for all images. Axiovision software (Zeiss) was used for morphometric analyses. Germinal centers were identified as PNA⁺PCNA⁺B220^{dim} cell clusters and follicles as clusters of B220^{bright} cells. The marginal zone (MZ) was identified as a line of MOMA⁺ staining in the peripheral region B220⁺ follicles. The extent of germinal center formation was calculated by dividing the total area occupied by GC by the total area of the tissue section. Neutrophil contacts with B and T lymphocytes in the spleen were quantitated based on the number of neutrophils (Ly6G^{bright} cells with PMN nuclear morphology) that appeared to be in contact with B220⁺ or CD3⁺ cells in the white pulp. Neutrophil localization relative to primary and secondary splenic follicles as well as to the marginal zone (MOMA⁺ line) was quantitated by positioning splenic follicles in a consistent fashion within the 200x field and measuring the distance between Ly6G⁺ cells and the follicle edge (B220⁺ cell clusters) or MOMA⁺ marginal zone line. Stains used for IHC: anti-B220 (RA3-6B2), PNA (peanut agglutinin), anti-PCNA (PC10), anti-MOMA (MOMA-2), anti-Ly6G (1A8), anti-CD3 (145-2C11).

ELISpot assay for antibody-secreting cells.

ELISpot was performed as described previously^{144, 145}. Briefly, in order to detect anti-dsDNA-secreting cells, 96-well MultiScreen plates (Millipore) were coated with poly-L-lysine and incubated with calf thymus DNA. Plates were coated with goat anti-mouse

IgG (Fc fragment specific, Jackson ImmunoResearch Laboratories) to detect total IgG-secreting cells. Cell suspensions from spleen, bone marrow and kidney were added to individual wells, starting with 5×10^5 cells in the top row and performing a 2-fold serial dilution. Cells were incubated overnight at 37°C and 5% CO₂. After incubation, plates were washed several times with 0.1% Tween 20 in PBS, incubated with alkaline phosphatase-goat anti-mouse IgG antibody (Southern Biotech), and finally developed with Vector Blue alkaline phosphatase substrate III kit (Vector Laboratories). Antibody-secreting cells were enumerated with a stereoscopic microscope (Cellar Technology Ltd.).

Purification of splenic neutrophils for RNA sequencing

Splenic neutrophils were purified by mechanical disaggregation of freshly isolated spleen without the use of erythrocyte lysis. Splenic neutrophils were isolated for RNAseq analysis pre-enrichment by magnetic negative selection (Miltenyi), followed by sorting of CD11b^{hi}Ly6G^{hi}Ly6b^{hi}Ly6C^{int} cells with doublet and dead (DAPI+) cell exclusion to obtain a purity of $\geq 97\%$ CD11b^{hi}Ly6G^{hi} cells, as determined by post-sort analysis. Neutrophils were kept cold (at 4°C) throughout the entire course of neutrophil isolation and handled gently to avoid mechanical activation. Antibodies used for FACS purification: anti-Ly6B (REA115), anti-Ly6C (HK1.4), anti-Ly6G (1A8), anti-CD11b (M1/70).

Low input RNA sequencing

Neutrophil total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen) and RNA quality assessed with the Agilent Bioanalyzer 2100 (Agilent) (n=6 mice per 3 age groups). 1ng of total RNA was pre-amplified with the SMART-Seq v4 Ultra Low Input

RNA Kit for Sequencing (Takara Bio USA). The quantity and quality of the subsequent cDNA was determined using the Qubit Fluorometer (Thermo Fisher Scientific) and the Agilent Bioanalyzer. 1ng of cDNA was used to generate Illumina compatible sequencing libraries with the NexteraXT (Illumina) library preparation kit. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina) at a concentration of 10pM per lane. Single-end reads of 100nt were generated for each sample and aligned to the organism specific reference genome. Sequenced reads were cleaned using Trimmomatic-0.32 before mapping to the mouse reference genome (GRCm38.p4) with STAR-2.4.2a. Raw read counts were obtained using HTSeq and gencode M6 mouse gene annotations. DESeq2 1.12.3 was used to perform data normalization. Following an expression level cutoff of 1 (rlog deseq norm counts), principle components analysis was performed using all transcripts. Additionally, a list of 130 genes of immunologic interest was compiled and differential analysis was conducted using deSeq. These include genes involved in neutrophil regulation of immune responses, both protective mediators and pro-inflammatory mediators, as well as genes involved in neutrophil responses to inflammation such as IFN regulated genes and cytokine signaling genes like the Stat family. The full transcriptome data set is available on GEO (Accession GSE97439).

Statistical analysis

Statistical analysis comparing among experimental groups was done using the Mann-Whitney U-test or Kruskal-Wallis ANOVA using GraphPad Prism and MultiExperiment Viewer software. Differential expression analysis of RNA sequencing

data was conducted using a Benjamini-Hochberg correction ($FDR \leq 0.05$). Principal components analysis was conducted using JMP Pro 13. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.4 Results

Neutrophils accumulate in the spleen and exhibit changing lymphocyte contacts during the progression of lupus

Under homeostatic conditions, neutrophils are present in SLO tissues at a frequency of 1-2% of total splenocytes, although their numbers can increase dramatically during an immune response¹⁴⁶. In order to characterize neutrophils in lupus and their impact on adaptive immunity, we first quantitated neutrophil infiltration of the spleen at several points over the course of lupus progression by FACS analysis of splenocytes from female NZB/W mice at 11, 23, 25, and 30 weeks of age. Between 23 weeks of age (around the onset of overt pathology, including proteinuria) and 30 weeks of age (advanced lupus), we observed a significant increase in both the frequency and total numbers of neutrophils infiltrating the spleen (Fig. 2.1A, 1B). Neutrophils were observed predominantly in the red pulp and interfollicular areas of the white pulp (Fig. 2.1C), and although we observe infiltration with the progression of disease, the average neutrophil proximity to B cell follicles did not change over the course of disease nor were they detected in GCs (data not shown).

In order to address the hypothesis that neutrophils are directly interacting with splenic B and T lymphocytes, neutrophil contacts with B and T cells were quantitated by IHC of spleens isolated from 11, 20, and 29 week-old NZB/W mice. Neutrophils were observed in close contact with T and B lymphocytes in the splenic white pulp during all

stages of disease (Fig. 2.2A). At 11 weeks, early in disease, the frequency of neutrophil contacts with T cells was 1.5-fold higher than contacts with B cells (Fig. 2.2B). By 29 weeks of age, this phenomenon is reversed, with nearly half of all Ly6G⁺ neutrophils in the white pulp in close contact with B220⁺ B cells, and only 20% of neutrophils in contact with CD3⁺ T cells. The changing frequency of neutrophil contacts with splenic lymphocytes over the course of disease suggests that the functional impact neutrophils have on splenic lymphocyte populations changes over the course of disease.

Analysis of the splenic neutrophil transcriptional program over the course of lupus progression

Because we observed significant changes in neutrophil frequency and localization relative to splenic B and T leukocytes in the spleen over the course of disease, we hypothesized that neutrophil function changes with disease progression. Because very little is known about the neutrophil effector program within the SLO in autoimmunity, we employed RNA sequencing as an unbiased approach to assess whether splenic neutrophils display a regulatory or pro-inflammatory transcriptional program, as well as determine whether this program changes over the course of disease. Neutrophils were pre-enriched and FACS sorted from NZB/W splenocytes at three time points, 14 weeks (early disease), 20 weeks (mid-disease), and 26 weeks (advanced disease)¹⁴⁷. Neutrophil mRNA was isolated and the transcriptome sequenced using an Illumina platform.

Principal components analysis of the entire neutrophil transcriptome revealed clear separation of neutrophils isolated at the three disease time points, indicating significant

changes in the neutrophil transcriptional program with lupus progression (Fig. 2.3A). Differential expression analysis (using a significance cutoff of $FDR < 0.05$) revealed several transcripts of immunologic interest (Fig. 2.3B), including $TNF\alpha$ (Tnf) and BAFF (Tnfsf13b), factors known to promote B cell auto-reactivity in SLE^{29, 148, 149}. Interestingly, these two factors show a relative decrease at 20 weeks of age, followed by an increase with more advanced disease (26 weeks) (Fig. 2.3B). Concomitantly, between 14 and 20 weeks, we observed an increase in pro-resolving and anti-inflammatory mediators including PD-L1 (Cd274), IL-1RA (Il1rn), and $TGF\beta$ (Tgfb1) (Fig. 2.3B). These results suggest that, during the onset and acceleration of auto-antibody production in NZB/W mice between 14 and 20 weeks of age, splenic neutrophils adopt a more regulatory transcriptional program.

The increase in protective, anti-inflammatory mediators by 20 weeks coincides with an increase in Stat3 and Socs3, transcription factors involved in induction of an anti-inflammatory neutrophil phenotype in response to prolonged exposure to an inflammatory cytokine milieu^{150, 151}. It is notable that expression of genes associated with restraining inflammation, including PD-L1 (Cd274), IL-1RA (Il1rn), and $TGF\beta$ (Tgfb1) is down regulated between 20 and 26 weeks. Concomitantly, genes involved in neutrophil responses to inflammation and activation increased during this time frame. This includes the alarmins S100a8 and S100a9¹⁵², which have largely although not exclusively been associated with proinflammatory functions^{153, 154}. The interferon regulated chemokines, CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), important for T cell chemoattraction and activation were also significantly up-regulated¹⁵⁵ at 26 weeks (Fig. 2.3B). Based on these findings, we hypothesize that neutrophils acquire a protective

transcriptional program between 14 and 20 weeks of age during the initial rise in auto-reactivity in NZB/W mice, but that the neutrophil phenotype shifts to a more activated state by 26 weeks (advanced disease).

Depletion of neutrophils after auto-reactivity is established does not alter disease

In order to examine whether neutrophils exert predominantly protective or deleterious effects in modulation of auto-reactivity in lupus, we depleted neutrophils continuously in female NZB/W mice using an anti-Ly6G (1A8) antibody or isotype control (polyclonal rat IgG) over a period from 25-30 weeks of age. Anti-Ly6G injection effectively depleted neutrophils, as quantitated by FACS analysis of splenocytes isolated at sacrifice (30 weeks) (Fig. 2.4A). However, neutrophil depletion from 25-30 weeks of age did not result in a significant alteration in the progression of disease, including proteinuria, serum anti-dsDNA levels, splenomegaly, or germinal center formation (Fig. 2.4B-E). These results suggest that neutrophils are not required for progression of autoimmunity once active disease is well established.

Depletion of neutrophils during onset of lupus accelerates development of autoimmunity

Although neutrophil depletion during established disease did not significantly alter the course, we next assessed whether neutrophils modulate adaptive immune responses at early stages of lupus. We depleted neutrophils as above, continuously in NZB/W mice over a 12-week period, from 14-26 weeks of age. The efficacy of depletion was assessed by FACS analysis of splenocytes (Fig. 2.5A), peripheral blood, and bone marrow (Supplemental Fig. 1A, 1B) isolated at sacrifice. Following anti-Ly6G treatment, mice

exhibited an early and significant rise in plasma anti-dsDNA levels compared to isotype treated mice and developed proteinuria at an accelerated rate (Fig. 2.5B, 5C). Comparison of kidney histopathology did not reveal a statistically significant difference between anti-Ly6G- and isotype-treated groups (data not shown). Depletion during this 12-week period also resulted in significantly increased splenomegaly (Fig. 2.5D) and enhanced germinal center formation (Fig. 2.6A, 6B).

In order to assess whether neutrophil depletion alters the frequency of other immune cells, FACS analysis was used to quantitate the frequency and absolute numbers of M1 and M2 macrophages, GC B cells, T follicular helper cells (Tfh), and FoxP3+ regulatory T cells (Tregs). Consistent with the finding that GC formation is significantly augmented with anti-Ly6G treatment, we found a significant increase in numbers of GC B cells and frequency of Tfh cells following neutrophil depletion. The increase in Tfh frequency was not a global effect on T cells as there was no significant change in FoxP3+ Treg frequencies or absolute numbers in the spleen (Supplemental Fig. 2A). We also did not observe significant alterations in frequency of monocyte/macrophage populations or a shift in polarization to an M1 versus M2 phenotype following neutrophil depletion (Supplemental Fig. 2B, 2C). Together these results suggest that neutrophil depletion during the onset of lupus results in acceleration in the progression of auto-reactivity and magnitude of the adaptive immune dysregulation.

Because the accelerated rise in serum anti-dsDNA levels seen with neutrophil depletion (Fig. 2.5C) may be attributable to either antibody production or antibody clearance (including precipitation of immune complexes), we quantitated the production

of both total IgG and anti-dsDNA IgG antibody secreting cells (ASC) directly by ELISPOT. Notably, total IgG and anti-dsDNA ASC increase significantly in spleen, kidney, and bone marrow following anti-Ly6G treatment (Fig. 2.7A-D). Additionally, there was enrichment in auto-reactive anti-dsDNA IgG ASC as a fraction of total IgG ASC in the bone marrow (Fig. 2.7E), suggesting that neutrophil depletion beginning at the onset of autoimmunity results in a systemic increase in auto-reactive B cells. Overall, these results indicate that neutrophils have a protective function during the early stages of lupus, but that this protective effect is lost by advanced stages of disease.

2.5 Discussion

Neutrophils play a key role in the innate immune response to infections, but have increasingly been recognized to contribute to autoimmunity in diverse ways. Somewhat unexpectedly given the dominance of literature (mostly *in vitro*) supporting a pro-inflammatory role for neutrophils in lupus^{48, 62, 138}, we find that depletion of neutrophils early in murine SLE profoundly accelerates disease. This was evidenced by significant enhancement of splenic GC reactions and generation of autoantibody secreting cells in SLO. Notably, autoantibody secreting cells accumulated in target tissue (kidney) as well as the BM suggesting systemic effects of neutrophil absence on the autoimmune process. The findings that late neutrophil depletion had no impact on disease, shift in splenic neutrophil interactions from T cells to B cells, and change in transcriptional profile over the course of the disease highlights the plasticity in neutrophil roles in SLE. Our results indicate a dominantly suppressive effect for much of the disease likely mediated by direct

interaction with T and B cells, whereas neutrophils acquire a more activated phenotype later in disease.

The finding that B cell autoimmunity is profoundly accelerated by neutrophil depletion starting early in disease strongly implicates a direct role for neutrophils in modulating the adaptive immune response in SLE. Although neutrophils have been shown to act in an immunosuppressive capacity during chronic inflammatory conditions such as malignancy or viral infection, the possibility that a pro-resolving or protective neutrophil is present in lupus has only recently been proposed^{78, 80, 81, 82, 105, 106}. In an amyloid-induced lupus model neutrophil depletion worsened disease similar to our findings. A regulatory effect for neutrophils was suggested to be mediated by ROS inhibition of NK cell IFN γ production at local sites of inflammation¹⁰⁶. Another study demonstrated that Gr-1^{hi}CD11b⁺ splenocytes suppress lupus pathogenesis in young male but not female NZB/W mice based on the finding that administration of anti-Gr-1 antibody *in vivo* increased anti-dsDNA antibody titers¹⁰⁵. Limitations of this study include the use of anti-Gr-1 antibody, which depletes both neutrophils and some monocyte populations^{156, 157, 158}. Ours is the first study in a well-accepted murine lupus model demonstrating that specific (anti-Ly6G) depletion of neutrophils accelerates disease via promotion of GC reactions.

Nonetheless, this finding should be considered in the context of literature implicating neutrophils as exerting a highly pathogenic impact on loss of tolerance in certain contexts. Neutrophils in both peripheral blood and bone marrow have been shown to express elevated amounts of BAFF, a factor known to underlie failure of auto-reactive B cell anergy^{29, 64, 65, 159}. Along these lines and in contrast to our findings, neutrophil depletion in a

B6.Fas^{lpr}/J/*Tnfrsf17*^{-/-} autoimmune model resulted in significant amelioration of autoimmunity, including reduced serum anti-dsDNA titers and splenic plasma cell frequency. This result depended on splenic neutrophil production of BAFF, which was shown *in vitro* to stimulate CD4⁺ T cell production of IFN γ —a critical mediator of auto-reactive germinal center formation^{64, 142}. The contrasting results in this model may be attributable to the strong dependence of the lupus phenotype on specific immune pathways, such as BAFF signaling. We do not rule out the possibility that neutrophils can augment the progression of auto-reactivity in the NZB/W model, particularly during advanced disease, where we observed a notable rise in transcription of BAFF and TNF α between 20 and 26 weeks. It is also possible that other neutrophil functions in addition to pro-inflammatory cytokine secretion, such as NETosis, are dominantly pro-inflammatory. On the other hand, it is interesting to note that when NETs were inhibited in the NZM lupus model vascular damage was ameliorated but anti-DNA antibodies actually did rise^{74, 75}, suggesting that even NETosis may have unexpected anti-inflammatory effects¹⁶⁰. It is also important to consider that the lack of effect of neutrophil depletion from 25-30 weeks of age in our experiments may be attributable to neutrophil effects being overwhelmed by a strong immune response and rapidly worsening autoimmunity in this period. Nonetheless, our findings clearly demonstrate that neutrophils are non-essential for progression of both auto-reactivity and tissue pathology during this time frame, regardless of the precise mechanisms of contribution to the disease.

Our analysis of neutrophil contacts in the spleen reveals that neutrophils are preferentially localized near T cells early in disease, with a shift toward predominant B cell

interactions with advanced disease. These data, in conjunction with the results of neutrophil depletion, suggest that neutrophils play a protective role via interaction with splenic T cells. Prior studies have investigated the effects of splenic neutrophils on lymphocyte proliferation and effector function *in vitro* but with apparently conflicting results. Thus, Triguñaite et al. reported that NZB/W splenic Gr-1^{high}CD11b⁺ cells suppressed B cell differentiation to antibody secreting cells *in vitro*, suggesting that splenic neutrophils play a protective role and consistent with worsening disease upon *in vivo* anti-Gr-1 antibody administration in male mice. Interesting differences were noted in the mechanisms of suppression by neutrophils isolated from young male vs. female mice, with an ROS and NO dependence only in neutrophils from female mice. Similar to our study, a plasticity in neutrophil function was suggested by the loss of the suppressive phenotype in neutrophils from older mice ¹⁰⁵. This same group also described Gr-1⁺ cell inhibition of Tfh differentiation *in vitro* and correspondingly increased Tfh with *in vivo* depletion of Gr-1⁺ cells, although the specificity of these findings to a neutrophil population is undetermined ¹⁶¹. In other studies, co-culture of neutrophils with T cells impaired Treg differentiation and promoted Th17 differentiation ⁹⁰, leaving an open question as to whether neutrophils are predominantly immunosuppressive. Additionally, it is unclear to what extent these *in vitro* assays recapitulate neutrophil-lymphocyte interactions *in vivo*. In particular, the results of *in vitro* culture of neutrophils are confounded by the fact that neutrophils are easily activated when isolated and cultured *ex vivo*, and thus neutrophil respiratory burst or neutrophil cell death *in vitro* may underlie the observed suppression of splenic lymphocyte proliferation. Our results significantly add to the literature by providing the first *in vivo*

evidence for a dominant inhibitory effect for neutrophils in lupus with significant increases in Tfh and GC B cells upon early neutrophil depletion. These data are compatible with neutrophil inhibition of Tfh differentiation, but it is also possible that inhibition of B cell differentiation plays a role given the contacts between neutrophils and B cells throughout the disease course.

Our results highlight that the influence of neutrophils on adaptive immune dysregulation depends on both the specific cell encounter and the phenotype of the neutrophil. This may have parallels in other chronic inflammatory conditions such as HIV infection, cancer, and hepatitis B, where neutrophils can adopt a granulocytic myeloid-derived suppressor cell (gMDSC) phenotype^{104 81, 82}. In addition to ROS production, neutrophils may acquire a suppressive phenotype via production of a wide variety of soluble and cell surface mediators, including Il-10 and nitric oxide^{94, 95, 96, 101, 102, 103}. Our work expands the potential mechanisms by which neutrophils regulate adaptive immune responses in SLO, including TGFβ1, IL-1RA, and PD-L1. PD-L1 is particularly interesting in light of its association with a gMDSC phenotype and a recent report demonstrating significant elevation in PD-L1 expressing neutrophils in human SLE, correlating with disease activity¹¹⁴. PD-L1 delivers inhibitory signals to activated T and B cells upon ligation of PD-1, and thus may provide a critical negative feedback mechanism to dampen autoimmune responses. Of note, Tfh express PD-1, and PD-L1-PD-1 interactions have been specifically shown to negatively regulate Tfh expansion¹⁶². Further, blocking PD-L1 in the NZB/W lupus mouse model increased splenic PD-1+ T cells, anti-DNA levels, and nephritis¹⁶³. Based on our data, we hypothesize that PD-L1 expression by splenic

neutrophils and inhibition of Tfh may be a key mechanism by which neutrophils suppress lupus disease development.

In conclusion, our findings demonstrate the plasticity of neutrophil phenotype and function in terms of both transcriptional profile in SLO and changing spatial localization relative to B and T cells, dependent on the stage and severity of the disease. In turn, this neutrophil plasticity has dramatic effects on B cell autoimmunity, with surprisingly dominant regulatory effects as evidenced by the promotion of GC reactions and auto-reactive PC generation in the setting of neutrophil depletion. Despite the apparent complexity of neutrophil contributions to the outcome of autoimmunity, it is increasingly clear that neutrophils can exert a strong influence on adaptive immune dysregulation in lupus, highlighting the need for further investigation into the factors that may tip the balance in neutrophil acquisition of a pro-inflammatory versus regulatory phenotype.

2.6 Figures

Figure 2.1. Neutrophils accumulate in the spleen over the course of lupus progression.

(A) Representative FACS plots showing frequency of Ly6G+CD11b+ neutrophils as a fraction of CD45+ splenocytes. (B) Quantitation of frequency (of CD45+Live) and numbers of Ly6G+CD11b+ splenocytes cells by FACS (n=4-5mice/group). C57BL/6 mice are 15-20-week old female mice in (A) and (B). (C) Representative IHC of spleen showing neutrophil localization relative to the marginal zone (MOMA+). 200x Mag. Analysis was done as described in Materials and Methods. n=5 mice per group. *p<0.05, **p<0.01 by unpaired Student's t-test. Data shown are the mean $\pm$ s.e.m.

Figure 2.2. Neutrophil contacts with splenic B and T lymphocytes in NZB/W spleen change over the progression of lupus. (A) Representative IHC showing neutrophil proximity to B220+ cells and CD3+ cells in lupus spleen at several stages in the progression of lupus. Insets show neutrophil localization in relation to CD3+ and B220+ cells. (B) Quantitation of neutrophils in contact with B220+ B cells and CD3+ T cells as a frequency of Ly6G+ cells per 200x field. Analysis was performed as described in Materials and Methods. n=5 mice per group. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney U-test comparing

frequency of neutrophils in contact with B220+ B cells and frequency of neutrophils in contact with CD3+ T cells. Data shown are the mean $\pm$ s.e.m.

Figure 2.3. Transcriptional profile of splenic neutrophils is consistent with acquisition of a protective phenotype as auto-reactivity accelerates and loss of this phenotype in advanced disease. (A) Principle components analysis reveals differential gene expression (cutoff >1 , norm (rlog) expression) over the course of disease. (B) Differential expression analysis was conducted for 130 select genes of immunologic interest based on neutrophil function and regulation of immune responses using deSeq. Expression heat map of normalized and rlog transformed mRNA counts quantitated via RNAseq for select genes that were differentially expressed among the 3 experimental groups (significance cutoff, $FDR < 0.05$, ANOVA (Kruskal-Wallis). $n=6$ /group).

Figure 2.4. Continuous depletion of neutrophils from 25-30 weeks of age (established disease) does not alter lupus disease progression. (A) Quantitation of the efficacy of neutrophil depletion by FACS analysis of the frequency of Gr-1hiCD11bhi splenocytes at sacrifice (30 weeks of age) following 5 weeks of treatment with anti-Ly6G (1A8) or isotype control. (B) Progression of renal disease was assessed by quantitation of proteinuria. Scoring: 0.5=trace, 1=0.3g/L, 2=1g/L, 3=3g/L, 4>20g/L. (C) Quantitation of serum anti-dsDNA IgG by ELISA at sacrifice. (D) Splenomegaly was assessed at sacrifice. (E) Splenic germinal center size & number were quantitated by immunohistochemistry as described in Materials and Methods. n=10 mice per group. Outliers $> \bar{x} \pm 2\sigma$ were excluded from analysis in (D) and (E). 'ns'=not significant, ***p<0.001 by Mann-Whitney U-test. Data shown are the mean $\pm$ s.e.m.

Figure 2.5. Continuous depletion of neutrophils from 14-26 weeks of age (disease onset) accelerates development of auto-immunity. (A) Quantitation of the efficacy of neutrophil depletion by FACS analysis of the frequency of Gr-1^{hi}CD11b^{hi} splenocytes at sacrifice (26 weeks of age) following 12 weeks of treatment with anti-Ly6G (1A8) or isotype control. (B) Progression of renal disease was assessed by quantitation of proteinuria. Scoring: 0.5=trace, 1=0.3g/L, 2=1g/L, 3=3g/L, 4>20g/L. (C) Serum anti-dsDNA was quantitated by ELISA at baseline (immediately before depletion began), and after 4, 6, 12 weeks of antibody treatment. (D) Splenomegaly assessed at sacrifice. Outliers $> \bar{x} \pm 2\sigma$ were excluded from analysis in (D) n=10 mice per group. *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U-test. Data shown are the mean $\pm$ s.e.m.

Figure 2.6. Germinal center formation is greatly accelerated with neutrophil depletion during the onset of autoimmunity. (A) Representative IHC of splenic germinal center formation in the isotype-treated versus anti-Ly6G-treated mice. 200x Mozaix. (B) IHC analysis was used to quantitate the extent of splenic germinal center formation. (C-D) FACS analysis was used to assess the frequency and absolute numbers of TFH cells and germinal center B cells (gating as described in Mat. & Met.). N=10 mice per group (B-D). Outliers $> x+2d$ were excluded from analysis in (C) and (D). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Mann-Whitney U-test. Data shown are the mean + s.e.m.

Figure 2.7. Changes in frequency of anti-dsDNA⁺ and IgG⁺ antibody secreting cells (ASC) in spleen, kidney, and bone marrow. ELISPOT quantitation of (A) absolute number of IgG⁺ ASC per whole tissue, (B) absolute number of anti-dsDNA⁺ IgG⁺ ASC

per whole tissue, (C) frequency of IgG+ ASC per 10⁶ cells, (D) frequency of anti-dsDNA+IgG+ ASC per 10⁶ cells. (E) Anti-dsDNA IgG-secreting cells as a fraction of the total IgG-secreting cells in bone marrow. n=10 mice per group. Outliers $> \bar{x} \pm 2\sigma$ were excluded from analysis. Zero value data points are not shown on graphs due to log scale but were included in the analysis. *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney U-test. Data shown are the mean $\pm$ s.e.m.

CHAPTER 3 Changes in the Transcriptomic Profile of Splenic Neutrophils over the Course of Lupus Progression in the NZB/W model

3.1 Abstract

Neutrophils have been traditionally characterized as short-lived effector cells that participate in the innate phase of the immune response. However, a growing literature has identified neutrophils' capacity to tailor their transcriptional and phenotypic program to respond to a specific inflammatory milieu and impact adaptive immune responses. Neutrophil functional plasticity can accelerate or dampen an ongoing immune response, particularly in chronic inflammatory conditions including malignancy and chronic viral infection. However, little is known about the neutrophil transcriptional changes over the course of autoimmunity. Here we used RNA sequencing to characterize the changes in transcriptional program of splenic neutrophils at several phases of disease progression in a murine model of systemic lupus erythematosus (NZBWF1). Over the course of disease, we identify complex changes in transcription of multiple immune mediators such as lymphotoxin-beta, programmed death ligand 1, and macrophage colony stimulating factor, suggesting that the neutrophil effector program undergoes substantial changes over the course of lupus progression. Changing gene networks include pathways associated with TLR signaling, the inflammasome pathway, chemokines, and ROS generation, and several novel pathways, such as metabolism and lipid mediators. Additionally, we find that at both early and late stages of lupus, the splenic neutrophil population shows enrichment of transcripts associated with immature neutrophils, similar to pathogenic neutrophil subsets observed in human lupus patients. We use analysis of

changes in genes associated with neutrophil maturation state to provide functional predictions associated with neutrophil maturation state. Based on this analysis, we predict that neutrophil maturation stage may be a significant determinant of neutrophil immunologic function. Overall, our analysis highlights the importance of disease stage and severity on neutrophils' transcriptional program and imply that the changing neutrophil effector program may be a significant contributor in the loss of immunologic self-tolerance in lupus.

3.2 Introduction

Neutrophils tailor their effector program to specific inflammatory states by deploying a variety of both pro-inflammatory or immunosuppressive mediators to coordinate both the acute and resolution phases of the immune response^{164, 165, 166, 167, 168, 169}. This plasticity in neutrophil function in response to different types of inflammation is mediated in part by rapid changes in transcriptomic program that are specific for the inflammatory stimulus encountered¹⁷⁰. While neutrophil transcriptomic changes have been best characterized in the context of acute response to injury and infection, the neutrophil transcriptional response to chronic inflammation such as occurs in autoimmune diseases such as systemic lupus remains relatively unexplored.

A current conflict in the lupus literature persists regarding whether neutrophils are protective or deleterious within various pathways of lupus pathogenesis. Neutrophils in lupus mediate tissue damage and organ dysfunction and have thus been identified as a potential therapeutic target^{62, 68}. In contrast, recent literature has implicated neutrophils as

having protective or immunosuppressive function, particularly in relation to loss of tolerance by the adaptive immune compartment^{90, 105, 106}. In accord with a regulatory function for neutrophils in SLE, we recently described that depletion of neutrophils from an early disease time point contributes to a loss of B cell tolerance, with accelerated GC formation in the spleen, auto-reactive plasma cell generation, auto-antibody production, and progression of end-organ disease (nephritis) (Chapter 2). Furthermore, we identified neutrophils in contact with both B and T lymphocytes in the spleens of autoimmune (NZB/W) mice. T cell interactions predominated earlier in disease and combined with the expansion of T follicular helper (TFH) and GC B cells upon early neutrophil depletion, led us to speculate that neutrophils may mediate regulation of disease via TFH suppression. However, the precise mechanisms by which neutrophils affect adaptive immune responses in SLE are not clear. Here, we delineate how the neutrophil transcriptional and phenotypic program changes over the course of lupus progression may offer in order to gain insight mechanisms of disease suppression vs. activation. Additionally, this analysis provides insight into conflicting literature on neutrophil contributions to lupus by predicting neutrophil phenotypic changes over the course of lupus progression.

Although the importance and origins of immunosuppressive neutrophils in lupus remains unclear, the observation that neutrophils can adopt an immunosuppressive phenotype is now well established in a variety of other inflammatory states, particularly in chronic inflammatory diseases such as malignancy, chronic viral (e.g. HIV, HBV) infection, multiple sclerosis, and rheumatoid arthritis^{78, 79, 80, 81, 82, 171, 172}. Several recent publications have suggested that granulocytic myeloid-derived suppressor cells (gMDSC)

occur in lupus, although this literature has provided differing conclusions on whether gMDSC are protective or deleterious toward loss of self-tolerance^{173, 174, 175, 176}. The effector program of immunosuppressive neutrophils has most typically been characterized by production of reactive oxygen species (ROS), IL-10, and nitric oxide (NO), as well as arginine depletion via arginase I activity¹⁷⁷. These factors enable neutrophils to suppress proliferation and cytokine production by T cells and NK cells¹⁷⁷, thereby exerting a potent influence on the outcome of malignancy and infection^{178, 179, 180}. Neutrophil production of pro- and anti-inflammatory mediators in lupus has not been fully profiled, particularly with respect to different stages of disease.

Here, we use RNA sequencing of splenic neutrophils at three stages of autoimmunity in the SLE prone female NZB/W murine model, early disease as autoantibodies are beginning to arise (14 weeks), mid-disease at a point of high autoantibodies but pre-nephritis (20 weeks), and late disease when autoantibodies have peaked and nephritis is accelerating (26 weeks) in order to better understand how the neutrophil phenotype changes in response to progressing autoimmunity. We also conducted an analysis of enrichment for gene signatures associated with neutrophil maturation status, which we predict underlies transcriptomic differences in neutrophils profiled at different stages of lupus.

3.3 Methods

Mice

Female NZBWF1/J mice were obtained from The Jackson Laboratory and housed in pathogen-free conditions. Mice were sacrificed at 14, 20, or 26 weeks of age. All animal experiments were conducted in accordance with the policies established by the Institutional Care and Use Committee at the University of Rochester.

Neutrophil purification

Fresh murine spleens were disaggregated mechanically and splenocytes immediately placed on ice. To obtain highly purified neutrophils, total splenocytes were loaded onto a percoll discontinuous density gradient and pre-enriched by neutrophil negative selection (Miltenyi). The resulting neutrophil enriched-cell population was then FACS sorted on DAPI-Ly6B^{hi}Ly6C^{int}CD11b^{hi}Ly6G^{hi} cells with doublet and dead (DAPI+) cell exclusion to obtain a purity of $\geq 97\%$ CD11b^{hi}Ly6G^{hi} cells, as determined by post-sort analysis (Appendix B, Figure S1). Isolated neutrophils were immediately placed in Qiagen RLTPlus buffer. Cells were kept at 4°C throughout isolation.

RNA purification and RNA sequencing

Neutrophil total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen). RNA quality was assessed with the Agilent Bioanalyzer 2100 (Agilent). 1ng of cDNA was used to generate Illumina compatible sequencing libraries with the NexteraXT (Illumina) library preparation kit. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina) at a concentration of 10pM per lane. Single-end reads of 100nt were generated for each sample and aligned to the organism specific reference genome. Sequenced reads were cleaned using Trimmomatic-0.32 before

mapping to the mouse reference genome (GRCm38.p4) with STAR-2.4.2a. Raw read counts were obtained using HTSeq and gencode M6 mouse gene annotations. DESeq2 1.12.3 was used to perform data normalization for data shown in heatmaps.

Bioinformatic analysis

Sequenced libraries were screened for quality and were filtered if fewer than 8000 genes were detected, which resulted in the removal of one library. Genes were filtered if fewer than 2 libraries expressed fewer than 5 counts, resulting in a total of 13058 genes in 17 libraries. Normalization and differential expression analysis was performed using the Limma package in R with voom quality weights. MultiExperiment Viewer software was used for creating heat maps and performing cluster analysis. Volcano plots were created using R and pathway analyses were conducted using Ingenuity Pathway Analysis Software and plotted using JMP Pro 13. Construction of gene signature transcriptional profiles was conducted using NCBI GEO data sets, as described below. All DEG lists derived from the differentially expression analysis of these GEO data sets included >150 genes.

3.4 Results

The splenic neutrophil transcriptional program changes substantially over the course of lupus progression

Neutrophils were purified *ex vivo* from the spleens of untreated female NZB/W mice at 14, 20, and 26 weeks of age (Fig 3.1A). RNA sequencing (Illumina) was conducted, and resulting transcriptomic data were cleaned and normalized, as described in the 3.3 Methods. The full transcriptomic data set is publically available as a NCBI GEO

Dataset (Accession GSE97439). To determine whether the neutrophil transcriptomic program shows significant differences over the course of lupus progression, neutrophil RNAseq libraries from the three disease points (14, 20, and 26 weeks of age) were compared by hierarchical clustering of the Pearson correlation matrices. This analysis reveals that the transcriptomes of splenic neutrophil populations cluster together on the basis of age (Fig 3.1B), indicating that neutrophils at each disease stage have a unique transcriptional profile.

Next we considered whether other measures of disease progression besides age may better predict the transcriptional changes in splenic neutrophils. Although lupus in the NZB/W model develops spontaneously according to a predictable time line, there is heterogeneity in the disease onset and rate of progression of auto-antibody production and nephritis. Besides age, several immunologic endpoints can be used to gauge disease progression, most commonly auto-antibody serum titers (cf. Chapter 2). We used a regression model to assess the degree to which the neutrophil transcriptomic program is associated with serum anti-dsDNA levels versus murine age alone.

Specifically, we considered an *unadjusted* one-factor ANOVA with “week” as an explanatory variable, and an *adjusted* analysis of covariance regression model with “week” and log2-transformed anti-DNA levels. We identified differentially expressed genes by considering twice the lesser of the adjusted and unadjusted moderated F-statistics at an FDR of 10%. This resulted in 3057 genes being identified as differentially expressed in one, or the other model. A volcano plot of the week 20-14 and week 26-20 log2-fold changes and p-values (t-test) are shown in Appendix B, Figure S2. These complementary

models returned generally similar estimates for the “week” effects. Importantly, relatively few genes showed significant associations with anti-DNA after removing the effect of “week,” suggesting a limited independent role of anti-DNA on the transcriptional phenotype. This result suggests that disease-related changes in the neutrophil transcriptome are most informatively described as a function of murine age, and thus additional differential expression analysis will focus on the 3 age groups.

Differential expression analysis reveals changes in genes involved with multiple diverse immunologic pathways including innate immune responses (TLR, PRR, inflammasome), cytokine signaling, and metabolic activity

In order to profile the changes in the neutrophil transcriptomic program over the course of lupus, we conducted a differential expression analysis (using the Limma moderated t tests), comparing splenic neutrophil populations between the 14- and 20-week time points, and between the 20- and 26-week time points. We find that a majority of differentially expressed genes (DEG) follow a v- or $\wedge$ -pattern (i.e. genes rising from 14 to 20 weeks and then decreasing from 20 to 26 weeks or vice versa), whereas transcripts changing in a monotonic pattern (i.e. genes rising from 14 to 20 weeks and rising again from 20 to 26 or vice versa) constitute a minority of DEGs. This is reflected in the Venn diagram (Figure 3.2A) which shows between 267 DEGs up-regulated between 14 and 20 weeks and 635 DEGs up-regulated between 20 and 26 weeks with only 15 genes overlapping. Similarly, there are 340 DEGs down-regulated between 14 and 20 weeks and 387 genes down-regulated between 20 and 26 weeks, with only 63 overlapping. DEG from the adjusted regression model are shown in parentheses in the Venn diagram, and indicate

patterns of gene expression change (i.e. a majority of differentially expressed genes (DEG) follow a v- or $\wedge$ -pattern) similar to the unadjusted model (Fig. 3.2A). This finding suggests that neutrophils at the 20 week time point are unique, which is consistent with our observation that autoimmune disease in the NZB/W model, often follows an arc, with splenomegaly, germinal center formation and auto-antibody production often peaking by 20-24 weeks (d.n.s. and Appendix B, Figure S3). However, as end organ pathology (nephritis) in this model progresses (24 weeks and beyond), a decline in immune function can be observed.

Ingenuity pathway analysis was conducted using all DEG (FDR 10%) from the 14-20 and 20-26 week comparison (DEGs shown in “volcano” plots Figure 3.2B). A cutoff was applied on the resulting pathways, and any pathways with ≤ 5 genes associated with each DEG set were eliminated. The remaining Top 35 pathways most significantly associated with the DEG sets (Fig. 3.2 C,D) show associations with a number of immunologic pathways, including cytokine signaling, adhesion and extravasation pathways, and pattern recognition receptor (PRR) signaling including TLR and inflammasome in both the 14-20 week and 20-26 week comparisons. The changes in these pathways is consistent with changing neutrophil responsiveness to PRR ligands and expression of inflammatory mediators (discussed further below). Additionally, pathways associated with nuclear receptor activity including LXR/RXR (liver x receptor and retinoic acid receptor), VDR (vitamin D receptor)/RXR, and PPAR (peroxisome proliferator-activated receptor) /RXR change significantly from 14 to 20 weeks (Fig 3.2C). The most significant nuclear receptor pathway in the 14-to-20 DEG analysis is the ‘LXR/RXR

activation' pathway, the activity of which is predicted to decline from 14 to 20 weeks. LXR signaling is generally thought to be anti-inflammatory; murine deficiency of LXR α and β causes a defect in apoptotic cell clearance, resulting in lupus^{182, 183}.

The top pathways in the 20 to 26 week comparison (Fig 3.2D) also include PRR and cytokine signaling but are uniquely characterized by pathways associated with energy metabolism, including significant changes in transcription of genes associated with oxidative phosphorylation, mitochondrial dysfunction, translation, cell cycle, and cell motility.

Immunologic pathways associated with immunosuppressive functions show significant changes over the course of lupus progression

We have previously demonstrated that neutrophil depletion in the NZB/W SLE mouse model starting early in disease (14 weeks) significantly accelerates disease (Chapter 2). In contrast, depletion late in disease (25 weeks) has no impact. Thus, we hypothesized that neutrophils may have high expression of immunosuppressive or pro-resolving mediators early in disease, with loss of these factors later in disease. One of the mechanisms by which neutrophils can suppress NK cell, T cell, and B lymphocyte proliferation and function (e.g. antibody production¹⁰⁵, Treg differentiation⁹⁰, IL-15 generation¹⁰⁶) is via ROS production. Transcription of enzymes involved with neutrophil generation of ROS follow a pattern consistent with this hypothesis, with Cybb [NOX2], Ncf1 [p47phox], and Pik3cg [PI3Kgamma] (Fig3.3A)—whose protein products facilitate ROS generation upon neutrophil activation, expressed at their highest early in disease and

decline with disease progression¹⁸⁴. Concomitant with this decline, expression of *Sod2* [Superoxide dismutase] and *Cat* [Catalase]—enzymes responsible for neutralizing ROS—rise with disease progression (Figure 3.3A), suggesting that neutrophil capacity for ROS production is high early in disease, but declines with disease progression.

Because neutrophils also regulate inflammation via production of pro-inflammatory and pro-resolving lipid mediators, we more closely examined expression changes in enzymes in the eicosanoid synthetic pathway. Of note, this was one of the top 35 pathways in the ingenuity analysis. Transcription of enzymes including *Ptgs2* (COX2), *Pla2g7* [Lp-PLA2], and *Pla2g15* [LYPLA3], enzymes responsible for synthesis of inflammatory lipid mediators is lowest at 14 weeks, significantly increases at 20 weeks, and then remains overall unchanged with disease progression (Fig. 3.3A). Additionally, *Alox5* [5-LOX] is highly expressed early in disease with *Alox5ap* [FLAP] rising with advanced disease. These enzymes have multiple functions and may be either pro- or anti-inflammatory depending on the context^{185, 186}. *Alox8*, *Alox12*, *Alox15*, and *Alox15b* were not differentially expressed at FDR 10% (Limma F-test) (d.n.s). The significant changes in expression of enzymes in the eicosanoid synthetic pathway was concomitant with an IPA-predicted decrease in the PPAR signaling pathway from 14 to 20 weeks of age (Fig 3.2C).

As genes associated with ROS production decrease, pro-inflammatory eicosanoids increase, and PPAR signaling decreases (all predicted to be associated with a transition from pro-resolving to inflammatory functions), genes in the PRR pathway increase (Fig 3.3A). This is depicted in the IPA signaling schematic shown in Figure 3.3B, where it can be clearly seen that an end result is activation of NFκB and transcription of pro-

inflammatory cytokines (c.f. below). With the rise expression of NFκB target genes (e.g. *Csf-1* [M-CSF], *Il1b* [IL-1β], and *Ltb* [lymphotoxin- β]^{187, 188, 189, 190}) between 14 and 20 weeks (Fig. 3.4A) is a concomitant IPA-predicted decrease in PPAR signaling. Changes in PPAR signaling may account for many of the transcriptional changes observed between 14 and 20 week splenic neutrophils. The PPARα and PPARγ transcription factors mediate inflammatory resolution and actively inhibit NFκB signaling under homeostatic conditions^{191, 192, 193}. Although neither *Ppara* nor *Pparg* transcripts show differential expression with lupus progression (d.n.s.), IPA predicts a net decrease in PPAR signaling between 14 and 20 weeks of age, based on a decrease in transcription of *Ncor1*, which inhibits NFκB¹⁹⁴, as well as an increase in transcription of both *Fos* and *Jun* [AP-1], Ap-1 target *Ptgs2* [COX2]¹⁹⁵, *Nfkb2* [NFκB] (Fig. 3.3A) and NFκB target genes¹⁹⁴.

Neutrophil transcription of immunologic mediators changes significantly over the course of lupus progression

In order to provide a broad profile of the transcription of additional immunologic mediators of interest in splenic neutrophils, as well as expression changes over time, a curated gene list was constructed (Fig. 3.4). Genes were chosen for analysis based on either their disease-relevance based on the current literature (e.g. *Tnfsf13b* [BAFF] and *Tnf* [TNFα]) or significant differential expression in at least one disease point comparison (e.g. chemokines). Also included here are several factors of immunologic interest that were expressed at levels above the expression cutoff (cf 3.3 Methods), but were not differentially expressed at FDR10% in the time point comparison. These factors include *Tnfsf13b* [BAFF], *Il6* [IL-6], and *Tnf* [TNFα] (Fig. 3.4A). Additionally, several inflammatory

mediators of immunologic interest, including IFN-I and IFN-II genes were nulls in this data set. Mediators of interest shown in Fig. 3.4 are categorized as predominantly pro-inflammatory or regulatory in order to discern functional pattern changes. However, the true in vivo effects of these mediators is likely highly dependent on a number of factors including the cell type encountered.

Splenic neutrophil expression of inflammatory mediators change over the course of disease, with a significant increase of *Csf1* [M-CSF], *Il1b* [IL-1 β], *Cd274* [PD-L1], and *Ltb* [Lymphotoxin- β] (*Tgfb1* was differentially expressed at FDR 14% [Limma F-test]) between 14 and 20 weeks (Fig3.4A,B). *Cd274* [PD-L1] is one of the most significantly upregulated genes at the 20 week time point and is interestingly known to be an anti-inflammatory neutrophil mediator in other contexts such as malignancy. There were also significant decreases in *Aldh2* [RALDH2] over this early to mid-disease time frame, an enzyme responsible for retinoic acid synthesis¹⁹⁶, as well as pro-resolving mediator *Anxa1* [Annexin A1]¹⁹⁷. Retinoic acid induces lymphocyte gut homing, but is also a known inducer of CD4⁺ T cell differentiation to Treg. Thus, decreased synthesis of RALDH2 between 14 and 20 weeks is consistent with loss of capacity to produce retinoic acid and its protective effects¹⁹⁶. From 20 to 26 weeks, many of the 14-20 week changes are reversed, including moderate upregulation of *Aldh2* [RALDH2], and a downregulation of *Cd274* [PD-L1], as well as *Csf1* [M-CSF].

The results of this analysis demonstrate that between 14 and 20 weeks of age, neutrophil expression of an array of chemokines including *Ccl3* [MIP-1 α], *Ccl4* [MIP-1 β], *Ccl6* [CCL6], *Ccl9* [MIP-1 γ], *Cxcl2* [GRO β], *Cxcl10* [IP-10] increases (Fig. 3.4C),

suggesting that neutrophils are transcriptionally primed for recruitment of other immune cell types. Between 20 and 26 weeks, changes in chemokine expression are mixed, with increased expression only of Cxcl9 and Cxcl11, chemokines known to be upregulated in response to IFN-I and IFN-II^{198, 199, 200}.

Because both the frequency and numbers of splenic neutrophils change over the course of lupus progression²⁰¹, we assessed whether neutrophil chemokine receptors undergo transcriptional changes. Neutrophil trafficking to and from peripheral lymphoid organs is mediated by their surface expression of chemokine receptors and integrins including LFA-1 PSGL-1, sphingosine-1-phosphate receptor (S1PR), L-Selectin, and P-Selectin, as well as PECAM ligand CD177^{202, 203, 204}. Several transcripts directly involved with neutrophil trafficking and cell-cell contact are differentially expressed over the course of lupus progression, including *Selplg* [PSGL-1], *Itgb2* [Integrin-β2/LFA-1], *Itgal* [CD11a/LFA-1], *Cxcr1* [CXCR1], *S1pr4* [S1PR4], *Sell* [L-Selectin] (Fig. 3.4D)²⁰⁵. Neither *Cxcr2* nor *Cxcr4*—factors mediating neutrophil migration to sites of infection and clearance (respectively)—were differentially expressed (d.n.s)²⁰⁶. These chemokine receptor changes are consistent with the changing frequency of neutrophils in the spleen observed in our previously published work²⁰¹.

Neutrophils show enrichment for immature granulocyte transcripts during early and late stages of lupus

A prior study by Bennett et al. 2003²² found that circulating leukocytes from human lupus patients are enriched for genes associated with neutrophil precursors, indicating

population is enrichment for neutrophil precursors^{48, 62, 135}. To assess whether splenic neutrophils in the NZB/W murine model are similarly enriched for immature neutrophil transcripts, we examined expression of genes known to be associated with neutrophil precursor differentiation and maturation, including transcription factor *Erg* [ETS-related gene] (Fig. 3.5A), which is associated with neutrophil differentiation²⁰⁷, and *Cebpe* [CEBP/ε], a transcription factor expressed predominately in myelocytes and metamyelocytes, and which regulates expression of genes encoding secondary and tertiary granule components²⁰⁸. Both expression of *Cebpe* as well as granular component transcripts including *Lyz1* and *Lyz2* [lysozyme], *Ltf* [lactoferrin], *Lcn2* [lipocalin-2], *Camp* [cathelicidin], *Mmp8* [collagenase], and *Mmp9* [gelatinase B]²⁰⁹ are highly expressed at 14 and 26 weeks (Fig. 3.5E), with lower expression at 20 weeks (Fig. 3.5A)²⁰⁸. Transcripts encoding azurophilic granule components, such as *Mpo* [myeloperoxidase], and *Elane* [neutrophil elastase], which are expressed at the earliest stages of neutrophil development²⁰⁹, were non-detectable in the splenic neutrophil transcriptome. These findings indicate that, similar to human lupus patients^{22, 62}, immature neutrophils are present in the circulating neutrophil pool at 14 and 26 weeks. However, the expression profiles of neutrophils at 14 and 26 weeks both shown enrichment for precursor transcripts, differences are observable in which types of granular transcripts are enriched. Neutrophils at 14 weeks show high expression of *Mmp9*, which is specifically associated with gelatinase (tertiary) granules, whereas at 26 weeks, specific (secondary) granule components *Lcn-2* and *Camp* show high expression (Fig. 3.5A), suggesting that neutrophils may be present at different stages of maturation, with 26 weeks showing the strongest left

shift associated with enrichment for mRNAs expressed during secondary granule formation^{210, 211}.

Although the upregulation of neutrophil precursor transcripts at 14 and 26 weeks suggests a left shift in the splenic neutrophil population, we do not find significant alteration (at FDR10%) in transcription factors known to regulate steady-state granulopoiesis (i.e. *Cebpa* [CEBP/α]) versus emergency granulopoiesis (*Cebpb* [CEBP/β]) (d.n.s.)²¹², which occurs during acute infection²¹³, suggesting that the neutrophil left-shift at 14 and 26 weeks is not attributable to emergency granulopoiesis.

Maturation stage has been strongly linked to neutrophil effector program in several disease models, including sepsis and malignancy²¹⁴. Neutrophil maturation status is associated with immunosuppressive function in malignancy, although it is unclear whether immature neutrophils in lupus are deleterious or protective toward loss of immunologic self-tolerance. In order to predict changes in neutrophil function which may be associated with changes in maturation state in lupus, we compared the transcriptomic program of lupus neutrophils to the transcriptomes of mature circulating and bone marrow-derived neutrophils, available via the Immunological Genome Project (GEO Data Set: GSE15907)²¹⁵. In order to identify gene sets that characterize mature circulating neutrophils versus immature marrow neutrophils, differential expression analysis between the transcriptomes of Immgen ‘GN_BL’ (healthy blood neutrophil) and ‘GN_BM’ (healthy marrow neutrophil) populations was conducted to generate sets of genes that are differentially expressed in immature marrow neutrophils relative to mature circulating neutrophils. This list of discriminating and differentially expressed genes were constructed

from each of these comparisons using cutoffs at $|\text{Log2FC}| = 1.5$ and FDR5%, and further partitioned into 2 gene sets of genes upregulated ('Mature') and downregulated (called 'Immature') in mature neutrophils relative to immature marrow neutrophils.

Expression of the immature (Fig. 3.5B) and mature (Fig. 3.5C) gene sets showed significant fluctuation within the splenic lupus neutrophil data set over the course of disease, consistent with elevated transcription of granular components (Fig 3.5A) observed at 14 and 26 weeks. However, at 20 weeks, neutrophils show considerable loss of immature transcripts (Fig. 3.5B) and enrichment for mature (Fig. 3.5C) circulating neutrophil gene expression. Pearson clustering of mature and immature neutrophil gene set expression (Fig. 3.5 B,C) revealed incomplete overlap between the immature neutrophil genes enriched at 14 weeks with immature genes enriched at 26 weeks, suggesting that although neutrophil precursors may be present in the splenic neutrophil population at both early and late time points, the transcriptional and functional profile of immature neutrophils at these time points may differ.

In order to gain insight into the functional implications of maturation-stage-related changes in the neutrophil transcriptional program, we characterized the functional pathway associations of each Pearson-clustered module in Fig. 3.5B, C, each of which comprises gene sets uniquely enriched at either 14 weeks, 26 weeks or both. Module genes that were differentially expressed (at $\text{FDR} < 10\%$) between 14 and 20 weeks or between 20 and 26 weeks were analyzed using IPA, and pathway analysis results were then compared for Modules A-D (Fig. 5).

Mod. A comprises BM-neutrophil-associated genes that show enrichment at both 14 and 26 weeks, and predominantly includes genes involved with metabolism (Fig 5 pathway comparison), such as *Hibadh*, which is involved in catabolism of valine²¹⁶ and *Adss*, which is involved in adenosine monophosphate biosynthesis^{217, 218, 219, 220}. Mod. A also includes transcripts encoding enzymes involved with lipid and cholesterol synthesis and metabolism, such as *Abhd5*^{221, 222, 223}, *Cnep1r1*²²⁴, *Lpgat1*²²⁵ (Fig 3.5. D,E). The elevation in transcripts encoding these metabolic enzymes is consistent with the significant changes expected to occur between actively differentiating and developing immature neutrophils and mature, terminally differentiated PMN. Although changes in both lipid metabolism and cell cycle-related gene expression are likely related to the active differentiation and maturation that occurs in neutrophil precursors, changes in these genes may have a significant impact on neutrophil immunologic function as well. For example, Mod. A gene *Inhba*, is involved in cell cycle^{226, 227} but also functions as an inflammatory mediator in response to TLR signalling^{228, 229, 230}. Mod. A also includes *Anxa3* [Annexin A3] and *Anxa5* [Annexin A5] are involved with neutrophil migration, degranulation, and responses to Ca^{2+} ^{231, 232, 233, 234}, as well as *Cybb* [NOX2], which is involved in superoxide production and neutrophil migration^{235, 236, 237}. Transcript encoding protease inhibitor *Serp1b1a* is also elevated at the early and late in disease (Fig 3.5. D,E)²³⁸. Thus, neutrophil maturation state may represent a significant determinant of the neutrophil effector program.

Mod. B comprises immature neutrophil-associated transcripts that are uniquely enriched at 26 weeks, includes pathways involved in immune response and cellular activation pathways, as well as functional genes involved in cellular activation endocytosis

and degranulation. Transcripts uniquely enriched at 26 weeks include *Lta4h* [Leukotriene A4 hydrolase] and *Mgst2* [Microsomal glutathione S], which mediate leukotriene biosynthesis^{239, 240, 241, 242}. Several genes involved in neutrophil activation and immune responses are also uniquely upregulated including *Camp* [Cathelicidin], which has a variety of immunologic functions, including regulating monocyte migration^{243, 244, 245}, *Cd63* [CD63], which is directly involved in neutrophil activation and migration²⁴⁶, *Lbp* [lipopolysaccharide binding protein], which is involved in TLR signaling (Fig 3.5. D,E)^{247, 248, 249}.

Mod C comprises genes associated with immature neutrophil genes enriched at 14 weeks. Similar to Module A, Mod. C includes genes involved predominantly in metabolism, including amino acid anabolic and catabolic pathways (e.g. *Got1* [aspartate aminotransferase]), glycogen synthesis (e.g. *Gbe1* [glucan branching enzyme 1]), urate biosynthesis (e.g. *Nt5e*), choline biosynthesis (e.g. *Pld1* [phospholipase D1]). Mod C also includes genes involved in immune function, such as *Ptgir* [Prostacyclin receptor]¹⁹⁷, which is involved in prostaglandin signaling, as well as *Syne1* [Nesprin-1] and *Syne2* [Nesprin-2], a nuclear envelope protein that the nucleus to the cytoskeleton (Fig 3.5. D,E)²⁵⁰.

Functionally, Mod. D transcriptional changes are predominantly associated with cell proliferation-associated genes, including *Egr1* [Early growth response-1] and *Adrb2* [adrenoceptor beta 1]^{251, 252, 253, 254}, as well as lipid metabolism (e.g. genes inflammatory and cellular pathways, including *Csf-1* (Fig. 3.4A), *Fos*, *Jun* (Fig 3.3A), *Socs3* (Fig. 3.5E), which are associated with STAT and PPAR signaling, as discussed above. The strong association of these transcripts with neutrophil maturation status indicates that the

neutrophil functional properties (e.g. capacity for STAT signaling) is strongly associated with neutrophil maturation state. Thus, enrichment for granulocyte precursors in the splenic neutrophil population may be a significant determinant of neutrophil effector function. Although there is some overlap between pathway associations associated with each module (e.g. lipid metabolism), both Mod. B and Mod. C contain different gene sets related to immune function, suggesting that neutrophil effector function may differ not only between immature and mature granulocytes, but also between subsets of immature granulocytes (Fig 3.7).

Additionally, we find that *Csf3r* [G-CSF-receptor], *Socs3* [Suppressor of Cytokine Signaling 3], which suppresses G-CSF signaling, and *Stat3* [STAT3], which mediates G-CSF-R signaling are significantly upregulated at 20 weeks compared to both 14 and 26 weeks (Fig. 3.5E), suggesting that the changes in the prevalence of precursor neutrophils in the spleen may be driven by changes neutrophil responsiveness to G-CSF signaling²¹².

The transcriptional changes in neutrophil is not consistent with a shift between N1 and N2 neutrophil effector program

Although neutrophil subsets have not yet been fully defined in lupus, the division of neutrophils into functionally pro-inflammatory ‘N1’ and anti-inflammatory ‘N2’ subsets has been used as a model of neutrophil phenotypic heterogeneity in malignancy^{51, 255}. In order to gain insight into whether the N1/N2 paradigm may be used to define the subsets of neutrophil in lupus, we therefore examined expression of genes associated with the two neutrophil functional programs in malignancy. N1 neutrophils are characterized by

expression of pro-inflammatory cytokines and chemokines, including TNF α , ICAM-1, FAS, and the N2 subset is characterized by elevated expression of CXCR4, VEGF, MMP9, CCL5, CCL2, and Arginase-1^{51, 255}. In the lupus neutrophil transcriptome, both Arg1 and Ccl2 transcripts were nulls, and of the N1/N2 genes examined, only *Icam1* and *Mmp9* are differentially expressed (Limma F-test, FDR<10%) (Fig. 3.6). Cluster analysis of the expressed N1/N2-associated factors revealed that expression of these factors N1 and N2 genes do not cluster together, but rather that neutrophils each disease time point show mixed expression of N1- and N2-associated genes. This finding suggests that the transcriptomic changes in splenic neutrophils over the course of lupus are not clearly characterized by an N1/N2 phenotypic classification.

A model of the changes in the neutrophil transcriptome over the course of lupus progression

At 14 weeks, the neutrophil population is enriched for neutrophil precursors displays an immunosuppressive phenotype including enhanced capacity for ROS generation, which inhibits T cell proliferation, as well as elevated expression of Annexin A1^{256, 257, 258}, protease inhibitor granulin, and RALDH (Fig. 3.7). The transition from 14 to 20 weeks is characterized by a rise in both pro-inflammatory mediators—M-CSF, IL-1 β , LIGHT, and lymphotoxin- β ²⁵⁹, which may contribute to monocyte maturation and GC formation. Neutrophils at 20 weeks also produce immunosuppressive mediators, including IL-1RA and PD-L1. Unique features of the 26 week time point include high expression of *Sod2* [superoxide dismutase 2] and *Cat* [catalase], which neutralize reactive oxygen species, as

well as high expression of IFN γ -responsive genes encoding CXCL9²⁶⁰ and CXCL11²⁶¹, which may influence monocyte and T cell trafficking within PLO.

3.5 Discussion

We have previously reported that depletion of neutrophils early in murine SLE profoundly accelerates disease with enhancement of splenic GC reactions and generation of autoantibody secreting cells in secondary lymphoid organs. This surprising finding, the fact that late neutrophil depletion had no impact on disease, and shift in splenic neutrophil interactions from T cells to B cells highlighted the plasticity in neutrophil roles in SLE and suggested a dominantly suppressive effect earlier in disease. However, the precise mechanisms of neutrophil suppressive effects, the balance with pro-inflammatory effector functions, and the factors that may dictate changes in neutrophil function over the course of disease remain unclear. Here we profile neutrophil function via RNA sequencing approaches to define the transcription of an array of inflammatory mediators and pathways. A main goal is for hypothesis generation regarding how neutrophils contribute to the progression of SLE, while also providing a resource for understanding how the neutrophil phenotype changes over the course of autoimmunity. Results reveal complex and dynamically changing gene networks throughout the course of disease, including a number of pathways associated with a regulatory neutrophil phenotype balanced against pro-inflammatory factors. Overall, these results highlight the importance of disease stage and severity on neutrophils' transcriptional program and imply that the changing neutrophil effector program may influence the course of lupus progression.

Common pathways dynamically changing throughout the course of disease include innate pattern recognition receptor signaling, cytokine/chemokine signaling, ROS pathways, and lipid mediators. Additionally, a number of transcripts associated with a regulatory neutrophil phenotype including IL-1RA, PD-L1, and TGF β peak at mid disease (20 weeks) but are subsequently down-regulated in concert with a rise in pro-inflammatory pathways including interferon-regulated chemokines CXCL9 and CXCL11. The predicted high capacity for ROS production at 14 weeks is of particular interest given its association with neutrophils in malignancy, which adopt a gMDSC phenotype and have the capacity to suppress T cell responses. This may be especially relevant at the early to mid-disease time points where we have previously shown that neutrophil-T cell interactions in the spleen dominate. A gMDSC-like immunosuppressive phenotype at 14 weeks is consistent with the worsening in disease that we noted with neutrophil depletion over this time frame. The resemblance of the neutrophil transcriptome at 26 weeks to 14 weeks may be related to the decline in immunologic function that can occur with advanced disease in the NZB/W model, and appears to be characterized by a loss of the acute inflammatory response suggested by high IL-1 β and NF κ B signaling at 20 weeks. Although it is unknown whether neutrophils from 26 week old have the capacity for T cell suppression, it is also possible that enrichment for immature granulocyte genes at 26 weeks may not represent a gMDSC phenotype, but rather a unique transcriptional phenotype associated with chronic inflammation. In humans, neutrophils present in chronic inflammatory states such as cystic fibrosis and juvenile idiopathic arthritis show a unique transcriptomic profile²⁶².

Paradoxically, concomitant with the rise in PD-L1, genes associated with ROS production decrease, pro-inflammatory eicosanoids increase, and PRR pathways increase, all predicted to be associated with a transition from pro-resolving to inflammatory functions. Indeed, the 20 week time point shows evidence of neutrophil activation, based on transcription of both *Nfkb2* [NFκB] and several of its gene targets, including *Il1b* [IL-1β]¹⁸⁷, *Il1rn* [IL-1RA]²⁶³, *Ltb* [Lymphotoxin-β]¹⁸⁸, *GROβ*²⁶⁴, *MIP1α*, *MIP1b*²⁶⁵, and *NOD2*²⁶⁶. Likewise, *Ptgs2* (COX2) transcription peaks around 20 weeks, consistent with neutrophil production of prostaglandins²¹⁵. Lupus features dysregulation of prostaglandin and thromboxane production, an imbalance that likely contributes to tissue damage^{267, 268, 269, 270}. It has been established in other settings that neutrophils can control the magnitude and localization of the immune response via inflammatory lipid mediators such as thromboxane A₂²⁷¹, as well as promoting resolution via leukotriene A₄, which can be converted to resolvins by platelets²⁷². However, neutrophil production of pro-inflammatory vs. anti-inflammatory prostaglandins in SLE remains under-characterized. Of particular interest in our data is the expression pattern of *Alox5* (5-LOX), an enzyme that converts arachidonic acid to lipxygenase A₄ (LXA₄). LXA₄ can have either pro-inflammatory or pro-resolving effects, depending on whether it is converted to leukotriene B₄ or to resolvins^{186, 273, 274}. Further work will be necessary to define whether the dominant effects of neutrophils on prostaglandin pathways in SLE are pro-inflammatory or anti-inflammatory and how this may change over the course of disease. Examination of the several other novel pathways that emerged in our analysis, including changes in nuclear

receptors during the early-to-mid disease transition and metabolic pathways from mid to late disease also merits further investigation.

The changing transcription of genes associated with known neutrophil effector functions, including TLR signaling, Ddx58 [RIG-1], Nod2 [NOD2], and eicosanoid synthetic enzymes suggests that the functional properties of neutrophils are changing over the course of lupus, particularly responsiveness to TLR ligands. However, the mechanisms underlying these changes is uncertain. Given the strong enrichment for genes associated with immature neutrophils both at 14 and 26 weeks of age, the factors driving the change in the neutrophil phenotype may be strongly dependent on the inflammatory milieu in the bone marrow. Additionally, it is likely that the marrow microenvironment exerts a strong influence on the transcriptional profile of circulating neutrophils because neutrophils have a short circulating half-life *in vivo*²⁷⁵. The finding that neutrophils in the NZB/W model show enrichment of genes associated with immature neutrophils recapitulates findings by Bennet et al. (2010) which showed enrichment of neutrophil precursor genes in PBMC transcriptomic data from human lupus patients²². The NZB/W strain may thus provide a suitable model in which to study the mechanisms of generation of abnormal neutrophils in lupus, as well as the effects of bone marrow inflammation on generation of functionally abnormal neutrophils⁶². The origin of immature neutrophils is unclear. Although left-shifting within marrow granulocytes has been identified in human lupus²⁷⁶ and may underlie the presence of immature neutrophils within the murine spleen, immature neutrophils may also be generated via extramedullary hematopoiesis and resident within the spleen, as was observed in *S. pneumoniae* infection²⁷⁷. Regardless of the origin

of the immature neutrophil subsets, however, strong evidence²⁷⁸ from malignancy models suggests that immature neutrophils have unique functional properties. Neutrophil maturation state may be the major determinant of the strong changes in transcriptional program we observe in splenic neutrophils in lupus. Since neutrophils demonstrate a short circulating half-life²⁷⁹ and the vast majority of the neutrophil pool is regularly renewed by the bone marrow, the neutrophil effector program is likely an *ad hoc* response to short-term exposure to the inflammatory milieu both during granulopoiesis in the marrow as well as a short period of circulation *in vivo*. However, it is unclear to what extent the phenotype of splenic neutrophils is determined by functional plasticity of individual circulating neutrophils post-egress from the marrow versus plasticity of the granulopoietic process in response to disease progression.

In conclusion, our data suggest that there is a strong relationship between the neutrophil transcriptional program and the stage and severity of lupus. Furthermore, there is evidence for the emergence of a regulatory neutrophil effector program as the disease evolves, likely mediated by the inflammatory milieu in the bone marrow and periphery. However, this is balanced by the variable kinetics of rise in pro-inflammatory pathways that may become dominant later in disease.

3.6 Figures

Figure 3.1. Splenic neutrophils from NZB/W mice show distinct transcriptional states in early, mid- and late-stage lupus. A) Experimental design: Splenocytes were FACS-sorted to obtain high purity DAPI-Ly6B+Ly6CintCD11b+LyGhi cells using female NZB/W mice at 14, 20, and 26 weeks of age. N=5-6mice/time point. B) Pearson clustering (2-way, ave. linkage) and heat map showing the global transcriptome (deseq rlog expression counts; expr. norm. by gene; expression cutoff = 1).

A

B

C

D

Figure 3.2. Changes in the splenic neutrophil transcriptome over the course of lupus follow an expression arc with disease progression, with the 20 week time point transcriptionally distinct from 14 and 26 weeks. A) Venn diagram showing the total number of DEG (Limma-moderated t-test, FDR 10%) that are up- or down-regulated between each time point. Values in brackets are derived from the adjusted model, and the

bottom right values indicate genes not in differentially expressed, at the indicated cutoff (FDR10%). B) Volcano plots showing DEG (orange) and non-DEG (green) for two time point comparisons (Limma-mod. F-test, FDR 10%). C-D) Ingenuity pathway analysis constructed using the a gene list input of the most significant DEG from C) the 14-20week comparison, or D) the 20-26 week comparison (Limma-moderated t-test, FDR 10%). A cutoff was applied to the pathway list such that DEG associated with each pathway is ≥ 6 . Top 35 most significant pathway associations are shown, with IPA predictions (z-test) regarding pathway activation with disease progression (red=increasing activation; grey no change; blue=decreasing activation; black=no prediction).

A

B

C

Figure 3.3. The neutrophil transcriptome shows significant changes in pathways associated with TLR/PRR signaling, eicosanoid synthesis, and oxidative burst at different stages of lupus. A) Heatmap shows expression changes in specific genes associated with the indicated biologic pathways. Expression is shown normalized by gene, and all genes shown are significantly differentially expressed by Limma F-test (FDR10%)., B-C) Changes in expression of genes associated with TLR signaling between B) 14-20 weeks and C) 20-26 weeks are represented IPA signaling schematics with colored fill showing significantly up-reg. (red) and down-reg. transcripts (green) (Limma-mod. F-test, FDR 10%).

D

Figure 3.4. Curated list of expression changes in genes of immunologic interest in splenic neutrophils over the stages of lupus. A) B) C) D) All genes displayed are significantly differentially expressed at FDR10% (Limma F test) unless otherwise indicated.

A

B

C

E

Figure 3.5. Functional Implications of Splenic Neutrophil Maturation Status in Lupus. A. Normalized expression of transcripts associated with granule development and differentiation of immature neutrophil subsets. B-C. Normalized expression of gene signatures associated with B. bone marrow neutrophils or C. mature circulating neutrophils. Expression modules were defined using a distance threshold (Pearson correlation co-efficient) of 0.65 for both BM neutrophils (Modules A-C) and peripheral blood (PB) neutrophils (Mod. D). D. Pathway analysis showing the top six most statistically significant Ingenuity pathway associations of each module (gene set) defined in B-C. E. Expression of curated gene lists associated with maturation state-specific functional predictions. A-E. Gene input for pathway analysis input were filtered using a FDR cutoff of 10% (Limma-moderated t-test, comparing pairs of disease time points), and pathway output was filtered so that the number of pathway-associated DEG is ≥ 6 . Heat

maps in A and B show a median centered z-score of normalized expression levels each gene.

Figure 3.6. Neutrophil transcriptional profile does not neatly fit the N1/N2 classification paradigm. Pearson clustering of N1 and N2-associated genes expressed by lupus neutrophils. Genes are Pearson clustered (dendrogram scale indicates Pearson distance). *differentially expressed or **not differentially expressed by Limma-moderated F-test comparing the three disease time points at FDR10%. Color scale displays z-score of normalized expression levels for each gene and is median centered.

Figure 3.7. Model of how changes in neutrophil transcriptional program affect may affect the progression of auto-immunity. A) Between 14 and 20 weeks, neutrophils transition from an immunosuppressive effector program at the early stages of disease to an activated participant in the immune response that occurs at 20 weeks. B) From 20 to 26 weeks, neutrophils transition from a mature, activated transcriptional state, and return to a phenotype that partially resembles the 14 week time point.

CHAPTER 4 Functional defects in circulating neutrophils are associated with bone marrow inflammation in lupus

4.1 Abstract

Systemic lupus erythematosus involves dysregulation of both innate and adaptive cell lineages of the hematopoietic system. Various factors underlie this dysregulation, including defects in cell clearance and dysregulated cytokine balance in the periphery. However, the contribution of bone marrow—the source of hematopoietic cells—to lupus pathology is not well characterized. Lupus bone marrow from both human patients and murine lupus models shows signs of pathology, including necrosis and inflammatory cytokine milieu, and these signs of tissue pathology have been linked to both cytopenias in lupus patients. Additionally, there is evidence that the bone marrow generates functionally abnormal leukocytes, including unique neutrophil populations that are cytotoxic to the endothelium and show functional impairments such as spontaneous NETosis and impaired phagocytic capacity. Here we examine whether the functional abnormalities observed in circulating neutrophils in lupus originate in the bone marrow in order to assess whether marrow pathology may underlie abnormalities in granulopoiesis.

4.2 Introduction

Systemic lupus erythematosus is a multi-organ autoimmune disorder that involves dysregulation of both innate and adaptive immune compartments. Although a variety of factors contribute to lupus pathogenesis, it remains unclear to what extent inflammation in the bone marrow and dysregulation in hematopoiesis contribute to the generation of

pathogenic and functionally impaired leukocytes in SLE. Here we examine whether dysregulation in the bone marrow of human lupus is associated with generation of functionally abnormal neutrophils in circulation.

Pathogenic and functionally abnormal neutrophils have been observed in circulation in peripheral blood of both human and murine lupus. These cells exhibit a number of abnormalities, including elevated production of TNF α , IFN γ , BAFF, and IFN-I, decreased phagocytic capacity, and spontaneous extracellular trap formation (NETosis) upon *ex vivo* isolation^{62, 64, 65, 69}. These neutrophil functional abnormalities contribute to tissue damage, including endothelial and renal damage and may contribute to atherosclerosis^{280, 281, 282, 283, 284, 285}. The relevance of neutrophil abnormalities to lupus pathogenesis is indicated by *in vivo* studies in two murine models of lupus, which showed that NETosis inhibition ameliorates renal, vascular, and skin pathology, as well as decreasing interferon signaling in both bone marrow and kidney^{284, 285}. Although, abundant evidence suggests that neutrophils in lupus mediate tissue damage, the origins and mechanisms underlying the generation of these pathogenic neutrophils remains unknown.

Several lines of evidence suggest functionally abnormal lupus neutrophils are generated as a result of dysregulated granulopoiesis in the bone marrow. For example, pathogenic neutrophils identified in the circulation by Denny et al. (2010)⁶², show enrichment for precursor neutrophils (cells with immature nuclear morphology). Evidence for left-shift in lupus (enrichment for neutrophil precursors) also includes transcriptomic data of both total human PBMC and total bone marrow, which shows enrichment for gene transcripts associated with neutrophil precursors^{133, 134}.

Currently, the impact of the bone marrow in SLE pathogenesis is incompletely understood. Current evidence for tissue dysregulation in lupus marrow primarily focuses on histopathologic studies. These studies have revealed heterogeneous forms of tissue pathology, but common observations include fibrosis, necrosis, hypocellularity, neoplastic outgrowth of myeloid cells (myelofibrosis), atypical localization of immature precursors, and an elevated proportion of plasma B cells^{119, 120, 121}. Marrow hypo-cellularity and necrosis are associated with peripheral cytopenias, including as anemia, thrombocytopenia, and neutropenia are common in SLE^{110, 122}. Cell death in the marrow is likely a major cause of SLE cytopenias and associated risk of infection (a prevalent cause of mortality in SLE)^{123, 124}. Although tissue dysregulation has been observed in SLE marrow, little is known about the effects of marrow dysregulation the generation of pathogenic and functionally abnormal hematopoietic cells. We hypothesize that inflammation within the bone marrow may contribute to both the underproduction of leukocytes and generation of functionally abnormal and pathogenic neutrophils in the peripheral blood.

Supporting this hypothesis, and elevated IFN-I signaling and elevated levels of TNF α have both been identified in lupus marrow^{65, 125}. Both of these factors may underlie elevated cell death or defects in hematopoiesis^{65, 125}. Conversely, little is known regarding which cells may drive inflammation in the marrow, although both neutrophils and monocytes were implicated in one study as a source of TNF α (a mediator of marrow necrosis)^{125, 126}. A limited number of studies have found the bone marrow to be of relevance to lupus pathogenesis due to abnormalities in hematopoiesis. Limited evidence has indicated defects in central tolerance in SLE¹⁰⁴, the frequency of developing B cell subsets

is altered in SLE, alterations associated with elevated IFN-I signaling in the bone marrow⁶⁵. Also of note, mesenchymal stem cells (MSC) support hematopoiesis in the marrow, but in lupus, MSC show deficiency in production of cytokines known to support hematopoietic competence (e.g. IL-6, IL-7)¹²⁷, as well as an enhanced capacity to stimulate transitional and mature naïve B cell differentiation to plasma cells¹²⁸. Hematopoietic stem cells in lupus marrow exhibit elevated apoptosis and proliferative defects^{129, 130}, associated with defects in hematopoiesis, including for example, decreased frequency and impaired functional maturation in NK cells¹³¹. Similar to NK cells, we hypothesize that defects in circulating, peripheral neutrophils (described in 1.5) originate with defects in granulopoiesis driven by bone marrow inflammation. Here we profile the inflammatory milieu in the bone marrow in lupus patients compared with healthy controls, and examine whether alterations in the marrow microenvironment is associated with neutrophil functional abnormalities in both bone marrow and circulating neutrophils.

4.3 Methods

Human subjects

All human tissues used in this study were obtained following written, informed consent from human volunteers in accordance with a protocol approved by the Institutional Review Board at the University of Rochester Medical Center. SLE patients were accepted as donors based on their meeting diagnostic criteria of the American College of Rheumatology or Systemic Lupus International Collaborating Clinics. SLE donors were matched with healthy controls based on age and sex.

Tissue collection

Bone marrow aspirates (BMA) and paired peripheral blood (PB) were obtained from each lupus patient and matched healthy control within a 1.5 hour period, and experiments were conducted in parallel for each pair of healthy and SLE donors. BMA was obtained from the iliac crest, and PB obtained from antecubital venipuncture. Both BMA and PB were collected into heparinized vacutainer tubes, with the exception of serum and Paxgene samples. Contamination of BMA by peripheral blood was minimized by needle tip adjustments during the aspiration procedure.

Neutrophil isolation

Neutrophils were isolated from PB and BMA via HetaSep (StemCell Technologies) erythrocyte sedimentation, followed by magnetic negative cell selection using the EasySep Human Neutrophil Enrichment Kit (StemCell Technologies), which yielded >97% CD15+CD66b+ cells, as determined by FACS analysis. Neutrophils were kept at 4°C where permitted by experimental protocol and handled gently throughout isolation to avoid cell activation.

Cytokine quantitation

To assess neutrophil capacity for cytokine production, isolated human neutrophils were cultured *ex vivo* in RPMI 1640/10%FBS. Neutrophils were incubated at 37°C and 5% CO₂. Neutrophils were allowed to adhere for 20 minutes before PMA (100ng/mL) was added to the stimulated culture condition as indicated. Cells were cultured for 16 hours before culture supernatants were removed and stored at -80°C. Cytokine quantitation was

then performed using Luminex (ThermoFisher) multiplex immunoassays. Cytokine concentrations in serum and bone marrow supernatant were quantitated using LEGENDPlex (Biolegend) immunoassays. Bone marrow supernatant was obtained by centrifugation of whole marrow aspirate, and recovery of the supernatant (i.e. bone marrow plasma).

Phagocytosis assay

Neutrophil phagocytic capacity was assessed using the pHrodo Green *E. Coli* bioparticle assay (ThermoFisher), in accordance with the manufacturer's instructions. In brief, whole blood or marrow was cultured with inactivated *E. coli* bioparticles conjugated to a pH-indicator fluorochrome. The frequency of phagocytic events per neutrophil was then detected using an ImageStream X (Amnis) imaging. Phagocytic capacity was determined in several stages of neutrophil development via initial gating Aqua-CD15+CD66b+ singlets (total neutrophils), followed by sub-gating of neutrophil developmental subsets. Neutrophil developmental subsets are classified as: early/precursor, CD11b-CD10-; mid/immature, CD11b+CD10-; and late/mature, CD11b+CD10+. Phagocytic capacity was quantitated using the Amnis IDEAS 'Spot Counting' feature, which counts the number of phagocytosed pHrodo bioparticles within each neutrophil ('phagocytic capacity'). These counts were then used to generate a frequency distribution of spots per neutrophil for each neutrophil population analyzed, with an associated distribution mean and median. Population phagocytosis mean and medians are then used as a metric of phagocytic capacity.

Statistics

For comparisons of SLE patient data with matched healthy controls and for comparisons of PB and BMA from the same patient, paired t-tests were used. For comparisons of non-paired data Mann-Whitney U-tests were performed. Statistical analyses were conducted using GraphPad Prism and JMP Pro 13 software. False discovery rates are given where large-scale regression screens were conducted.

4.4 Results

Lupus patients display an abnormal inflammatory milieu in bone marrow

In order to determine whether neutrophil functional abnormalities may be driven by abnormalities in the milieu of inflammatory mediators in the bone marrow of lupus and healthy control. Bone marrow aspirates (BMA) were obtained from lupus patients and matched healthy controls (HC), alongside paired peripheral blood from the same patients, using a study design outlined in Figure 4.1. The bone marrow tissue cytokine and chemokine profile was then quantitated in the bone marrow (BM) supernatant (SN) samples from lupus patients and healthy controls (Fig. 4.2). Principal components analysis (PCA) revealed differences in the overall chemokine milieu in lupus marrow as compared to healthy control marrow (Fig. 4.2A), suggesting that the chemokine milieu in the SLE patients may be abnormal. However, PCA of the cytokine milieu (produced using the cytokines indicated in Fig. 4.2) did not reveal clear differences between HC and SLE bone marrow (Figure 4.2B). Comparison between individual inflammatory mediators in lupus versus healthy controls revealed that the most consistent differences in both serum and BM

SN were G-CSF levels, which were significantly elevated in lupus patients over healthy controls (Figure 4.2C). Because G-CSF is one of the most potent regulators of neutrophil development, function, egress from the marrow^{286, 287}, elevated G-CSF may be associated with dysregulation in granulopoiesis. Additionally, paired analysis of the cytokine milieu (Fig. 4.2D,E and Appendix C, Figure S2) in SLE versus HC BM SN revealed that a subset of lupus patients show significantly altered concentrations of pro-inflammatory mediators, including IP-10, as well as decreased levels of IL-6, a cytokine known to support hematopoietic competence and myeloid cell development²⁸⁸, as well as lower IL-1 β (Fig. 4.2D), another factor known to affect neutrophil development and function^{289, 290, 291, 292, 293}. Additionally, a subset of lupus patients show elevated CXCL5, CXCL1 in their peripheral blood (Fig. 4.2E), factors which promote neutrophil mobilization from the marrow^{294, 295}. Additionally, SLE patients showed significant alterations in their marrow IL-10, IP-10, and TNF α

In order to assess whether elevation in inflammatory mediators in lupus peripheral blood (described in prior literature^{296, 297}), is also reflected in lupus bone marrow, we examined the relationship between serum and peripheral blood concentrations of chemokines that mobilize neutrophils—CXCL1 and CXCL8—interferon-inducible chemokine IP-10, and found a significant association between serum and bone marrow concentrations of these chemokines (Fig. 4.3), suggesting that the inflammatory milieu in the periphery is reflected in the bone marrow.

The inflammatory milieu in the bone marrow is associated with neutrophil capacity for cytokine production.

In order to determine whether the inflammatory milieu within the bone marrow is associated with neutrophil effector function in SLE, we assessed whether inflammatory mediator concentrations in the bone marrow predict neutrophil production of inflammatory mediators using regression analysis. We find that several cytokines and chemokines in lupus bone marrow (BM) supernatant (SN) are significantly associated with *ex vivo* cytokine production by lupus bone marrow neutrophils (Fig. 4.4). BM SN concentrations of IFN γ , IL-1 β , and IL-10 concentrations are significantly associated with neutrophil capacity for BAFF production by isolated bone marrow neutrophils, whereas BM SN IL-6 predicts neutrophil propensity to secrete IL-8 upon *ex vivo* PMA stimulation (Fig. 4.4A). Additionally, BAFF production by BM neutrophils cultured *ex vivo* in media alone is significantly associated with BM SN concentrations of IL-23, IL-6, and IL-10 (Fig. 4.4B). Although BAFF production by neutrophils following PMA stimulation (Fig. 4.4A) is negatively associated with the concentrations of several BM SN cytokines, the degree BAFF production by unstimulated BM neutrophils is positively associated with BM SN IL-10, IL-6, and IL-23 concentrations (Fig. 4.4B). Although the reasons for this different response are unclear, these findings suggest an association of neutrophil capacity for inflammatory mediator production with the inflammatory milieu.

Neutrophil capacity for cytokine production and phagocytic capacity in the peripheral blood is also apparent in developing neutrophils in the marrow.

In order to assess whether the defects in phagocytic capacity in the circulating neutrophil population in lupus are apparent in developing neutrophils in the marrow¹¹⁸, we used imaging cytometry to quantitate the phagocytic capacity of neutrophil populations at

various stages of development in the bone marrow as well as in mature circulating neutrophils. We find that phagocytic capacity in the total neutrophil population in the bone marrow has a significantly lower phagocytic capacity in lupus compared to matched healthy controls (Fig. 4.6A). Additionally, we found this impaired phagocytic capacity could be attributed to significant differences in the mature neutrophil subset in the bone marrow (Fig. 4.6B), rather than to differences in earlier precursor subsets, which did not show statistically significant differences in the comparison of SLE and HC (d.n.s.). Likewise, we observe a reduced phagocytic capacity in SLE in the total circulating neutrophil pool (Figure 4.6C). We hypothesize that this reduced phagocytic capacity may be attributable to premature egress of marrow neutrophils due to alterations in the milieu of neutrophil chemoattractants. Consistent with this hypothesis, we do find a negative association between neutrophil phagocytic capacity in SLE marrow and marrow concentrations of CXCL2, a chemoattractant which promotes neutrophil egress from the marrow (Fig. 4.6D). Similar to neutrophil capacity for inflammatory mediator production, we observe that neutrophil capacity for phagocytosis in developing bone marrow neutrophil populations is significantly associated with neutrophil phagocytic capacity in the periphery, supporting the idea that defects in phagocytic capacity are established during granulopoiesis in the marrow (Fig. 4.6E).

Additionally, we asked whether bone marrow neutrophil capacity for inflammatory mediator production is reflected in the cytokine production capacity of circulating peripheral blood neutrophils. Using regression analysis to assess whether neutrophil capacity for inflammatory mediator production by developing BM neutrophils predicts

inflammatory mediator production in circulating PB neutrophils, we found that neutrophil capacity for production of BAFF (Fig. 4.5) by in immature marrow neutrophils is significantly associated with propensity of mature PB neutrophils to produce BAFF.

Together, these results support the model that abnormalities in neutrophil effector function are observable in immature neutrophil populations in the marrow and that neutrophil effector function is predicted by the concentrations of inflammatory mediators within the marrow, implicating the bone marrow in the generation of functionally abnormal neutrophils in SLE.

4.5 Discussion

Although the contribution of secondary and tertiary lymphoid tissues to SLE have been well-characterized as an aspect of lupus pathogenesis, the contribution of inflammation within the bone marrow inflammation to lupus pathogenesis. The results in this chapter identify an altered inflammatory milieu within the bone marrow of lupus patients, as well as associated neutrophil functional abnormalities within the developing neutrophil compartment in the marrow. These functional abnormalities have several implications for lupus pathogenesis, including providing on possible explanation for generation of functionally abnormal neutrophils in lupus peripheral blood. The observation that defects in neutrophil effector function in the peripheral blood is also observed in neutrophil effector function within the marrow and may have multiple underlying mechanisms, including genetic mechanisms that directly affect neutrophil capacity to acquire effector function. However, our regression analyses showing that the marrow

inflammatory milieu (which can fluctuate with lupus flare or remission) significantly predicts neutrophil effector function—including phagocytic capacity and cytokine production—is consistent with the idea that inflammation within the bone marrow underlies generation of functionally abnormal neutrophils. This study also supports future mechanistic studies examining whether inhibition of cytokine signaling *in vivo* in a murine lupus model would restore homeostatic generation of functionally normal neutrophils.

Hematopoiesis within the bone marrow is regulated by peripheral inflammation, and in acute inflammation, elevated neutrophil death in inflamed peripheral tissues can increase serum concentrations of G-CSF, resulting in emergency granulopoiesis within the bone marrow²⁹⁸. Our finding that G-CSF is significantly elevated in both the peripheral blood and bone marrow may underlie the granulocyte left-shift observed at the transcriptional level in both bone marrow and peripheral blood of lupus patients with active disease^{133, 134}. Additionally, we find that a subset of lupus patients show alterations in the bone marrow cytokine and chemokine milieu. The effects of chronic inflammation on hematopoiesis are largely uncharacterized in lupus, but we hypothesize that the alterations in cytokine milieu drive hematopoietic dysregulation and cytopenias, and may contribute to damage to the bone tissue itself, thereby contributing to the elevated propensity for osteoporosis and bone fracture in lupus patients²⁹⁹.

Additionally, our finding that bone marrow inflammation correlates with neutrophil effector function has several implications. Our observation that phagocytic capacity is reduced in both bone marrow and circulating neutrophils, and that phagocytic capacity correlates inversely with concentrations of the major regulator of neutrophil egress,

CXCL2, suggests that functional impairment of circulating neutrophils may simply be attributable to premature egress from the marrow. Lupus patients exhibit increased susceptibility to infection, and defects in phagocytosis originating in the bone marrow may contribute to this infection risk.

Lastly, our observation that lupus marrow displays significant alterations in inflammatory milieu as well as functionally abnormal neutrophils has implications for not only the generation of cytotoxic and functionally impaired granulocytes, but also may affect the lymphopoiesis and monopoiesis as well, and contribute to generation of auto-reactive or activated immune cells that drive lupus pathogenesis. Thus we propose that the bone marrow pathology is a central and previously underappreciated aspect of lupus pathogenesis.

4.6 Figures

Figure 4.1. Study design: Assessment of the association of marrow inflammation and development of functionally aberrant circulating neutrophils. A) A total of 10 patient pairs were analyzed in this study. SLE patients were paired with healthy controls based on age, sex, and ethnicity. B) BMA and paired peripheral blood were collected for each person (2 tissues x 2 patients = 4 samples), and C) experiments were run for each of the four samples in parallel (i.e. immunoassays run on the same plate, phagocytic capacity assessed using imaging cytometry within the same run).

Figure 4.2. The milieu of cytokines and chemokines is altered in SLE compared to healthy controls in both serum and bone marrow supernatant (SN). A) Principal

components analysis showing difference in patient serum and bone marrow supernatant levels of chemokine concentrations [CXCL2 (GRO β), CXCL8 (IL-8), CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP1 α), CXCL5 (ENA78), CXCL1 (GRO α)] and B) Principal components analysis showing difference in patient serum and bone marrow supernatant levels of cytokine concentrations [IL-1 β , IFN γ , IL-10, IL-12p70, IL-17A, IL-23, IL-6, TNF α , G-CSF, GM-CSF]. n=10 patients per sample group. C) G-CSF levels were determined in PB and BM. Data are pooled from historic Luminex (ThermoFisher) immunoassay results and LegendPlex (Biolegend) assays for patients analyzed in this study. n=19 HC, n=28 SLE. D-E) SLE versus NC cytokines and chemokine concentrations were compared in D) BM SN samples and E) PB serum samples. Wilcoxon matched pairs signed rank tests. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.005, ****p $\leq$ 0.001 n.s.=not significant.

Figure 4.3. Concentrations of chemokines in the peripheral blood predict chemokine concentrations in the bone marrow of SLE patients. Serum and BM SN chemokine concentrations are plotted here for SLE patients. p-values based on linear regression.

A

B

Figure 4.4. Inflammatory mediator concentrations in bone marrow (BM) supernatant (SN) correlate with cytokine production by BM neutrophils. Regression analysis is used to predict an association between SLE BM SN inflammatory mediator concentrations and neutrophil capacity for cytokine production, as measured following *in vitro* stimulation of neutrophils with A) PMA, or B) media alone. p-value based on linear regression. FDR = Benjamini-Hochberg false discovery rate.

Figure 4.5. Comparison of neutrophil phagocytic capacity between SLE patients and paired healthy controls. Phagocytic capacity was assessed using imaging cytometry, where neutrophils were gated as described in 4.3 Methods. A) Mean spot count is plotted for total bone marrow neutrophil; B) mature bone marrow neutrophils; and C) total

peripheral blood neutrophils. n=11 patients per group. Paired t-test. * $p \leq 0.05$. D) Phagocytic capacity plotted against CXCL2 concentration in the bone marrow SN. P-value based on linear regression. E) Linear regression comparing the phagocytic capacity of mature bone marrow neutrophils versus total peripheral blood neutrophils, with healthy control (blue), and SLE (red). F) Neutrophil capacity for BAFF production upon PMA stimulation in the marrow plotted against BAFF produced by neutrophils in peripheral blood. p-value based on linear regression.

CHAPTER 5 Discussion

5.1 Neutrophils in acute versus chronic inflammation

The studies described in this thesis examine how neutrophils contribute to lupus pathogenesis. However, this work also has relevance to the broader model of neutrophil phenotypic plasticity and contributions to the progression of different types of inflammatory states. The classical model of neutrophil participation in the immune response is largely based on studies of neutrophil responses to acute inflammation. In an acute inflammatory response to a bacterial infection, neutrophil responses follow a patterned response that involves initial amplification of the inflammatory response, clearance of the pathogen, and then resolution of inflammation. This type of response largely relies on short term effector functions such as phagocytosis, respiratory burst, degranulation or NET release.

However, chronic inflammatory diseases such as lupus are characterized by failure to fully resolve inflammation. Although the role of neutrophils in chronic inflammatory states is incompletely understood, the balance of neutrophil pro-inflammatory and pro-resolving mediators may help tip the balance between disease flare or quiescence in autoimmune disorders such as lupus. We hypothesize that the neutrophil effector program in SLE may not be accurately modeled as simply pro-resolving or pro-inflammatory, according the paradigm of acute inflammation. Rather, the effector program of neutrophils in chronic inflammation may be unique to specific chronic inflammatory states. Our data suggests a dynamic balance between inflammatory and suppressive roles for neutrophils in

lupus (Figure 5.1). We hypothesize that the ultimate failure of neutrophil immunosuppressive capacity with advanced disease—indicated by both the neutrophil depletion results in late-stage disease (Chapter 2) as well as changes in neutrophil transcriptomic program (Chapter 3)—is one of several immunologic defects that allows the systemic failure to resolve inflammation that occurs in SLE.

Although an abundance of evidence now indicates neutrophil functional plasticity³⁰⁰, neutrophil subsets with corresponding effector programs remain incompletely defined. The phenotype of neutrophils in chronic inflammation has been well described in malignancy, where neutrophils are known to suppress adaptive immune responses through use of a phenotypic program known as a granulocytic myeloid derived suppressor cell. The notion that gMDSC are present in lupus has been proposed in recent literature³⁰¹, but it remains unclear whether lupus neutrophils can accurately be classified as gMDSC. The best method for classification of neutrophil subsets remains a matter of debate in the literature, and even in malignancy, gMDSC is a broad term that covers a heterogeneous population of cells, which may contain granulocytes with immature and mature nuclear morphology, and with different developmental origins including the granulopoiesis in the bone marrow or extramedullary hematopoiesis^{302, 303}. Additionally, the gMDSC phenotype can be reversibly induced in mature neutrophils by the peripheral cytokine milieu or via differentiation from monocytic MDSC³⁰⁴. Immature neutrophils have greater plasticity and acquire this pro-inflammatory phenotype in response to cytokines such as GM-CSF and IFN- γ ³⁰⁵, and have the capacity to differentiate into cells with DC-like properties³⁰⁶. Understanding the role of neutrophils in the propagation and regulation of lupus and other

chronic inflammatory states will require a fuller description of the diversity of neutrophil subpopulations and their distinct effector programs. Additionally, it is possible that chronic and systemic inflammation in lupus drives generation of SLE-specific neutrophil subsets attributable to tissue pathology within the bone marrow.

5.2 The contribution of this work to the lupus field

Despite the recent findings that gMDSCs may arise in both murine and human SLE, whether they exert protective¹⁰⁶ or promoting¹⁴¹ effects on loss of self-tolerance remains a critical unresolved question. Moreover, there is an abundance of literature implicating neutrophils as pro-inflammatory mediators in the disease both as a source of immunogenic antigen in the form of NETosis and cytokines such as BAFF and Type I IFN that could promote B cell responses. Thus, therapeutic targeting of neutrophil effector function in SLE will require reconciliation of this conflicting literature. We hypothesized that neutrophils may have different effects on the adaptive immune system depending on their tissue localization, cell-cell interactions, disease stage and severity, and the specific murine model of SLE examined. In Chapter 2, we elucidate the role of disease stage and severity as a determinant of neutrophil effects on SLE pathogenesis. We find that neutrophil depletion early in murine SLE profoundly accelerates disease, with promotion of GC reactions and generation of autoreactive plasma cells, while neutrophil depletion at later stages of lupus had no effect on the rate of disease progression. These findings indicated both that the influence neutrophils exert on lupus pathogenesis changes over the course of disease progression and that neutrophils at early stage of lupus have a protective, regulatory phenotype. This work provides clarification in the context of conflicting literature by

examining the importance of disease stage and severity to the neutrophil phenotype, by demonstrating that neutrophil effects on lupus pathogenesis can change. The results of Chapter 2, showing that neutrophils are predominantly protective, also highlights an important distinction between neutrophil influence on lupus pathogenesis in affected tissue, where neutrophils may mediate tissue damage, versus neutrophil effects on the loss of immunologic self-tolerance by the adaptive immune system, in which neutrophils may have the capacity for immunosuppression. The idea that neutrophils are protective toward loss of self-tolerance in lupus is a recent and unexpected finding, and the mechanism of this immunosuppression remains unclear. Because immunosuppressive neutrophil subsets (gMDSC) have been identified as determinants of immunologic quiescence and disease outcome in malignancy, we drew comparisons between the two in the Chapter 3 transcriptomic profile of splenic neutrophils.

In Chapter 3, we profiled changes in the splenic neutrophil transcriptome over the course of lupus in order to generate hypotheses regarding how neutrophils may be influencing adaptive immune responses at different stages of disease. This transcriptomic profiling addressed one possible interpretation of the neutrophil depletion results in Chapter 2 by determining whether neutrophil depletion at late-stage lupus fails to alter the course of lupus progression because neutrophils have changed phenotypically or because immunosuppressive neutrophils are present throughout lupus progression but are simply overwhelmed by accelerating inflammation. Results demonstrate robust changes in the neutrophil transcriptome, suggesting that the differing outcomes of neutrophil depletion are the result of differences in the neutrophil phenotype at different stages of lupus. The

robust changes in the neutrophil transcriptome over the course of disease include diverse immunologic pathways that would be expected to impact suppressive vs. pro-inflammatory functions, such as ROS, eicosanoids, cytokines/chemokines, and surface molecules associated with gMDSC function (PD-L1). Thus, the transcriptomic experiment provides a number of testable hypotheses regarding the neutrophil effector program and likely neutrophil effects on lymphocytes. In combination with the profound disease acceleration observed in the setting of neutrophil depletion starting early in disease and the lack of effect late in disease, our data supports the concept that neutrophils can adjust their effector program depending on the milieu, including acquisition of immunosuppressive and pro-resolving function. Chapter 3 proposes several immune mediators that may drive the protective effects of neutrophils in early-stage lupus, including ROS (Fig. 5.1), similar to immunosuppressive neutrophils in malignancy. However, we found that with disease acceleration, neutrophils exhibit a transcriptional profile consistent with an acute inflammatory response, including elevated expression of both regulatory and pro-inflammatory mediators. Neutrophil expression of these immune mediators may exert a significant influence on the progression of auto-reactivity, both because of the proximity between neutrophils and splenic lymphocytes observed in Chapter 2, as well as the numbers of circulating neutrophils, which likely exert a significant impact on the systemic cytokine milieu as well as recruitment of other leukocytes to the PLO. The RNA sequencing on a bulk-sorted splenic neutrophil population provides insight into the overall changes in the neutrophil inflammatory phenotype.

The Chapter 3, we present transcriptomic evidence for the presence of immature neutrophils within the lupus spleen and compared murine lupus neutrophils at different stages of disease to neutrophils in other inflammatory states including infection and malignancy. Neutrophils in lupus are enriched in gene signatures associated with precursor neutrophils both early and late in disease, suggesting a left shift in the peripheral neutrophil population. We hypothesize that the precursor transcriptome may reflect premature egress from the bone marrow due to inflammation, including granulopoiesis-promoting cytokines such as G-CSF, as described in Chapter 4. Immature neutrophils have a greater degree of functional and phenotypic plasticity than terminal differentiated mature PMN, and we hypothesize that the state of maturation is a strong determinant of both neutrophil effector program at the time of egress from the marrow as well as a determinant of neutrophil functional plasticity in response to peripheral inflammation. In the neutrophil transcriptomes 14 and 26 weeks, we find only partial overlap between the immature neutrophil transcripts, a difference that we hypothesize are attributable to the presence of neutrophils at different stages of maturation at 14 versus 26 weeks, as well as transcriptomic evidence that these differences are reflected in neutrophil immunologic function. Overall, these studies provide insight into how neutrophils may be changing functionally, and provide a strong rationale for ongoing study of the impact of neutrophils on adaptive immunity as a function of disease stage.

The Chapter 3 finding that precursor neutrophils are present in peripheral lymphoid tissue is of significant experimental interest because it identified that the NZB/W model displays egress of neutrophil precursors similar to that observed in human lupus^{22, 134}. The

NZB/W model may provide a suitable experimental system for studying both the underlying mechanisms of immature neutrophil generation as well as the effects of immature granulocytes on lupus pathogenesis. Given the enrichment in the neutrophil precursor transcripts in both the NZB/W model and human lupus, we reasoned that the bone marrow is an important compartment to examine more closely. However, the impact of this milieu on neutrophil function has not previously been examined, nor the relationship between developing bone marrow neutrophils and abnormal peripheral blood neutrophils (such as enhanced propensity for NETosis⁷⁷).

In Chapter 4, we examined whether functionally abnormal neutrophils in the circulation in lupus originate in the abnormal bone marrow micro-environment. We observed striking alterations in cytokine and chemokine expression in the lupus marrow, including factors known to impact neutrophil development, migration, and function, such as TNF³⁰⁷, G-CSF, GM-CSF, and IL-6 (Figure 5.1). Even more notably, the abnormal BM milieu correlated with functional abnormalities in both marrow and peripheral neutrophils, including altered phagocytosis, as well as propensity for BAFF production. Overall, these results suggest that dysregulation in the bone marrow may be an under-appreciated contributor to lupus pathogenesis and dysregulated granulopoiesis (Figure 5.1).

5.3 Implications for future research

The results described here raise several additional questions that should be addressed in future studies, with the goal of generating a comprehensive model of how neutrophils influence adaptive immune dysregulation and identifying potential therapeutic

targets. In Chapter 2, we described neutrophils in close proximity to both B and T cells in lupus peripheral lymphoid tissues, with dominant T cell interactions earlier in disease and increasing B cell contacts over the course of disease progression. We hypothesize that neutrophils within the spleen release mediators which directly influence adaptive immune responses, including direct effects on T and B cell responses. We find that the neutrophil transcriptome (Chapter 3) is enriched in regulatory mediators such as PD-L1, TGF- β , IL-1RA, and enzymes involved in ROS generation during early stage lupus. In contrast, with further disease progression there is a rise in expression of IFN regulated chemokines, IL-1 β , lymphotoxin- β , BAFF, and TNF, which would be predicted to mediate more pro-inflammatory function. An important future direction is to validate expression of these factors in splenic neutrophils *in vivo* at the protein level, as well as the direct impact of neutrophils on B and T cell function *in vitro* assays. It will be critical in these studies to take into account the changing phenotype of neutrophils over the course of lupus progression and compare neutrophil function at different stages of disease.

Prior literature has examined the effects of neutrophils on B and T lymphocyte function as discussed in Chapter 1, but with some conflicting results. It is also important to recognize that *in vitro* assays with neutrophils may be confounded by rapid neutrophil death upon *ex vivo* isolation and thus fail to recapitulate neutrophil activity *in vivo*. Examination of neutrophil inflammatory mediator production *in situ* using immunohistochemistry is another important future direction to provide critical validation of neutrophil inflammatory mediator production *in vivo* as well as data on *in situ* cell-cell interactions. Although Chapter 2 demonstrates changing cellular contacts of neutrophils

with B and T lymphocytes, we have yet to define the precise mechanism of suppression of GC reactions. As previously noted this could be mediated by direct effects on T cell proliferation, T cell differentiation, or the B cell compartment. Given the dominant T cell contacts early in disease, increase in PD-L1 mRNA expression in neutrophils at a time when neutrophil depletion accelerates disease, and known role of PD-L1 binding to PD-1 in suppressing Tfh differentiation, we speculate that direct effects on Tfh are critical. Although PD-L1 can suppress Tfh differentiation, this effect has been shown to be mediated by B cells and DCs^{162, 308}. Thus, a priority is to demonstrate elevated PD-L1 expression by lupus neutrophils and PD-L1-PD-1 mediated suppression of Tfh.

In Chapter 4, we demonstrated that functional defects in circulating neutrophils in human lupus likely arise in part due to alterations in granulopoiesis in the bone marrow. Future studies should investigate the mechanisms underlying dysregulated granulopoiesis in lupus using murine lupus models. Based on our observation that splenic neutrophils in the NZB/W mice are enriched in transcripts associated with immature neutrophils, this is likely a good model for studying the impact of the bone marrow micro-environment on generation of functionally abnormal neutrophils in lupus.

Overall, our findings support the hypothesis that neutrophils have the capacity for either a deleterious or protective influence on the development of auto-reactivity, dependent on the stage and severity of disease, tissue localization, and dominant cellular interactions. Moreover, the BM is one of the key sites that may drive changes in neutrophil phenotype and function (Figure 5.1). Additional studies to further validate our proposed model of how neutrophils influence adaptive immune responses may provide more specific

therapeutic targets. Current therapeutics used to treat lupus, including steroids, are broadly immunosuppressive with significant side effects. Neutrophil function may be targeted via more specific approaches such as NETosis inhibition using PAD4 inhibitors *in vivo*. If the net-effect of gMDSC in lupus is protective, G-CSF—an inducer of gMDSC¹⁸⁰—may be used to promote development of immunosuppressive neutrophils. G-CSF administration has been demonstrated to be effective (at specific doses) in both the NZB/W³⁰⁹ and MRL/lpr³¹⁰ lupus models, and has been used as a treatment for neutropenia in lupus patients. Although there is concern that G-CSF may cause disease flare³¹¹, administration of G-CSF in specific doses may prove to be predominantly protective in lupus.

5.4 Figure – Model of neutrophil participation in lupus pathogenesis

Figure 5.1. Model Illustrating Hypothesized Mechanisms by which Functionally Abnormal and Pathogenic Neutrophils are Generated and Neutrophil Effects on Loss of Self-tolerance. A) An aberrant cytokine milieu in the bone marrow—including chronically elevated G-CSF, TNF α , and IFN-I alters neutrophil acquisition of effector function during granulopoiesis, as well as the rate of neutrophil generation and egress from the marrow. B) Functionally abnormal neutrophils egress from the marrow and contribute to lupus pathogenesis in the periphery via excess generation of BAFF and IFN-I. C) Circulating neutrophils show impaired phagocytic capacity, which may contribute to susceptibility to infection. D) Circulating neutrophils exhibit spontaneous NET generation, which is cytotoxic to endothelium. E) Neutrophils migrating into peripheral lymphoid tissue (e.g. spleen) secrete both pro- and anti-inflammatory mediators, such as IL-1 β , IL-1RA, and M-CSF, factors known to influence monocyte maturation and antigen presentation. F) Neutrophils express regulatory factors such as PD-L1 and ROS, which can

inhibit T cell proliferation. G) Neutrophils generate BAFF and Lymphotoxin β within the spleen, which facilitates B cell activation and GC formation, particularly at later phases of disease when neut.:B cell interactions occur at high frequency. Abbr: PM – promyelocyte; M – myelocyte; MM – metamyelocyte; Band – band cell; PMN – mature polymorphonuclear cell; ROS – reactive oxygen species; GC – germinal center; Mono – monocyte; BAFF – B cell activating factor; IFN-I – Type I interferon; PD-L1 – programmed death ligand 1; G-CSF – granulocyte colony stimulating factor; M-CSF – monocyte colony stimulating factor; TNF – tumor necrosis factor.

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APPENDICES

Appendix A - Supplemental Figures Associated with Chapter 2

Appendix A - Supplemental Figure 2.1. Efficacy of neutrophil depletion with 1A8 (Anti-Ly6G). Quantitation of the efficacy of neutrophil depletion by FACS analysis of the frequency of Gr-1^{hi}CD11b^{hi} (A) peripheral blood and (B) bone marrow at sacrifice (26 weeks of age) following 12 weeks of treatment with 1A8 or isotype control. ****p<0.0001 by Mann-Whitney U-test. Data shown are the mean $\pm$ s.e.m.

Appendix A - Supplemental Figure 2.2. Splenic leukocyte subset frequencies and numbers following treatment with anti-Ly6G or isotype control in NZB/W mice 14-26 weeks of age. FACS analysis was used to assess the frequency of (A) Tregs, (B) M1 inflammatory monocytes, and (C) M2 non-classical monocytes. 'ns'=not significant by Mann-Whitney U-test. Data shown are the mean $\pm$ s.e.m.

Appendix B - Supplemental Figures Associated with Chapter 3

Appendix B - Supplemental Figure 1. Post-sort data for splenic neutrophil FACS purification. Pre-sort data (left) and post-sort data (right) are shown here for splenic neutrophils purified for RNA sequencing. Sample shown here is from the 20 week time point.

Appendix B - Supplemental Figure 2. Transcriptomic changes predicted by anti-dsDNA level versus week of age. Differential expression of all adjusted for anti-dsDNA levels (left side); DEG expression adjusted for both anti-dsDNA level and week for the 14-20 week comparison (middle), and the 20 to 26-week comparison (right). Differential expression was assessed by ANOVA.

Appendix B - Supplemental Figure 3. Splenomegaly over the course of lupus progression in untreated female NZB/W mice.

Appendix C - Supplemental Figures Associated with Chapter 4

Lupus patient data				Matched healthy control data		
Age (years) at Date of BMA	Ethnicity	Gender	Total SLEDAI Score	Age (years) at Date of BMA	Ethnicity	Gender
49	NH, AA	F	4	39	NH, C	F
25	NH, AA	F	8	51	NH, C	F
56	NH, C	F	2	52	NH, C	F
50	NH, C	F	2	44	NH, C	F
46	NH, C	F	4	51	NH, C	F
62	NH, AA	F	0	58	NH, C	F
33	NH, AA	F	2	37	NH, C	F
49	NH, AA	F	8	46	NH, AA	F
33	NH, AA	F	4	36	NH, C	F
51	H, C	F	4	50	NH, C	F
51	H, C	F	6	31	NC, C	F

Appendix C - Supplemental Figure 1. Patient information for SLE patients and matched healthy controls. Paired patient data is shown in each row. SLE patient disease status is measured here by SLE disease activity index (SLEDAI). Abbv: African American (AA), Caucasian (C), Non-hispanic (NH), Hispanic (H).

A

B

Appendix C - Supplemental Figure 2. Comparison of cytokines and chemokine concentrations between SLE and HC Serum and bone marrow supernatant (SN). Inflammatory mediators were quantitated by immunoassay for A) BM SN samples and B) peripheral blood serum samples. Paired Wilcoxon signed rank tests. n.s.=not significant.