

Pro- and Anti-inflammatory Neutrophils in Lupus

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Abstract

A hallmark of SLE is the presence of elevated levels of circulating anti-nuclear autoantibodies specific towards chromatin, histones or dsDNA. Understanding the regulation of antibody production is therefore of utmost importance in understanding lupus pathogenesis. Spearheaded by the identification of accumulating immunosuppressive neutrophils in cancer patients, the nature and function of neutrophils have expanded from a uniform pro-inflammatory cell population to a heterogeneous population of cells with pro-inflammatory or immunosuppressive capacities. While much is known about pro-inflammatory neutrophils and the likely pathogenic function of such cells in lupus, a potential role for immunosuppressive neutrophils in protecting genetically predisposed individuals has only recently emerged. For example, SLE-derived neutrophils spontaneously produce type I interferons (IFN α), strongly associated with disease development, release chromatin-containing neutrophil extracellular traps (NETs), potentially functioning as a source of nuclear auto-antigen, and may activate B cells in a T cell independent fashion. In contrast, levels and functions of regulatory neutrophils (Nregs) involved in T cell-dependent B cell differentiation and germinal center reactions, are dysregulated in female lupus-prone mice during disease development. Here we review data supporting a role for both pro- and anti-inflammatory neutrophils in lupus.

Keywords: Systemic lupus erythematosus; Autoantibody; Neutrophils; Pro-inflammatory; Immunosuppression

Abbreviations

ANA: Anti-Nuclear Antibodies; APRIL: A Proliferation Inducing Ligand; BAFF: B cell Activation Factor; BM: Bone Marrow; CNS: Central Nervous System; GC: Germinal Center; ds: double stranded; IC: Immune Complex; IL: Interleukin; IFN: Interferon; LDG: Low Density Granulocyte; NET: Neutrophil Extracellular Trap; Nreg: Regulatory Neutrophil; SLE: Systemic Lupus Erythematosus; T_{FH}: T Follicular Helper Cell

Introduction

Systemic Lupus Erythematosus (SLE) is characterized by elevated levels of antinuclear antibodies (ANA) and circulating immune complexes (IC) known to deposit in organs such as the skin, kidney, heart, and lung, promoting mononuclear cell infiltration and tissue damage. The development of SLE is attributed both environmental triggers and genetic predisposition, and presents with an overwhelming female bias [1]. Since pathogenic autoantibodies characterize the disorder, decades of research have focused on the function and dysregulation of autoreactive B cells. However, since the discovery of elevated levels of neutrophil-associated gene transcripts in peripheral blood mononuclear cell samples from SLE patients as compared with healthy controls [2], an increasing number of studies have investigated the role of neutrophils in the pathogenesis of SLE.

Neutrophils are the most abundant circulating leukocyte. The cells are short-lived, granule-rich and constitute the primary defense against microbial and fungal infections [3]. During infections, circulating neutrophils are recruited to the site of infection by

chemotactic cytokines. The classic effector functions of neutrophils include phagocytosis and the release of antimicrobial proteins, reactive oxygen species (ROS), metalloproteinases (MMPs) and other endopeptidases such as trypsin and neutrophil elastase [4]. While the functions of these effector mechanisms are detrimental to pathogens, these enzymes may also harm the host resulting in tissue damage and necrosis. More recent evidence suggests that neutrophils can also function as antigen presenting cells [5,6], as a source of autoantigen and type I interferon (IFN α) in autoimmunity [7-10], or even as negative regulators of T and B cell responses during systemic inflammation [11-14]. In this review we discuss evidence from human and mouse studies supporting a key role for pro- and anti-inflammatory neutrophils in lupus pathogenesis.

Pro-inflammatory Neutrophils in Accumulate in SLE Patients and Mice with Lupus-like Disease

Despite chronic neutropenia and hyper-susceptibility to bacterial infections [15-17], a significant proportion of SLE patients display elevated levels of immature neutrophils and a pronounced granulopoiesis gene expression signature in cells from both peripheral blood and bone marrow exudates [2,18,19]. In addition, neutrophils are known to infiltrate target organs such as the skin, kidney or vasculature in SLE patients [20-22], although the pathogenic nature of such infiltration remains unknown. The population of neutrophil-like cells accumulating in SLE patients can be divided into at least two subpopulations based on their granularity: high density (mature granulocytic) neutrophils and low density (immature monocytic) granulocytes (LDGs) [2,22]. The functional capacities of both cell subsets are dysregulated in SLE patients however it is not yet clear whether such defects are in themselves causative in disease development [7,8].

Likewise, in virtually all murine models of SLE, myeloid lineage cells accumulate in the circulation, spleen and/or target organs (CNS, kidneys, lungs and vasculature) as disease progresses [23-26]. The appearance of elevated numbers of CD11b⁺ cells most often correlates with the development of other disease markers such as ANA production and/or kidney infiltration, and thus, these cells are generally believed to exert pro-inflammatory functions. CD11b expression is shared among many cell subsets and additional surface markers along with nuclear morphology analyses are commonly used to define neutrophil populations. Thus, accumulating neutrophil-like cells co-express the surface marker Ly6C and show a monocytic nuclear morphology in female autoimmune (NZB x NZW)F1 mice [27,28], while the dominant population of neutrophil-like cells in protected male (NZB x NZW)F1 mice express Ly6G and a multilobe nuclear morphology [28] (please see below for more details). In MRL/lpr lupus-prone mice and pristane-induced lupus models both Ly6C⁺ and Ly6G⁺ cell populations accumulate [23,29,30], while neither of these two markers are present on accumulating myeloid cells in BXSB mice [31-33].

Neutrophil-associated Effector Functions are Abnormal in SLE Patients and Mouse Models of Lupus

Phagocytosis

Both adult and juvenile-onset SLE patients and patients with chronic granulomatous disease are known to have an increased risk of developing lupus, present with elevated levels of apoptotic neutrophils [34-38]. Under non-inflammatory conditions, neutrophils have a short life span ending with spontaneous apoptosis and subsequent removal by phagocytes [39]. During inflammation, however, neutrophils are kept alive to help fight off the infection. It is therefore conceivable that apoptotic neutrophils accumulate in SLE patients, not as a result of increased apoptosis, but rather, due to impaired clearance of apoptotic bodies by professional phagocytes including macrophages and neutrophils themselves. Defective phagocytosis may stem from SLE-associated gene polymorphisms affecting the expression and/or function of Fc-receptors (FcγRIIa, FcγRIIb and FcγRIII) or complement receptor 3 (CR3; CD11b/CD18) [40-42], all of which are involved in macrophage and neutrophil-dependent phagocytosis.

Production of reactive oxygen and nitrogen species

A major effector mechanism of neutrophils is their ability to produce and release antimicrobial products and reactive oxygen species (ROS) upon encounter of pathogens. Correlating with the accumulation of neutrophils, patients with active SLE display elevated production of ROS by circulating neutrophils and increased levels of oxidative damage in kidney biopsies [43-46]. Abnormal oxidative protein modifications have also been associated with disease development in MRL/lpr lupus-prone mice and increased immunity in rabbits [47,48]. Increased ROS production could be a result of accumulating neutrophils as described above, or a consequence of decreased expression or inhibited function of superoxide dismutase (SOD1). SOD1 is a critical antioxidant involved in the consumption of free oxygen radicals that has been shown to be significantly reduced in SLE patients with active disease [49,50]. Alternatively, a polymorphism in the gene neutrophil cytosolic factor 2 (NCF2) encoding for p67^{phox}, a critical component of the NADPH oxidase and thus involved in the production of ROS, has been found in several cohorts of SLE patients [51,52].

Cytokine production

While the mechanisms of tissue damage are not completely characterized, cytokine and chemokine production by inflammatory myeloid cells have been observed in both SLE patients and several of the murine models of lupus. As such, many cytokines have been associated with the initiation and progression of lupus including interleukin-6 (IL-6), IL-10, IL-17, IL-18, IL-21, tumor necrosis factor-alpha (TNFα) and interferon-alpha (IFNα) [53,54].

IFNα has received significant attention as PBMCs from SLE patients often express elevated levels of IFNα-induced gene transcripts [2,55,56]. IFNα is induced in response to a broad range of signals, but most efficiently in response to intracellular toll-like receptors: TLR3, TLR7, TLR8 and TLR9 [57]. Interestingly, chronic TLR7-stimulation was recently shown to induce myelopoiesis leading to monocyte and neutrophil accumulation in an IFNα-dependent manner [58]. The most potent IFNα-producing cell type is the plasmacytoid dendritic cell [59]; however neutrophils can produce IFNα under chronic inflammatory conditions as well [22,60]. This is true in humans, as well as in mice, and has recently been reviewed elsewhere [61].

B cell manipulating factors such as IL-6, IL-21, BAFF and APRIL, can all be produced by neutrophils and have been associated with lupus pathogenesis. IL-6 is a powerful regulator of B cell differentiation and antibody production and manipulation of IL-6 levels affect lupus pathogenesis in both female (NZB x NZW)F1 mice and pristane-induced lupus [62-64]. IL-21 is required for functional T follicular helper (T_{FH}) cell differentiation and the formation of germinal centers (GC), and can be produced by neutrophils during chronic inflammation. In SLE, such IL-21 may also lead to T-cell independent B cell activation and differentiation [11]. Interestingly, these cells also produced A Proliferation Inducing Ligand (APRIL), crucial for plasma cell survival. Further evidence for communications between neutrophils and B cells comes from studies showing that tissue infiltrating, G-CSF-triggered neutrophils produce and secrete BAFF under inflammatory conditions [65], while BM neutrophils from SLE patients produce both APRIL and BAFF supporting early B cell development and plasma cell survival [66].

Finally, in two recent studies of pristane-induced lupus, bone-marrow derived neutrophils (Ly6G^{hi}Ly6C^{low}) were reported to produce significant levels of TNFα and IL-17A, resulting in myelopoiesis and the accumulation of neutrophil-like cells [67,68]. Interestingly, TNFα have been associated with many neutrophil effector functions including ROS production, while IL-17A is emerging as a key cytokine controlling both T and B cell dependent immune responses [69,70].

NETosis

Neutrophils are known to release extracellular traps, known as neutrophil extracellular traps or NETs, as a mechanism to immobilize pathogens [4]. This process leads to the subsequent death of the neutrophil; a process known as NETosis [71]. From a lupus perspective, NETs offer an enticing source of nuclear antigen, as these structures consist of strands of elastase covered with antimicrobial proteins, such as MPO and LL37, alongside histones and free DNA [4,21] and excellently reviewed in [61,72]. In support hereof, recent data show that LL37/DNA-containing NETs effectively activate plasmacytoid DCs via TLR9 cross linking resulting in IFNα production [7]. Interestingly, the production of NETs by neutrophils can also be induced by many inflammatory signals including IFNα and

IC [7,8]. Finally, it has been shown that low density granulocytes (LDGs) from SLE patients spontaneously undergo NETosis *in vitro* [21,60], while NETs have been observed in cultures of Ly6C^{high}CD11b⁺ neutrophils from pristane-treated lupus mice [73], further supporting a role for NETs in lupus pathogenesis.

In addition to the spontaneous and chronic generation of NETs, dysregulated removal of NETs may play a role in lupus pathogenesis [74]. Removal of NETs relies on DNaseI activity, and impaired DNaseI activity has been associated with kidney damage in lupus nephritis patients and mouse models of SLE [74-76]. Moreover, the presence of DNaseI inhibitors and anti-NET antibodies preventing DNaseI from binding and degrading the NETs, have been identified in serum samples from SLE patients [74], further adding to the pathogenicity of NETs.

Anti-inflammatory Properties of Neutrophils in Lupus

The biological importance of immunosuppressive tumor-infiltrating Ly6G⁺ and Ly6C⁺ myeloid cells was spearheaded by the identification of ROS-dependent T cell suppression in cancer patients [77] and numerous studies have since then explored the tumor-promoting function of these cells in both mouse models and cancer patients [78]. Recently, immunosuppressive functions of neutrophils have been described in several studies of systemic inflammation and autoimmunity including sepsis and SLE [11,12]. Still, the mechanism(s) of suppression utilized by these cells remain to be identified.

We recently reported the presence and function of populations of regulatory Ly6G⁺Gr1^{high}CD11b⁺ and Ly6C^{high}Gr1^{low}CD11b⁺ neutrophils in the (NZB x NZW)F1 mouse model of SLE [13,28]. Since, lupus presents with an overwhelming female bias, we hypothesized that such regulatory neutrophils could be a mechanism preventing disease in otherwise genetically predisposed males. Consistent with a sex-dependent immunosuppressive function, we found that *male* (NZB x NZW)F1 mice express increased numbers of Gr1⁺ cells throughout life [28] (Table 1). In young <9 week old lupus-prone male, as well as female, (NZB x NZW)F1 mice, Ly6G⁺Gr1^{high}CD11b⁺ and Ly6C^{high}Gr1^{low}CD11b⁺ neutrophils were found to be immunosuppressive towards B and T cells, respectively. As *female* mice aged, however, the cells lost their immunosuppressive capability both *in vitro* and *in vivo* [13,28]. A similar loss of function was also observed in males, albeit not until much later in life. Interestingly, over time Ly6C^{high}Gr1^{low}CD11b⁺ neutrophils became immunostimulatory to both T and B cells, resembling the function of pro-inflammatory LDGs as described above.

In addition to the temporal difference in immunosuppression between male and female-derived Nregs, our studies also showed that Ly6G⁺Gr1^{high}CD11b⁺ and Ly6C^{high}Gr1^{low}CD11b⁺ neutrophils not only targeted different lymphoid cell populations *in vitro*, but also utilized different immunosuppressive mechanisms [13,28]. For example, female Gr1^{high}CD11b⁺ cells depended on cell-cell contact and utilized ROS/NO as their mechanism of inhibition in B cell differentiation assays, while male cells exerted their suppressive capacity via an unknown secreted, soluble factor ([28] and unpublished results). These data are consistent with a study showing that immunosuppressive Gr1-CD11b⁺ myeloid cells from MRL/lpr mice suppress T cell proliferation in an arginase-1-dependent manner [79], and the observation that MRL/lpr mice defective in ROS-production display an accelerated disease profile [80].

	Gr1 ^{high} CD11b ⁺ cells	Gr1 ^{low} CD11b ⁺ cells
Expression levels (spleen)	Elevated in males 4-26 weeks of age	Elevated in females (>9 wks)
Immunosuppressive <i>in vitro</i>	Yes (females only <16 wk)	Yes (females only <9 wk)
Cellular target	B cell differentiation	T cell differentiation and proliferation
Cell-Cell contact	No (males); Yes (females)	ns*
Effector mechanism	Unknown (males); ROS/NO (females)	ns*
Immunostimulatory <i>in vitro</i>	No	Yes (female ≥ 9wk old; males >16 wk old)
All Gr1⁺ cells		
Immunosuppressive <i>in vivo</i>	Yes (males)	No (females ≥ 9 wks of age)
Cellular target	GC reaction via control of TFH cell differentiation (males)	n/a
*ns: not studied; n/a: not applicable [13,28]		

Table 1: Neutrophil-like cells differ in numbers and functions between protected males and disease-prone females in (NZB x NZW)F1 lupus mice.

Several lines of evidence suggest that the observed immunosuppressive function in male lupus-prone mice is reminiscent with the normal behavior of such cells. For example, studies in non-autoimmune mice have shown that neutrophils can limit immune reactions in response to inflammation or immunization ([81,82] and our unpublished observation). In addition, complement factor 4 produced by myeloid cells may regulate spontaneous GC reactions in self-reactive B cell receptor transgenic mice [14]. We therefore suggest that the chronic inflammatory milieu developing in female (NZB x NZW)F1 mice affect a population of regulatory neutrophils (Nregs) either via the induction of apoptosis (or maybe even NETosis) [4,83] or via the induction of a differentiation program driving the development of non-immunosuppressive cells such as dendritic cells, macrophages or mature neutrophils, as previously suggested [83-85].

Summary

There is still much to learn about pro- and anti-inflammatory neutrophils, their effector functions and role in the pathogenesis of SLE. We here suggest a model in which immunosuppressive neutrophil-like cells (Nregs) constitute a normal regulatory component involved in the control of adaptive immune responses (Figure 1). We suggest that the normal function of Nregs is to control T_{FH} cell differentiation and GC reactions either directly via the production of immunomodulatory cytokines affecting differentiation of T and B cells or via the production of chemokines affecting the assembly of GCs during T-dependent antibody responses. During systemic inflammation (including infections, cancer, and SLE) Nregs are targeted to either die, hereby promoting access to nuclear autoantigens, or differentiate into pro-inflammatory dendritic cells,

macrophages and mature neutrophils driving T and B cell activation and eventually autoantibody production. Future studies identifying factors targeting and changing the function of Nregs during chronic

inflammation (including SLE) may provide additional targets for early intervention therapy in lupus-susceptible individuals.

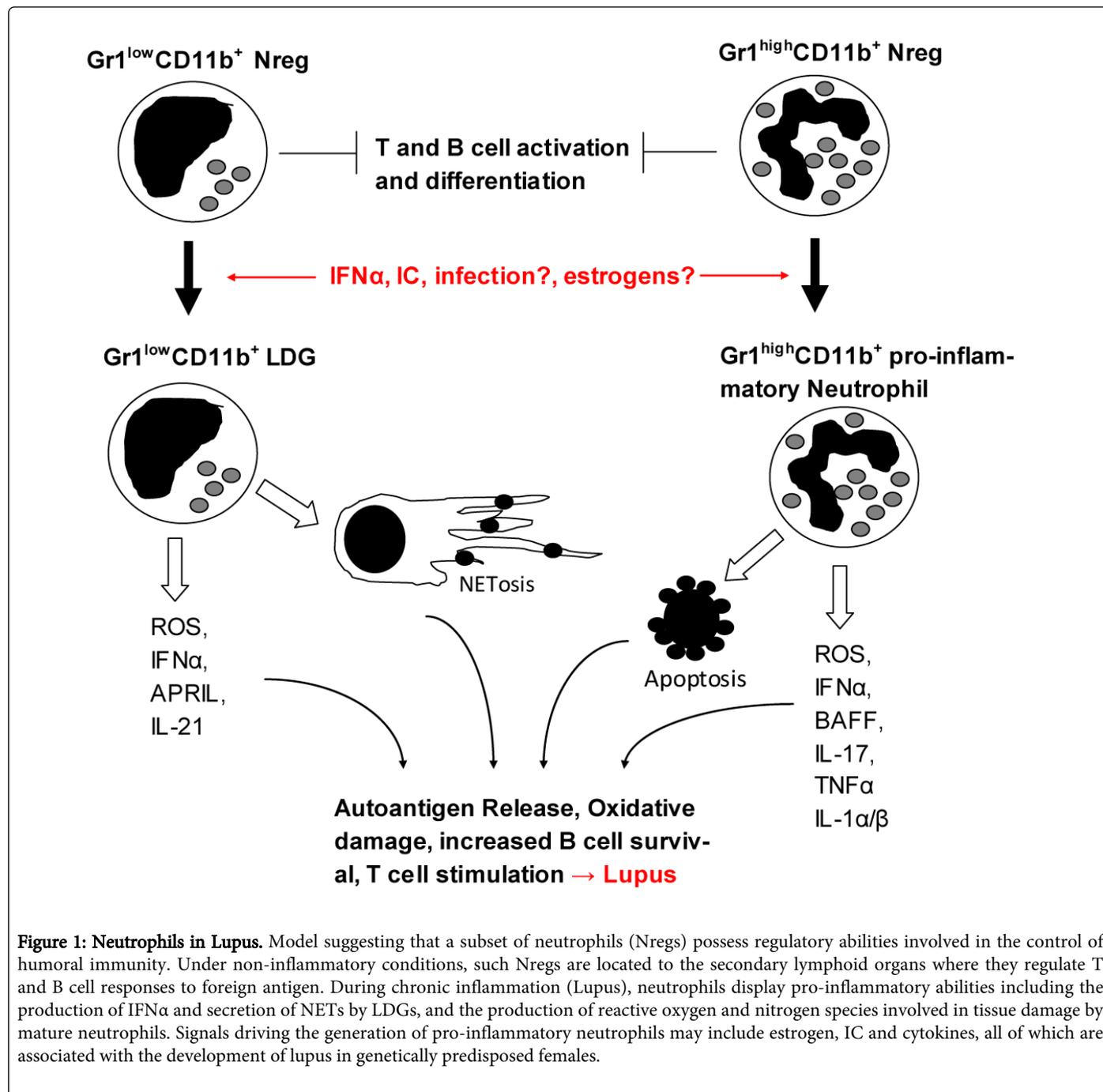


Figure 1: Neutrophils in Lupus. Model suggesting that a subset of neutrophils (Nregs) possess regulatory abilities involved in the control of humoral immunity. Under non-inflammatory conditions, such Nregs are located to the secondary lymphoid organs where they regulate T and B cell responses to foreign antigen. During chronic inflammation (Lupus), neutrophils display pro-inflammatory abilities including the production of IFNα and secretion of NETs by LDGs, and the production of reactive oxygen and nitrogen species involved in tissue damage by mature neutrophils. Signals driving the generation of pro-inflammatory neutrophils may include estrogen, IC and cytokines, all of which are associated with the development of lupus in genetically predisposed females.

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