Original Article

Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age-Related Immune Impairment

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Abstract

Aging negatively impacts immunity, resulting in inefficient responses to vaccinations and infections. Fibroblastic reticular cells (FRCs) are the major stromal cell subset in lymph nodes (LNs) and play an intricate role in the orchestration and control of adaptive immune responses. Although stromal cells have a major impact on immune responses, the impact of aging on LN stromal cells remains unclear. Quantitative analysis of LN stromal cells by flow cytometry revealed that there are no significant differences in the number of stromal cells in young and aged LN at steady state but after influenza infection aged FRCs have delayed expansion as a result of reduced proliferation. Aged LNs also produce reduced levels of homeostatic chemokines, which correlates with reduced homing of naive T cells. Image analysis reveals that young and aged T-cell zone FRCs have similar morphology at steady state and after infection. Furthermore, aged FRCs did not appear to be a contributing factor in the reduced proliferation of young T cells transferred into aged LNs after influenza infection. These results demonstrate that aging alters LN stromal cell response to challenge and these age-related changes may be an underlying contributor to impaired immune responses in the elderly people.

Keywords: Immunity, Influenza, T cells

Influenza is a highly problematic and often lethal infection for the elderly people (1) and the decline in immune system function with aging, collectively termed “immunosenescence,” contributes greatly to this increased susceptibility to influenza infection (2). Intrinsic age-related defects in adaptive immunity have been well characterized, with a strong focus on the T-cell lineage (3,4). Both CD4⁺ and CD8⁺ T-cell responses to influenza infection have been reported to be altered with aging. During influenza infection, aged mice exhibit delayed influenza nucleoprotein (NP)-specific CD4⁺ and CD8⁺ T-cell responses in the lungs when compared to responses in young mice (5,6). This correlates with the delayed clearance of virus from aged lungs (7,8). While intrinsic age-related defects in the adaptive immune system have been extensively studied, age-related changes in secondary lymphoid organs are less well understood and may also contribute to and exacerbate problems with T-cell immunity and age.

Adoptive transfer studies have been the key to dissecting the impact of the aged environment on T-cell-mediated responses. In these

studies, young fully functional donor T cells are transferred into either young or aged hosts, which are then subsequently challenged to assess function (9–11). This approach analyzes the influence of T-cell-independent factors in the secondary lymphoid organ micro-environment on the development of antigen-specific T-cell responses. These studies have provided a striking insight into the deleterious impact of the aged secondary lymphoid organ environment on T-cell responses. Importantly, these studies demonstrated that young donor T cells have delayed entry and priming and reduced effector function in aged lymph nodes (LNs) and spleens when compared to young (9–11). Yet it remains to be determined which factors in the aged secondary lymphoid organs hinder young T-cell responses.

Stromal cells in secondary lymphoid organs such as LN are non-hematopoietic cells of mesenchymal origin (12). These cells were once thought to solely function as the structural skeleton of the LN, mediating architectural organization, but are now appreciated to be involved in many aspects of both innate and adaptive immune responses

(13–15). There are four major subsets of stromal cells in a LN: fibroblastic reticular cells (FRCs), lymphatic endothelial cells (LECs), blood endothelial cells (BECs), and double negative cells (DNCs). LECs and BECs line lymphatic and blood vessels, respectively, functioning to facilitate transport of fluid, cells, and antigen into and out of LN (16,17). DNCs are a rare and poorly understood subset of LN stromal cells that are thought to be contractile FRC-like pericytes (18). FRCs are the major stromal cell subset of the LN and play an intimate role in the initiation, orchestration, and control of adaptive immune cell functions (15,19). At homeostasis, FRC-produced CCL19 and IL-7 promote the survival of naive T cells. With regards to influenza infection, Denton and coworkers demonstrated that LN FRCs are required to initiate adaptive immunity to influenza infection (20). FRCs produce homeostatic chemokines (CCL19 and CCL21) that recruit and position lymphocytes and dendritic cells (DCs) in LN (21,22). FRCs also create a conduit network controlling the migration of T cells, DCs, and antigen in the LN paracortex and medulla (23). During the response to an infection, FRCs have been shown to elongate and expand, which is thought to increase space available in the LN for the lymphocytes that are proliferating in response to antigenic challenge (24–26). Apart from enhancing immune cell interactions and survival, FRCs have been shown to control T-cell-mediated immunity, through a NOS2-dependent pathway (27–29).

Despite the in-depth involvement of FRCs in the development and control of T-cell-mediated immunity, it is unclear how aging impacts the number and function of these cells. Conflicting reports exist regarding the number and function of stromal cells at homeostasis in aged mice (30–32). It remains to be determined how these cells respond to challenge. We hypothesize that age-related changes in FRCs in the aged lung-draining mediastinal LN (MLN) could be one underlying factor in the attrition of T-cell responses to influenza infection in aged mice. In this study, we rigorously characterize how aging impacts FRC number and morphology at homeostasis and during influenza infection. We also determine how aging impacts FRC functional ability to produce homeostatic chemokines important for T-cell recruitment into the LN and tested the ability of aged FRCs to inhibit T-cell proliferation.

Materials and Methods

Mice and Influenza Infection

Young (2–4 months) male C57BL/6 mice were acquired from Jackson Laboratories. Aged (19–21 months) male C57BL/6 mice were acquired from the National Institute on Aging colony at Charles River Laboratories. CD45.1⁺ F5 T-cell receptor (TCR) transgenic mice, specific for a NP peptide from H17 influenza, on a RAG^{-/-} background were received as a generous gift from Dr. Linda Cauley at the University of Connecticut School of Medicine and were used in experiments at 2 months of age. Mice were housed under specific pathogen-free conditions in sterilized, individually vented, HEPA-filtered cages in the UConn Health animal facility. All mice were housed in the UConn Health animal facility for 1 month prior to use to normalize for microbiome differences. Mice were infected via intranasal administration with either 400EID₅₀ A/Puerto Rico/8/1934 (PR8) or 600EID₅₀ E61-13-H17 (H17) influenza. After infection, mice were monitored and weighed daily and sacrificed if they showed visible signs of distress or if their weight dropped below 70% of the starting weight. Mice were sacrificed at time points indicated via CO₂ asphyxiation. The UConn Health Animal Care and Use Committee reviewed and approved all experimental procedures using animals.

LN Stromal Cell Digestion and Flow Cytometry and Histology of LN

Detailed methods can be found in the [Supplementary Data](#).

T-Cell Transfer

Detailed method can be found in the [Supplementary Data](#).

CCL19/CCL21 ELISA

MLNs were dissected, flash frozen, and stored at –80°C until processing. MLNs were homogenized in 400 µL of tissue protein extraction reagent with ethylenediaminetetraacetic acid (Invitrogen) and Halt protease and phosphatase inhibitor cocktail (ThermoFisher) and then centrifuged at 1,000g for 10 minutes. Supernatants were aliquoted and frozen at –80°C until analysis was performed. CCL21 was measured using mouse CCL21/6kine DuoSet ELISA (R&D Systems) according to manufacturer's instructions on MLN homogenates diluted 1:3 in reagent diluent. CCL19 was measured using mouse CCL19/MIP-3 beta DuoSet ELISA (R&D Systems) according to manufacturer's instruction on undiluted MLN homogenates. Chemokine concentrations were normalized to total protein concentration determined using Pierce BCA Protein Assay (ThermoFisher).

FRC-Mediated T-Cell Proliferation Inhibition and T-Cell Survival Assays

Detailed methods can be found in the [Supplementary Data](#).

Statistical Analysis

Statistical significance was determined by Student's *t* test, one-way or two-way analysis of variance (ANOVA), repeated measures ANOVA, or Mantel–Cox log rank test as specified in the figure legends. Statistical analyses were performed with Prism 6 software (GraphPad Software). Differences were considered significant at *p* < .05.

Results

Altered Kinetics of Aged LN Stromal Cell Expansion

To evaluate if stromal cells are an underlying contributor to impaired immune responses during influenza infection in aged mice, we first sought to determine how stromal cells respond in young and aged mice. Following PR8 influenza infection, aged mice exhibit decreased survival ([Supplementary Figure 1A](#)) and increased weight loss ([Supplementary Figure 1B](#)) after infection when compared to young, in agreement with prior studies (7,8).

In order to determine how aging impacts the number of LN stromal cells during influenza infection ([Figure 1A](#)), we examined the kinetics of stromal cell responses in both the lung-draining MLN and the non-draining popliteal LN (as a control to ensure that digestion was standard across time points). While some reports have suggested that aged LNs fail to expand to the same extent as young LNs after immune challenge (11), our results showed that young and aged MLNs expanded with similar kinetics and no significant differences were observed in total cell number at homeostasis or at any time point after influenza infection in young and aged MLNs or peripheral lymph nodes (PLNs) ([Figure 1B](#)). In order to quantify LN stromal cell numbers, a slightly modified version of a published protocol for digestion of LNs for stromal cell analysis was employed (19). With minor modifications, we were able to digest LNs with high viability ([Figure 1C](#)) and achieved similar frequencies of

Figure 1. Reproducible, high validity stromal cell digestion reveals altered stromal cell expansion kinetics in aged mediastinal lymph nodes (MLNs) after influenza infection. (A) Experimental design: Young and aged C57BL/6 mice were infected with PR8 influenza and sacrificed at time points indicated. (B) Total cell numbers of stromal cell digested MLNs (draining lymph node) and peripheral lymph nodes (PLNs) (non-draining lymph node) after infection with PR8 influenza. (C) Viability of lymph nodes after stromal cell digestion. (D) Digested lymph node cells from young (top) and aged (bottom) were gated on viable Ter119 negative cells to eliminate erythrocytes. Dot plots show gating strategy for stromal cell populations; numbers on plots show percentages within the total gated population. PDPN⁺CD45⁺ cells were divided into stromal cell populations based on PDPN and CD31 staining (right dot plots): fibroblastic reticular cell (FRC) in green, lymphatic endothelial cell (LEC) in blue, blood endothelial cell (BEC) in red, and DN in black. Number of (E) total stromal cells, (F) FRCs, (G) LECs, and (H) BECs in young and aged MLNs and PLNs after influenza infection. The frequency of proliferating Ki67⁺ (I) FRCs, (J) LECs, and (K) BECs after infection in draining (MLN) and non-draining lymph nodes (PLN) was assessed by flow cytometric analysis. Data are pooled from two independent experiments, $n = 10$ –18 mice per group, per time point. Statistical significance was determined by two-way analysis of variance with Tukey post-test. Error bars = $\pm$ SEM. ** $p < .01$, *** $p < .001$, **** $p < .0001$.

stromal cell populations (Figure 1D) to what has been reported (19). Upon quantification of the total number of CD45⁺Ter119⁺ stromal cells in MLNs and PLNs at homeostasis, no significant differences in young and aged samples were observed. Day 10 post-influenza infection has been shown to be the peak of stromal cell expansion (20) and aged MLNs had significantly fewer total stromal cells compared to young MLNs at this time point (Figure 1E). By day 12 post-infection, the aged MLN stromal cell numbers were equal to that of the young MLNs, suggesting a delayed expansion in aged LNs. The total stromal cell population was further differentiated into FRCs (PDPN⁺CD31⁺CD21/CD35⁺), LECs (PDPN⁺CD31⁺), and BECs (PDPN⁺CD31⁺). At homeostasis in both MLNs and PLNs, these populations were not significantly different in number in young and aged LNs (Figure 1F–H). After infection, there were significantly fewer aged FRCs and LECs at day 10 post-infection; but this difference was not apparent by day 12 when the aged numbers equaled that of the young for both FRCs and LECs (Figure 1F and G). BECs had different expansion kinetics with their numbers being similar in young and aged LN up to day 10 post-infection, but higher in aged MLNs at day 12 post-infection (Figure 1H). Importantly, PLN total stromal cell, FRC, LEC, and BEC numbers were not significantly different from the non-infected time point at any day post-influenza infection (Figure 1E–H), confirming that the digestions were consistent and the increase in MLN FRC, LEC, and BEC numbers was not due to a technical artifact. We next sought to determine the mechanism behind the decreased numbers of FRCs and LECs in aged LNs at day 10 post-infection.

Decreased Proliferation Contributes to a Reduced Number of FRCs and LECs After Influenza Infection

It is possible that the decreased number of FRCs and LECs at day 10 post-infection in aged MLNs may be due to either decreased proliferation or increased death or a combination of both. Upon analysis of proliferation by Ki67 staining, there was a significantly lower frequency of proliferation on day 10 post-infection in FRC (Figure 1I), LEC (Figure 1J), and BEC (Figure 1K) populations in aged MLNs when compared to young, correlating with decreased cell numbers at this time point. Analysis of cell death in MLN FRCs (Supplementary Figure 2A), LECs (Supplementary Figure 2B), and BECs (Supplementary Figure 2C) showed no significant differences throughout the course of influenza infection. These results suggest that the mechanism behind the decreased number of FRCs and LECs at day 10 post-infection is in part due to decreased proliferation and not an increased rate of cell death. Thus, we next wanted to determine if the structure of the FRC network was altered with age.

Preserved T-Cell Zone FRC Network Morphology in Aged LN

It is well accepted that aging disrupts the architectural organization of secondary lymphoid organs. Several studies have described that aged LNs have poorly defined B-cell follicles that merge into the T-cell paracortex (11,32,33). To determine if similar architectural disorganization existed in aged MLNs at homeostasis and after influenza infection, confocal microscopy was performed. At homeostasis, there was mild disruption of B-cell follicles in aged MLN (Figure 2A, bottom left) when compared to young MLN (Figure 2A, top left). After influenza infection, aged MLNs showed the dramatic loss of B-cell follicle organization (Figure 2A, bottom right), while B cells in young MLNs were confined to well-organized follicles (Figure 2A, top right). Since FRCs are important for homeostasis and maintenance of

Figure 2. Disrupted lymph node architecture but not T-cell zone fibroblastic reticular cell (FRC) morphology in aged mice. (A) Mediastinal lymph node (MLN) from a naive (non-infected) young mouse (top left) shows organized B-cell follicles, while B-cell follicles in an aged naive MLN (bottom left) show mild disruption. Fourteen days after influenza infection, B-cell follicles in an aged MLN (bottom right) become highly disrupted, while young MLN B-cell follicles remain organized (upper right). Images shown are representative of five young and five aged mice at each time point from two experiments performed independently. Yellow-B220 (B cells), Red-CD31 (blood vessels), Blue-Lyve-1 (lymphatic vessels), and Purple colocalization of CD31 and Lyve-1 (lymphatic vessels). Scale bar = 200 μm . (B) Representative confocal image of stromal cells in a young non-infected MLN denoted by ER-TR7 in yellow. The red insert is a magnification of the T-cell zone FRCs, which are quantified in the remainder of the figure. (C) The image analysis pipeline for quantifying

B-cell follicle organization (34), we next sought to determine if the FRC network was preserved in aged LN at homeostasis and after influenza infection. Confocal image quantification of the T-cell zone FRC network in young and aged LNs (Figure 2B–D) revealed no difference in density (Figure 2E), network length (Figure 2F), connectivity (Figure 2G), or in the number of branch points (Figure 2H) at homeostasis or after influenza infection. After influenza infection, both young and aged FRC network density (Figure 2E) and length (Figure 2F) decreased, supporting previous data that after lipopolysaccharide challenge FRCs become less tense and elongate (35). These data demonstrate that the T-cell zone FRC network is preserved with age and that it responds similarly to influenza challenge, with no age-related differences. Interestingly, upon examination of the interface between the T-cell zone and B-cell follicles, we found striking differences in FRC architecture between young and aged MLNs (Supplementary Figure 3). In young LN, there was a clear delineation between where the FRCs processes ended and the B-cell follicle began (Supplementary Figure 3A–G). In aged MLNs, this distinction was lost (Supplementary Figure 3H–N). These data suggest that T-cell zone FRC architecture is preserved with age, but FRC architecture is disrupted at the T-cell zone–B-cell follicle interface, which may contribute to the disruption of B-cell follicular architecture. We next went on to determine the effects of aging on FRC function.

Reduction in Homeostatic Chemokines and Altered High Endothelial Venule Architecture Correlates With Reduced T-Cell Homing Into Aged MLNs

FRCs produce homeostatic chemokines CCL19 and CCL21, which facilitate the localization and homing of CCR7⁺-expressing cells in the LN (21,22). Previous research has shown that young donor T cells exhibit decreased homing into aged LNs (11). To determine if this also holds true for the MLN, young F5 TCR transgenic CD8⁺ T cells, which have a TCR specific for the influenza NP peptide 366–374 (NP_{366–374}/D^b) from the H17 influenza virus (36), were transferred into young and aged hosts (Figure 3A) and their entry into the MLN was quantified. There was a higher frequency and number of young donor CD8⁺ T cells in the peripheral blood of aged hosts when compared to young (Figure 3B–D). This correlated with decreased frequency and numbers of donor cells in aged MLN when compared to young (Figure 3E–G). These data suggest that there is a LN intrinsic defect that results in decreased recruitment of T cells.

Thus, we next determined if this decreased T-cell entry in aged MLNs correlated with reduced levels of homeostatic chemokines. At homeostasis, aged MLNs produced lower concentrations of both FRC-produced homeostatic chemokines CCL21 (Figure 3H) and CCL19 (Figure 3I). The location of CCL21 appeared to be similar in

the FRC network. Z stack images of four 1- μm steps were taken using the 40x objective lens of the Zeiss LSM780 (left-panel). Imaris imaging software was used to create a digital rendering of the staining called an isosurface (middle) to quantify FRC volume and segmentation. The filament tracer tool in Imaris software was used to map the FRC network (right) to determine the network length and number of FRC branch points. (D) Representative FRC images from young (top) and aged (bottom) non-infected (left), day 7 post-infection (middle), and day 10 post-infection (right) MLNs. No significant difference was discovered in the FRC volume (E), segmentation (F), network length (G), or branch points (H) in young and aged mice at steady state and after infection. Statistical significance was determined using two-way analysis of variance with Tukey post-test. * $p < .001$. $n = 10$ young, $n = 10$ aged mice per time point. Data shown are pooled from two experiments performed independently. Error bars = $\pm$ SEM. Scale bar = 20 μm . * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

Figure 3. Decreased homing of young T cells into aged lymph nodes correlates with decreased homeostatic chemokines and altered high endothelial venule (HEV) structure. (A) Experimental outline for T-cell transfer. F5 T-cell receptor (TCR) transgenic CD8⁺ T cells were transferred into young and aged C57BL/6 mice and their presence in blood and mediastinal lymph node (MLN) was quantitated after 30 minutes. (B) Representative flow plot, (C) frequency, and (D) number (cells/μl of blood) of young transferred CD8⁺ T cells in young or aged blood. (E) Representative flow plot, (F) frequency, and (G) total number of young transferred CD8⁺ T cells in each young and aged MLN. (H) CCL21 protein concentration in naive and day 3 post-infection MLNs. (I) CCL19 protein concentration in naive MLNs. (J) Naive MLNs stained for CCL21. (K) Representative images of HEVs in naive MLNs. (L) Quantification of HEV thickness in naive MLNs. (M) ICAM-1 gMFI of blood endothelial cells (BECs) from naive MLNs. (N) PECAM-1 gMFI of BECs from naive MLNs. (B–G) Data are from one representative of three experiments performed independently, $n = 4$ mice per group. (H, I) Data are pooled from two independent experiments, $n = 13$ –20 mice per group. (J, K) Representative

both young and aged MLNs and did not extend into the B-cell follicles (Figure 3J). Since FRCs have been shown to respond to immune challenge by temporarily decreasing their production of CCL21 (37,38), we went on to determine if aged FRC responded to infection in a manner similar to young. Three days after infection, young MLNs exhibited decreased production of CCL21, but aged MLN production of CCL21 was not altered by infection (Figure 3H). Thus, we next sought to explore a mechanism behind the reduced homeostatic chemokine concentrations in aged MLNs.

Lymphotoxin beta receptor (LTβR) expressed on FRCs is important for survival and maintenance of these cells. FRCs, which express low levels of LTβR, produce reduced quantities of CCL19 and CCL21 (12). Thus, we sought to determine if aged FRC had decreased LTβR expression, which could lead to reduced homeostatic chemokine production. Contrary to our hypothesis, we found that both young and aged FRCs expressed similar levels of LTβR at steady state and day 3 after influenza infection (Supplementary Figure 4), ruling decreased LTβR expression out as a possible mechanism for the reduced homeostatic chemokines. These data show that aged stromal cells produce less homeostatic chemokines and are less responsive to challenge when compared to young stromal cells. Importantly, these changes in FRC chemokine production may contribute to the reduced homing of young T cells into aged LNs.

Another important component for T-cell entry into LNs are high endothelial venules (HEVs). Since HEVs are the physical entry sites for lymphocytes into the MLN (17), the influence of age on HEV morphology was examined. HEVs in aged naive MLNs appear less cuboidal and are thinner when compared to those in young MLNs (Figure 3K and L). This thinning of HEVs was also observed in aged inguinal LN (Supplementary Figure 5). ICAM-1 is an important adhesion molecule on HEVs and BECs which binds to LFA-1 on T cells to mediate arrest during transendothelial migration (39). Interestingly, there was no significant difference in ICAM-1 expression on young or aged BECs (Figure 3M). Additionally, we examined PECAM-1, another adhesion molecule, and saw no differences in its expression between young and aged BECs (Figure 3N). Taken together, these data show changes in stromal cell function and morphology, which could contribute to impaired homing of T cells into aged LNs.

Reduced Priming of Young T Cells in Aged MLNs

Another way that aged FRCs could impact T-cell responses is by influencing initial T-cell priming. Since previous studies reported defective priming of young T cells in aged popliteal LNs (9), the response of young donor T cells in the MLN during influenza infection was examined. We used an adoptive transfer approach with F5 TCR transgenic CD8⁺ T cells. Young carboxyfluorescein succinimidyl ester (CFSE)-labeled CD45.1⁺ F5 CD8⁺ T cells were transferred I.V. into young and aged CD45.2⁺ hosts, which were rested overnight and then infected with H17 influenza (Figure 4A). Three days after infection, the response of the transferred cells was assessed by flow cytometry. This time point was selected because it was the

images from one of $n = 6$ –10 mice per group, scale bar = 200 μm. (L) Each data point is the average thickness of three HEVs from one mouse. Each HEV was measured at five different points and the average thickness was taken, $n = 6$ –10 mice per group. (M, N) Data are representative from one of two experiments performed independently, $n = 5$ –8 mice per group. (C, D, F, G, I, L–N) Statistical significance was determined by two-tailed t test. (I) Statistical significance was determined by two-way analysis of variance with Tukey post-test. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Error bars = $\pm$ SEM.

Figure 4. Reduced proliferation and activation of young influenza-specific CD8⁺ T cells in aged lymph nodes after infection. (A) Experimental outline for adoptive transfer. F5 T-cell receptor (TCR) transgenic CD8⁺ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), transferred into young and aged C57BL/6 mice which were then infected with H17 influenza. Donor T-cell division (CFSE dilution) and activation (CD69 and CD44 expression) were assessed by flow cytometric analysis on day 3. (B) Cell division on day 3 indicated by CFSE dilution on donor F5 CD8⁺ T cells in young (gray) or aged (white) mediastinal lymph nodes (MLNs); flow plot (left) and quantification (right). (C) CD69 and (D) CD44 expression on donor T cells in young (gray) or aged (white) MLNs. Fluorescence minus one (FMO) represented by black lines in (C) and (D). Data are one representative experiment of two performed independently; $n = 4-5$ mice per group. Statistical significance was determined by a two-tailed t test. * $p < .05$, ** $p < .01$. Error bars = $\pm$ SEM.

peak of F5 T cell proliferation in young hosts (Supplementary Figure 6A–C). The young F5 cells in aged MLNs exhibited reduced proliferation when compared to those in young MLNs (Figure 4B). Furthermore, fewer F5 cells in the aged host expressed the activation markers CD69 (Figure 4C) and CD44 (Figure 4D) when compared to donor cells in young hosts. By day 4 post-infection, nearly all of the F5 T cells had proliferated (Supplementary Figure 6D) and upregulated CD44 (Supplementary Figure 6F) in both the young and aged hosts. Interestingly, a higher frequency of the young F5 cells in the aged hosts expressed CD69 compared to in the young host at day 4 post-infection (Supplementary Figure 6E). These data show that the initial priming of the CD8⁺ T-cell response is delayed in aged MLNs after influenza infection.

DC Response to Influenza Is Preserved With Age

Since the priming of young T cells was impaired in aged hosts, we next sought to determine if DC responses were altered in the aged mice. We found that at homeostasis and 3 days after influenza infection, there were no significant differences in the number of total

DCs in young and aged MLNs (Supplementary Figure 7A and B). The number of DCs that migrated into the MLN from the lung after influenza infection was significantly reduced in aged MLNs (Supplementary Figure 7C and D).

These migratory DCs localized to the T-cell paracortex, near the transferred young F5 T cells (Supplementary Figure 7D). These results show that aging reduces the number of migratory DCs but does not alter their location in MLNs after infection. These results suggest that reduced DCs responses may contribute to the delayed proliferation of young T cells in aged LN. To examine this further, we assessed if FRCs were played a role in this proliferation defect.

FRCs Maintain Their Inhibitory Capacity With Age

FRCs have been shown to reduce the proliferation of T cells in culture via production of nitric oxide (NO) (27) and this may be one mechanism involved in the reduced proliferation of T cells in aged LN as shown in Figure 4. The inhibitory capacity of young versus aged FRCs was tested using the system published by Lukacs-Kornek and coworkers in which CFSE-labeled CD8⁺ T cells were stimulated in cultures with or without FRCs (27). Since aged T cells have intrinsic proliferation defects (40), we used only young T cells in our co-culture system to test the capacity of aged and young FRCs to inhibit T-cell proliferation. Young polyclonal CD8⁺ T cells stimulated in cultures without FRCs had high rates of proliferation as expected (Figure 5A–C). Interestingly, both young and aged FRCs suppressed the division of these young CD8⁺ T cells equally (Figure 5A–C).

Since FRC-produced NO could also impact T-cell responses, we went on to determine if young and aged FRCs produced similar quantities of NO. As reported by Lukacs-Kornek and coworkers (27), we found that FRCs only produced NO in the presence of activated T cells, not when they are cultured alone (Figure 5D). Importantly, young and aged FRCs produced equal amounts of NO when cultured with stimulated young CD8⁺ T cells (Figure 5D), in agreement with their ability to equally suppress T-cell proliferation (Figure 5A–C). These experiments have the major caveat of being *in vitro*. Since changes in aged FRCs may be lost during culture, an *in vivo* approach was also employed to analyze nitric oxide synthase 2 (NOS2) expression. NOS2, the protein responsible for NO production, was examined in FRCs from both MLNs and PLNs at homeostasis and after influenza infection (27,28,41). Both young and aged MLN FRCs upregulate NOS2 expression after influenza equally, with no age-related differences (Figure 5E). Taken together, these results demonstrate that in our *in vitro* assay, aged FRCs do not exhibit suppressive capacities that are greater than young FRCs when it comes to direct suppression of T-cell proliferation. Furthermore, since FRCs produce important T-cell survival factors, IL-7, IL-15, and CCL19 (23), we also examined whether young and aged FRCs had the same capacity to promote T-cell survival, using the *in vitro* system in which we tested FRC-mediated T-cell proliferation inhibition. The results of our co-culture found that both young and aged FRCs equally enhance the survival of naive T cells (Supplementary Figure 8), ruling out the possibility that age-related changes in FRCs impact T-cell survival at homeostasis.

Discussion

Aging has a profound impact on immunity resulting in reduced responses to influenza infection. In this study, we addressed the hypothesis that age-related changes in LN stromal cells could be an underlying factor hindering the development of protective T-cell

Figure 5. Aged fibroblastic reticular cells (FRCs) maintain their ability to suppress T-cell proliferation. Polyclonal CD8⁺ T cells from young C57BL/6 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and then cultured with or without T-cell receptor (TCR) stimulation (anti-CD3/anti-CD28 microbeads) for 48 hours with either young FRCs, aged FRCs, or no FRCs at a 10:1 ratio (T cell/FRC). The ability of FRCs to suppress T-cell proliferation was measured by CFSE dilution of young CD8⁺ T cells, representative flow plots shown in (A). (B) Division index of CD8⁺ T cell in the FRC co-culture. (C) Percent of young CD8⁺ T cells that divided in co-culture. (D) Nitric oxide in the co-culture supernatants was quantified using the Griess reagent system measuring nitrite levels. (E) *In vivo* NOS2 mean fluorescence intensity (MFI) was measured on FRCs at homeostasis and after influenza infection in draining (mediastinal lymph node [MLN]) and non-draining (peripheral lymph node [PLN]) lymph node sections. MFI was normalized to fluorescence minus one (FMO) at each time point listed. (A–D) Each point represents one experimental replicate, which is the average of three technical replicates, $n = 3$ experiments. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey post-test. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. (E) Combined data from two experiments performed independently, $n = 8$ –16 mice per group. Statistical significance was determined by two-way ANOVA with Tukey post-test. No significant differences were found in young or aged PLNs at any time point tested. ** $p < .01$, *** $p < .001$, **** $p < .001$. Error bars = $\pm$ SEM.

immunity to influenza infection in a mouse model. The results presented provide the most comprehensive analysis of aged LN stromal cells to date at homeostasis and after infection. Our quantification of stromal cell numbers at homeostasis align with a study published by Turner and coworkers which found that aged LN (pooled cervical, axillary, brachial and inguinal) had similar numbers of FRCs, LECs, and BECs (32). This study, however, had a very high frequency of

DN. Up to 70% of all of the stromal cells in young LN were the DN fraction. Becklund and coworkers found that aged LNs either have fewer FRCs than young LN, although statistical significance was not proven for this claim (31). Davies and coworkers found fewer total stromal cells in aged cervical LN compared to young, but this decrease was mainly due to the reduction of the DN population (30). Like Turner and coworkers (32), this study also found the major stromal cell population in both young and aged LN to be the DN fraction. DN cells are widely accepted to be a small population of stromal cells in the LN, generally amounting to well less than 20% of the of the total stromal cell population (30). Artificial increases in the DN population can occur when CD45-positive immune cells downregulate CD45 during the process of death, often due to harsh or inadequate digestion protocols (19). Stromal cell digestion techniques are technically challenging and need to be optimized before reliable results can be obtained (19). The digestion protocol reported in this manuscript, is a high viability, high recovery method that was consistently reproducible, across experimental time points. This consistency allowed us to reliably quantify LN stromal cell expansion after influenza infection.

After antigenic challenge or infection, LN stromal cells expand and proliferate (12,24,42). Our studies determined that after influenza infection, aged stromal cells have different kinetics of expansion when compared to young. The peak of LEC and FRC expansion in aged MLNs is delayed compared to young MLNs, in part due to decreased proliferation. BECs in aged MLNs have similar expansion kinetics but are actually increased in number in aged MLNs at day 12 post-infection. The exact signals that mediate FRC proliferation are still being elucidated. It has been reported that mechanical strain caused by the trapping of naive T and B cells could be an early trigger for proliferation (24,26). Aging decreases the number of naive T cells in the periphery (30,43). This in combination with reduced homing of T cells into aged LN due to decreased homeostatic chemokines could contribute to the reduced stromal cell proliferation. In addition, it has been reported that aged LN become fibrotic which could decrease their ability to expand and thus dampen stromal cell proliferation (43). DCs are also thought to be required for stromal cell proliferation (24,26). We have shown that the total number of DCs in the aged LN is not altered at steady state and at 3 days post-infection, but the number of DCs migrating from the lungs into the draining LN is decreased. This reduced homing of DCs may also play a part in the reduced stromal cell proliferation.

The biological importance of stromal cell expansion is not entirely clear since there is currently no way to selectively inhibit their proliferation. Depletion of FRCs prior to the initiation of adaptive immunity has a profound negative impact on the adaptive immune response to influenza infection, while depletion later on during infection has little impact (20). Thus, it is still unclear what impact age-related differences in later FRC proliferation might have on the adaptive immune response during infection. It is possible that decreased numbers of FRCs and LECs at day 10 post-infection could alter the subsequent generation of immunological memory in aged mice by decreasing the entry of new T cells destined to become memory T cells into the LN late during the immune response (44). In fact, it is well established that memory CD8⁺ T cells generated in aged mice have reduced functionality and protective capacity, and stromal cells may contribute to this defect (45).

Apart from differences in expansion kinetics, we also found that some aspects of FRC functionality differed with age. FRCs produce homeostatic chemokines CCL19 and CCL21 which are important for recruitment and motility of CCR7-expressing cells in the LN

(21,22). At homeostasis, we found that both CCL19 and CCL21 were reduced in aged MLNs. We also determined that after influenza infection, young FRCs downregulated the production of CCL21, a process described by Mueller and coworkers to be important for limiting the entry of new immune cells into the LN to promote the priming of existing immune cells (37). Interestingly, this reduction in CCL21 concentration did not occur in aged LN, suggesting that aged FRCs are not as responsive as young FRCs to challenge. FRCs require signaling through LT β R to produce homeostatic chemokines (12). We did not find reduced expression of LT β R on aged FRCs at homeostasis or 3 days post-infection, suggesting that this is not the mechanism behind the reduction in homeostatic chemokine expression. It is possible that aged immune cells could have decreased expression of the heterotrimeric lymphotoxin beta, which could lead to the observed reductions, but this remains to be determined.

Reduction in homeostatic chemokine concentration correlates with reduced homing of young naive T cells into the aged LN. Since fewer naive T cells are entering aged LN, this could dramatically diminish the adaptive immune response to influenza infection or delay the kinetics of the response. Thinning HEV morphology, which we have reported in aged MLNs, suggests that changes in HEVs may also contribute to diminished homing of T cells into aged LN. DCs are important for HEV maintenance and functionality (46) but we did not find a reduction in DC number in aged LN, ruling them out as the mediator of the altered HEV structure. The cuboidal shape of HEVs is also partly attributed to the presence of naive lymphocytes (17). Aging dramatically reduces the numbers of naive T cells in the periphery, this could contribute to the thin HEV morphology in aged LNs (4). Additionally, it is well established that the vasculature stiffens with age due to the accumulation of arteriosclerotic plaques and altered extracellular matrix proteins (47). These changes may also impact HEV morphology. HEV changes are not limited to mouse aging models since it has been reported that aged humans have reduced numbers of HEVs in LN (48).

A hallmark of the aged immune system is disruption of secondary lymphoid organ architectural organization. Along with disruption of HEV morphology, LN display characteristic blending of B-cell follicle and T-cell paracortex in both humans and mice (48,49). Along with B-cell follicle resident follicular DCs (FDCs), FRCs are also critical for maintenance of B-cell follicular organization and homeostasis (34). When FRCs are depleted from LN, the distinction between the B-cell follicle and T-cell zones is lost, recapitulating the morphological defects of aging (34). Our results suggest that FRCs may contribute to the morphologic disruption found in aged LN, in part due to altered localization, not reduction of numbers. We found that after influenza infection, FRCs in aged LN extend into the B-cell follicle, unlike young LN where FRCs stop abruptly before the B-cell follicle. Another factor which contributes to follicular disorganization of aged LN are changes in FDCs. At homeostasis, FDC morphology is relatively maintained in aged LN, but after antigenic challenge, their structure becomes disrupted (32,33). The concentration of FDC-produced chemokine CXCL13 is not different at homeostasis in young and aged LN but after West Nile infection aged LNs have reduced concentrations (11). These changes in FDCs may also be a factor in the disorganization of aged LN B-cell follicles.

Unlike B-cell follicles, we found that morphologically the T-cell zone FRC network remains intact with age and during influenza infection. There were no differences in FRC network lengths, the number of FRC segments, or the number of FRC branch points, indicating that the FRC "highway" within the LN remains intact with aging. In addition, similar to previous reports, we found that after infection, the density of the FRC network decreases in both young

and aged LNs, which may be in part due to relaxation of the FRC network tension (35).

FRCs are also known to control the proliferation of activated T cells via NO production (27) and an age-related increase in production of NO could be one mechanism responsible for reduced T-cell responses in aged LNs. Interestingly, our results showed that is most likely not the case, with young and aged FRCs having similar inhibitory capacity and production of NO. *In vivo* studies linking FRCs to T-cell proliferation suppression have used models of self-reactivity, but whether or not this mechanism is important during viral infections remains to be determined. We determined that after influenza infection, FRCs in young and aged MLNs upregulate expression of NOS2, correlating with our results showing preserved suppressive functionality. These data also present the possible role of FRC-mediated T-cell proliferation control during influenza infection. Why young influenza-specific T cells have delayed proliferation in aged LN is likely the result of numerous factors, including the reduced migration of DCs from the lungs to draining LN as reported here, and age-related accumulation of regulatory T cells (50).

In contrast to their ability to inhibit T-cell responses, FRCs also produce important factors contributing to T-cell survival including IL-7 and CCL19 (23). Our results suggest that aged FRCs maintain their ability to support the survival of naive T cells *in vitro*. These data align with published studies showing that both LN IL-7 protein and mRNA transcript are maintained with age (31,43).

Overall, these data expand our understanding of the aged immune system and elucidate the possible relationship of age-related changes in stromal cells to alterations in adaptive immune function. Our results presented here suggest that age-related changes in LN stromal cells may have the largest impact on the initiation of the immune response to influenza infection, and maybe a factor contributing to delayed T-cell responses to this virus. Of equal importance, we also have developed an appropriate protocol for further examination of how aging impacts the function of LN stromal cells. This is just the beginning to enhance our understanding of the impact of stromal cells on the aged immune response.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None reported.

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