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Publication Date
2012

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The role of Foxo3 in neutrophil homeostasis

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Hannah Kang

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2012
The thesis of Hannah Kang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012
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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Steve Hedrick for allowing me to join his lab and giving me an amazing research experience, which opened my eyes to what it is really like to do research. I know that as a PI and the department chair, you are extremely busy with your daily responsibilities, but I appreciate you for spending time to help me with every single one of my practice talks, meeting with me every Tuesday afternoons to review my data, and encouraging me to use you as an easily accessible resource if any questions arise. I have learned so much about immunology and research during the past two years and without you, I would not have had the opportunity to do so. Erica, I do not know where to begin to thank you because you have done so much for me. Every time I thanked you for helping me with something in lab, you would respond enthusiastically, “It’s my job!” Being my mentor was really a fulltime job for you in addition to your already over-the-top work schedule. You were there to teach me every technique I learned in lab, to answer all of the questions, and to guide my project into a possible publication in the future. You have been the most crucial person in my academic career by far and I have not learned so much science from anyone else but you in my entire life. Without you I could not have been able to accomplish anything described in this thesis. Thank you so much Erica for devoting two years to my growth in research and actively opening doors of new opportunities for me. I have been extremely lucky to have such a caring, generous, and encouraging mentor.

Carol you are an incredible lab manager, who is also a mother figure in our lab everyone loves and cannot live without. You were the first person who taught me how to
do PCR in lab and without you I would have not known the genotypes of the hundreds of mice I used during the past two years. Rodrigo, you are probably the most lively and happy person I know. In addition to patiently helping me with the Foxo3 intracellular stain, thank you for changing my stressful days into happy ones and bringing such a joyful spirit to lab everyday. Jenn, I absolutely loved sharing benches with you. I enjoyed our random talks, your hilarious sense of humor, and your very cool, laid back personality. Your presence and encouraging words have been extremely comforting and thank you for being a great listener whenever I needed someone to vent to about my random struggles in life.

Dr. Maripat Corr, thank you for giving me the ever-so-valuable K/BxN serum. I could not have examined arthritis in the Foxo3\textsuperscript{Lex} and Foxo3\textsuperscript{f/f}LysMCre\textsuperscript{+} mice without your generosity and collaboration. Robert and Eva from the Murre lab, thank you for teaching me how to master the complicated myeloid progenitor staining. Ed from the Goldrath lab, thank you for teaching me how to use the Olympus microscope upstairs in the Jamora lab. Without you, I would not have been able to obtain the beautiful neutrophil images. I would also like to thank past lab members, Fong, Kevin, and Laura, who have also contributed to my priceless experience in lab. Everyone has been so kind and generous, providing a wonderful learning environment for me. I have learned something new everyday in the last two years. Thank you everyone for this amazing experience—it has truly been a blessing.
ABSTRACT OF THE THESIS

The role of Foxo3 in neutrophil homeostasis

by

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Master of Science in Biology
University of California, San Diego, 2012
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Foxo transcription factors are critical mediators and have a highly conserved role in regulating hematopoietic homeostasis by limiting physiologic oxidative stress. A loss in Foxo3 causes a striking myeloproliferation, induced in part by increased accumulation of reactive oxygen species (ROS), that amplifies that Akt/mTOR signaling pathway. Here we found that a Foxo3-deficient mouse strain, Foxo3\textsuperscript{Lex}, exhibited increased neutrophil production from an expansion of myeloid progenitor cells: CMPs, Pre GMs, and GMPs. Such neutrophilia and increase in progenitor cells were more apparent and exaggerated in the spleen, in comparison to the BM of Foxo3\textsuperscript{Lex} mice. Interestingly, although neutrophils
are known to exaggerate conditions of neutrophilic inflammation, Foxo3^{Lex} mice, exhibited normal conditions of peritonitis and reduced severity of rheumatoid arthritis despite their increased neutrophil numbers.

The increase in splenic neutrophils also compelled us to examine their possible novel role as B cell-helper neutrophils, which have been recently identified as neutrophils in the marginal zone of the spleen that activate B cells to generate an antimicrobial immunoglobulin defense. In conclusion, Foxo3 appears to regulate and promote the proper balance of neutrophil populations to avoid overproduction possibly from exaggerated granulopoiesis, especially in the spleen.
INTRODUCTION

The innate immune system and neutrophils

The innate immune system in multicellular organisms serves as the first line of host defense, providing immediate protection against common infections. The innate immune cells: granulocytes, macrophages, mast cells, and immature dendritic cells, are crucial nonspecific effector cells that recognize infection and orchestrate the removal of the infectious agent. They also play a critical role in initiating the adaptive immune response, which arrives 4-7 days after infection, and supplies specific protection against the same pathogenic infection.

Neutrophils are the most abundant of the circulating leukocytes, and are readily mobilized, terminally differentiated cells. Along with eosinophils and basophils, neutrophils form a class of polymorphonuclear (PMN) cells, named for their segmented nuceli. Neutrophils also exhibit a very short half-life. In vivo labeling in humans with the use of 2H2O under homeostatic conditions reveals an average median half-life of 3.8 days in humans and 12.5 hours in mice (1). Neutrophils are normally absent in healthy tissues, during an infection however, chemotactic factors activate neutrophils to “roll” along the blood vessel and be recruited to the inflamed tissue through chemotaxis(2). This process of neutrophil rolling is initiated by the neutrophil homing molecule, L-selectin, which interacts with adhesion molecules of the blood endothelium, and later contributes to neutrophil activation (3). Once neutrophils enter the tissue, chemoattractants such as N-formyl-methionyl-leucyl-phenalanine (fMLP) and
complement component C5a, cause neutrophils to migrate towards the exact site of infection, where neutrophils can exert their anti-microbial functions (2).

Neutrophils have three main techniques for fighting off infections: 1) phagocytosis of microbial particles or microorganisms 2) release of anti-microbial granules and 3) production of neutrophil extracellular traps (NETs) (4). Like macrophages, neutrophils are phagocytic cells that engulf complement-coated bacteria. Once they internalize the bacteria in a phagosome, neutrophils secrete reactive oxygen species (ROS) into the phagosome to activate proteases and hydrolytic enzymes to kill the bacteria. Along with mast cells and other PMN cells, neutrophils contain cytoplasmic granules, vesicles that store antimicrobial products. Neutrophils have various types of granules that store different cytotoxic proteins, such as lactoferrin and myeloperoxidase, capable of destroying foreign microorganisms during degranulation. Neutrophils can also utilize their granule proteins together with chromatin to create extracellular fibrous traps, NETs, which are effective in localizing antimicrobial factors, degrading virulence factors, and destroying bacteria (4). Once they have played their role in creating an inflammatory response, neutrophils are mostly removed by macrophages by phagocytosis.

**Foxo transcription factors**

Foxo transcription factors have a highly conserved role in regulating transcription of genes involved in cell differentiation, cell cycle arrest, cell survival and DNA repair (5). Four Foxo members have been identified in mammals: Foxo1, Foxo3a, Foxo4, and Foxo6. FOXO proteins are found usually inside the cell nucleus where it is activated. However in the presence of growth factors or insulin, phosphatidylinositol-3-OH kinase (PI3K) becomes activated, inducing phosphorylation of downstream protein effectors like
the serine/threonine kinase Akt. Activated Akt subsequently phosphorylates FOXO, triggering its export from the nucleus to the cytoplasm where FOXO can become degraded. On the other hand, in the presence of oxidative stress, activation the Jnk mitogen-activated protein kinase, cause FOXO nuclear localization, which permits activated FOXO proteins to exert their diverse physiologic functions.

Recently, Foxo transcription factors have been shown to regulate specialized functions in cells of the immune system. Foxo1 controls naïve T cell homeostasis by detecting the presence of growth factors and regulating critical genes required for T cell homing and survival (6). Foxo3 is found to be involved in the dynamics of T cell population expansion and contraction. For example, Foxo3 controls T cell survival by moderating IL-6 production by dendritic cells (7). Foxo3-null mice strains have also been shown to have greater proportions of total splenic dendritic cells and neutrophils. The increase in splenic neutrophil numbers seen in Foxo3-null mice has not been fully investigated.

**Foxo transcription factors and hematopoiesis**

Homeostasis of mammalian tissues is provided by tissue-specific stem cells and their ability to self-renew and differentiate into new mature cells. To maintain tissue functionality and ensure their sustenance throughout an organism’s lifetime, tissue-specific stem cells must be protected from oxidative stress, cell cycle progression, and cellular aging. Foxo1, Foxo3, and Foxo4 transcription factors have been shown to maintain the hematopoietic stem cell (HSC) pool in response to physiologic oxidative stress and play a critical role in the self-renewal of HSCs (8). Foxo-deficient mice, with a conditional deletion in Foxo1, Foxo3, and Foxo4, exhibit an expansion of myeloid and
erythroid cells, increased extramedullary hematopoiesis from the spleen, and decreased maturation of B and T cells. They also show aberrant cell cycle regulation of HSCs with a marked increased of HSCs in the S/G2/M phases, increased ROS levels in HSCs from changes of ROS gene expression, and increased cell-autonomous apoptosis of HSC. The lack of these cell cycle and apoptosis phenotypes in single and double-knockout out mice, suggest that all three Foxo alleles are required to mediate quiescence and survival of HSCs. Specifically, a deficiency of Foxo3a in mice, generates age-dependent neutrophil proliferation in both the BM and spleen, even after induced myelosuppression, indicating the Foxo3a’s possible role of negatively regulating granulopoiesis (9). AKT and ERK hyperphosphorylation in HSCs of Foxo3-deficient mice suggests that Foxo3a regulates granulopoiesis by downregulating AKT/ERK pathways in stem cells. Although these Foxo3a-deficient mice exhibit marked neutrophilia, the frequency and differentiation potential of HSCs in the BM are normal (10). Instead, the loss of Foxo3a impairs the self-renewal capacity and the maintenance of quiescence in HSCs.

Foxo3 also regulates intracellular reactive oxygen species (ROS) levels in HSCs. Loss of Foxo3 causes increased levels of ROS in HSCs, and subsequently amplifies the AKT/mTOR pathway (11). In turn, this enhanced signaling causes exaggerated cytokine production, which contributes to the myeloproliferative syndrome seen in Foxo3-deficient mice. Such results allude to Foxo3’s role in ROS suppression to maintain HSC homeostasis and quiescence.

**Splenic granulopoiesis**

Neutrophils are constantly being replenished during homeostasis, and much of the BM is devoted to their production, however neutrophils can also be produced by
extramedullary hematopoiesis of the spleen (12). In a genetic mouse model of lung adenocarcinoma, the spleen serves as an extramedullary site for producing tumor-associated macrophages and tumor-associated neutrophils (TAMs and TANs). Increased myeloid progenitor cells such as common myeloid progenitors (CMPs) and granulocyte macrophage progenitors (GMPs) is seen in the spleen but not in the BM, indicating that the spleen may enhance TAM and TAN populations by regulating the release of myeloid progenitors to tumors. Consistent with this notion, spleens from human patients with chronic inflammatory disorders have significantly increased numbers of splenic GMPs, which contribute to the increased production of monocytes and neutrophils in vivo during cancer progression.

The mechanism of splenic granulopoiesis, whether it is by an expansion of stem cells in the spleen or by migration from the BM, has been open to question. Rats treated with growth factors, stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF), exhibit markedly increased splenic granulopoiesis (13). These growth factors work synergistically to increase the number of granulocytic precursors (myeoblasts to metamyelocytes) and cause a dramatic expansion of circulating neutrophils. In mice, a conditionaly deletion of an antiapoptotic gene, c-FLIP, in myeloid cells, causes a striking alteration in hematopoiesis from the BM to the spleen (14). This shift is shown to be cytokine-mediated. Along with splenomegaly, neutrophilia in the spleen and blood, and increased myeloid progenitor populations (LSK cells, CMPs, GMPs, and MEPs) in the spleen, c-FLIP<sup>ff</sup> LysM-Cre mice exhibit increased serum levels of cytokines IL-1β, G-CSF, and MIP-1α. G-CSF alone has shown to be responsible for increased neutrophil production in an IL-1 independent signaling pathway. In addition, the accumulation in
splenic neutrophils seen in c-FLIP<sup>ff</sup> LysM-Cre mice accounts for the entire increase in splenic cellularity, and in contrast to the increased myeloid progenitors populations seen in the spleen, the BM displays a loss of Lin<sup>-</sup> cells and some progenitor populations. These findings suggest that the BM is mostly dedicated to neutrophil production and splenic granulopoiesis works to compensate for the decrease in BM hematopoiesis during defects in cell differentiation and proliferation.

**K/BxN serum transfer model of arthritis and neutrophils**

KRN transgenic mouse (K/BxN) model has been effectively used to study arthritis due to their efficiency in developing spontaneous arthritis (15). Serum from K/BxN mice is used to reliably induce RA in recipient mice because K/BxN mice produce autoantibodies against glucose-6-phosphate isomerase (GPI), a protein present in joints. The recognition of GPI on cartilage surfaces induces migration of neutrophils to the joints (16). Fc receptors for IgG are required for RA induction and in this model it has been shown that the common γ signaling chain of Fc receptors are required for arthritis. Various additional molecules required for neutrophil functions have been shown to be involved in the pathogenesis of RA (17). Consequently neutrophils are an important component of this RA model and it is probable that an increase in neutrophils leads to an increase in severity of this model of RA.
RESULTS

Analysis of Ly-6G$^{hi}$ CD11b$^{hi}$ cells of Foxo3$^{\text{Lex}}$ mice

*Foxo3$^{\text{Lex}}$ mice have increased numbers of Ly-6G$^{hi}$ CD11b$^{hi}$ cells*

Our lab has previously shown that several strains of Foxo3 null strain mice have increased numbers of Gr-1$^{hi}$ CD11b$^{hi}$ cells in the spleen (7). However in another Foxo3-deficient mice strains, Foxo3$^{\text{Trap}}$ mice, exhibit normal numbers of Gr-1$^{hi}$ CD11b$^{hi}$ (18). Due to this discrepancy, Gr-1$^{hi}$ CD11b$^{hi}$ cells in Foxo3$^{\text{Lex}}$ mice were examined and the increase in numbers of these cells was confirmed. Foxo3$^{\text{Lex}}$ mice, Ly-6G$^{hi}$ CD11b$^{hi}$ cells were quantified in the spleen, bone marrow, and peripheral blood of Foxo3$^{\text{Lex}}$ mice. Gr-1 is a myeloid differentiation antigen and Ly-6G is one of several types of the Gr-1 marker, predominantly expressed on neutrophils (19).

In the bone marrow, Foxo3$^{\text{Lex}}$ mice had about 8 million Ly-6G$^{hi}$ CD11b$^{hi}$ cells, a ~2 fold increase in cell number compared to wild-type B6 mice (Fig. 1). In the spleen, Foxo3$^{\text{Lex}}$ mice had about 3 million Ly-6G$^{hi}$ CD11b$^{hi}$ cells, a ~6 fold increase in cell number compared to wild-type B6 mice (Fig. 1). In the peripheral blood, Foxo3$^{\text{Lex}}$ mice had about 1000 Ly-6G$^{hi}$ CD11b$^{hi}$ cells / µl, a slight increase in cell number but not a significant increase compared to wild-type B6 mice, which were ~15 weeks old (Fig. 1).
Figure 1: Foxo3<sup>Lex</sup> mice have increased numbers of Ly-6<sup>G</sup><sup>hi</sup> CD11b<sup>hi</sup> cells

Ly-6<sup>G</sup><sup>hi</sup> CD11b<sup>hi</sup> cell populations were gated from CD11c<sup>-</sup> cells to exclude dendritic cells. Cell populations of wild-type and Foxo3<sup>Lex</sup> mice from the (A) BM, spleen, and blood were compared. (B) Ly-6<sup>G</sup><sup>hi</sup> CD11b<sup>hi</sup> cell numbers in the BM, spleen, and blood were quantified. Data are mean (***p < 0.0005).

**Ly-6<sup>G</sup><sup>hi</sup> CD11b<sup>hi</sup> cells are neutrophils**

To determine whether Ly-6<sup>G</sup><sup>hi</sup> CD11b<sup>hi</sup> cells are neutrophils, Ly-6<sup>G</sup><sup>hi</sup> cells were purified from the spleens of both Foxo3<sup>Lex</sup> and B6 mice. The purity of Ly-6<sup>G</sup><sup>hi</sup> cells from the splenocytes was ~80% (data not shown). All cells from one purification were put on one slide and the cells were stained with Giemsa. When the purified cells were visualized under the confocal microscope, most of the cells were identified as neutrophils for their
multilobulated nuclei, and small almost invisible granule content (Fig. 2A, B). Splenocytes from Foxo3\textsuperscript{Lex} mice visually had higher numbers of neutrophils as well.

**Figure 2: Ly-6\textsuperscript{hi} CD11b\textsuperscript{hi} cells are neutrophils**

Ly-6G\textsuperscript{+} cells were purified from spleens of (A) Foxo3\textsuperscript{Lex} and (B) wild-type mice. Multilobulated nuclei of neutrophils were observed in Giemsa stain stained slides.

**Analysis of increased neutrophil proportions in Foxo3\textsuperscript{Lex} mice**

*Foxo3\textsuperscript{Lex} mice have increased production of neutrophils*

The reason why Foxo3\textsuperscript{Lex} mice have increased neutrophil numbers was unknown. It was unclear whether Foxo3\textsuperscript{Lex} mice have increased cell numbers due to increased
neutrophil production or longer neutrophil half-life. Neutrophil may accumulate from their increased production in hematopoietic sites like the BM and spleen, or from the reduced rate of neutrophils apoptosis and clearance. To investigate this question, a Bromodeoxyuridine (BrdU) cell proliferation assay was done to examine the kinetics of neutrophil production in vivo.

At day 0, Foxo3\textsuperscript{Lex} and B6 mice were administered BrdU via their drinking water. Each day, mice from each genotype (n=2) and a control mouse, which was not administered BrdU, were tail bled and sacrificed (Fig. 3A). In both the blood and spleen, Foxo3\textsuperscript{Lex} mice exhibited higher numbers of BrdU\textsuperscript{+} neutrophils from at least day 4 (Fig 3B).

**Figure 3: Foxo3\textsuperscript{Lex} mice exhibit increased production of neutrophils**

(A) Kinetics of neutrophil production were examined by conducting a BrdU assay where BrdU was administered through the drinking water until day 5. (B) BrdU\textsuperscript{+} neutrophils were quantified daily from the blood and spleens of wild-type (n = 2), Foxo3\textsuperscript{Lex} (n = 2), and control mice that did not receive BrdU (n = 1).

**Foxo3\textsuperscript{Lex} mice have increased numbers of myeloid progenitor cells**

It has been previously stated that the increased frequency of neutrophils and monocytes seen in Foxo3-deficient mice from the same strain as Foxo3\textsuperscript{Lex} mice,
correlated with an expansion of myeloid progenitor (Lin\(^-\) IL7R\(\alpha\) Sca-1\(^-\) c-Kit\(^+\)) cells in the BM\(^{11}\). A loss of Foxo3 exhibited an increased in reactive oxygen species (ROS) concentration in Lin\(^-\) IL7R\(\alpha\) Sca-1\(^-\) c-Kit\(^+\) cells, a population of common myeloid progenitor cells \(^{11}\).

To determine if the increase of neutrophil production in Foxo3\(^{Lex}\) mice was due to a possible increase their myeloid progenitor cells numbers and to further define the precursors increased, various myeloid precursor populations were quantified. Progenitor populations that participate in neutrophils development are the Lin\(^-\) Sca-1\(^+\) c-Kit\(^+\) (LSK) cells, which constitute the hematopoietic stem cell (HSC) and multipotent progenitor (MPP) populations, which differentiate into common myeloid progenitors (CMP, Lin\(^-\) c-Kit\(^+\) Sca-1\(^-\) IL7R\(\alpha\)), which differentiate into Pregranulocyte-macrophage progenitors (Pre GM, FcgR\(^{low}\) CD105\(^-\) CD150\(^-\)), which further differentiate into granulocyte-macrophage progenitors (GMP, CD41\(^-\) FcgR\(^+\) CD150\(^-\)), which ultimately produce primarily granulocytes and/or macrophages.

Foxo3\(^{Lex}\) mice exhibited no significant difference in LSK cell numbers. However they had increased numbers of CMPs in the BM and spleen: there was \(\sim\)1.5 fold increase and a \(\sim\)3 fold increase of these cells numbers in the BM and spleen, respectively, of Foxo3\(^{Lex}\) mice (Fig. 4A, B). These mice also exhibited increased numbers of Pre GMs: there was a \(\sim\)1.5 fold increase and a \(\sim\)2.5 fold increase in the BM and spleen, respectively, of Foxo3\(^{Lex}\) mice (Fig. 4C, D). For GMPs, there was a \(\sim\)2 fold increase in cell number of the spleen of Foxo3\(^{Lex}\) mice, however there was no significant increase in cell number in the BM (Fig 4E, F).
Figure 4: Foxo3^{Lex} mice exhibit an expansion of myeloid progenitor cells
(A) CMPs (c-Kit^+ Sca-1^-) were gated from Lin^- cells and (B) quantified from the BM and spleens of Foxo3^{Lex} and wild-type mice. (C) Pre GMs (FcgR^{low} CD105^- CD150^-) were gated from Lin^- c-Kit^- Sca-1^- CD41^- CD16/32^- cells and (D) quantified from the BM and spleens of Foxo3^{Lex} and wild-type mice. (E) GMPs (CD41^- FcgR^+ CD150^-) were gated from Lin^- c-Kit^- Sca-1^- CD41^- cells and (F) quantified from the BM and spleens of Foxo3^{Lex} and wild-type mice. (B, D, F) Data are mean frequency (*p < 0.05, **p < 0.005, ***p < 0.0005).
Figure 4 continued.
Other cell progenitor populations that do not participate in myeloid development have been examined (data not shown). Cell numbers of premegakaryocyte erythrocyte progenitors (Pre MegE, FcgR$^{\text{low}}$ CD105$^{-}$ CD150$^{+}$) were increased in the spleen but not the BM of Foxo3$^{\text{Lex}}$ mice. Lin$^{-}$ Sca-1$^{+}$ c-Kit$^{+}$ (LSK) cells constitute the hematopoietic stem cell (HSC) and multipotent progenitor (MPP) populations. No significant difference in LSK cell numbers was observed between Foxo3$^{\text{Lex}}$ mice and wild-type mice. Megakaryocyte progenitors (MkP, Lin$^{-}$ c-Kit$^{+}$ Sca-1$^{-}$ CD41$^{+}$), which produce megakaryocytic erythroid cells, were normal in cell number in the BM and spleen of Foxo3$^{\text{Lex}}$ mice. Pre CFU-E cells (FcgR$^{\text{low}}$ CD105$^{+}$ CD150$^{-}$), which give rise to erythroid cells, exhibited normal cell numbers in the BM and spleen of Foxo3$^{\text{Lex}}$ mice as well.

**Foxo3 expression levels of Pre CFU-E cells differ from other myeloid progenitor populations**

Another strain of Foxo3-deficient mice called the Foxo3$^{\text{ff}}$ LysMCre$^{+}$ mice, posses a deletion in Foxo3 in only myeloid lineages. To determine at which progenitor stage LysMCre acts to delete Foxo3, an intracellular staining of Foxo3 was done in various progenitor populations of Foxo3$^{\text{ff}}$ LysMCre$^{+}$ mice. In the earlier progenitor population, CMPs, Foxo3 was not deleted in the cells of the BM and spleen of Foxo3$^{\text{ff}}$ LysMCre$^{+}$ mice (Fig. 5A). In myeloid precursor populations, Pre GMs and GMPs, Foxo3 was deleted in splenocytes but not in the BM cells of these mice (Fig. 5A). In other precursor populations, Pre MegEs and Pre CFU-Es, Foxo3 was deleted in both the cells of the BM and spleen of Foxo3$^{\text{ff}}$ LysMCre$^{+}$ mice (Fig. 5A).
Comparing the expression level of Foxo3 in these cell progenitor populations, it was also observed that Pre CFU-E cells exhibited higher levels of Foxo3 expression (Fig. 5B). This may be related to abnormalities of peripheral blood, such as decreased erythrocyte lifespan and rate of maturation, seen in Foxo3-deficient mice (20).

**Figure 5: mFoxo3a expression in Foxo3*"LysMCre*" myeloid progenitor cells**

(A) mFoxo3a intracellular expression of various myeloid progenitor populations were examined in the BM and spleens of both wild-type (black) and Foxo3*"LysMCre*" mice (gray). (B) mFoxo3a expression of Pre CFU-E cells (red) were compared to those of CMP, Pre GM, GMP, and Pre MegE cells (all gray) in the BM and spleens of both wild-type and Foxo3*"LysMCre*" mice.
Conditions of peritonitis in Foxo3\textsuperscript{Lex} mice

\textit{Foxo3\textsuperscript{Lex} mice have no significant difference in their conditions of peritonitis}

It previous studies, it has been shown that Foxo3a-deficient mice known are Foxo3\textsuperscript{Trap} mice, are resistant to two models of neutrophilic inflammation: antibody-mediated arthritis and peritonitis (21). Instead of an increase in neutrophil numbers, these Foxo3a-deficient mice, which are from a different mouse strain as Foxo3\textsuperscript{Lex} mice, exhibit normal neutrophil numbers. Due to this difference in phenotype seen between these Foxo3-deficient strains, it was hypothesized that Foxo3\textsuperscript{Lex} mice might exhibit exaggerated conditions of arthritis and peritonitis from the neutrophilia seen in these mice. To observe conditions of peritonitis in Foxo3\textsuperscript{Lex} mice, proteose peptone was used to induce inflammation in the peritoneum and peritoneal neutrophils were quantified. Proteose peptone was used instead of thioglycollate because proteose peptone generated consistent inflammation, while the induction of inflammation by thioglycollate varied with separate trials and the length of the period the thioglycollate solution was aged.

First, an experiment showing the time course of neutrophils entering the peritoneum after an i.p injection of proteose peptone was done (Fig. 6A). It was determined that in B6 mice, neutrophils enter the peritoneum by 4 h post proteose peptone injection, and neutrophils numbers begin to plateau 6 h post injection. Consequently, peritoneal cells were collected 6 h post i.p proteose peptone injection for all experiments observing the condition of peritonitis in Foxo3\textsuperscript{Lex} mice.

After inflammation induction in the peritoneal cavity, Foxo3\textsuperscript{Lex} mice exhibited an influx of neutrophils into the peritoneum and contained \textasciitilde 2 million peritoneal neutrophils,
similar to the number of peritoneal neutrophils seen in wild-type B6 mice during peritonitis (Fig. 6B). Foxo3\textsuperscript{Lex} mice did not exhibit a significant difference in the number of peritoneal neutrophils as compared to that of wild-type mice during peritonitis – this presented a contradiction to the resistance of peritonitis seen in Foxo3-deficient mice in the previous study (21).

**Figure 6: Foxo3\textsuperscript{Lex} mice exhibit normal conditions of peritonitis**
(A) Timepoint of neutrophils homing to the peritoneum of wild-type mice post 10% proteose peptone injection. (B) Peritoneal neutrophils from wild-type and Foxo3\textsuperscript{Lex} mice were gated from CD11c<sup>−</sup> cells to exclude dendritic cells, and then quantified. Data from the graph are mean numbers. (C) FasL surface expression of peritoneal neutrophils from wild-type (black) and Foxo3\textsuperscript{Lex} mice (gray) were observed. (D) As a FasL control, upregulated FasL expression was seen in activated T cells (gray, CD69<sup>hi</sup> CD44<sup>hi</sup> CD8<sup>+</sup>) in comparison to naïve T cells (black, CD69<sup>−</sup> CD44<sup>med</sup> CD8<sup>+</sup>).

**Peritoneal neutrophils of Foxo3\textsuperscript{Lex} mice have no significant difference in FasL expression**

Foxo3a has previously shown to regulate neutrophil survival during inflammation by inhibiting Fas ligand (FasL) surface expression of neutrophils (21). FasL, type II
membrane protein of the tumor necrosis factor (TNF) family, is primarily known for its role in the induction of apoptosis in cells bearing the Fas receptor, such as activated T cells, and Foxo proteins have shown to be able to bind and inactivate the Fasl promoter (22).

To examine the effect of the absence of Foxo3 in Foxo3\textsuperscript{Lex} mice on neutrophils FasL expression during inflammation, FasL surface expression of peritoneal neutrophils was observed during peritonitis. It was shown that peritoneal neutrophils of Foxo3\textsuperscript{Lex} mice had no significant difference in FasL expression as that of wild-type mice (Fig. 6C). FasL expression was seen in activated CD69\textsuperscript{hi} CD44\textsuperscript{hi} CD8\textsuperscript{+} T cells as a FasL control (Fig. 6D).

**Conditions of rheumatoid arthritis in Foxo3\textsuperscript{Lex} and Foxo3\textsuperscript{f/f} LysMC\textsuperscript{re+} mice**

*Foxo3\textsuperscript{Lex} mice exhibit reduced severity of RA*

Increased neutrophils are known to exaggerate inflammatory conditions like rheumatoid arthritis (RA). Early RA patients have shown to have lower levels of neutrophil apoptosis. The delay in neutrophil clearance causes apoptotic neutrophils to undergo secondary necrosis, which in turn induces macrophage production of pro-inflammatory cytokines (23). Since Foxo3\textsuperscript{Lex} mice experience dramatic neutrophilia, it was hypothesized that mice deficient for Foxo3 might exhibit exaggerated conditions of RA. However Foxo3\textsuperscript{Trap} mice, exhibit resistance to arthritis (21). To test the hypothesis and investigate conditions of arthritis in Foxo3\textsuperscript{Lex} mice, they were induced with RA using the K/BxM serum transfer model of RA.
Arthritis was induced in Foxo3\textsuperscript{Lex} and wild-type mice and the progression of arthritis was observed by measuring ankle thickness and assigning clinical scores of ankles and wrists each day in a blinded fashion. Through these experiments, it was found that Foxo3\textsuperscript{Lex} mice exhibited reduced severity of RA compared to wild-type mice (Fig. 7A). According to clinical scores of the ankles and wrists, wild-type mice progressively developed arthritis and generated maximum thickness of the ankle at about day 10 post K/BxN serum transfer. At day 10, the amount of change in ankle thickening wild-type mice experienced compared to day 0 was ~5 times that of Foxo3\textsuperscript{Lex} mice (Fig. 7A).

\textit{Foxo3}\textsuperscript{ff} LysMC\textsuperscript{Cre}\textsuperscript{+} mice exhibit reduced severity of RA

Neutrophils have been identified as critical mediators of inflammation during the K/BxN serum transfer model of arthritis. To determine if the reduced severity of arthritis seen in Foxo3\textsuperscript{Lex} mice is myeloid cell intrinsic, K/BxN serum was transferred into Foxo3\textsuperscript{ff} LysMC\textsuperscript{Cre}\textsuperscript{+} mice, a strain of mice that have the loxP-flanked target gene, Foxo3, deleted by LysMC\textsuperscript{Cre} in only myeloid cell lineages.

When arthritis was induced in Foxo3\textsuperscript{ff} LysMC\textsuperscript{Cre}\textsuperscript{+} and wild-type mice, ankle measurements and clinical scores of their paws were taken everyday during the 13-day time course. Preliminary results from this experiment indicated that Foxo3\textsuperscript{ff} LysMC\textsuperscript{Cre}\textsuperscript{+} mice, similar to Foxo3\textsuperscript{Lex} mice, exhibited reduced severity of arthritis compared to wild-type mice (Fig 7B). At day 11, the amount of change in ankle thickening wild-type mice experienced compared to day 0 was ~4.5 times that of Foxo3\textsuperscript{ff} LysMC\textsuperscript{Cre}\textsuperscript{+} mice. However, this phenotypic difference between wild-type and Foxo3\textsuperscript{ff} LysMC\textsuperscript{Cre}\textsuperscript{+} mice was seen only
in male mice (data not shown). The K/BxN serum transfer experiment with Foxo3^{f/f} LysMCre^{+} mice was conducted once.

Figure 7: Foxo3^{Lex} and Foxo3^{ff} LysMCre^{+} mice exhibit reduced severity of arthritis
(A) Ankle measurements and clinical scores of the paws of wild-type (black) and Foxo3^{Lex} mice (grey) were taken daily after K/BxN serum transfer. (B) Ankle measurements of wild-type (black) and Foxo3^{ff} LysMCre^{+} mice (grey) were taken as well. (C) Images of ankles of wild-type and Foxo3^{ff} LysMCre^{+} mice on day 7.

Analysis of splenic neutrophil function of Foxo3^{Lex} mice

Foxo3^{Lex} mice increased proportions of marginal zone B cells

In previous recent studies a novel function of splenic neutrophils has been discovered, which has served to challenge the theory that neutrophils are exclusive to the innate immune system. Neutrophils in the peri-marginal zone area of the spleen interact with marginal zone (MZ) B cells, stimulating these B cells to undergo immunoglobulin class switch, somatic hypermutation, and antibody production. Through mechanisms involving cytokines BAFF, APRIL, and IL-21, neutrophil induction of B cell
hypermutation has been shown to generate preimmune immunoglobulins to T cell-independent antigens \textit{in vivo} in humans. In patients with severe congenital neutropenia (SCN), who have lower neutrophil numbers, exhibit normal percentages of total CD19$^+$ B cells and naïve B cells, but possess lower numbers of circulating MZ B cells (24).

To begin to determine whether splenic neutrophils of Foxo$^{3\text{lex}}$ mice display B-cell helper neutrophil functions, MZ B cells in the spleens of Foxo$^{3\text{lex}}$ mice were first examined. MZ B cells in mice are identified as CD19$^+$ IgM$^{\text{hi}}$ IgD$^{\text{lo}}$ CD93$^{\text{lo}}$ CD23$^{\text{med}}$ cells. In the spleens of Foxo$^{3\text{lex}}$ mice, total MZ B cell numbers were normal, but the percentage of MZ B cells in the total splenic B cell population was significantly increased compared to that of wild-type mice (Fig. 8A, B). Transitional T1 and T2 B cells, which mature into MZ B cells were also examined. T1 B cells, which are CD19$^+$ IgM$^{\text{hi}}$ IgD$^{\text{lo}}$ CD93$^{\text{hi}}$ CD23$^{\text{lo}}$, and T2 B cells, which are CD19$^+$ IgM$^{\text{hi}}$ IgD$^{\text{lo}}$ CD93$^{\text{hi}}$ CD23$^{\text{hi}}$, were both significantly decreased in percentage in total splenic B cells of Foxo$^{3\text{lex}}$ mice (Fig. 8A, B).
Figure 8: Foxo3\textsuperscript{Lex} mice exhibit an expansion of MZ B cell proportion and a decrease in transitional B cells

(A) MZ B cells (CD93\textsuperscript{lo} CD23\textsuperscript{med}) and T1 (CD93\textsuperscript{hi} CD23\textsuperscript{lo}) and T2 (CD93\textsuperscript{hi} CD23\textsuperscript{hi}) transitional B cells were gated from CD19\textsuperscript{+} IgM\textsuperscript{hi} IgD\textsuperscript{lo} cells. (B) Data are mean frequency (%). (*p < 0.05 and **p < 0.005).

**Splenic neutrophils of Foxo3\textsuperscript{Lex} mice are similar to B cell helper neutrophils**

B cell helper neutrophils in humans have significant differences in surface marker expressions as that of circulating blood neutrophils. In comparison to blood neutrophils, B cell helper neutrophils have higher expression of molecules that limit toll-like receptor (TLR) signaling such as CD11b and CD24, and markers that display immune activation such as CD27, CD40L, CD86, and CD95 (Fas) (24). B cell helper neutrophils also have lower expression levels of adhesion molecules such as CD62L (L-selectin), CD62P (P-selectin), ICAM-1, and ICAM2. To determine if splenic neutrophils of Foxo3\textsuperscript{Lex} mice exhibit B cell helper-like phenotypes, surface expressions of these markers of wild-type and Foxo3\textsuperscript{Lex} splenic neutrophils were analyzed in comparison to that of wild-type and Foxo3\textsuperscript{Lex} blood neutrophils.
Consistent to human B cell helper neutrophils, splenic neutrophils of both wild-type and Foxo3^Lex^ mice in comparison to their blood neutrophils, had increased expression of CD24 and CD40L and decreased expression of CD62P and ICAM-1 (Fig. 9A). There were no significant differences of expression levels between splenic neutrophils of wild-type and Foxo3^Lex^ mice (Fig. 9A). Similarly, there were no significant differences of expression levels between blood neutrophils of wild-type and Foxo3^Lex^ mice (Fig. 9A).

Furthermore, unlike human B cell helper neutrophils, splenic neutrophils of both wild-type and Foxo3^Lex^ mice expressed no difference in CD27 surface levels from those of their blood neutrophils (Fig. 9B). Instead of having higher expression of CD95 (Fas) as seen in human marginal zone neutrophils compared to the blood, splenic neutrophils of both wild-type and Foxo3^Lex^ mice exhibited lower CD95 expression (Fig. 9B). For adhesion molecules, CD62P and ICAM-2, splenic neutrophils of wild-type and Foxo3^Lex^ mice had higher expression compared to the blood – opposite from what was seen in human neutrophils (Fig. 9B). Additionally, wild-type neutrophils had higher expression levels of both adhesion molecules compared to Foxo3^Lex^ mice (Fig. 9B).
Figure 9: Splenic neutrophils from Foxo3^Lex^ and wild-type mice exhibit differences from their blood neutrophils in surface expressions of markers used to phenotype human B cell-helper neutrophils.

The comparison of surface expression on splenic and blood neutrophils of Foxo3^Lex^ and wild-type mice displayed (A) similarities and (B) differences from the comparison of human blood and B cell-helper neutrophils expressions. Data represent surface expression of markers on wild-type splenic (black) and blood (gray) neutrophils, and Foxo3^Lex^ splenic (red) and blood (pink) neutrophils.
DISCUSSION

Foxo transcription factors have a highly conserved role in regulating cell cycle arrest and stress resistance (5). They are also critical mediators of HSC survival and quiescence by limiting oxidative stress (8). Here we demonstrated that Foxo3 regulates neutrophil numbers by possibly preventing overproduction of neutrophils from increased granulopoiesis. First we confirmed the presence of neutrophilia in Foxo3Lex mice by 1) identifying appropriate surface markers for neutrophils and 2) observing increased neutrophil numbers in the BM and spleens of Foxo3Lex mice. Next, we observed increased corporation of BrdU in splenic and blood neutrophils of these mice, signifying the occurrence of increased production of neutrophils. However, there is a caveat to this experiment. Conclusions were drawn based on the assumption that neutrophils do not undergo both increased self-production and increased half-life, which was another hypothesis to why Foxo3Lex mice experience neutrophilia. Also during BrdU incorporation, although Foxo3Lex mice generated higher BrdU+ neutrophils counts, they exhibited decreased percentages of BrdU+ neutrophils out of their total neutrophil population in the blood and spleen (data not shown). Despite the discrepancy between BrdU+ neutrophil percentage and number, it was concluded that Foxo3Lex mice likely experience increased neutrophil production simply from the detection of increased numbers of proliferating cells.

It has been previously noted that an ROS accumulation in myeloid progenitor cells and increased frequency in these cells of the BM contribute to myeloproliferation in Foxo3-deficient mice that are the same strain as Foxo3Lex mice (11). In this study instead of examining one early myeloid progenitor population of Lin− IL7Rα− Sca-1− c-Kit+ cells,
various progenitor populations that participate in myeloid development, specifically neutrophil production, were quantified in Foxo3^{Lex} mice. The increased numbers of CMPs and Pre GMs in both the BM and spleens of Foxo3^{Lex} mice, are consistent with the notion that Foxo3 works to prevent unbalanced expansion of myeloid progenitor cells and limit increased proliferation of one or more myeloid cells. However, the increased number of GMPs seen in spleens of Foxo3^{Lex} mice was not displayed in the BM. A similar phenotype in which GMP numbers are increased in the spleen but not the BM was seen in a mice model of lung adenocarcinoma (12). In this model, the spleen has been shown to be an important reservoir for monocyte and granulocyte production in both mice and humans. These results may indicate that there may be differences in Foxo3 control of hematopoietic functions of the spleen and BM. They might also suggest that extramedullary hematopoiesis in the spleen may be critical for neutrophil accumulation in Foxo3^{Lex} mice, more so than the BM. Furthermore, an increase in cell numbers of premegakaryocyte erythrocyte progenitors (Pre MegE, FcgR^{low} CD105^{−} CD150^{+}) was observed in the spleen but not the BM of Foxo3^{Lex} mice. This result may be related to abnormalities of peripheral blood, such as decreased erythrocyte lifespan and rate of maturation, seen in Foxo3-deficient mice (20).

Although Foxo3^{Lex} mice exhibited increased neutrophil production, they did not display exaggerated conditions of neutrophilic inflammations. In contrast to previous findings in the literature (21), the loss in Foxo3 in Foxo3^{Lex} mice does not alter the ability of neutrophils to accumulate to the peritoneum during peritonitis. Additionally, normal expression levels of FasL on peritoneal neutrophils significantly reduce the possible occurrence of FasL-mediated apoptosis of neutrophils in Foxo3^{Lex} mice. Such results may
indicate that Foxo3 does not affect neutrophil accumulation in the peritoneum in this model of sterile inflammation. Given the presence of neutrophilia in Foxo3\textsuperscript{Lex} mice and the critical role of neutrophils in the pathogenesis of arthritis in K/BxN mice (17), it was hypothesized that Foxo3\textsuperscript{Lex} mice would exhibit exaggerated conditions of RA after induction of arthritis through K/BxN serum transfer. Contrary to the hypothesis however, Foxo3\textsuperscript{Lex} mice exhibited reduced severity of RA compared to wild-type mice. The preliminary result of the reduced severity of RA seen also in Foxo3\textsuperscript{f/f} LysMCre\textsuperscript{+} mice may indicate that the decreased severity of this disease in Foxo3\textsuperscript{Lex} mice may be myeloid cell intrinsic. This reduced severity correlates with the resistance to K/BxN-mediated arthritis in Foxo3\textsuperscript{Trap} mice used in previous studies, despite the discrepancies in conditions of peritonitis observed between Foxo3\textsuperscript{Trap} mice and Foxo3\textsuperscript{Lex} mice (21). Several speculations can be made on why there is increased resistance to RA in Foxo3\textsuperscript{Lex} mice. Neutrophils of Foxo3\textsuperscript{Lex} mice may express different complement receptors compared to wild-type mice, causing anomalies in the function of neutrophils to create an inflammatory response. Neutrophils of Foxo3\textsuperscript{Lex} mice may also exert immunosuppressive functions, similar to myeloid-derived suppressor cells (MDSCs), a Gr-1\textsuperscript{hi} CD11b\textsuperscript{hi} cell population that contributes to anti-inflammatory responses, tumor escape, and immune suppression during chronic inflammation (25). Expansion of splenic MDSCs have shown to inhibit T cell and NK cell cytotoxicity in tumor-bearing mice (26). Although we confirmed that a loss of Foxo3 causes reduces severity of arthritis, the mechanism of how this phenotype occurs is unknown.

The spleens of Foxo3\textsuperscript{Lex} mice appeared to be critical extramedullary sites for neutrophil production. The ~6 fold increase in neutrophils numbers and the increase in
myeloid progenitor cells, like CMPs, PreGMs, and GMPs, led to the investigation of splenic neutrophil functions. MZ B cell induction to class switch recombination, somatic hypermutation, and IgM secretion, is a novel and extremely intriguing function of MZ neutrophils, named B cell-helper neutrophils (24). To determine whether Foxo3\textsuperscript{Lex} splenic neutrophils may exert B cell-helper neutrophil-like functions, phenotypes of splenic and blood neutrophils were compared. Wild-type and Foxo3\textsuperscript{Lex} splenic neutrophils had similar surface expression levels of CD24, CD40L, CD62P, and ICAM-1 as human B cell-helper neutrophils in the splenic marginal zone. However, their CD27, CD95, CD62L and ICAM-2 expression did not correlate with what was observed in human B cell-helper neutrophils. These similarities and differences between Foxo3\textsuperscript{Lex} splenic neutrophils and B cell helper neutrophils may be due to the different model organisms used, mouse and human respectively, for these cell types.

B cell-helper neutrophils have shown to induce MZ B cell to undergo class switch recombination to IgG and IgA in response to T cell-independent (TI) antigens (24). To examine splenic neutrophil activation of MZ B cells in Foxo3\textsuperscript{Lex} mice, steady-state immunoglobulin titers produced in response to a TI antigen lipopolysaccharide (LPS), will be observed. Furthermore, B cell-helper neutrophils are recruited to the spleen by translocation of microbial bacteria in humans (24). Germ free mice have also shown to contain fewer B cell-helper neutrophils. This led to the speculation if mucosal commensals of Foxo3\textsuperscript{Lex} mice are required for the increased number of splenic neutrophils and myeloid progenitor cells. To investigate this question, Foxo3\textsuperscript{Lex} mice are treated with broad-spectrum antibiotics, which will allow observation of the effect on MZ neutrophils by the absence of a microbiome. By use to immunohistochemistry, defining
the location of Foxo3^{Lex} splenic neutrophils within the spleen, whether it is the MZ or the red pulp, will furthermore aid in comparing Foxo3^{Lex} splenic neutrophils to B cell-helper neutrophils.

In conclusion, Foxo3 appears to regulate and promote the proper balance of neutrophil populations to avoid overproduction possibly from exaggerated granulopoiesis, especially in the spleen. This study confirms Foxo3’s critical role in regulating myeloid lineages and sheds light on its potential role in mediating progenitor cell development to specialized B cell-activating splenic neutrophils. This study and future studies could help to understand how extramedullary hematopoiesis is controlled in natural and disease states. An important next step will be to determine if the increase in myeloid progenitor cell numbers is cell intrinsic or extrinsic.
MATERIALS AND METHODS

Mice

Mice work was done in accordance to the UCSD Institutional Animal Care and Use Guidelines. C57BL/6J (B6) mice were obtained from the Jackson Laboratory. Foxo3\textsuperscript{Lex}, also known as Foxo\textsuperscript{Kca} mice, were developed by an insertional mutagenesis, which generated a germline deletion in Foxo3 and has been previously described (7). These mice were fully backcrossed to C57BL/6J mice.

Cell Purification and Flow Cytometry

To collect peritoneal cells, 1 ml of 10\% proteose pepone was injected intraperitoneally into mice. Cells were then collected 6 h post injection through a peritoneal washout, where 10 ml of pre-chilled PBS (phosphate buffered saline) was injected into the peritoneal cavity and recollected (27). To obtain blood cells, mice were tail bled and blood was collected in heparin-covered tubes. 50 µl of blood was used for each stain and red blood cells were lysed by RBC lysis buffer. Bone marrow and splenic cells were obtained by single-cell suspension in HBSS (Hank’s Buffered Salt Solution) and 0.5\% FCS (fetal calf serum). Splenic red blood cells were lysed by Akt lysis buffer as well. All cells were kept in ice to decelerate cell death and resuspended in PBS and 0.5\% FCS. Cells were counted using the hemocytometer or the Beckman Coulter Counter.

To measure \textit{in vivo} proliferation of neutrophils, 0.8 g/L BrdU was administered to mice via drinking water in from day 0. Water bottles were covered in aluminum foil to prevent light exposure and the administration of BrdU water was stopped at day 5. Blood
cells and splenocytes were stained with BrdU following the protocol from the APC BrdU Flow Kit (BD Biosciences).

For cell surface staining, Fc receptor was blocked using the unconjugated mouse antibody CD16/32. The following conjugated antibodies were used to phenotype various cell populations: BAFF, B220, c-Kit, CCR7, CD3, CD4, CD5, CD8, CD11a, CD11b, CD11c, CD15, CD16/32, CD24, CD27, CD31, CD41, CD44, CD45.2, CD62L (L-selectin), CD62P (P-selectin), CD69, CD86, CD95 (Fas), CD105 (Endoglin), CD127 (IL-7Ra), CD150 (SLAM), Dx5, FasL, Flk-2, Flt-3, F4/80, ICAM-1, ICAM-2, IgD, IgM, Ly-6C, Ly-6C/G, Ly-6G, MHC class II, Sca-1, Ter119, and TLR2. Apoptotic cells were stained with 7AAD or propidium iodide (PI). All antibodies were purchased from Abcam, BD Pharmingen, BioLegend, and eBioscience.

For cell intracellular staining of Foxo3, instructions from the BD Phosflow intracellular staining kit (BD Biosciences) was followed and the unconjugated monoclonal Foxo3a antibody was used with the PE-conjugated secondary goat anti-rabbit IgG antibody.

All samples were run by BD Biosciences FACSCaliber or LSRFortessa. Data analysis were done by using the FlowJo software.

**K/BxN serum transfer**

75 µl of K/BxN serum was injected intraperitoneally on days 0 and 2 (28). 75µl instead of the recommended dose, 100 µl of sera, was used to be able to view any possible reduced severity of RA. Mice ankle thickening was measured using an electronic caliper (Mitutoyo Corporation). Clinical scores of ankles and wrists were attained by following
a protocol described in previous studies (28). For later possible immunofluorescence staining, mice were sacrificed on the last day of the experiment and their ankle tissues were skinned, flash frozen, and stored at -80°C.

**Microscopy**

To view the histology of neutrophils, splenocytes were first stained with the following biotinylated antibodies: B220, CD4, CD8, CD11c, Dx5, MHC class II, and Ter119. After staining with Strepavidin beads, cells were purified through the AutoMACS Separator (Miltenyi Biotec), and the negative population was collected. These cells were then stained with Ly-6G, and the positive population was collected during a second purification.

The collected cells were resuspended in 100 µl PBS and 0.5% FCS, and coated on glass slides by using the cytopsin centrifuge (Harlow Scientific). The slides were air-dried for 1 h and cells were stained with Giema after cell fixation using the staining protocol (Ricca Chemical Company). Microscopy images were taken using the Olympus confocal microscope.

**Immunohistochemistry**

Spleens were flash frozen in an OCT medium with 2-methyl butane. Organs were sent to the Histology Core of the UCSD Moores Cancer Center for sectioning and H&E staining.
REFERENCES


