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ORIGINAL ARTICLE

Aged neutrophils accumulate in lymphoid tissues from healthy elderly mice and infiltrate T- and B-cell zones

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Abstract

The average age of the human population is rising, leading to an increasing burden of age-related diseases, including increased susceptibility to infection. However, immune function can decrease with age which could impact on processes that require a functional immune system. Aging is also characterized by chronic low-grade inflammation which could further impact immune cell function. While changes to neutrophils in blood during aging have been described, little is known in aging lymphoid organs. This study used female C57BL/6J mice comparing bone marrow (BM), spleen and lymph nodes from young mice aged 2–3 months (equivalent to 18 human years) with healthy elderly mice aged 22–24 months (equivalent to 60–70 human years). Neutrophil proportions increased in BM and secondary lymphoid organs of elderly mice relative to their younger counterparts and presented an atypical phenotype. Interestingly, neutrophils from elderly spleen and lymph nodes were long lived (with decreased apoptosis via Annexin V staining and increased proportion of BrdU^{neg} mature cells) with splenic neutrophils also demonstrating a hypersegmented morphology. Furthermore, splenic neutrophils of elderly mice expressed a mixed phenotype with increased expression of activation markers, CD11b and ICAM-1, increased proinflammatory TNFα, yet increased anti-inflammatory transforming growth factor-beta. Elderly splenic architecture was compromised, as the marginal zone (required for clearing infections) was contracted. Moreover, neutrophils from elderly but not young mice accumulated in lymph node and splenic T- and B-cell zones. Overall, the expansion of functionally compromised neutrophils could contribute to increased susceptibility to infection observed in the elderly.

INTRODUCTION

Polymorphonuclear neutrophils are key elements of the innate immune system and a first line defense against foreign agents. They primarily originate from hematopoietic stem cell precursors from the bone marrow (BM).[1] Under physiological conditions, storage of neutrophils also occurs in the lungs, spleen and liver.[1] In the presence of pathogens, neutrophils rapidly migrate from blood to the site of infection, become activated and mediate the clearance of microbes through production of proteolytic granule enzymes, antimicrobial peptides, reactive oxygen species, chemokines and cytokines, which in turn recruit and regulate the response of other immune cells, such as macrophages, dendritic cells and T lymphocytes in a context-dependent manner.[1] Once the pathogen has been eliminated, neutrophils spontaneously undergo apoptosis and are eventually cleared by macrophages in the liver, BM and spleen.

Neutrophils were previously thought to be short-lived cells; however, recent studies show that their lifespan is extended in response to damage-associated molecular

patterns, pathogen-associated molecular patterns and proinflammatory cytokines such as TNFα.[1] Recent studies also highlight a previously unrecognized role that neutrophils play in shaping adaptive immune responses in secondary lymphoid organs. For example, in a murine influenza model, neutrophils were capable of antigen presentation via MHC class I[2] and, during chronic HIV infection, they have been shown to mediate immunosuppression via upregulation of programmed death ligand-1 (PD-L1[3]). Neutrophils also have the capacity to indirectly influence T-cell activation through production of pro- or anti-inflammatory cytokines.[4]

During healthy aging, immune function can decline in a phenomenon known as immunosenescence.[5] It is thought immunosenescence contributes to the increasing incidence of infections, autoimmune diseases and cancers in the elderly population. Interestingly, aging is also associated with the onset of chronic low-grade inflammation termed "inflammaging"[6] characterized by increased levels of inflammatory cytokines, for example, TNFα and IL-6. However, in healthy aging, anti-inflammatory transforming growth factor-beta (TGFβ) and IL-10 also increase.[7]

Immunosenescence has been extensively studied in the context of the adaptive immune system, highlighting the declining function of T and B cells.[8] Several studies also indicate that aging impacts on innate immunity.[9,10] The higher susceptibility to bacterial infections in older humans strongly supports the hypothesis of a suboptimal response by neutrophils. A number of studies in both humans and mice show that several neutrophil functions decline with aging, such as altered migration, reduced phagocytosis and apoptosis.[9] However, other studies have shown that neutrophils have a pre-activated basal state prior to introduction of inflammatory stimuli, suggesting that neutrophils may contribute to inflammaging.[10] The majority of these studies examined neutrophils isolated from blood and in the context of experimental disease models. However, their localization and function in lymphoid organs during healthy aging has yet to be determined.

Lymphoid organs are the main anatomical structures where immune reactions occur following early pathogen exposure.[11] Splenic and lymph node white pulp contain mainly T cells surrounding the central arteriole and B-cell follicles responsible for activating the immune responses,[11] while red pulp consists of mainly macrophages and granulocytes responsible for the clearance of old platelets, erythrocytes and apoptotic cells.[12,13] The two compartments are separated by the marginal zone (MZ), in which macrophages remove pathogens and dead blood cells from the blood.[14] The ability of an individual to mount an efficient immune response depends partially on the integrity of this structural architecture that guarantees appropriate antigen presentation between antigen presenting cells and effector cells.[15] In the spleen, neutrophils are normally confined to the red pulp.[13] Following *in vivo* stimulus of the immune system by administration of lipopolysaccharide, neutrophils migrate from the MZ to the white pulp leading to activation of naïve T cells.[16] Similarly, neutrophils also home to T cell areas in lymph nodes following an infection.[17] Recently, disruption of the splenic microanatomy and lymph node structure has been described in healthy elderly animals.[18] However, the role of neutrophils in lymphoid organs during healthy aging has yet to be established.

We hypothesized that chronic low-grade inflammation that occurs during healthy aging (inflammaging) results in changes to neutrophil function in secondary lymphoid organs, possibly via an autocrine feedback loop. Here we show that neutrophils increase in BM and secondary lymphoid organs of elderly mice. Neutrophils were found to infiltrate white pulp in the absence of exogenous stimuli, were long-lived and presented with an atypical phenotype. The expansion of functionally compromised neutrophils may produce pleiotropic effects, including compromised organ function, delayed resolution of inflammation in response to tissue damage and could contribute to the increased susceptibility to infections in the elderly.

RESULTS

Neutrophils increase in lymphoid organs of healthy elderly mice

To confirm the presence of inflammaging in our elderly mice cohorts, we first analyzed circulating levels of proinflammatory cytokines. Similar to previous studies,[19] IL-6, TNFα and MCP-1 were elevated in the plasma of elderly compared with young C57BL/6J mice (Figure 1a). While inflammation is also associated with neutrophil accumulation in secondary lymphoid organs, very few studies have systematically examined the role of neutrophils in healthy lymphoid tissues during aging. Therefore, we stained BM, lymph node and spleen samples with antibodies against F4/80, CD11b and Ly6G and identified neutrophils as viable F4/80^{neg}CD11b^{+} Ly6G^{+}SSC^{hi} cells (flow cytometry gating strategy in Figure 1b). The percentage of neutrophils was significantly increased in elderly C57BL/6J BM, spleen and lymph nodes (Figure 1c) relatively to their younger counterparts. To ensure that this was not strain specific we also analyzed Balb/c mice and similar results were observed (Figure 1d).

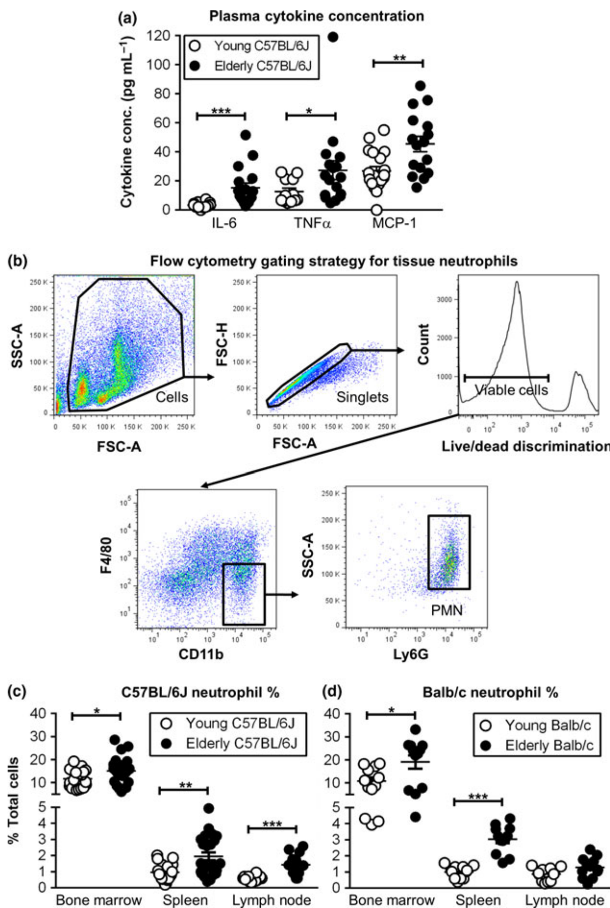

Figure 1. Neutrophils increase in secondary lymphoid organs of healthy elderly mice. Proinflammatory cytokines were analyzed in plasma from healthy young and elderly C57BL/6J mice **(a)**. Polymorphonuclear neutrophils (PMN) were analyzed by flow cytometry (gating strategy shown in **b** for spleens, BM and lymph nodes from healthy young and elderly C57BL/6J **(c)**, and Balb/c mice **(d)**, after staining for F4/80, Ly6G, CD11b, viability. Data from individual mice (12–20 mice/group pooled from 5 or 6 experiments) and mean ± s.e.m. are shown, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. [The color version of this figure can be viewed at www.wileyonlinelibrary.com/journal/icb]

Hypersegmented, aged neutrophils are present in healthy elderly lymphoid organs

In order to confirm that these cells were neutrophils we performed cell sorting on $F4/80^{neg}CD11b^{+}Ly6G^{+}SSC^{hi}$ from young and elderly C57BL/6J mice and analyzed cell nuclear morphology. All of the $F4/80^{neg}CD11b^{+}Ly6G^{+}SSC^{hi}$ cell nuclei displayed characteristic segmented neutrophil nuclei (Figure 2a). Interestingly, we observed an increased percentage of cells from elderly spleen and bone marrow that exhibited hypersegmented nuclei with more than five nuclear lobes (Figure 2b).

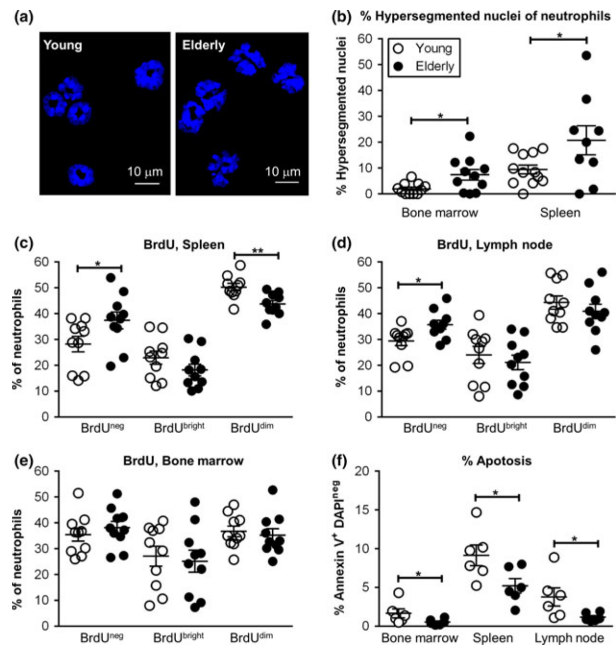

Figure 2. Neutrophils display an extended lifespan in secondary lymphoid organs of healthy elderly mice. $F4/80^{neg}Ly6G^{+}CD11b^{+}SSC^{hi}$ cells were sorted from either healthy young or elderly C57BL/6J mice and stained with nuclei dye, Hoechst 33258. Representative confocal microscopy images shown in **(a)**. The percentage of hypersegmented nuclei was determined from young and elderly C57BL/6J BM and spleen samples **(b)**. Mice were injected with BrdU and 48 h later, $F4/80^{neg}Ly6G^{+}CD11b^{+}SSC^{hi}$ from spleen **(c)**, BM **(d)** and lymph nodes **(e)** were stained for BrdU incorporation to identify aged, mature ($BrdU^{neg}$), less mature ($BrdU^{bright}$) and immature neutrophils ($BrdU^{dim}$). Samples were stained with Annexin V and DAPI and apoptotic cells reported as Annexin $V^{+}/DAPI^{neg}$ **(f)**. Data from individual mice (9–12 mice/group pooled from 4 or 5 experiments for **a** to **e**; 6 mice/group from 2 experiments for **f**) and mean ± s.e.m. are shown, **P* < 0.05, ***P* < 0.01. [The color version of this figure can be viewed at www.wileyonlinelibrary.com/journal/icb]

Previous studies have suggested that hypersegmented neutrophils may have an extended lifespan.[20] Therefore, to determine neutrophil lifespan we analyzed samples from young and elderly mice for incorporation of the nucleoside analogue BrdU which identifies aged, mature ($BrdU^{neg}$), less mature ($BrdU^{bright}$) and immature neutrophils ($BrdU^{dim}$).[21] Spleen and lymph nodes from healthy elderly mice exhibited an increase in the percentage of $BrdU^{neg}$ aged, mature neutrophils compared with young mice (Figure 2c and d, respectively), with no differences in the BM (Figure 2e). We also observed no difference in the percentage of $BrdU^{neg}$ aged, mature neutrophils in the blood of elderly mice compared with young mice (Supplementary figure 1). It is also possible that the increased number of neutrophils observed from elderly mice may be due to delayed apoptosis, which can occur following tissue infiltration at sites of inflammation. Similarly, flow cytometry analysis revealed a

decrease in F4/80^{neg}CD11b^{+}Ly6G^{+}SSC^{hi}AnnexinV^{+}DAPI^{neg} apoptotic neutrophils in lymphoid organs from elderly mice compared to their young counterparts (Figure 2f). Taken together, these data suggest that neutrophils from elderly mice are longer lived than their younger counterparts and this may account for their increased proportions in secondary lymphoid organs.

Splenic macrophages from healthy elderly mice exhibit decreased phagocytosis

Phagocytosis of neutrophils by splenic macrophages represents a crucial step in the resolution of inflammation.[22] It is possible that altered clearance of neutrophils by macrophages during aging leads to the increased proportion of neutrophils observed in secondary lymphoid organs, particularly in the spleen. Therefore, we assessed the phagocytic capacity of splenic macrophages from young and elderly mice by fluorescent bead uptake. As shown in Figure 3, splenic macrophages from elderly mice showed decreased phagocytic ability compared with young mice. Decreased clearance may also contribute to the increased proportion of neutrophils in elderly healthy spleen samples.

Neutrophils infiltrate splenic white pulp zones in healthy elderly mice

Remodeling of splenic microanatomy frequently occurs during inflammatory processes and is considered crucial for an effective immune response and for neutrophil clearance.[15] However, splenic microarchitecture is altered in elderly mice including reduction in the MZ.[18] We therefore immunostained spleens and lymph nodes to evaluate whether disrupted lymphoid microanatomy was accompanied by altered distribution of neutrophils. Similar to previous studies of healthy elderly spleen sections, we observed a reduction in the MZ size on images (Figure 4, asterix) and neutrophils increased in the red pulp, which could be attributed to decreased phagocytosis by splenic macrophages (Figure 3). Moreover, a small proportion of neutrophils were also seen in the MZ and white pulp T- and B-cell zones in healthy elderly spleens (Figure 4b and c, white arrows). Interestingly, there was a large amount of heterogeneity in healthy elderly spleens, despite no obvious macroscopic or pathological disease being present in any mice. Similar results were also observed for lymph nodes from elderly mice (Supplementary figure 2).

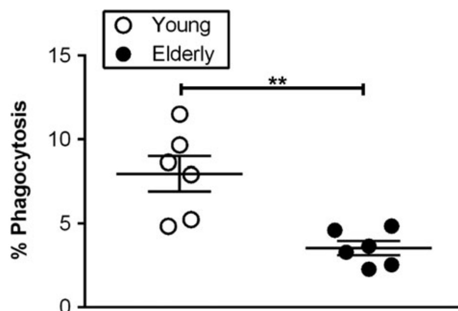

Figure 3. Splenic macrophages from healthy elderly mice have decreased phagocytosis. Splenic macrophages from healthy young and elderly C57BL/6J mice were incubated with fluorescent beads. Uptake was analyzed by flow cytometry using F4/80 antibody to identify macrophages which had undergone phagocytosis. Data from individual mice (6 mice/group pooled from 3 experiments) and mean ± s.e.m. are shown, $^{**}P < 0.01$.

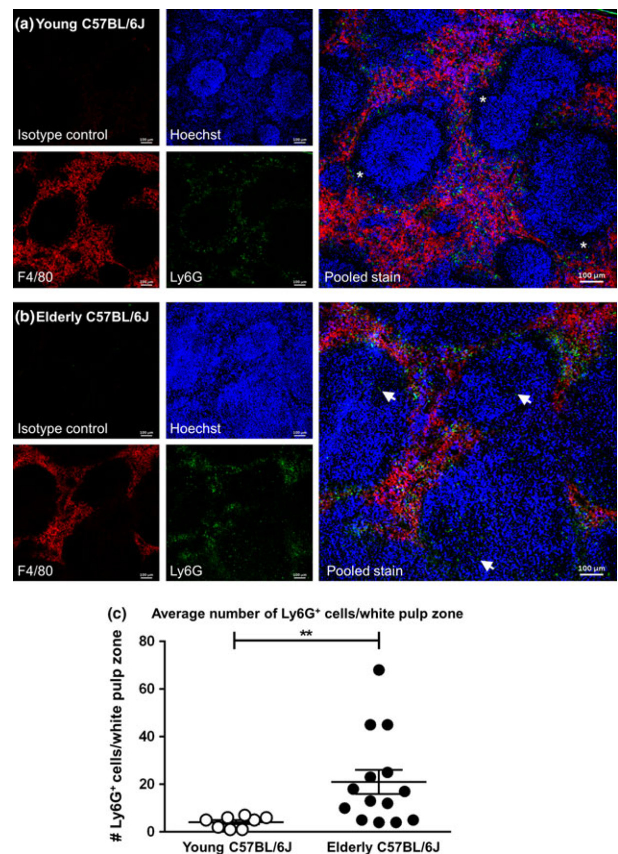

Figure 4. Neutrophils infiltrate T- and B-cell zones in healthy elderly spleens. Immunofluorescence staining for red pulp macrophages (F4/80; red), neutrophils (Ly6G; green), nuclei (Hoechst 33258; blue) and combined negative isotype control was performed on 10 µm spleen cryosections. Confocal microscopy images are shown from young and elderly C57BL/6J mice (**a** and **b,** respectively). *Highlights splenic marginal zones in young mice, which contracts in elderly mice. Arrows show neutrophil infiltration into splenic T- and B-cell zones in elderly mice. The average number of Ly6G^{+} neutrophils PMN was calculated from at least six well-orientated white pulp zones per mouse **(c)**. Data from individual mice (8 young mice and 14 elderly mice pooled from 5 experiments) and mean ± s.e.m. are shown, $^{**}P < 0.01$.

Neutrophils in lymphoid organs from healthy elderly mice exhibit characteristics associated with chronic inflammation

CD11b and ICAM-1 (CD54) are transmembrane glycoproteins involved in neutrophil migration and activation. In steady state, they are localized intracellularly on the surface of cytoplasmic granules, which mobilize upon cellular activation resulting in their increased expression on the cell surface.[23,24] We observed a significant increase in the percentage of both CD11b and ICAM-1 expressing cells in lymph nodes from healthy elderly mice compared with young mice (Figure 5a, b). Similarly, ICAM-1 expression increased in spleens from elderly mice and a slight, but not significant, increase in CD11b expression was observed. There was no change in elderly BM samples (data not shown). These data suggest that age-related changes primarily occur within neutrophils in secondary lymphoid organs following exit from the BM.

To further assess neutrophil phenotype and likely function in secondary lymphoid organs of elderly mice we first measured markers associated with direct T-cell interaction (PD-L1, MHC class I and MHC class II). Neutrophils expressed little PD-L1 (Supplementary figure 3a) and no MHC class II (data not shown) across all tissues examined with no difference between the age groups. MHC class I also did not differ between the two age groups in BM and spleen, whilst a slight, but not significant, increase was observed in elderly lymph nodes (Supplementary figure 3b). Following this, we examined intracellular pro-/anti-inflammatory cytokine expression (TNFα, IL-12, IL-10 and the C-terminal proregion latency associated peptide of TGFβ LAP). TNFα and TGFβ LAP were increased in splenic neutrophils of elderly compared to young mice (Figure 5c). In elderly lymph nodes, TGFβ LAP was also significantly increased in neutrophils (Figure 5d). There was no difference in IL-12 or IL-10 expression in BM between young and elderly mice (Supplementary figure 3c). Overall, these data suggest that neutrophils infiltrating secondary lymphoid organs in healthy elderly mice exhibit an altered function, which is likely to influence innate and adaptive immune responses.

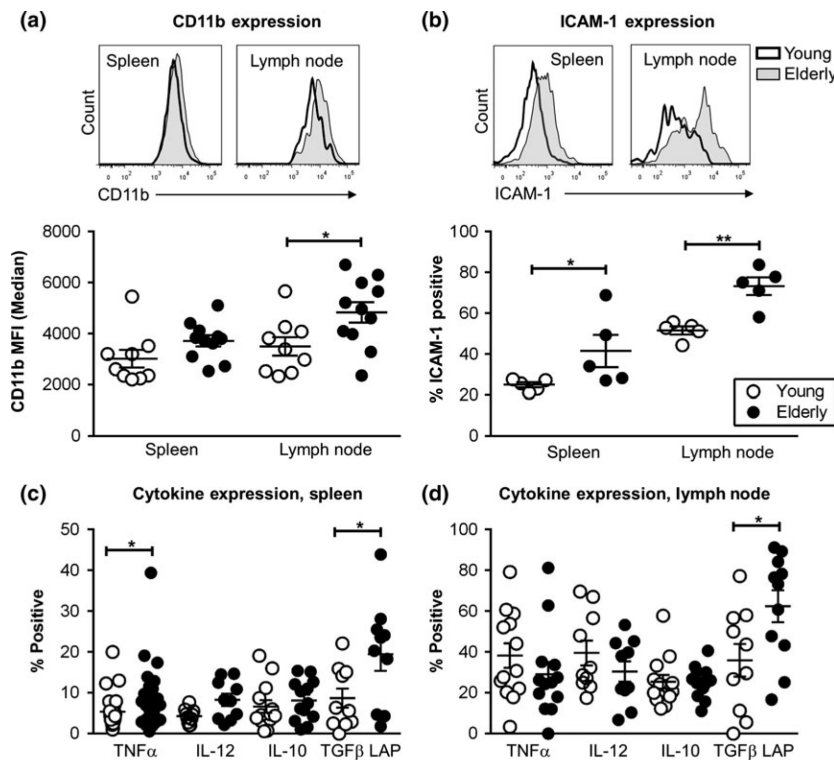

Figure 5. Neutrophils exhibit a distinct phenotype in secondary lymphoid organs of healthy elderly mice. Spleen and lymph node from healthy young and elderly C57BL/6J mice were stained for F4/80, Ly6G, viability, CD11b **(a)**, CD54/ICAM-1 **(b)**, TNFα, IL-12, IL-10 and TGFβ LAP and analyzed by flow cytometry (Spleen in **c** and lymph nodes in **d**). Data from individual mice (9–11 mice/group pooled from 3 experiments for **a**; 5 mice/group from 2 experiments for **b**; 10–20 mice/group pooled from 5 or 6 experiments for **c** and **d**) and mean ± s.e.m. are shown, $^*P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

Increased rates of mortality and morbidity in elderly humans are mainly caused by opportunistic bacterial, fungal and viral infections,[25] which are also found in patients with neutropenia or granulocyte defects,[26,27] underlining the importance of operative neutrophils for the immune system. Deterioration in organ functionality and structure also contributes directly and indirectly to the systemic low grade levels of inflammatory cytokines found in elderly hosts.[18] However, there are no published data of neutrophil distribution in secondary lymphoid organs of healthy elderly hosts that could impact responses to pathogens. In this study, we characterized the proportion, localization, phenotype and function of neutrophils in BM, spleens and lymph nodes from of elderly healthy mice (aged 22–24 months).

We found a significant increase in neutrophils in BM of the elderly mice, consistent with a shift in the hematopoietic stem cell pool toward the myeloid lineage described during aging.[28] As a consequence, and in accordance with previous studies,[29] we found this increase in the BM was accompanied by increased total numbers of neutrophils in both spleen and lymph nodes of elderly mice. This rise in numbers may be a compensatory mechanism for the reduced efficiency of these cells in fighting infections. For example, studies have shown that neutrophils from elderly mice and humans have diminished phagocytic ability.[9,30] In addition, we observed impaired age-related phagocytic capacity of splenic mouse macrophages, which could reflect their failure to efficiently clear neutrophils, resulting in the accumulation of these cells.

We observed increased infiltration of neutrophils in healthy elderly mouse spleens and lymph nodes. Neutrophils are known to accumulate in spleen and lymph nodes in response to inflammatory stimuli[16,17] and it is possible that neutrophils in the elderly migrate into these areas as a result of inflammaging and low levels of inflammatory cytokines. For example, neutrophils from elderly hosts retain their chemotactic ability to infiltrate injured tissues,[31,32] but they are unable to migrate correctly.[33] This could result in collateral damage due to the presence of activated cells in the tissue, leading to an autocrine feedback loop whereby this response is exacerbated thereby inducing further inflammaging.

Under physiological conditions, white pulp access is generally restricted to lymphocytes and dendritic cells with neutrophils almost entirely confined to the red pulp.[13] However, neutrophils infiltrated these areas in healthy elderly lymphoid organs. It is possible that along with inaccurate migration, the progressive loss of MZ macrophages during aging, which represents the main barrier between white pulp and red pulp, leads to infiltration. Moreover, under normal conditions neutrophil half-life is limited to ~6–10 h in the circulation before undergoing spontaneous apoptosis.[1] However, we saw an increase in lifespan in tissues combined with decreased apoptosis in elderly neutrophils consistent with observations at sites of inflammation.[1,34] For example, proinflammatory mediators can prolong neutrophil survival,[34] exacerbating inflammation, leading to tissue damage and compromised organ dysfunction.

Neutrophils can impact other immune cells through a direct contact-dependent crosstalk.[35,36] However, we observed no changes in expression of PD-L1, MHC class I and MHC class II molecules associated with direct T-cell interaction. Interestingly, elderly neutrophils exhibited a hypersegmented nuclear morphology, a process dependent on mTOR pathway,[37] which mediates several other neutrophil functions including chemotaxis and cytokine production. Nuclear hypersegmentation is also a distinct characteristic of antitumoral neutrophils characterized by a marked cytotoxic and proinflammatory phenotype.[4,38] Consistent with this, we observed upregulation of the cytoplasmic granule integrin CD11b, and the ICAM-1 endothelial adhesion molecule by neutrophils from elderly mice, suggesting increased degranulation.[39] CD11b is normally required for their extravasation and recruitment to the site of infection, in accordance with the increased rolling observed in leukocytes from aged donors.[40] However, in the lymph node and spleen, elderly neutrophils also displayed upregulation of TGFβ, concomitant with upregulated TNFα expression in the spleen. Overall, this suggests neutrophils exhibit a heightened pro-/anti-inflammatory basal state in lymphoid organs during healthy aging. In healthy spleens, neutrophils deliver suppressor signals to T cells to maintain tolerance in sites constantly exposed to antigens, whilst inducing activation of MZ B cells.[41] In the context of inflammaging, this dual function might become amplified in both directions. Furthermore, engagement of CD11b by neutrophils in the presence of TNFα programs dendritic cells into potent T cell activating antigen presenting cells,[35] and ICAM-1 enhances NK cell activation and phagocytosis,[42,43] suggesting an active crosstalk with elderly neutrophils. However, these cells also resemble a sub-set of human neutrophils described by Pillay and colleagues with increased CD11b and ICAM-1 with suppressive effects on T-cell proliferation.[44] Interestingly, ICAM-1^{high} neutrophils were also found in patients with chronic inflammatory disorders and were less prone to apoptosis,[45] suggesting a potential pathogenic role for this cell subset whose protracted longevity may contribute to exacerbation and protraction of the inflammatory response during aging.

While some features of the immune system are similar between mice and humans,[46] one difference relates to the number of neutrophils in the blood. In mice, neutrophils represent only 10–25% of leukocytes, whereas neutrophils in human blood represent 50–70% of leukocytes.[46,47] However, few studies have examined age-related changes to neutrophils in lymphoid tissues from humans. Our studies show that neutrophil proportions increase in elderly BM, spleen and lymphoid tissues from mice and it is possible that studying elderly hosts may be more representative of some human diseases, particularly age-related diseases.

In conclusion, this study shows that neutrophils increase in lymphoid organs of healthy elderly female mice and infiltrate T- and B-cell lymphoid areas in the absence of any pathological disease. Compared to young mice, neutrophils from elderly spleens and lymph nodes expressed a distinct phenotype, with extended lifespan, increased surface expression of ICAM-1 and CD11b, concomitant with increased TGFβ. Together, our observations support the hypothesis of age-related changes in the distribution and phenotype of neutrophils, potentially as a result of inflammaging. Prolonged low-grade neutrophilia during aging may eventually lead to tissue damage, further inflammation and result in the development of age-related diseases.

METHODS

Mice

Female Balb/c and C57BL/6J mice aged 2–3 months (young), 17–18 months (Balb/c) or 22–24 months (C57BL/6J) were obtained from Animal Resources Centre (Murdoch, Western Australia) and maintained under specific pathogen free conditions, with 3–5 mice/cage on corn-cob bedding at Curtin University and the University of Western Australia animal facilities. Any mouse with a palpable mass, enlarged organ (including lymph nodes and spleens), weight loss more than 15% from 12 months, or change in body condition from 3[48] were excluded so that only healthy elderly mice were examined. Animals were maintained in a standard light/dark cycle and all sample collection performed in the morning. All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes as per Curtin University and the University of Western Australia Animal Ethics Committee.

Flow cytometry

To ensure cell viability and limit neutrophil activation *ex vivo*, BM, spleen, axillary and inguinal lymph nodes were collected into ice-cold PBS containing 1% BSA (Sigma-Aldrich, St Louis, Missouri, USA) and 2% FCS (Hyclone, GE Healthcare Life Sciences, Utah, USA). BM cells were isolated via gently flushing tibia and femur bones with a 29-gauge needle. Tissue samples were disaggregated into single-cell suspensions by gentle dispersion between two frosted glass slides. Viability of $CD11b^{+}F4/80^{neg}Ly6G^{+}$ neutrophils was $95.9 \pm 0.7\%$ for BM and $86.3 \pm 1.1\%$ for tissue samples.

For surface staining (and subsequent cell sorting) all steps were performed on ice in the dark. Combinations of the following anti-mouse primary antibodies (all from Biolegend, San Diego, California, USA; details in Supplementary table 1) were incubated for 30 min: anti-CD11b), anti-F4/80, anti-Ly6G, anti-CD54, anti-PD-L1, anti-MHC class I and anti-MHC class II. Following surface staining, samples were washed twice in PBS/2%FCS following by one wash in PBS and then incubated with either Zombie NIR™ or Zombie Green™ Fixable viability dye (Biolegend, diluted 1:400 in PBS) for 15 min.

For analysis of apoptosis by flow cytometry following surface staining samples were resuspended in Annexin V binding buffer (BD Biosciences, San Jose, California, USA) and then incubated with Annexin-V-FITC and 4′,6′-Diamidine-2′-phenylindole dihydrochloride (DAPI, 1 μg mL^{-1}, Sigma-Aldrich) for 15 min at room temperature. Cell apoptosis was reported as the percentage of Annexin $V^{+}/DAPI^{neg}$ cells.

For intracellular staining, samples were processed in buffer containing Brefeldin A (1 μg mL^{-1}, Biolegend), fixed in 1% paraformaldehyde (Sigma-Aldrich) for 15 min, followed by permeabilization for 15 min with PBS/2% FCS solution containing 0.1% saponin (Sigma-Aldrich). Cells were washed twice then stained with anti-TNFα, anti-IL-10 and anti-TGFβ latent associated protein (all from Biolegend; details in Supplementary table 1).

Following staining, cells were washed twice and resuspended in PBS/2% FCS for acquisition on either a BD FACSCanto II or BD LSR Fortessa using FACSDiva software (BD Biosciences), followed by analysis using FlowJo software (TreeStar, Oregon, USA). Relevant matched isotype controls were included as negative controls and fluorescence minus one controls were included as gating controls.

Neutrophil morphology

Viable $F4/80^{neg}CD11b^{+}Ly6G^{+}SSC^{hi}$ cells from BM and spleen samples were sorted into ice-cold PBS/20%FCS using a FACSJazz cell sorter with FACSort software (BD Biosciences). Samples were sorted based on purity which resulted in >95% $F4/80^{neg}CD11b^{+}Ly6G^{+}SSC^{hi}$ cell recovery. Sorted samples were prepared with an FCS underlay and centrifuged onto poly-l-lysine coated slides at 800 RPM for 10 min using a cytospin laboratory centrifuge (MPW Med. Instruments, Warsaw, Poland). Slides were allowed to air dry, then stained with Hoechst 33258 (Sigma-Aldrich; 1 μg mL^{-1}) for 5 min, washed three times in PBS and mounted with Prolong™ Gold Antifade mountant (Life Technologies, Carlsbad, California, USA). Images were acquired using a Nikon A1+ point scanning confocal microscope with NIS elements confocal software (Nikon Instruments, Tokyo, Japan). Images were collected using a Plan Apo 60× objective lens (N.A. 1.40) with laser scanning using 405 nm (450/50 filter). The percentage of hypersegmented neutrophils was determined

from at least 30-cell differential leukocyte counts/sample. Lymph nodes were not analyzed because of limited cell numbers.

BrdU pulse labeling and staining

Mice were injected intravenously via the tail vein with 200 μL BrdU (2.5 mg/mouse, Biolegend) 48 h prior to flow cytometry analysis. After 48 h, spleen, BM and lymph nodes were processed and stained for surface markers as described previously. Following surface staining, samples were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice in PBS/2% FCS and then permeabilized for 15 min with 0.5% Triton X diluted in PBS. Following permeabilization, samples were incubated for 1 h at 37°C with 20 μg DNase/sample (Sigma-Aldrich, diluted in HBSS with Ca^{2+} and Mg^{2+}) and then stained with anti-BrdU-Alexa Fluor® 647 (Biolegend) at room temperature for 20 min. Cells were then washed twice and resuspended in PBS/2% FCS for flow cytometry analysis as described previously.

Cytokine bead array

Concentrations of TNFα, IL-6, IL-10, IFNγ, IL-12 and MCP-1 in plasma samples were measured using a BD mouse inflammation cytokine bead array (BD Biosciences) as per the manufacturer's instructions.

Phagocytosis assay

Spleens were disaggregated into single-cell suspensions by gentle dispersion between two sterile frosted glass slides. Cells were cultured for 4 h at 37°C in RPMI (Life Technologies) supplemented with 10% FCS, L-glutamine, penicillin/streptomycin (Life Technologies) to promote adherence of macrophages. Following culture, non-adherent populations were removed by washing with PBS. The remaining cells were then incubated at 37°C with fluorescent yellow-green latex beads (1-μm size, Sigma-Aldrich) for 15 min. Cells were then stained for flow cytometry analysis as described previously and macrophages identified as $F4/80^{+}CD11b^{+}Ly6G^{neg}$.

Immunofluorescence

Spleen and lymph node samples were embedded in OCT freezing medium (Tissue-Tek, ProSciTech, Queensland, Australia) and immediately placed at −80°C. Cryosections (10 μm) were fixed with ice-cold acetone (Hurst Scientific, Forrestdale, Western Australia) at 4°C for 10 min and then air-dried. Sections were blocked with PBS/10%FCS/1%BSA for 1 h at room temperature and then incubated with primary antibodies anti-F4/80 and anti-Ly6G overnight at 4°C in the dark (both from Biolegend, details in Supplementary table 1). Following incubation, slides were washed three times with PBS and stained with Hoechst 33258 for 5 min. Slides were washed again three times with PBS before being mounted in Prolong™ Gold Antifade mountant. Relevant matched isotype controls were included as negative controls.

Images were acquired using a Nikon A1+ point scanning confocal microscope with NIS elements confocal software. Images were collected using a Plan Apo 20× objective lens (N.A. 0.75) with sequential laser scanning using 405 nm (450/50 filter), 488 nm (525/50 filter) and 640 nm (700/75 filter) and then analyzed using NIS elements advanced research software (Nikon). Equivalent thresholds were applied across images and regions of interest were generated for either whole lymph nodes or splenic white pulp zones. For spleen samples, a minimum of six well-orientated white pulp zones were examined per mouse and the number of $Ly6G^{+}$ objects within each white pulp zone was calculated.

Statistical analysis

Statistical significance was calculated using GraphPad PRISM 6 (California, USA). The Student's *t*-test and Mann–Whitney *U*-test were used to determine differences between two populations. *P*-values of < 0.05 were considered statistically significant.

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CONFLICT OF INTEREST

There are no conflicting financial interests with regard to this submission.

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