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Attrition of T Cell Zone Fibroblastic Reticular Cell Number and Function in Aged Spleens

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ABSTRACT

Aging has a profound impact on multiple facets of the immune system, culminating in aberrant functionality. The architectural disorganization of splenic white pulp is a hallmark of the aging spleen, yet the factors underlying these structural changes are unclear. Fibroblastic reticular cells comprise one stromal cell subset in the spleen that is important for maintenance of architectural organization, yet it remains to be determined how aging impacts these cells. In this study, we sought to determine how aging impacts splenic T cell zone reticular cell (TRC) numbers, morphology, and function. Using a mouse model of aging, we found that aged naive spleens have fewer TRCs than young spleens. This reduction in TRC number correlated with reduced CCL19 and CCL21 concentrations in aged spleens, which may contribute to impaired homing of T cells. CCL21 in both young and aged spleens localized with TRCs. Aged TRCs extended marginally into B cell follicles and may contribute to the blending of the T cell zone and B cell follicles in aged spleens. The described age-related changes in TRC number and function may be an underlying factor contributing to impaired immune system function with age.


INTRODUCTION

Organization is a frequently overlooked component of an effective immune response (1). The likelihood of low-frequency, Ag-specific T cells interacting with the APC displaying their cognate Ag is amplified by multiple layers of organization, including secondary lymphoid organs (2–4). The spleen is one such organ located in the upper right portion of the abdomen (5). The spleen is attached to the vasculature (6) and is an important defense against bloodborne pathogens like encapsulated bacteria (7, 8). Blood enters into the spleen through blunt-ended central arterioles, which empty into the marginal sinus surrounding the splenic white pulp (6). From the marginal sinus, immune cells can use bridging channels to enter into the T cell zone (9). The T cell zone is surrounded by highly segregated B cell follicles in which germinal centers can develop and promote the production of high-affinity, class-switched Ab responses (10, 11).

Nonhematopoietic stromal cells provide the framework and directional cues to optimize the organization of immune cells in the splenic white pulp (12–14). In the spleen, there are multiple stromal cell subsets that localize to specific areas (15, 16). The splenic red pulp is populated with CXCL12-producing fibroblasts, which can attract CXCR4-expressing effector T cells and plasma cells away from the white pulp (17, 18). Red pulp fibroblasts also facilitate clearance of dying RBCs and direct blood flow (16). Blood vessels, including central arterioles, are lined with the stromal cell subset blood endothelial cells (BECs), which facilitate entry of cells and Ag into the splenic marginal zone (16). Fibroblastic reticular cells (FRCs) are the most abundant variety of stromal cell in the splenic white pulp (19). Multiple subsets of FRCs exist that localize to specific regions of the splenic white pulp and have distinct functional characteristics. FRCs can collectively be identified by their expression of the peptidoglycan podoplanin (PDPN), lack of expression of the vasculature marker CD31, and lack of
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hematic lineage maker CD45 (16, 20). Marginal zone reticular cells are a MADCAM-1–expressing FRC subset that localize to the marginal zone. These cells produce CXCL13 and may facilitate trafficking of Ag into B cell follicles (21). T cell zone reticular cells (TRCs) form a lattice-like conduit network in the bridging channels and T cell zone upon which Ag, lymphocytes, and dendritic cells traffic (9, 14, 22). TRCs produce the homeostatic chemokines CCL19 and CCL21, which bind to CCR7–expressing cells to direct them into the T cell zone (12, 23, 24). A specialized subset for FRCs called follicular dendritic cells (FDCs) are located in the B cell follicles. FDCs produce the chemokine CXCL13, which interacts with CXCR5 on B cells and T follicular helper cells to direct them to the B cell follicle (25). FDCs also trap Ag and provide cues to help B cells in the production of high-affinity Abs (26–28).

It is well established that aging leads to disorganized splenic architecture, characterized by merging of cells in the B cell follicles and T cell zone (29–33). Yet it is less clear how aging impacts the stromal cells underlying this organization (34). Although several studies have described age-related attrition of FDC function and morphology (35–38), it largely remains to be determined how aging impacts splenic TRCs. In this study, we sought to characterize how aging impacts the number, morphology, and function of splenic TRCs to further our understanding of the aging immune system.

Our results reveal that aged spleens have fewer TRCs and decreased T cell zone areas. Concomitant with the reduction of TRC number, homeostatic chemokine levels are reduced in aged spleens, correlating with impaired recruitment of young T cells. Interestingly, CCL21 largely colocalizes with TRCs and appears to extend slightly into the B cell follicles in aged but not young spleens. Reduction of TRC number and homeostatic chemokine concentrations may contribute to altered splenic architecture and impaired T cell homing found in aged spleens.

MATERIALS AND METHODS

Mice

Aged (18–20 mo) male C57BL/6J mice were obtained from the National Institute of Aging colony at Charles River Laboratories. Young male C57BL/6J mice (2–4 mo) were obtained from The Jackson Laboratory. CD45.1–F5 RAG knockout (KO) mice expressing a transgenic TCR specific for a nucleoprotein (NP) peptide from H17 influenza were received as a generous gift from Dr. L. Cauley at the University of Connecticut School of Medicine and were used in transfer experiments at 2 mo of age (39). All mice were kept under specific pathogen-free conditions in sterilized, individually ventilated, high-efficiency particulate air–filtered cages. Mice were sacrificed by CO2 asphyxiation. The Institutional Animal Care and Use Committee at UConn Health reviewed and approved all experimental procedures using animals.

ELISA

Spleens were harvested, flash frozen, and stored at −80°C. Spleens were homogenized in 1 ml of T-PER (Thermo Fisher) with 5 μM EDTA (Invitrogen) and 1× Halt protease and phosphatase inhibitor mixture (Thermo Fisher). Homogenates were centrifuged at 10,000 × g for 10 min, and supernatants were aliquoted and frozen at −80°C until analysis. CCL21 was measured using mouse CCL21/6Ckine DuoSet ELISA (R&D Systems). CCL19 was measured using mouse CCL19/MIP-3 beta DuoSet ELISA (R&D Systems). Chemokine concentrations were normalized to total protein concentration, determined using Pierce BCA Protein Assay (Thermo Fisher).

T cell transfer

CD8+ T cells were isolated from pooled spleens and lymph nodes of CD45.1–F5 RAG KO mice using MojoSort Mouse CD8 T cell Isolation Kit (BioLegend) according to the manufacturer’s instructions. Before transfer, the purity of the isolated CD8+ T cells was confirmed at 85% or above. A total of 2.3 × 10^6 CD8+ T cells in 200 μl PBS were injected via the tail vein into each mouse. Mice were sacrificed exactly 30 min after transfer for analysis.

Immunofluorescence

Spleens were fixed overnight in medium containing 0.05 M phosphate buffer, 0.1 M l-lysine (pH 7.4), 2 mg/ml NaNO3, and 10 mg/ml paraformaldehyde and then dehydrated with 30% sucrose in phosphate buffer (33). Spleens were frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek), and 20-μm sections were cut using a CM1850 Cryostat (Leica). Sections were blocked for 30 min with 2% goat serum and stained overnight at 4°C in a humidified chamber with the following primary Ab mixture: anti-PDPN clone 8.1.1 unconjugated (Origene) (1:300 dilution), anti-B220 PE-Dazzle AF594 clone RA3-6B2 (BioLegend) (1:100 dilution), anti-CD31 AF647 clone MEC13.3 (BioLegend) (1:100 dilution), and anti-CD8α BV421 clone 53.6.7 (BioLegend) (1:100 dilution) in 2% goat serum. Sections were then washed in PBS and stained for 2 h at room temperature with goat anti-hamster AF488 (Invitrogen) (1:400 dilution). For detection of CCL21, sections were blocked as described and stained overnight with biotinylated anti-CCL21 Ab (R&D) (1:120 dilution) in 2% goat serum with 0.3% Triton X-100 (Sigma-Aldrich). After PBS washes, CCL21 signal was amplified using Alexa Fluor 594 Tyramide SuperBoost Kit and streptavidin–Alexa Fluor 594 (BioLegend) (1:100 dilution). Staining, slides were mounted with Immu-Mount (Thermo Fisher) and coverslipped. Images were acquired using the Zeiss 780 Laser Scanning Microscope (air objective 20× Plan-Apochromat with numerical aperture 0.5 or water objective 10× C-Apochromat with numerical aperture 0.45; Carl Zeiss) using multichannel frame scans. The ZEN Black software (Carl Zeiss) was used for image acquisition. For full spleen images, the ZEN Black tile scan function was used to stitch individual 10× images together. PDPN, CD31, CD8α, and B220 areas were quantified by creating an isosurface of the staining of 20× images, and image processing was performed using Imaris 8.1 software (Bitplane).

Splenic stromal cell digestion/flow cytometry

To quantify the number of stromal cells in the spleen by flow cytometry, spleens were cut into 2.5-mm pieces and then enzymatically and mechanically digested as previously

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described (40). The chopped spleens were placed in 2 ml of enzyme mix composed of RPMI 1640 with 0.2 mg/ml of Collagenase P (Roche), 0.8 mg/ml Dispase II (Roche), and 0.1 mg/ml DNAse I (Sigma-Aldrich) and incubated for 20 min in a 37°C water bath, inverting every 5 min. The chopped spleens were gently pipetted up and down three times using a 1-ml pipette tip. The fragments were allowed to settle for 30 s, and the supernatant containing the cells released by digestion was collected into a tube containing 10 ml of ice-cold FACS buffer made from 2% FBS, 5 mM EDTA, and PBS and was centrifuged for 4 min at 300 × g and 4°C. Two milliliters of freshly prepared enzyme mix was added to the pellet and incubated for an additional 10 min in the 37°C water bath. The spleens were then vigorously pipetted for 30 s using a 1-ml pipette tip. Fragments were again allowed to settle for 30 s, and the digested cells in the supernatant were added to 10 ml of FACS buffer and centrifuged. Two milliliters of new enzyme mix was added to the pellet and then incubated in the 37°C water bath. Every 5 min until the spleens were completely digested (no visible aggregates), the cells were vigorously pipetted for 30 s. On average, the aged spleens took ~5–10 min longer to digest than the young spleens. RBCs were lysed for 5 min at room temperature in ACK Lysing Buffer (Thermo Fisher Scientific). After digestion, cell number and viability were quantified using the Cellometer Auto 2000 (Nexcelom Biosciences) with acridine orange and propidium iodide. Five million viable splenocytes were stained for flow cytometric quantification of splenic stromal cell populations. Cells were stained in an Ab mixture of 100 μl in FACS buffer: anti-CD45/CD32 clone 93 Fc block unconjugated (Thermo Fisher), anti-PDPN Alexa Fluor 594 clone 8.1.1 (BioLegend), anti-CD45 PerCPcy5.5 clone 30F-11 (BioLegend), anti-CD31 APC clone MEC13.3 (BioLegend), and Carboxylic Acid Succinimidyl Ester Alexa Fluor 350 (Life Technologies). Fluorescence minus one controls were created for each Ab and used to set gates for analysis. Without fixation, samples were acquired immediately on an LSRII flow cytometer (BD Biosciences). Flow cytometry data analysis was performed using FlowJo software (Tree Star).

**Statistical analysis**

Data was tested for normality using the F test. For normally distributed data, an unpaired two-tailed t test was used to determine significance. For data with unequal variances, analysis was performed using the Mann–Whitney U test. Significance was set at \( p \leq 0.05 \). Statistical analysis was performed using GraphPad Prism 6 software.

**RESULTS**

**Reduction of FRC numbers in aged naive spleens**

Despite the well-characterized disruption of splenic white pulp architecture with age, it is unclear how aging impacts the splenic stromal cell, which is critical for architectural organization. We first sought to quantify the number of stromal cells in both young and aged spleens. Using a rigorous mechanical and enzymatic digestion, stromal cells were isolated out of spleens and quantified using flow cytometry (40). No significant differences were found in either the total number of cells (Fig. 1A), CD45− stromal cells (Fig. 1C), BECs (Fig. 1E), or double-negative (CD3+PDPN−) splenic red pulp fibroblasts (double-negative cells [DNc]) (Fig. 1F) in young and aged spleens. Interestingly, aged spleens had significantly fewer FRCs (Fig. 1D) as gated in Fig. 1B. The total number of CD45+ immune cells in young and aged spleens was also not significantly different (Supplemental Fig. 1).

Although flow cytometry is a powerful tool, digestion of stromal cells from spleens is an imperfect method and may underrepresent the true number of cells present. To further validate the reduction of FRC numbers and to determine if TRCs were reduced in aged spleens, quantitative confocal microscopy was used. T cell zone FRCs were identified by their expression of PDPN (Fig. 2A, left) and their localization under CD8+ T cells in the T cell zone (Fig. 2A, right). A digital isosurface rendering of the PDPN staining was created using Imaris imaging software (Fig. 2A, middle). Quantification of the PDPN isosurfaces revealed that the area of aged splenic TRCs in the white pulp were smaller than their young counterparts (Fig. 2B), confirming flow cytometric quantification (Fig. 1D). Full spleen images (Fig. 2C) provide further support to the reduction of TRC area in aged spleens. In agreement with the reduction in PDPN area in the splenic white pulp, there was also a reduction in the area of CD8+ staining (Supplemental Fig. 2A, 2B), correlating loss of TRCs with reduced T cell zone area. Neither the area of the B cell zone (Supplemental Fig. 2A, 2C) or the area of BECs (Supplemental Fig. 2A, 2D) showed significant reduction in young compared with aged spleens. Full spleen images (Fig. 2C) provide further support to the reduction of the T cell zone in aged spleens. These results provide evidence that aged spleens have reduced numbers of TRCs but preserved numbers of other stromal cell subsets. We next sought to determine if TRC function was altered with age.

**Attrition of aged splenic TRC function**

TRC-produced homeostatic chemokines CCL19 and CCL21 are important cues directing the localization and entry of immune cells into the T cell zone of the splenic white pulp (12, 23, 24). Mice deficient in CCL19 and CCL21 have severe deficiencies in the recruitment of T cells into the spleen (41). It has been previously reported that young CD4+ T cells have reduced recruitment into aged spleens compared with young spleens after Ag challenge (29, 42). To determine the impact of aging on CD8+ T cell recruitment, we transferred young CD8+ T cells into young and aged hosts and examined homing after 30 min, a time point where exit was unlikely (7) (Fig. 3A, 3B). In agreement with other studies, we found a similar reduction in the frequency (Fig. 3C) and number (Fig. 3D) of young transferred CD8+ T cells in aged spleens when compared with young spleens. Yet it remains unclear how aging impacts the concentrations and localization of homeostatic chemokines, which are important for recruitment of T cells into the spleen. Quantification of CCL21 (Fig. 3E) and CCL19 (Fig. 3F) protein levels from homogenized spleens revealed a reduction in the concentrations of both chemokines in aged spleens compared with young. Thus, the reduced homeostatic chemokine concentrations in aged spleens may contribute to the impaired recruitment of young CD8+ T cells from the vasculature into aged spleens.

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We have previously shown that following challenge with OVA protein and alum adjuvant, aged spleens exhibit altered CCL21 localization, spreading from the T cell zone into the B cell follicles (29). Because antigenic challenge has been shown to dramatically alter homeostatic chemokine concentration and localization (43), we next sought to determine if CCL21 localization in young and aged spleens at homeostasis was equally disrupted. Because of low concentrations, CCL19 was not detectable by microscopy in young or aged spleens, even with the use of amplification reagents (data not shown). CCL21 in both young and aged spleens localized with PDPN+ TRCs (Fig. 4A, 4C). Aged TRCs appeared to extend marginally into the B cell follicles, where there was less overlap in young spleens (Fig. 4A, 4C). Of note, despite blocking, some nonspecific punctate background staining for CCL21 was found in the splenic red pulp surrounding the B cell follicles (Fig. 4B). These data suggest that aging reduces TRC-produced homeostatic chemokine concentration, which localize near TRCs and may contribute to the reduced recruitment of young T cells into aged spleens.

DISCUSSION

Aging is a multifactorial process that has profound effects on immune system function. Despite the extensive characterization of the aging immune system, many facets have yet to be rigorously cataloged and described. Several reports have implied that the environment of aged secondary lymphoid organs hinders the development of robust T cell responses (33, 42, 44, 45). We have previously reported that young OVA-specific CD4+ T cells transferred into aged hosts have delayed proliferation, reduced expansion, and impaired differentiation into T follicular helper cells compared with those transferred into young hosts following OVA immunization (29). In the aged hosts, the donor cells had impaired localization to the splenic T cell zones of the white pulp 18 h after immunization. This was in part due to altered localization of CCL21 (29). Although this report hinted at TRC dysfunction, it was not directly examined. We also found that the aged splenic environment is rich in active TGF-β and promotes the differentiation of young CD4+ T cells into regulatory T cells, which may contribute to impaired T cell and B cell immunity (33).

Studies have shown similar findings for young CD8+ T cells transferred into aged mice. Young influenza-specific clone 4 CD8+ T cells, when transferred into young or aged hosts that had been i.v. infected with influenza virus, had reduced expansion and decreased IFN-γ production in aged spleens (46). Another study found that after i.v. Listeria infection, young CD8+ T cells transferred into aged mice had reduced expansion in the spleen. This study linked poor CD8+ T cell responses to reduced CD8α dendritic cell responses and did not consider the implications of the disorganized splenic architecture or stromal cell dysfunction (44).

In this current study, we sought to directly examine how aging impacts one component of the splenic microenvironment: stromal
FIGURE 2. Smaller T cell zone FRC areas in aged spleens.

(A) Image analysis pipeline for splenic FRC quantification. Representative images from young (top) and aged (bottom) splenic white pulp. FRCs were identified by expression of PDPN (left). A digital rendering of the PDPN staining was created using Imaris isosurface function (middle). Combined image of CD8α (yellow), B220 (blue), CD31 (red), and PDPN (green) (right). Scale bar, 50 μm. (B) PDPN area quantification was accomplished by averaging the area of four different white pulp 20 x z stack images (1-μm step size, four steps) per mouse (six mice per group) (C) Longitudinal images of entire spleens from naive young (top) and aged (bottom) mice. Scale bar, 200 μm. Data are representative of n = 6 mice per group. Error bars = ±SEM. Statistical significance was determined by two-tailed t test. **p < 0.01.

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The disorganized architecture of the splenic white pulp led us to hypothesize that aging may influence FRC number and function because of their important role in orchestration of splenic architecture (12, 19). We found that aged spleens had fewer TRCs, which results in smaller TRC area in the T cell zone of the splenic white pulp. We also described how aged spleens have decreased concentrations of homeostatic chemokines CCL19 and CCL21, which is corroborated by previous studies (29). We found that CCL21 localizes near FRCs, which extend marginally into the B cell follicles of the aged splenic white pulp and may contribute to architectural disruption. CCL21 localization in aged spleens at homeostasis did not appear to be as dramatically altered after antigenic challenge as previously reported, which may correlate with more dramatic changes in architecture after challenge (29). We also found that decreased homeostatic chemokine concentrations correlated with reduced homing of young T cells into aged compared with young spleens. Along with reductions in CCL19 and CCL21 concentrations, other alterations in aged spleens, like changes to splenic arteries, may contribute to T cell homing defects and remain to be examined.

FRCs are functionally important for the organization of the splenic white pulp but are most likely only one component contributing to the age-related changes in the spleen. Changes in chemokine receptor expression on immune cells and their ability to respond to chemotactic gradients has also been observed and may add to altered architecture (33, 47–51). The mechanism behind why there are fewer FRCs in aged spleens also remains to be determined. One possible explanation may be that there are

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**FIGURE 3. Reduced homeostatic chemokines in aged spleens correlate with impaired recruitment of young T cells.**

The ability of aged and young spleens to recruit young T cells was tested using the experimental design outlined in (A). Transferred young T cells were identified in young or aged spleens by gating on lymphocytes, single cells, viable cells, CD45+ cells, and (B) CD8α+CD45.1+ cells. (C) Frequency and (D) number of young transferred CD8+ T cells in young or aged spleens. (E) CCL21 and (F) CCL19 levels were quantified in homogenized naive spleens and normalized to total protein. (B–D) Data are one representative experiment of three performed independently (n = 4–5 mice per group). (E and F) Data are pooled from two independent experiments (n = 18–20 mice per group). Error bars = ±SEM. Statistical significance was determined by two-tailed t test. *p < 0.05, ****p < 0.0001.
interruptions to important maintenance signals via lymphotoxin β receptor (52–56). Whether aging impacts lymphotoxin β receptor expression on stromal cells or lymphotoxin β expression on immune cells remains to be determined.

Apart from their role in architectural organization, recent studies have elucidated roles for FRCs in the control (57, 58), survival (59, 60), and initiation (41) of T cell responses. It remains to be determined if aging impacts any of these functional attributes of FRCs. Whether lymph node stromal cells experience similar changes to splenic stromal cells also remains unclear (38, 61–63). On a broader scale, it also remains to be determined if splenic and lymph node FRCs are of similar origin and composition. Erosion of secondary lymphoid organ stromal cell function may have far-reaching effects on multiple facets of the aged immune system, and despite the advances made in this article, we are still in the infancy of understanding how aging impacts these cells.

**DISCLOSURES**

The authors have no financial conflicts of interest.

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