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Neutrophils regulate humoral autoimmunity by restricting interferon gamma production via the generation of reactive oxygen species

Xinfang Huang[1,2,7], **Jingjing Li**[1,7], **Stephanie Dorta-Estremera**[1,3], **Jeremy Di Domizio**[1,8], **Scott M. Anthony**[1,3], **Stephanie S. Watowich**[1,3], **Daniel Popkin**[4], **Zheng Liu**[5], **Philip Brohawn**[5], **Yihong Yao**[5], **Kimberly S. Schluns**[1,3], **Lewis L. Lanier**[6], and **Wei Cao**[1,3,*]

[1]Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030

[2]Department of Rheumatology, Renji Hospital, Shanghai Institute of Rheumatology, Shanghai Jiao Tong University School of Medicine, Shanghai 200001, China

[3]The University of Texas Graduate School of Biomedical Sciences, Houston, Texas 77030

[4]Department of Dermatology, Case Western Reserve University, Cleveland, OH 44106

[5]MedImmune, LLC, Gaithersburg, MD 20878

[6]Department of Microbiology and Immunology and the Cancer Research Institute, University of California at San Francisco, San Francisco, CA 94143

Summary

Here we examine the mechanism by which plasmacytoid dendritic cells (pDCs) and type I interferons promote humoral autoimmunity. In an amyloid-induced experimental autoimmune model, neutrophil depletion enhanced anti-nuclear antibody development, which correlated with heightened IFN-γ production by natural killer (NK) cells. IFN-α/β produced by pDCs activated NK cells via IL-15 induction. Neutrophils released reactive oxygen species (ROS), which negatively modulated the levels of IL-15 thereby inhibiting IFN-γ production. Mice deficient in NADPH oxidase 2 produced increased amounts of IFN-γ and developed augmented titers of autoantibodies. Both pDC-IFN-α/β pathway and IFN-γ were indispensable in stimulating humoral autoimmunity. Male NZB/W F1 mice expressed higher levels of superoxide than their female lupus-prone siblings, and depletion of neutrophils resulted in spontaneous NK cell and

*Correspondence: wcao@mdanderson.org, wei.cao.phd@gmail.com.

[7]Co-first author

[8]Present address: Department of Dermatology, University Hospital CHUV, 1011 Lausanne, Switzerland

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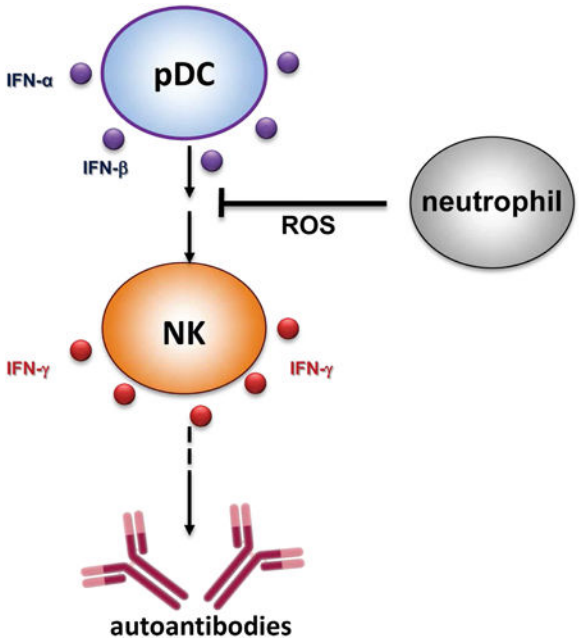
autoimmune B cell activation. Our findings suggest a regulatory role for neutrophils *in vivo* and highlight the importance of an NK-IFN-γ axis downstream of pDC-IFN-α/β pathway in systemic autoimmunity.

Graphical abstract



Introduction

Aberrant innate immune responses play a critical role in promoting the autoimmune adaptive immune response and exacerbate disease pathogenesis. A type I interferon (IFN-α, β, ω, τ, IFN-I) molecular signature is found in patients with systemic lupus erythematosus (SLE), a heterogeneous systemic disease with autoantibodies to nuclear antigens (ANA) and double-stranded DNA (dsDNA) (Ronnblom and Pascual, 2008, Kono et al., 2013, Lipsky, 2001). An IFN-I-stimulated gene (ISG) profile is significantly correlated with the levels of anti-dsDNA antibody and disease severity. IFN-α/β triggered by cells sensing nucleic acids is increasingly implicated as a key factor in antibody-mediated autoimmunity.

pDCs are a unique dendritic cell subset that specializes in rapid production of high amounts of IFN-I upon sensing RNA or DNA by endosomal TLRs, thereby functioning as an immediate early IFN-I producer during viral infections (Gilliet et al., 2008). In SLE, pDCs serve as a major source of aberrant IFN-I in response to immune complexes (ICs). These complexes are comprised either of autoantibodies to chromatin and ribonucleoprotein complexes, or of DNA-containing neutrophil extracellular traps (NETs) induced by autoantibodies (Gilliet et al., 2008, Caielli et al., 2012). In recent years using various genetic and cell-type specific ablation strategies, several groups have demonstrated that pDCs play a pivotal role in autoantibody development and disease progression *in vivo* (Di Domizio et al.,

2012a, Baccala et al., 2013, Rowland et al., 2014, Sisirak et al., 2014). However, how pDCs and IFN-I instruct autoimmune responses is not clear.

Neutrophils are abundant innate immune cells that rapidly infiltrate sites of infection or injury to provide host protection against microbes (Mayadas et al., 2014, Nauseef and Borregaard, 2014). SLE patients display defects in clearing apoptotic neutrophils and have aberrant neutrophils in the periphery, which constitute signature lupus erythematosus (LE) cells (Pisetsky, 2012, Caielli et al., 2012). NETs formed by IFN-I- or autoantibody-activated neutrophils stimulate pDCs to secrete IFN-I, which further promotes the generation of mature antigen-presenting cells and activates autoreactive B cells to enhanced autoantibody production *in vitro* (Caielli et al., 2012). In the kidney of lupus patients, netting neutrophils can induce tissue damage; SLE patients with impaired DNAse I function or failure to dismantle NETs have an increased incidence of lupus nephritis (Hakkim et al., 2010). In lupus-prone mice, inhibition of peptidylarginine deiminase, a key enzyme required for NET formation, protected against vascular, kidney, and skin damage (Knight et al., 2013). Separately, patients from a subgroup of vasculitides, a systemic disease with inflammation of blood vessels, develop anti-neutrophil cytoplasmic antibodies (ANCA), which are also present in some SLE patients. It was shown that ANCA induction can be initiated by NETs through transfer of cytoplasmic neutrophil antigens to dendritic cells (DCs) (Sangaletti et al., 2012).

However, several recent studies have revealed that neutrophils can inhibit systemic autoimmunity *in vivo*. Defective ROS production, and presumably NETosis, as a result of phagocyte NADPH oxidase 2 (NOX2) deficiency paradoxically exacerbates lupus development in MRL-Fas^{lpr} mice, an observation consistent with the increased incidence of lupus in patients with X-linked chronic granulomatous disease (Campbell et al., 2012). Moreover, Trigunaite *et al.* recently reported that Gr-1^{+} cells protect male NZB/W F1 mice from developing autoantibodies and lupus-like disease (Trigunaite et al., 2013). Therefore, how neutrophils participate in autoimmune pathogenesis versus protection remains an unresolved question.

We recently have established an animal model in which activation of pDCs and IFN-I production promotes the development of a lupus-like syndrome in healthy mice (Di Domizio et al., 2012a). Accompanying pDC activation, prominent neutrophilia was induced. In this study, we have investigated the contribution of neutrophils and their possible interactions with pDCs in instigating autoantibody development with an attempt to reveal the key pathways promoting humoral autoimmunity.

Results

Neutrophils negatively regulate pDC-mediated autoantibody development

Amyloid fibrils are stable insoluble aggregates of misfolded proteins and amyloidogenic proteins can act as danger-associated molecular patterns (DAMP), triggering NLRP3 inflammasome activation (Masters and O'Neill, 2011). Native proteins form amyloid fibrils by transiting through a state of amyloid precursors, which readily form amyloid in the presence of nucleic acids and glycosaminoglycan (Di Domizio et al., 2012b). We prepared

amyloid using human serum albumin (HSA), an abundant protein with native globular structure without apparent immune stimulatory function, and HSA with DNA or with heparin (Di Domizio et al., 2012a). When incubated with bone marrow-derived macrophages (BMDM), HSA amyloids invariably induced the secretion of IL-1β (Figure 1A). In contrast, no significant amount of IL-1β was detected from the BMDM cultured with native HSA or denatured HSA protein. This result indicates that different forms of amyloid fibrils can similarly function as DAMP.

As reported previously, DNA-containing amyloid can trigger selective infiltration of IFN-I-producing pDCs into peritoneal cavity after *i.p.* inoculation (Di Domizio et al., 2012a). We injected BALB/c mice with HSA amyloid containing DNA (precipitate formed by mixing amyloid precursor of HSA protein and genomic DNA of *E. coli*; we will refer it simply as amyloid hereafter) or a mixture of a comparable amount of native HSA and DNA (referred as control). High amounts of IL-1β transcript were detected in peritoneal exudate cells (PECs) harvested 18 hrs after inoculation of amyloid (Figure 1B). Neutrophilia represents a hallmark of IL-1β-mediated inflammation. Accordingly, significant numbers of neutrophils infiltrated the peritoneal cavity of mice that received amyloid, which peaked at 6 hrs after injection (Figure 1C). Apparently, IL-1β stimulates the neutrophilia, as *Il1r*$^{-/-}$ mice had drastically reduced infiltrating neutrophils after amyloid inoculation (Figure 1D).

Residing in the peritoneal cavity of naïve mice are two subsets of macrophage, *i.e.* large peritoneal macrophage (LPM) and small peritoneal macrophage (SPM) (Ghosn et al., 2010, Cain et al., 2013, Okabe and Medzhitov, 2014, Gautier et al., 2014). Amyloid inoculation resulted in the disappearance of LPM and slight increase of SPM in PECs (Figure S1A). Injection of clodronate encapsulated in liposomes prior to amyloid inoculation depleted LPM, but not SPM (Figure S1A-B). Although LPM depletion reduced recruitment of neutrophils and dendritic cells, it did not diminish the expression of IL-1β induced by amyloid (Figure S1B-D). Among PEC populations, both SPM and neutrophils transcribed IL-1β (Figure S1E), suggesting that the resident SPM, other than LPM, is likely the primary source of IL-1β upon sensing amyloid.

Given the dual infiltration of pDCs and neutrophils and the importance of these cells in systemic autoimmunity, we expected that neutrophils might facilitate a pDC-mediated humoral autoimmunity that is triggered by immunization with DNA-containing amyloid (Di Domizio et al., 2012a). To examine the functional role of neutrophils, we pre-injected BALB/c mice with anti-Ly6G mAb (clone 1A8), which selectively and transiently depleted neutrophils *in vivo* (Figure 1E). Unexpectedly, mice without neutrophils at the time of amyloid inoculation developed a heightened ANA response (Figure 1F and 1G) and increased antibodies reactive to tissue antigens (Figure 1H). We have observed that, in addition to ANA, amyloid-immunized BALB/c mice quickly developed ANCA with a cytoplasmic staining pattern (Figure S2). Different from ANA, ANCA was reduced in mice injected with anti-Ly6G, suggesting that infiltrating neutrophils are probably involved in ANCA development (Figure S2). Therefore, our data revealed an unexpected regulatory role of neutrophils in pDC-mediated ANA development.

Neutrophils do not affect IFN-α/β but rather regulate IFN-γ production

To understand the mechanism underlying the surprising observation, we examined whether neutrophils affect pDC infiltration and/or IFN-I production. The number of pDCs in the peritoneal cavity 24 hrs after amyloid injection was reduced in the absence of neutrophils, whilst the number of conventional dendritic cells (cDCs) remained unaffected (Figure 2A). However, the overall expression of ISGs in PECs, a functional readout of IFN-I, was not significantly affected in the absence of neutrophils (Figure 2B).

Next, we searched for the genes whose expression is affected by neutrophils and found that PECs from mice received anti-Ly6G prior to amyloid inoculation invariably expressed high levels of IFN-γ, *i.e.* type II interferon (IFN-II) (Figure 2C). Consistently, *Ifi30, Ciita,* and *Irf1,* genes sensitively affected by IFN-II, were upregulated in the same group (Figure 2C). Of note, the transcripts of *Ifng* and *Ifi30* were detected in all the mice that received amyloid in the presence of neutrophils. We also found consistently that neutrophil depletion caused a significant increase in IFN-γ protein secretion (Figure 2D). By contrast, IL-12 p40 production was unaffected by neutrophils. Granulocyte colony-stimulating factor (G-CSF) can potently mobilize neutrophils from bone marrow and increase the number of peripheral neutrophils. Mice that received G-CSF had elevated numbers of neutrophils and decreased IFN-γ levels in the peritoneal cavity after amyloid inoculation (Figure 2E), revealing a negative correlation between neutrophil presence and production of IFN-γ. Collectively, our findings indicate that neutrophils might downregulate IFN-γ production, but have no significant impact on IFN-I-mediated activation amidst an inflammatory response that can result in autoimmunity.

Infiltrating NK cells produce IFN-γ

To identify the cellular source of IFN-γ that is subjected to neutrophil regulation, we sorted peritoneal cells after amyloid inoculation and performed quantitative PCR analysis. NK cells uniquely expressed high levels of IFN-γ transcripts (Figure 3A). Kinetic analysis revealed a selective infiltration and a further expansion of NK cells in response to amyloid (Figure 3B). Consistent with the transcript analysis, peritoneal NK cells from amyloid-inoculated mice produced elevated IFN-γ protein detectable by intracellular staining (Figure 3C). No IFN-γ was detected in other peritoneal leukocytes (not shown). In addition to IFN-γ secretion, the infiltrating NK cells display a more mature phenotype, *i.e.* a higher percentage of NK cells expressing CD11b but lacking expression of CD27 (Figure S3A).

To examine the requirement of NK cells for IFN-γ production, we injected C57BL/6 (B6) mice with anti-NK1.1 mAb to deplete NK cells prior to the inoculation of amyloid and examined the amount of IFN-γ in the peritoneal fluid. As expected, NK cell depletion severely abolished IFN-γ production, but had no effect on IL-12 p40, which is produced by myeloid cells (Figure 3D). IL-15 is a key cytokine that is required for NK cell development (Sun and Lanier, 2011). *Il15ra*$^{-/-}$ mice failed to produce IFN-γ in response to amyloid, which correlates with the absence of peritoneal NK cells (Figure 3E). Taken together, our findings suggest that NK cells are the primary producer of IFN-γ during the innate immune response to amyloid.

Neutrophils inhibit IFN-γ response via ROS production

To understand how neutrophils control IFN-γ production, we further characterized these cells amidst the amyloid-induced peritonitis. The infiltrating neutrophils expressed IL-1β (Figure S1E) and upregulated the surface expression of CD80 and MHC class II (Figure 4A). Activated neutrophils can potently generate ROS. We detected increased levels of superoxide in neutrophils harvested from the peritoneum of amyloid-inoculated mice by staining with a specific fluorescent probe dihydrorhodamine 123 (DHR; Figure 4B). In addition, peritoneal fluid harvested from amyloid-inoculated mice contained significant amounts of hydrogen peroxide, consistent with the ROS production (Figure 4C, left). Furthermore, high amounts of myeloperoxidase (MPO) were detected in the peritoneal fluid after amyloid inoculation (Figure 4C, right). Hence, infiltrating neutrophils are highly activated and produce effector molecules that might affect the immune response by other cells.

To reveal the role of neutrophils in regulating the cytokine response, we harvested PECs 10 hrs after inoculation of amyloid and subsequently depleted neutrophils by using anti-Ly6G antibody-coated beads. After culture of the PECs with amyloid for 24 hrs, significantly higher amounts of IFN-γ, but not IL-12 p40, were detected in the culture depleted of neutrophils compared with the cultures containing neutrophils (Figure 4D). This result confirms a potent regulatory effect by neutrophils on IFN-γ production. Because several molecules produced by neutrophils are capable of inhibiting immune responses (Nauseef and Borregaard, 2014, Mayadas et al., 2014), we tested small molecule inhibitors against ROS, arginase, iNOS, and MPO in the culture of PECs upon re-stimulation with amyloid *in vitro*. Intriguingly, IFN-γ production was enhanced solely by the inhibition of ROS with N-acetyl-L-cysteine (NAc) and catalase, but not by blocking the other molecules (Figure 4E). Consistently, no significant induction of arginase, iNOS or IL-10 by amyloid was detected *in vivo* (Figure S3B).

NOX2 is primarily expressed by phagocytes and is responsible for oxidative burst in neutrophils (Sareila et al., 2011). We thus compared neutrophils isolated from wildtype (WT) and *Nox2*$^{-/-}$ B6 mice for their ability to modulate IFN-γ production in neutrophil-depleted PECs *in vitro*. Although as expected, WT neutrophils actively suppressed IFN-γ production, neutrophils lacking *Nox2* lost the ability to downregulate IFN-γ (Figure 4F), indicating an essential function of ROS in the regulatory activity of neutrophils. Conversely, IFN-γ production by *Nox2*$^{-/-}$ PECs were dose-dependently inhibited by the addition of H_2O_2 in culture (Figure 4G). Lastly, *Nox2*$^{-/-}$ mice secreted higher amounts of IFN-γ in comparison with WT mice in the peritoneal fluid after amyloid inoculation *in vivo* (Figure 4H). In summary, our results reveal that neutrophils potently regulate the NK cell-mediated IFN-γ response through the production of ROS.

ROS regulates IFN-γ by controlling IFNα/β-induced IL-15

Neither human or mouse NK cells respond directly to amyloid by producing IFN-γ *in vitro* (not shown). During the early phase of mouse cytomegalovirus infection, pDCs produce IFN-I and promote transient NK cell activation and cytotoxicity *in vivo* (Swiecki et al., 2010). To investigate the role of pDC-dependent IFN-I pathway in NK cell activation, we

inoculated amyloid into *Ifnar*$^{-/-}$ mice as well as *feeble* mice, which carry a mutant *Slc15a4* gene and are selectively defective in IFN production by pDCs (Blasius et al., 2010). Both strains produced significantly lower IFN-γ than WT mice (Figure 5A), suggesting a prominent role of IFNα/β produced by pDCs in provoking NK cell activation.

IL-12 and IL-18 can potently activate NK cells. However, amyloid did not induce IL-12 p70 or IL-18 production *in vivo* (Figure S3C). We thus seek to elucidate the mechanism underlying IFN-γ production by NK cells. PECs were first harvested 10 hrs after inoculation of amyloid, and then NK cells were isolated from the PECs by flow cytometric sorting. When sorted NK cells were added back to NK cell-depleted PECs, they secreted significantly higher amounts of IFN-γ in the culture after re-stimulation with amyloid (Figure 5B). In contrast, no elevated IFN-γ production was detected in the cultures when NK cells were placed in a separate chamber of a transwell from the PECs, suggesting a requirement of cell-cell contact for NK cell activation. IL-15 is a cytokine that is trans-presented by IL-15Rα on the surface of cDCs to promote the development and effector function of NK cells (Sun and Lanier, 2011, Lucas et al., 2007). Interestingly, *Il15* transcription was elevated in peritoneal cells from amyloid-inoculated mice (Figure 5C). Furthermore, IL-15 levels in PECs positively correlated with IFN-γ^{+} NK cells in *Ifnar*$^{-/-}$ mice and *feeble* mice (Figure 5D), suggesting a strong link between IL-15 and NK cell activation.

We then examined the infiltration of NK cells in the presence or absence of neutrophils after amyloid inoculation, but did not detect a significant difference in the number of NK cells in the peritoneal cavity (Figure 5E, left). However, neutrophil depletion induced a drastic increase of the percentage of IFN-γ^{+} NK cells (Figure 5E, right), suggesting that neutrophils suppress the activation of NK cells, rather than influencing their migration. It has been shown that IFNα/β can prime NK cells by activating cDCs to produce IL-15 (Lucas et al., 2007). Therefore, we isolated peritoneal cDCs and examined their gene expression. Intriguingly, cDCs expressed higher levels of ISGs and IL-15 in the absence of neutrophils; while in comparison, no upregulation of IL-15 and ISGs was detected in Ly6C^{+} monocytes (Figure 5F), suggesting a selective negative regulation of cDCs by neutrophils.

To further validate the function of IL-15 on NK cell activation, we performed co-culture experiments where WT NK cells were incubated directly with NK cell-depleted PECs from WT or *Il15ra*$^{-/-}$ B6 mice. After re-stimulation, only accessory cells from WT mice supported the IFN-γ production by NK cells (Figure 5G), indicating a requirement of IL-15 in NK cell activation. In contrast, IL-12 p40 was not significantly regulated by neutrophils or IL-15 in cDCs or PECs. Furthermore, when gene expression of PECs was examined, ROS-deficient *Nox2*$^{-/-}$ mice expressed highly elevated IL-15 (Figure 5H), which correlates positively with the increased IFN-γ production *in vivo* (Figure 4H). Therefore, our findings strongly suggest that ROS negatively modulates IL-15 expression, which leads to suppression of NK cell-mediated IFN-γ production.

pDC-IFNα/β pathway and IFN-γ are mutually critical for promoting humoral autoimmunity

T follicular helper (T_{FH}) cells are a specialized T cell subset essential for germinal center responses and increased numbers of T_{FH}-like cells have been detected in SLE patients

(Craft, 2012, Tangye et al., 2013). We investigated the generation of T_{FH} cells induced by IFN-γ and pDC-produced IFN-α/β in a culture system. Enriched splenic $CD11c^+$ DCs containing both pDCs and cDCs were co-cultured with Bcl6 reporter $CD4^+$ T cells (Kitano et al., 2011) in the presence of CpG A, a TLR9 agonist that induces an IFN-I response from pDCs, or/and IFN-γ for 3 days. CpG A effectively stimulated the generation of $Bcl6^+$ T_{FH} cells (Figure 6A). Although IFN-γ alone failed to increase the number of Bcl6-expressing in T cells, IFN-γ significantly enhanced the development of $Bcl6^+$ T_{FH} cells when added to CpG A-containing cultures. This result illustrates a synergy between IFN-I and IFN-II to promote T_{FH} differentiation.

Because *Nox2*$^{-/-}$ mice produced higher levels of IFN-γ than WT B6 mice in response to amyloid (Figure 4H), we examined their ability to support the development of humoral autoimmunity induced by amyloid. As shown in Figure 6B, anti-histone IgG developed early and in higher titers in the *Nox2*$^{-/-}$ mice in comparison with WT B6 mice after immunization with amyloid. Consistently, amyloid-immunized *Nox2*$^{-/-}$ mice displayed an elevated ANA response (Figure 6C), suggesting that *Nox2* deficiency, which is associated with exacerbated IFN-γ production, facilitates the onset of humoral autoimmunity. In contrast, mice lacking IL-1 receptor, which had diminished neutrophil infiltration (Figure 1D), developed autoantibodies similarly as WT B6 mice after immunization with amyloid (Figure S4A). Further analysis revealed that *Il1r*$^{-/-}$ mice produced generally normal levels of IFNγ after amyloid inoculation (Figure S4B), which was accompanied by dampened infiltration of pDCs and NK cells (not shown). Therefore, seemingly neutrophils and IL-1 differentially affect IFNγ levels and autoantibody development.

Lastly, we immunized *feeble* mice, which have defects in pDC-mediated IFN-α/β production, or IFN-γ-deficient mice and found that the pDC-IFN-α/β pathway and IFN-γ are both required by amyloid to break humoral immune tolerance (Figure 6D and Figure 6E). Collectively, our studies have revealed a critical role of IFN-γ and a close interplay between two IFN families in stimulating humoral autoimmunity.

Neutrophils prohibit B cell autoimmunity in male lupus-prone mice

F1 progeny of New Zealand Black × New Zealand White (NZB/W) mice spontaneously develop an autoimmune syndrome with remarkable similarity to human SLE. We compared young B6 and age-matched NZB/W mice and detected somewhat lower number of splenic neutrophils in the NZB/W strain (Figure 7A, left). In humans, many types of autoimmune pathology display strong female predominance (Fish, 2008). Likewise, female NZB/W mice develop an accelerated lupus-like disease compared with male NZB/W mice. Further examination revealed a selective decrease in the number of neutrophils in female NZB/W in comparison with female B6 mice (Figure 7A, right). Analysis of the plasma samples revealed higher levels of circulating H_2O_2 in young male compared with female mice in both strains (Figure 7B, left). By contrast, the amount of circulating MPO was not significantly different (Figure 7B, right), which echoes the discordant *in vivo* production between MPO and H_2O_2 observed earlier (Figure S1C). Although no overt difference was detected in the number of splenic NK cells (Figure S5A), male NZB/W mice contain significantly higher numbers of NK cells, when compared with B6 males (Figure 7C). In

general, NZB/W mice harbored the elevated numbers of mature NK cells (Figure 7D). Strikingly, all NK cells from NZB/W mice, regardless of maturation status or sex, express significantly increased levels of the activating NK cell receptor NKp46 (Figure 7E and Figure S5B). Therefore, neutrophils and NK cells seemingly are differentially regulated between young lupus-prone and healthy mice, which are also influenced by gender.

A recent study suggested that Gr-1^{hi}CD$11b^{+}$ cells suppress lupus pathogenesis in young male but not female NZB/W mice, based on the observation that continuous administration of anti-Gr-1 antibody increased both anti-dsDNA antibody titers and deposition of IgG immune complexes in the kidney (Trigunaite et al., 2013). To examine whether anti-Gr-1-treatment affects NK cell function, we administrated a single injection of anti-Gr-1 antibody to young male NZB/W mice and detected the appearance of IFN-γ^{+} NK cells in the spleen 4 days later (Figure S6A). As expected, injection of anti-Gr-1 antibody depleted both neutrophils and Ly6C^{+} myeloid cells (Figure S6B). To compare with the earlier findings from amyloid-induced autoimmunity model, we tested the impact of transient neutrophil depletion in young NZB/W mice. Specifically, we injected anti-Ly6G mAb *i.p.* once into male NZB/W mice, which selectively depleted neutrophils (Figure S6C). $CD3^{-}NK1.1^{+}$ NK cells isolated 2 days from the spleen of neutrophildepleted mice transcribed significantly higher levels of *Eomes* and *Prdm1* (also known as Blimp1), two transcriptional factors highly expressed by NK cells, and more *Ifng,* indicating strong NK cell activation (Figure 7F). Four days after mAb injection, B cells isolated from mice injected with anti-Ly6G significantly enhanced the expression of genes critical for B cell survival (*Bcl2* and *Akt1*), immunoglobulin gene recombination (*Aicda,* which encodes activation-induced cytidine deaminase, and *Adar* which encodes RNA-specific adenosine deaminase), costimulation (*Cd40* and *Cd80*), and terminal differentiation (*Xbp1* and *Irf4*) (Figure 7G).

To evaluate the functional consequence of neutrophil depletion, we isolated B cells from male NZB/W mice after a single anti-Ly6G mAb injection and cultured them with TLR7 agonist R848. B cells from neutrophil-depleted mice produced significantly higher levels of autoreactive IgG (Figure 7H). Furthermore, male NZB/W mice depleted of neutrophils continuously developed increased serum autoantibodies against ssDNA and dsDNA *in vivo* (Figure 7I). To apprehend the underlying mechanism, we isolated neutrophils from spleen of naïve male and female young NZB/W mice and detected the elevated expression of genes whose products are components of neutrophil primary granules (*Mpo* and *Prtn3*, which encodes serine protease enzyme 3) or involved in immune suppression (Cd274 and *Pdcdlg1* encodes PD-L1 and PD-L2, respectively) (Figure 7J). Collectively, these observations reveal a compelling regulatory function of neutrophils in curtailing B cell autoimmunity in male lupus-prone mice.

Discussion

Although autoimmune responses are potently restrained by regulatory lymphocytes, how innate immune cells regulate autoimmunity is less well understood. In this study, we have revealed a remarkable role played by neutrophils in limiting the magnitude of IFN-α/β-stimulated autoimmunity and maintaining B cell tolerance in male lupus-prone mice.

The data we have obtained from *in vitro* culture and *in vivo* analysis of different mouse strains and treatments strongly suggest a role of ROS in neutrophil function under inflammatory conditions. A heterogeneous group of highly reactive molecules that oxidize targets in a biologic system, ROS are important for host defense against invading pathogens. *Nox2*-derived ROS also regulate immune responses and cell proliferation under various conditions, contributing to the resolution of inflammation (Sareila et al., 2011). In humans and mice alike, NOX2 mutation in $gp91^{phox}$ results in X-linked chronic granulomatous disease, which renders the patients simultaneously prone to serious infections by microbial pathogens, sterile chronic inflammation, and occasionally SLE (Campbell et al., 2012, Sareila et al., 2011). Consistently, a mutation in neutrophil cytosolic factor 2 (*NCF2*), which results in a reduction of *Nox2* activity and ROS production, confers substantially increased SLE (Jacob et al., 2012). Interestingly, rodents bearing *Ncf1* mutation are susceptible to autoimmune arthritis (Hultqvist et al., 2004). Our results are consistent with the report showing that *Nox2* inhibits the pathogenesis of lupus in MRL-Fas^{lpr} mice (Campbell et al., 2012). Further, our findings have revealed that *Nox2* controls IFN-γ production, which promotes autoimmune responses (Figure S7).

The functional contribution by neutrophils in autoimmune pathogenesis is seemingly multifaceted (Mayadas et al., 2014, Nauseef and Borregaard, 2014). The inhibitory function of neutrophils we have revealed is closely associated with inflammation-induced activation. In another inducible lupus model, neutrophils infiltrate the peritoneal cavity in response to pristane, hydrocarbon oil capable of inciting chronic inflammation and autoimmunity (Lee et al., 2011). Interestingly, depletion of neutrophils significantly ameliorated diffuse pulmonary hemorrhage in these mice (Shi et al., 2014). Of note, we did not detect neutrophil activation or death in response to amyloid upon examination of both human and mouse neutrophils, nor observed NET formation *in vitro* or *in vivo* (not shown). Therefore, how NETosis impacts autoantibody development in amyloid-induced autoimmune model remains uncertain. The observation that neutrophil depletion abolished ANCA (Figure S2) nevertheless implies a role of neutrophils in facilitating neutrophil-specific autoantibody response *in vivo*. Separately, we have demonstrated that IL-1 did not affect ANA development in the amyloid-inducible autoimmune model, despite its critical role in neutrophil recruitment (Figure 1D and S4). This and the fact that $Il1r^{-/-}$ mice were defective in general leukocyte recruitment but maintained normal IFNγ levels indicate a dispensable role of IL-1 in regulating the cellular cascade critical for humoral autoimmunity. Although such result was unexpected, inflammasome activation has been shown to impact SLE ambivalently (Shaw et al., 2011, Yin et al., 2013).

Neutrophils and NK cells engage in a bidirectional crosstalk under many conditions (Costantini and Cassatella, 2011, Vivier et al., 2011). In steady-state, neutrophils critically facilitate terminal NK cell maturation (Jaeger et al., 2012). In the spleen of NZB/W mice, the homeostasis of neutrophils and NK cells is jointly influenced by gender: young males have more neutrophils and significantly higher number of NK cells than female mice. The elevated number of neutrophils in male NZB/W mice has been shown to be controlled by testosterone, evidenced by the effects of castration and hormone supplementation (Trigunaite et al., 2013). Therefore, an increased number of neutrophils may facilitate a

larger population of NK cells in these mice. In healthy mice, a clear sex difference exist in the immune cell populations, and during acute inflammation, more neutrophils infiltrate the peritoneal cavity of male mice than females, indicating intrinsic difference between the genders (Scotland et al., 2011). Similar to our observation in mice, plasma hydrogen peroxide production reportedly is significantly higher in men than women; and intriguingly, high H_2O_2 levels are correlated with reduced renal and glomeruli dysfunction (Lacy et al., 2000). Therefore, it would be interesting to examine whether the ROS-mediated protective mechanism is breached in male SLE patients.

In response to various stimuli *in vitro*, human pDCs engage with NK cells through IFN-α/β, costimulatory molecules, and NK receptor-ligand interactions (Gilliet et al., 2008). In mice, pDCs are essential for the activation and expansion of NK cells shortly after certain viral infections (Swiecki et al., 2010). Additionally, TLR9-activated pDCs within melanomas can secrete IFN-I, which recruits and activates NK cells, and induces tumor regression *in vivo* (Liu et al., 2008). Here we have shown that the pDC-IFN-I pathway stimulates NK cell activation via induction of IL-15 presentation by cDCs. Therefore, a powerful innate immune activation cascade involving pDC-IFN-α/β-cDC-IL-15-NK-IFN-γ is likely operational under diverse physiological conditions – viral infection, autoimmune inflammation, and anti-cancer immune responses. ROS-producing neutrophils intercept this pathway by downregulating the expression of IL-15.

The contribution of NK cells to systemic autoimmunity remains uncertain (Tian et al., 2012, Fogel et al., 2013). Decreased NK cell numbers or impairment of NK cell-mediated cytotoxicity has been observed in many autoimmune disorders; however, such findings do not correlate with the accumulation of NK cells in inflamed tissues. Nevertheless, NK cells have been directly linked to the pathogenesis of organ-specific autoimmunity and chronic NK cell lymphocytosis is associated with autoimmune syndromes (Fogel et al., 2013). Importantly, genotype combinations of Killer cell Immunoglobulin-like receptors and their HLA class I ligands that favor NK cell activation predispose individuals to certain autoimmune disorders (Fogel et al., 2013). Furthermore, genetic polymorphisms in the activating NK cell receptor NKp30 that results in reduced gene transcription conveys protection from primary Sjogren's syndrome (pSS), whereas NKp30-dependent IFN-γ secretion by NK cells is significantly elevated in Sjogren's patients (Rusakiewicz et al., 2013). These observations collectively suggest a potential involvement of NK cells in promoting systemic autoimmunity. In pre-autoimmune NZB/W mice, we have shown that NK cells are more mature and express high levels of the activating receptor NKp46. Intriguingly, depletion of neutrophils in male NZB/W mice rapidly stimulated NK cells, which preceded the activation of autoimmune B cells. Early attempts to study the involvement of NK cells in lupus-prone mice were hampered by the concurrent depletion of iNKT cells after injection of NK1.1 mAb, a serious complication because iNKT cells affect autoimmune development (Novak and Lehuen, 2011). A detailed study using NK cell-specific knockout mice is necessary to definitively establish the role played by NK cells in lupus pathogenesis.

Despite signaling through distinct receptors, IFN-I and IFN-II induce largely overlapping interferon-stimulated genes as a result of similar JAK-STAT signaling. Interestingly, both

IFN-I and IFN-II are implicated in SLE: SLE patients have elevated levels of circulating IFN-γ and IFN-γ-induced molecular signature; and like IFN-α, patients receiving IFN-γ treatment occasionally develop lupus-like disease (Pollard et al., 2013, Chiche et al., 2014). In pSS, the presence of pDCs and NK cells correlates with the dual signature of IFN-I and IFN-II in the salivary glands (Rusakiewicz et al., 2013, Gottenberg et al., 2006). Furthermore, polymorphism of *IFNG* favoring elevated gene expression and combinational polymorphisms of the gene encoding IFN-γ receptor are associated with increased SLE susceptibility and lupus nephritis (Kim et al., 2010). Consistently, transgenic mice overexpressing IFN-γ develop ANA and lupus nephritis (Seery et al., 1997). Lupus-prone mice, such as MRL-Fas^{lpr} and NZB/W, invariably require IFN-γ signaling for disease pathogenesis and as do mice in pristane-induced and chemically induced lupus models (Pollard et al., 2013). However, the mechanism by which IFN-γ promotes autoimmune progression remains elusive. Our study demonstrates a sequential link between type I and type II interferons and reveals a dual obligation of these IFNs in humoral autoimmunity. We have identified NK cells as a source of IFN-γ downstream of pDC activation and demonstrated that IFN-γ can boost pDC-induced T_{FH} development. Lee *et al.* reported that autoimmune $Roquin^{san/san}$ mice overproduce IFN-γ and have exaggerated T_{FH} development and a dysregulated germinal center response (Lee et al., 2012). Further detailed characterization is needed to fully elucidate the involvement of NK cells in facilitating B cell development and the synergistic interplay between IFN-I and IFN-II.

Various therapeutic strategies have been developed with an aim to block the function of IFN-I in SLE (Thanou and Merrill, 2014). Our findings suggest that a successful treatment regimen should also attempt to block IFN-II or target both IFN families, if enhanced susceptibility to infection can be managed. To harness the strategy deployed by the male mice with genetic predisposition to autoimmunity, specific NOX2 agonists may provide a means to restore the B cell tolerance and reduce systemic inflammation in patients. Strategies to target NK cells and other IFN-γ-producing helpers might also benefit the effort.

Experimental Procedures

Mice

All experiments were conducted with sex- and age-matched mice. Animal studies were approved by the Institutional Animal Care and Use Committees of University of Texas MD Anderson. C57BL/6, BALB/cByJ, $Ifng^{-/-}$ (B6.129S-$Ifng^{tm1Ts}$), $Nox2^{-/-}$ (B6.129S6-$Cybb^{tm1Din}$/J), NZB/W (NZBWF1/J), and B6-Fas^{lpr} (B6.MRL-Fas^{lpr}/J) mice were purchased from The Jackson Laboratory. Dr. W. Overwijk (University of Texas M.D. Anderson Cancer Center, Houston, TX) provided $Infar1^{-/-}$ C57BL/6 mice, and Dr. T Okada (RIKEN, Research Center for Allergy and Immunology, Yokohama, Japan) provided $Bcl6^{yfp/yfp}$ C57BL/6 mice. $Il15ra^{-/-}$ and *feeble* C57BL/6 mice were described previously (Castillo et al., 2009, Blasius et al., 2012). All animal experiments were conducted on 8-12 weeks old mice unless otherwise specified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Mice pre-depleted of neutrophils develop more autoantibodies after pDC activation
- pDC-IFN-α/β pathway stimulates NK cells to produce IFN-γ by inducing IL-15
- ROS released by neutrophils decreases IL-15 thus inhibits IFN-γ production
- Neutrophils in male NZB/W F1 mice suppress NK cell and autoimmune B cell activation

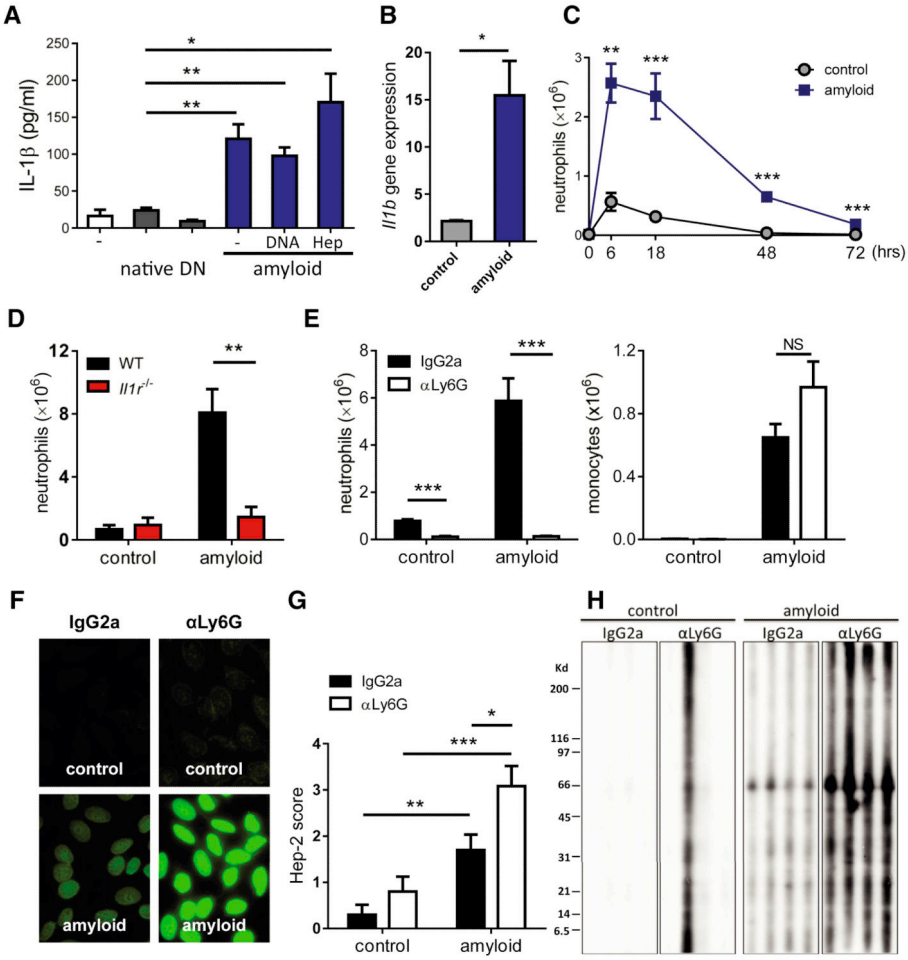

Figure 1. Neutrophils infiltrate and modulate autoantibody development in an inducible autoimmune model

(A) Secretion of IL-1β by BMDM stimulated with different forms of HSA for 2 hrs. DN: denatured HSA; Hep: heparin. Representative result (mean ± SD) of 3 experiments is shown. **P* <0.05, ** *P* <0.005.

(B) Relative *Il1b* gene expression in peritoneal cells of BALB/c mice 18 hrs after *i.p.* injection. Control groups received 70 μg native HSA and 30 μg DNA, and amyloid groups received 70 μg amyloid-precursor of HSA and 30 μg DNA (n = 4 from 2 experiments).

(C) Kinetics of neutrophil infiltration into the peritoneal cavity. Cell numbers were obtained by flow cytometry analysis on $Ly6G^{hi}CD11b^{hi}$ population (n = 6 from 2 experiments).

(D) The numbers of neutrophils in the peritoneal cavity of wildtype (WT) or *Il1r*$^{-/-}$ B6 mice 18 hrs after *i.p.* injection (n = 5 from 2 experiments).

(E) BALB/c mice were injected with 300 μg of anti-Ly6G or control rat IgG2a and, then 24 hrs later with control or amyloid *i.p.*. Peritoneal neutrophils and monocytes were analyzed 18 hrs later (n = 6 from 3 experiments).

(F) Anti-nuclear IgG in the sera of BALB/c mice that were pre-treated with anti-Ly6G mAb or control IgG2a and, then immunized with amyloid or control. Shown is immunofluorescence staining of fixed Hep-2 cells. Images were acquired at 40× magnification.

(G) Summary score of Hep-2 staining of immunized BALB/c sera (n = 12 from 2 experiments)
(H) Western blot analysis of sera of immunized BALB/c mice as in F and G on SDS-PAGE-separated mouse muscle proteins. Each line represents a serum sample from one mouse. Molecular weight markers in kilodalton are indicated on the left.
(B-E, G) Data represent mean and s.e.m. **P* <0.05, ***P* <0.01, and ****P* <0.001. NS: not significant. See Figure S1, S2 and S7.

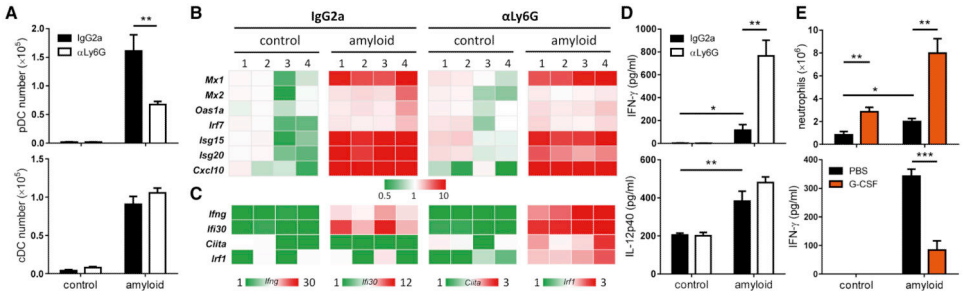

Figure 2. Depletion of neutrophils does not affect IFN-I but increases IFN-γ

(A) pDCs and cDCs present in PECs of BALB/c mice 24 hrs after inoculation of amyloid or control. Mice received pre-injections of either anti-Ly6G or control rat IgG2a 24 hrs prior to the inoculation. Cell numbers were obtained by flow cytometric analysis by gating $CD11c^{med}B220^{+}PDCA1^{+}$ as pDCs and $MHCII^{+}CD11c^{hi}F4/80^{-}$ as cDCs (n = 6 from 3 experiments).

(B) Expression of ISGs by PECs of BALB/c mice pre-injected with anti-Ly6G or IgG2a, and then inoculated with control or amyloid and harvested after 18 hrs. Heat-map is shown.

(C) Expression of IFN-γ and IFN-γ-stimulated genes in the same samples as in (B).

(D) Amounts of cytokines in the peritoneal fluid in the same samples as in (A).

(E) Number of neutrophils and levels of IFN-γ in the peritoneal cavity of BALB/c mice pre-injected with G-CSF or PBS for 24 hrs, and then inoculated with control or amyloid and harvested after 18 hrs (n = 4 from 2 experiments).

(A, D, E) Data represent mean and s.e.m. **P* <0.05, ***P* <0.01, and ****P* <0.001.

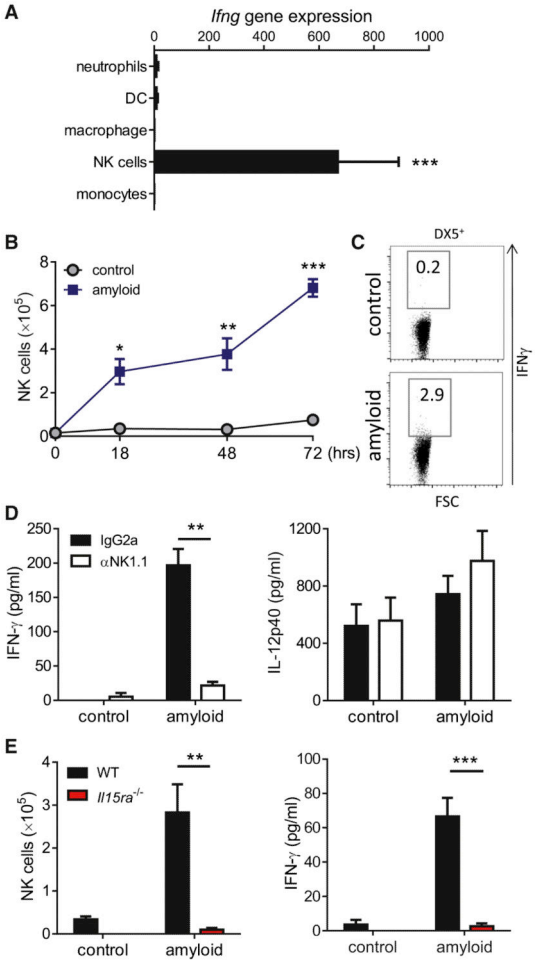

Figure 3. Amyloid induces NK cell infiltration and IFN-γ production

(A) Relative levels of *Ifng* transcript in different leukocytes isolated from peritoneal cells 18 hrs after amyloid inoculation (n = 4 from 2 experiments).

(B) Kinetics of NK cell infiltration into the peritoneal cavity (n = 10 from 2 experiments).

(C) Intracellular staining for IFN-γ protein in NK cells in peritoneal cells harvested 18 hrs after amyloid inoculation. Cells were stained for NK cell markers ($CD3^{-}DX5^{+}$) and IFN-γ, and then analyzed by flow cytometry. A representative sample is shown (n = 8).

(D) Levels of cytokines in the peritoneal fluid of mice pre-injected with anti-NK1.1 mAb or control IgG2a, and then inoculated with control or amyloid and harvested after 18 hrs (n = 8 from 3 experiments).

(E) Number of NK cells and levels of IFN-γ in the peritoneal cavity of WT or *Il15ra*$^{-/-}$ B6 mice 18 hrs after inoculation of control or amyloid (n = 7 from 3 experiments).

(A, B-E) Data represent mean and s.e.m. **P* <0.05, ***P* <0.01, and ****P* <0.001. NS: not significant. See Figure S3 and S7.

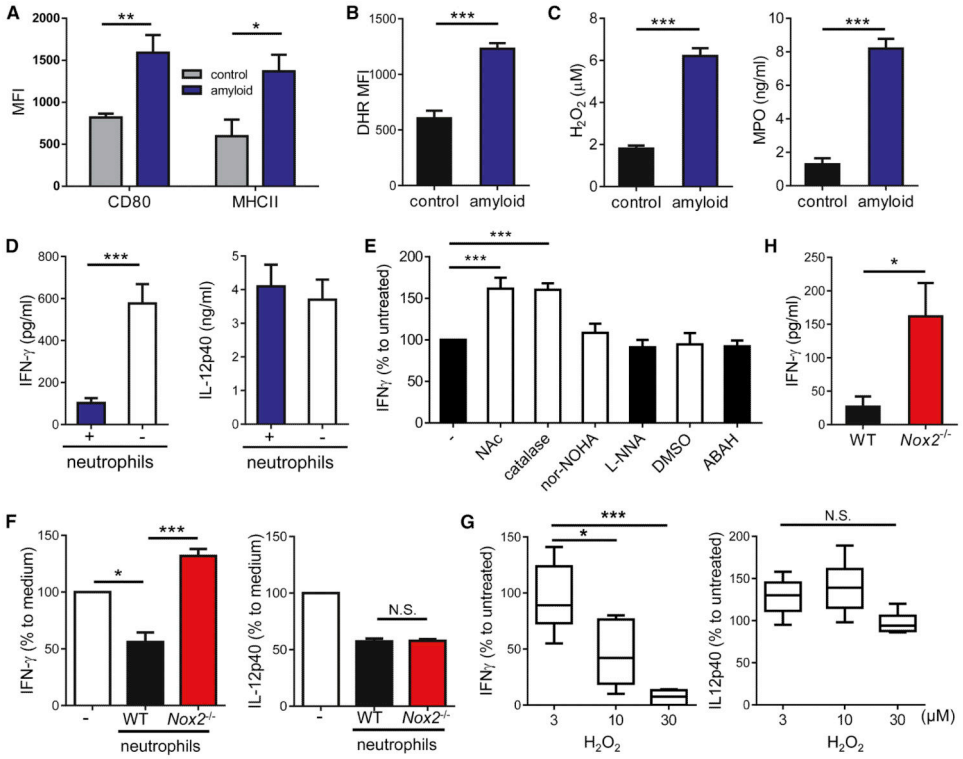

Figure 4. Neutrophils inhibit IFN-γ response via ROS

(A) Expression of CD80 and MHC class II on peritoneal neutrophils 6 hrs after inoculation of amyloid or control. Mean fluorescence intensity (MFI) was determined by flow cytometric analysis (n = 6 from 3 experiments).

(B) MFI of DHR levels in peritoneal neutrophils as in A.

(C) The levels of H_2O_2 and MPO in the peritoneal fluid harvested 18 hrs after inoculation of amyloid or control (n = 6∼15 from 3 experiments).

(D) PECs (+) or PECs depleted of neutrophils (-) were cultured in the presence of 10 μg/ml amyloid for 24 hrs. The cytokines in the culture supernatant were analyzed by ELISA (n = 10 from 3 experiments).

(E) PECs harvested from amyloid-inoculated mice were cultured with 10 μg/ml amyloid in the absence or presence of different inhibitors for 24 hrs. The levels of IFN-γ relative to culture with no inhibitor were calculated (n = 8 from 4 experiments).

(F) PECs harvested from amyloid-inoculated C57BL/6 mice were depleted of neutrophils *in vitro* (-). Separately, neutrophils were harvested from WT or *Nox2*$^{-/-}$ B6 mice after amyloid inoculation and added to the (-) PECs. The admixed cells were then cultured with 10 μg/ml amyloid for 24 hrs. The levels of cytokines relative to culture without neutrophils were calculated from ELISA analysis (shown are results from 4 experiments).

(G) PECs harvested from amyloid-inoculated *Nox2*$^{-/-}$ mice were cultured with 10 μg/ml amyloid in the absence or presence of different concentrations of H_2O_2 for 24 hrs. The levels of IFN-γ relative to culture with medium alone were calculated (n = 6 from 2 experiments).

(H) Levels of IFN-γ in the peritoneal cavity of WT or *Nox2*$^{-/-}$ B6 mice 10 hrs after inoculation of control or amyloid (n = 5∼6 from 2 experiments).

(A-H) Data represent mean and s.e.m. *P <0.05, **P <0.01, and ***P <0.001. N.S.: not significant. See Figure S3 and S7.

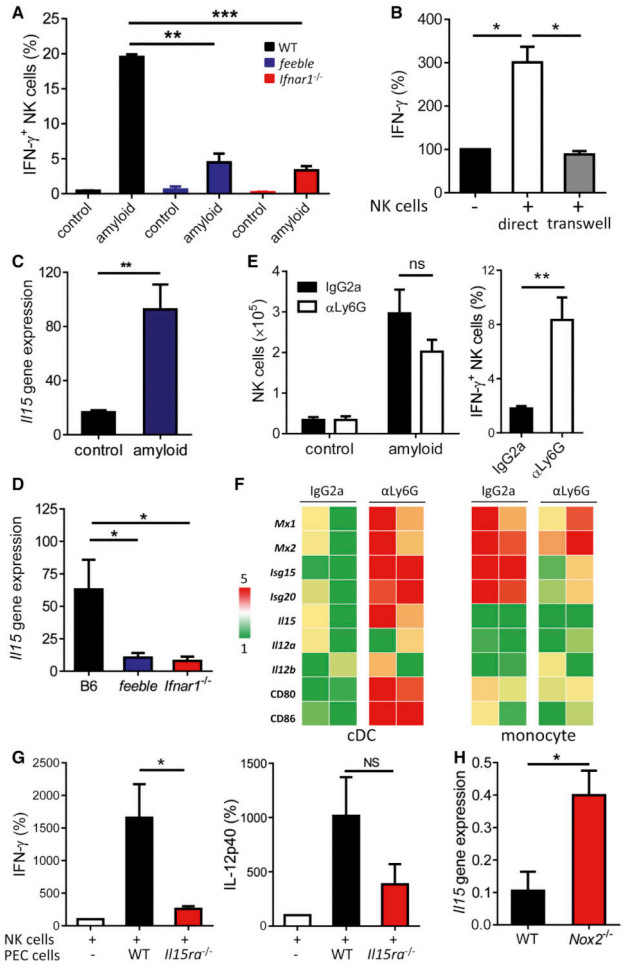

Figure 5. ROS restricts IFN-γ response by regulating IL-15 expression

(A) Percentage of NK cells producing IFN-γ in peritoneal cells from WT, *feeble*, or *Ifnar*$^{-/-}$ B6 mice 18 hrs after inoculation of amyloid or control. Cells were stained for NK cell markers and IFN-γ, and then analyzed by flow cytometry (n = 6 from 3 experiments).

(B) PECs from amyloid-inoculated B6 mice were depleted of NK cells *in vitro* (-). NK cells were added back to the (-) PECs either directly or in Trans wells to reconstitute to their original percentage in PECs. The mixed cells were cultured with 10 μg/ml amyloid for 24 hrs. The levels of IFN-γ relative to (-) culture were calculated after ELISA analysis. Data represent results from 3 experiments.

(C) Expression of IL-15 transcripts by PECs 18 hrs after inoculation of control or amyloid (n = 6 from 2 experiments).

(D) Expression of IL-15 transcripts by peritoneal cells of WT, *feeble* or *Ifnar*$^{-/-}$ B6 mice 18 hrs after inoculation of amyloid (n = 6 from 2 experiments).

(E) Number of NK cells in the peritoneal cavity of mice pre-injected with anti-Ly6G or control IgG2a, and then inoculated with control or amyloid and harvested after 18 hrs (left) and the percentage of IFN-γ^{+} NK cells from mice inoculated with amyloid (right) (n = 5 from 2 experiments).

(F) Transcript expression of ISGs and cytokines by cDCs or monocytes sorted from peritoneal cells harvested 6 hrs after inoculation of amyloid. The heat-map is shown.

(G) Peritoneal NK cells isolated from amyloid-inoculated B6 mice were cultured in medium (-), with NK-depleted PECs from WT mice, or with PECs from *Il15ra*$^{-/-}$ B6 nice, and then stimulated with 10 μg/ml amyloid for 24 hrs. The levels of cytokines relative to (-) culture were calculated after ELISA analysis. Data represent results from 6 experiments.
(H) Expression of IL-15 transcript by peritoneal cells of WT or *Nox2*$^{-/-}$ B6 mice 18 hrs after inoculation of amyloid (n = 6 from 2 experiments)
(A-E, G) Data represent mean and s.e.m. **P* <0.05, ***P* <0.01, and ****P* <0.001. NS: not significant. See Figure S3 and S7.

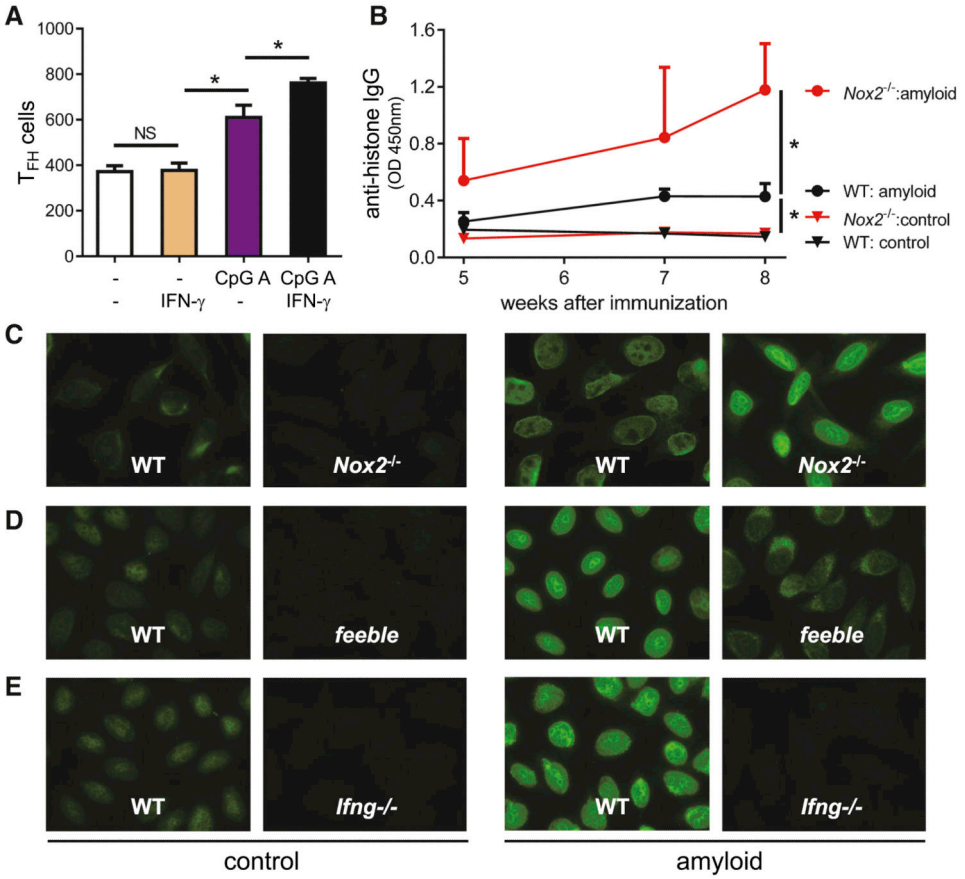

Figure 6. pDC-IFNα/β pathway and IFN-γ are critical for humoral autoimmunity

(A) Numbers of T_{FH} cells in the mixed culture of *Bcl6*^{fyp} $CD4^+$ T cells and $CD11c^+$ DCs (T:DC ratio=10:1). Flow cytometric analysis was performed on live cells and gated on the $CD4^+CD44^+ICOS^+CXCR5^+Bcl6^+$ population. Data represent results from 4 experiments.

(B-C) Levels of IgG against histone (B) and ANA (C) in the sera of WT or *Nox2*$^{-/-}$ B6 mice immunized with amyloid or control (n = 8∼9). Sera were diluted 1:500 in (C).

(D) Anti-nuclear IgG detected in sera of WT or *feeble* B6 mice immunized with amyloid or control. Sera were diluted 1:100 (n = 8).

(E) Anti-nuclear IgG detected in sera of WT or *Ifng*$^{-/-}$ BALB/c mice immunized with amyloid or control. Sera were diluted 1:200 (n = 9).

(A-B) Data represent mean and s.e.m. **P* <0.05, ***P* <0.01, and ****P* <0.001. (C-E) A representative sample is shown. See Figure S4 and S7.

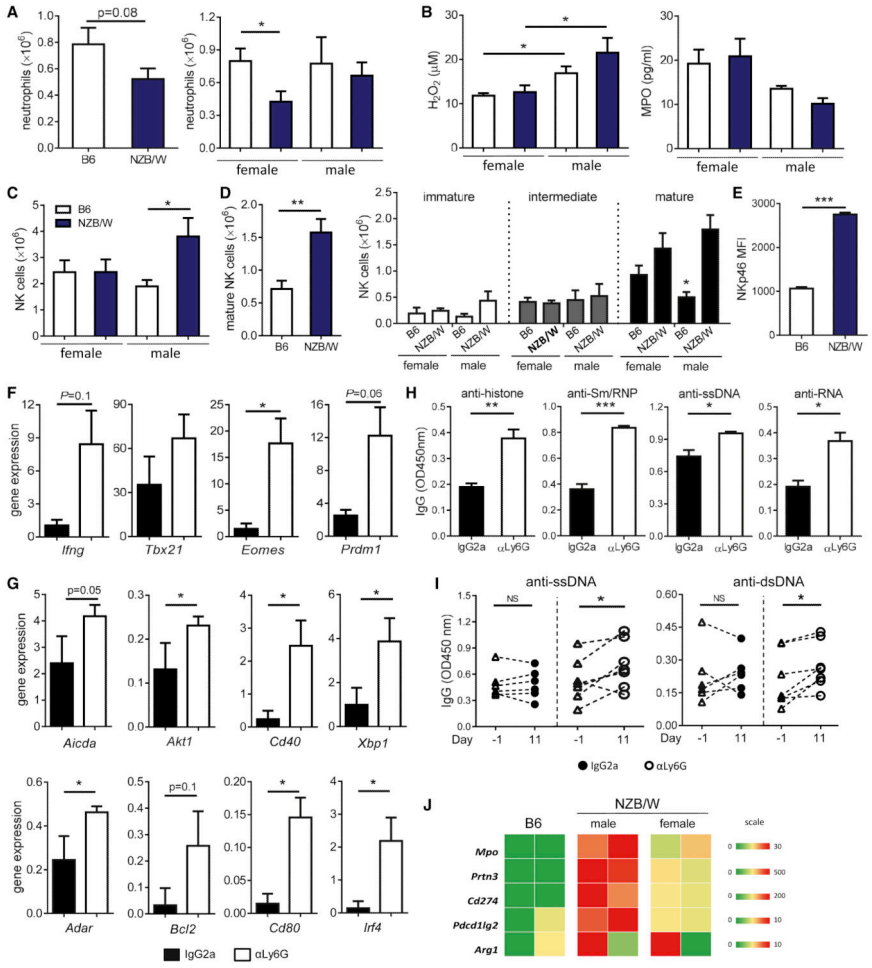

Figure 7. Neutrophils control autoimmune B cells in male NZB/W mice

(A) Numbers of neutrophils in the spleen of naïve B6 and NZB/W mice aged 6-9 weeks (n = 8∼10 (left) or 4∼6 (right) from 2 experiments)

(B) Levels of H_2O_2 and MPO in the plasma of young naïve B6 and NZB/W mice as in (A).

(C) Numbers of NK cells in the spleen of young naïve B6 and NZB/W mice as in (A).

(D-E) Phenotypes of splenic NK cells in young naïve B6 and NZB/W mice as in (A) based on the expression of CD27 and CD11b (D) and NKp46 (E). $CD11b^{+}CD27^{-}$ cells are defined as the mature NK cell population, $CD11b^{+}CD27^{+}$ as intermediate, and $CD11b^{-}CD27^{+}$ cells as the immature NK cell population.

(F-G) Relative gene expression by NK cells (F) or B cells (G) isolated from spleen of young NZB/W mice 2 days (F) or 4 days (G) after receiving anti-Ly6G mAb or control IgG2a (n = 4 from 2 experiments).

(H) The levels of autoreactive IgG in the culture supernatant of B cells isolated from young NZB/W mice 14 days after receiving anti-Ly6G mAb or control IgG2a. Cells were cultured with 1 μg/ml R848 for 7 days. A representative result from 3 experiments is shown (n = 3).

(I) The levels of autoreactive IgG in the sera of male NZB/W mice 1 day before and 11 days after receiving anti-Ly6G mAb or control IgG2a continuously (n = 7).

(J) Transcript expression by neutrophils sorted from the spleen of young naïve B6 and NZB/W mice. The heat-map is shown.

(A-I) Data represent mean and s.e.m. *P <0.05, **P <0.01, and ***P <0.001. NS: not significant. See Figures S5 - S7.