Alterations in Marginal Zone Macrophages and Marginal Zone B Cells in Old Mice

Shirin Z. Birjandi, Jill A. Ippolito, Anand K. Ramadorai and Pamela L. Witte

J Immunol published online 9 February 2011
http://www.jimmunol.org/content/early/2011/02/08/jimmunol.1001271
Alterations in Marginal Zone Macrophages and Marginal Zone B Cells in Old Mice

Shirin Z. Birjandi, Jill A. Ippolito, Anand K. Ramadorai, and Pamela L. Witte

Marginal zones (MZs) are architecturally organized for clearance of and rapid response against blood-borne Ags entering the spleen. MZ macrophages (MZMs) and MZ B cells are particularly important in host defense against T-independent pathogens and may be crucial for the prevention of diseases, such as streptococcal pneumonia, that are devastating in older patients. Our objective was to determine whether there are changes in the cellular components of the MZ between old and young mice. Using immunocytochemistry and a blinded scoring system, we observed gross architectural changes in the MZs of old mice, including a reduction in the abundance of MZMs surrounding the MZ sinus as well as disruptions in positioning of mucosal addressin cell adhesion molecule 1 (MAdCAM-1)⁺ sinus lining cells and metallophilic macrophages. Loss of frequency of MZMs was corroborated by flow cytometry. A majority of old mice also showed reduced frequency of MZ B cells, which correlated with decreased abundance of MZM in individual old mice. The spleens of old mice showed less deposition of intravenously injected dextran saccharide particles within the MZ, likely because of the decreased frequency in MZMs, because SIGN-R1 expression was not reduced on MZM from old mice. The phagocytic ability of individual MZMs was examined using Staphylococcus aureus bioparticles, and no differences in phagocytosis were found between macrophages from young or old spleens. In summary, an anatomical breakdown of the MZ occurs in advanced age, and a reduction in frequency of MZM may affect the ability of the MZM compartment to clear blood-borne Ags and mount proper T-independent immune responses.

The Journal of Immunology, 2011, 186: 000–000.

More than 12.9% of the U.S. population is over the age of 65 (U.S. Census Bureau, 2009 [http://quickfacts.census.gov/qfd/states/00000.html]) and life expectancy continues to make rapid gains as a result of advancements in modern healthcare. However, older people suffer greater risks of long-term complications and are still more susceptible to diseases than young and middle-aged individuals because of the effects of a weakened immune system. One of the leading causes of death in persons aged 65 years and older is invasive pneumococcal disease (1, 2). Vaccines for the prevention of pneumococcal disease show a decline in immune protection in the elderly when compared with the young (3, 4). This finding is possibly caused by the reduced ability of the aged immune system to deal with bacteria load and mount an immune response to the T-independent (TI) Ag components that compose the vaccine. Therefore, understanding how the immune system changes with age is critical for implementing better therapies and vaccines for the prevention of age related disease.

Blood-borne Ags are detected by in vivo imaging to initially enter the spleen in relation to other immune organs (5). Blood-borne bacteria, viruses, parasites, and other Ags enter a compartmentalized area of the spleen, the marginal zone (MZ), where they are sequestered by specialized MZ macrophages (MZMs) and MZ B cells (6, 7). MZMs are highly phagocytic cells and are responsible for rapid clearance of blood-borne TI Ags and debris (8–10). MZ B cells are also well known for their ability to respond to TI Ags by rapidly generating an Ab response (11–13). Notably, the Ag components of vaccines important for the prevention of bacterial pneumonia are TI (14). Thus, examining the MZ compartment, especially MZMs and MZ B cells, in older patients may provide an explanation for increased susceptibility and decreased efficacy of vaccines for bacterial diseases.

MZMs are capable of binding TI Ags through specific cell surface receptors. Two important MZM cell surface receptors are: macrophage receptor with collagenous structure (MARCO, a scavenger receptor) and specific intracellular adhesion molecule-grabbing nonintegrin receptor 1 (SIGN-R1) (15). MARCO binds to Staphylococcus aureus (15, 16) and Escherichia coli (16); SIGN-R1 (a homolog of human DC-SIGN) (17) binds the capsular polysaccharide of Streptococcus pneumoniae and also to the polysaccharide dextran (9, 18–20). Once MZMs bind Ag, they introduce it to closely associated MZ B cells (21). MZMs and MZ B cells have a direct intercellular interaction via MARCO expressed on MZMs with an undetermined ligand on MZ B cells (22–24). MZMs and MZ B cells are positioned around the outer border of the MZ sinus facing the red pulp of the spleen (7). Positioned around the inner border of the MZ sinus are the metallophilic macrophages (MMMs), which face the white pulp of the spleen (7). The anatomic microarchitecture of the MZ can be seen as critical for the proper binding and clearance of blood-borne pathogens.

Previous studies of the lymphoid architecture of old mice focused on disruption of the white pulp areas, T cell-rich periarteriolar lymphoid sheath, and B cell follicles, when compared with young mice (25–29). Because little is known about changes in the structure and cellular components of the MZ with age, the purpose of this study was to compare this important immune
compartment in old and young mice. We determined that the splenic tissue of most old mice have an overall alteration in the microarchitecture of the MZ, including decreased frequencies of MZMs and MZ B cells and disruptions in positioning of the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expressing cells and MMMs. The reduction of MZMs was particularly striking, and in individual aged animals the reduced abundance of MZMs in old mice correlated with decreased frequency of MZ B cells. The consequence of altered MZMs in old mice was reflected in reduced binding of blood-borne dextran particles by the MZMs. However, the phagocytic function of individual MZMs was found to be unaffected by age, suggesting that a decrease in the MZM compartment rather than cell intrinsic defects may be a key factor in the decreased immune response to TI Ags reported with age.

Materials and Methods

Animals
Female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) or Charles River (Wilmington, MA) through a contract with the National Institute of Aging at 17–18 mo of age and from Harlan Laboratories at 6–8 wk of age. Upon receipt, the animals were housed at the animal research facility (Loyola University Medical Center, Maywood, IL) under specific pathogen-free conditions until being sacrificed. Old animals with any visual abnormalities such as enlarged spleens and presence of tumors were not used in these studies.

Tissue preparation
For H&E staining, spleens were fixed in 10% buffered formalin and processed using an automatic tissue processor, dehydrated through graded alcohols, cleared in xylene, and infiltrated with paraffin. Paraffin sections were sectioned on a microtome (4 µm) and paraffin sections were stained with H&E. Tissues were viewed and photographed with a Leitz Diaplan microscope (W. Nuhnbaum, McHenry, IL). For frozen sections, one third of each spleen was flash-frozen in Tissue-Tek O.C.T. freezing medium (Sakura, Torrance, CA) and stored at −80°C. Frozen spleens were cryosectioned (6–8 µm) onto Superfrost Plus microscope slides. Splenic sections were fixed in acetone for 10 min prior to immunocytochemistry or immunofluorescence staining protocols.

Immunofluorescence and confocal microscopy
Cryosections of spleen were fixed and incubated with Super Block (ScyTek Laboratories, Cache, UT) for 5 min according to manufacturer’s protocol. Sections were incubated at room temperature with primary Abs for 1 h followed by respective secondary Abs for 30–45 min. To identify MZ sinus lining cells, sections were stained with unlabelled rat anti-mouse MAdCAM-1 or unlabelled rat IgG2a followed by goat anti-rat Alexa Fluor 633 (Invitrogen, Carlsbad, CA). To reveal MMM, sections were incubated with FITC conjugated rat anti-mouse CD169 (MOMA-1; AbD Serotec, Oxford, U.K.) or with rat IgG2a FITC. To reveal MZMs, sections were stained with unconjugated Armenian hamster anti-mouse SIGN-R1 (BD Pharmingen) followed by goat anti-hamster IgG1 (Biorad, Hercules, CA). To reveal MZ and MZ sinus lining cells, sections were stained with unconjugated rat anti-mouse MAdCAM-1 or unlabeled rat IgG2a followed by goat anti-rat Alexa Fluor 633 (Invitrogen, Carlsbad, CA). To reveal MMM, sections were incubated with FITC conjugated rat anti-mouse CD169 (MOMA-1; AbD Serotec, Oxford, U.K.) or with rat IgG2a FITC. To reveal MZMs, sections were stained with unconjugated Armenian hamster anti-mouse SIGN-R1 (BD Pharmingen) followed by goat anti-hamster IgG1 (Biorad, Hercules, CA). To reveal MZ and MZ sinus lining cells, sections were stained with unconjugated rat anti-mouse MAdCAM-1 or unlabeled rat IgG2a followed by goat anti-rat Alexa Fluor 633 (Invitrogen, Carlsbad, CA).

Assessment of MAdCAM-1+ MZ sinus lining cells
MZ sinus lining cells were revealed by immunofluorescence staining of frozen spleen sections from the middle third of the spleen. In young animals, the MAdCAM-1 stain forms a thin line outlining the outer white pulp. To compare staining in spleens from aged mice, a tiled image (original magnification ×25) of a complete cross section of spleen (6–8 µm) from each follow aged animal was captured using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) and viewed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). The microscope was recorded on a Zeiss LSM 510 confocal microscope (Carl Zeiss). Using LSM image browser software and the polyline drawing and measurement tools, lines were drawn along the MAdCAM-1+ cells surrounding each white pulp area, and measurements of the total length of MAdCAM-1+ cells enveloping all white pulp areas in a single section were recorded. Disrupted lengths were then discriminated and the length of the affected area was measured by identifying areas of dispersed, noncompact MAdCAM-1+ staining. Relative proportions of the disrupted areas versus the total length of the lines covered by MAdCAM-1+ cells were calculated.

In vivo uptake of dextran by MZM
Using a protocol previously described by Ato et al. (31), 200 µl dextran-FITC (500,000 m.w. Invitrogen) was injected i.v. in the tail vein of mice (100 µg/ml) in sterile PBS. After 45 min, the mice were sacrificed and

spleens were harvested. One third of each spleen was placed in Tissue-Tek O.C.T. freezing medium for immunofluorescence analysis, and the remaining 2/3 was used for flow cytometric analysis (described below). For colocalization of MARCO with dextran-FITC positive cells, cryosections were incubated with unconjugated anti-mouse MARCO followed by donkey anti-rodent IgG Dylight 488 as described above. Slides were mounted with fluorescent mounting medium and viewed using a Zeiss LSM 510 confocal microscope.

**Phagocytosis assay using ex vivo treatment with pHrodo**

*S. aureus BioParticles*

Young and old mice were injected with dextran-FITC as described above. Spleenocytes were harvested and 4 x 10^6 cells in 1 ml/well were allowed to adhere onto four-well chamber slides (Nunc Labtek) for 1 h in 37°C incubator. Nonadherent cells were removed and pHrodo S. aureus BioParticles (pHrodo; Molecular Probes Invitrogen) were added at 100 μg/ml in HBSS (Life Technologies Invitrogen) containing 20 mM HEPES (Life Technologies Invitrogen) for 1 h in an incubator at 37°C. Cells were then viewed using a Leica TCS SP5 two-photon confocal microscope (Leica Microsystems) equipped with a heated chamber at 37°C. For each animal, dextran^+ pHrodo^+ MZMs were imaged live (original magnification ×5000). Three criteria were needed to qualify as a MZM: first, cells were dextran^+; second, cells had macrophage morphology; and third, cells were pHrodo^+. Images were made at approximately the center of the cell. Phagocytosis of pHrodo by MZMs was semiquantitated using ImageJ version 1.43u software. For each animal, individual dextran^+ pHrodo^+ MZMs were evaluated for the mean fluorescence intensity (MFI) of pHrodo or dextran. The relative size (area in arbitrary units) of each MZM analyzed was also determined using ImageJ software. The number of pHrodo^+ phagocytic compartments per representative image for individual MZM was also counted. Phagocytic compartments were also counted on Z-stack images of selected cells to verify that counts using the representative images approximated the average number of compartments per image for an entire cell. For each young mouse, 7–10 MZM images were analyzed, and 4–5 MZM images were analyzed for each old mouse. Fewer MZMs were detectable in young mice.

**Flow cytometric analysis**

Spleenocytes were dispersed in approximately half to two thirds of each spleen by gently pressing the tissue between two frosted glass slides. The dispersed cells were collected in RPMI 1640 with 10% FCS, and small clumps were allowed to settle in a conical tube and discarded. Erythrocytes were lysed with 0.8% ammonium chloride solution in PBS and incubated on ice for 4 min. For four-color analysis of MZ B cells, 1 x 10^6 cells per sample were incubated with rat anti-mouse CD23-FITC (B3B4; eBioscience), rat anti-mouse B220-PE (RA3-6B2; Southern Biotech), rat anti-mouse CD21/35-PE, and rat anti-mouse IgM-PE-Cy5.5 (B1/41; eBioscience) for 20 min on ice, per a protocol modified from Wen et al. (32). Cells were then washed, fixed in 1% paraformaldehyde, and analyzed using FACS Canto II with Tree Star FlowJo software version 7.5.5 (Becton Dickinson, Mountain View, CA). MZ B cells were identified as CD23^+ B220^+ CD21/35^high IgM^high.

For MZM characterization, spleen cells were treated with Collagenase D (Roche Diagnostics, Indianapolis, IN) for 1.5 h at 37°C, and single-cell suspensions of splenocytes were recovered after gentle filtration of the minced tissue through a fine nylon mesh. The erythrocytes were lysed, and the cells were washed and resuspended in RPMI 1640. For two-color analysis to identify MZMs, 1 x 10^6 cells per sample were pretreated on ice with rat anti-mouse CD16/32 FcR-block (FcR4G8; AbD Serotec) and subsequently incubated with either allopurinolin-Cy7 labeled rat anti-mouse CD11b (M170; eBioscience) or biotinylated CD11b (M170; eBioscience) and Armenian hamster anti-mouse SIGN-R1-Alexa Fluor 647 (22D1; eBioscience) for 20 min on ice. Streptavidin-PerCP (16904; BD Pharmingen) was used as a second-step reagent.

**Statistical analysis**

Statistics were calculated using GraphPad Prism software (version 5.01). Mann–Whitney U test, Unpaired t test, and Pearson correlation were applied where appropriate.

**Results**

**Morphologic disruption of the splenic MZ areas in old mice**

A substantial literature attests to the high degree of morphologic organization of the MZ region in young animals (7, 33, 34). In consultation with a pathologist, two morphologic criteria were selected to discriminate the degree of morphologic disruption in the MZ areas of aged spleens. Sections of H&E-stained spleens from young and old mice were scored independently by two investigators who were blinded to the identity of the specimens (Fig. 1A). The criteria used were 1) the interface distortion, defined as the boundary between the white pulp area and the inner edge of the MZ, and 2) the percent radius involvement, defined as the percentage of interface distortion protruding toward the center of the white pulp. Each criterion was given a score ranging from 0 to 4 (severe to minimal). Four MZ areas were scored for each spleen. A sum of scores for both criteria was made for each MZ area, and this sum was averaged to yield a value for each individual spleen (Fig. 1B). The MZs from young mice (2–6 mo;
Altered in the cells of the MZ region in spleens of old mice revealed by immunofluorescence staining

Because the MZ is a compartment composed of different macrophage types and B cells that are highly organized for the function of detecting and clearing Ags from the blood, our goal was to determine which cells, if any, or cellular positioning within the MZ changed in old age. Staining was included for MAdCAM-1+ MZ sinus lining cells to provide a landmark, because both MZMs and MMMs relate in position to the MZ sinus. Similar to numerous reports of spleens from young mice, MAdCAM-1+ stain (purple or red) revealed continuous, thin rings of MZ sinus lining cells surrounding white pulp areas (Fig. 2A, 2B, top panels). In contrast, the MAdCAM-1+ cells exhibited a less even, more diffused distribution, and they often appeared as multiple layers of cells oriented toward the white pulp in spleens from old mice (Fig. 2A, 2B, bottom panels). In spleen sections from old mice, ~90% of the linear portion of the MAdCAM-1+ stain appeared visually altered compared with MAdCAM-1+ stain in spleens from young mice (Fig. 2A, far right panel; n = 5 for both age groups, p = 0.0001). In spleens from young mice, MMMs (green) were closely aligned with the MAdCAM-1+ MZ sinus lining cells and formed a continuous line of cells on the follicular side of the MZ sinus (Fig. 2B, middle top panel), consistent with published reports (7). Again, in the spleens from old mice contrasted with young, the MMMs were aligned along the MZ sinus but were highly diffused into the white pulp-follicle area (Fig. 2B, middle bottom panel). The diffused distributions of MAdCAM-1+ MZ sinus lining cells and MMMs were consistently observed in old mice.

Another pronounced difference between old and young spleens was in the distribution of the MZMs, marked by expression of either MARCO or SIGN-R1. MZMs in young spleens formed continuous rings on the outer edge of the MZ sinus (Fig. 2C, left and middle upper panels), as reported by others (7). However, in spleens from many old mice, MZMs appeared to be greatly reduced and aligned in discontinuous patches along the MZ sinus (Fig. 2C, left and middle lower panels). In summary, we observed several changes in the positioning of cells specific to the MZ region, including the MAdCAM-1+ sinus lining cells, MMMs, and MZMs. We next assessed the degree of alteration in the MZM population within individual old animals, because of the critical importance of MZM in clearance of blood-borne bacteria (7, 20) and because more variability was observed between aged individuals in disruption of MZMs than was observed in MAdCAM-1+ cells and MMMs.

Reduction in MZMs in the spleens of old mice revealed by immunocytochemical staining and flow cytometry

Sections of young and old spleens were stained using Ab to MARCO (brown) to mark MZMs and Ab to MAdCAM-1 (blue) to mark the MZ sinus lining cells. Fig. 3A (top panel) shows the typical, consistent pattern observed in young spleens: the MZM population was present in a continuous line of cells adjacent to the sinus lining cells. However, the distribution of MZMs in the spleens of old mice was highly variable, ranging from a young-like array of MZMs to a negligible stain for MZMs. Many spleens from old mice had notably decreased staining for MARCO (Fig. 3A, middle panel; representative of intermediate reduction). In these spleens, the expression of MARCO was localized in patches rather than in a continuous line of cells around the MZ sinus. A semiquantitative scoring was devised to compare the MZM distribution in young versus old spleens. For each spleen, four well-oriented white pulp areas were graded blind for the relative abundance of MARCO+ MZMs present and the continuity of MARCO+ MZM positioning around the MZ sinus. Scores ranging from 1 to 3 were given for each criterion graded (worst to best; Fig. 3A shows representative tissue sections for each score). An average of scores is shown for individual animals (Fig. 3B). Expression of MARCO+ MZMs showed a statistically significant decrease in old tissue compared with young tissue (n = 13 young, n = 15 old, p = 0.0012). In some animals, serial sections were stained for MARCO and SIGN-R1 by immunocytochemistry to confirm that reduction of MARCO expression reflected a decreased presence of MZMs. Although less intense than for MARCO, SIGN-R1 staining reflected a similar pattern of reduced abundance and patchy stain (data not shown; example of immunofluorescent cos- tain is shown in Fig. 2C).

To further explore the interpretation that MZMs were reduced in old mice, MZMs were quantified using flow cytometric analysis. Many reports agree that MZMs express MARCO and SIGN-R1 (9, 15, 18). Although anti-MARCO Ab worked well in immunocytochemistry, anti-SIGN-R1 staining was easier to distinguish in flow cytometry. MZMs comprise a minor subset of dispersed spleen cells; therefore, another marker was used to aid in gating the population. Published reports indicate that MZMs lack expression of the typical macrophage marker F4/80 (9) and express negligible CD11b (35). Using flow cytometric analysis, we found that the SIGN-R1+ population was consistently identified within a population of large cells that expressed very low levels of CD11b (Fig. 4A). The frequency of SIGN-R1+ MZMs of total splenocytes was then determined for individual young versus old animals. Fig. 4B shows that there was a significant decrease in the average frequency of MZMs in old mice when compared with young mice (n = 14 young mice, n = 15 old mice, and p = 0.0163). Although 60% of the old mice had MZM frequency less than that of all the young mice, we observed again that the frequency of MZMs in some old mice overlapped with the MZM frequency in young animals. In a smaller, independent study to ascertain absolute cell number, we found that young mice (n = 8) had 0.81 ± 0.10 × 10⁶ MZMs per spleen versus 0.29 ± 0.01 × 10⁶ MZMs per spleen in old mice (n = 4).

Interestingly, there was a significant increase in SIGN-R1 expression on MZMs from old mice (p = 0.0088; Fig. 4C). However, this increase in SIGN-R1 may be attributed to an increase in MZM size with age, because MZMs from old mice were found to have higher forward scatter values when compared with young MZMs (p = 0.0006; Fig. 4D). The results indicated a decline in abundance of and area covered by MZMs in the MZs of aged splenic tissue.

Functional differences in binding of blood-borne particles by MZMs in old mice

Because an important function of MZMs is the clearance of blood-borne Ags, we asked whether reduced binding would be a consequence of the changes in MZMs with age. Based on previous reports that dextran particles bind to SIGN-R1 and are rapidly cleared from the blood by MZMs (9, 18), experiments were......
performed using FITC-labeled dextran to mimic the capture of blood-borne Ag in vivo (9, 31). Dextran-FITC was administered i.v. into young and old mice. After 45 min, the spleens were frozen in OCT for visualization of dextran binding to cells in tissue sections. Binding of dextran-FITC to MZMs in spleens from both young and old mice was confirmed by costaining with anti-

FIGURE 2. Changes in the cellular compartments of the MZ region of young and old mice revealed by immunofluorescence. Representative images of frozen spleen sections (6–8 μm) stained for cells within various compartments of the MZ and analyzed by confocal microscopy. A. Left panels show staining for MAdCAM-1+ MZ sinus lining cells (purple) and low-power images (original magnification ×25, tiled images) of representative cross-sections of entire spleens. Middle panels show high-power (original magnification ×25) at the boundary of the white pulp (WP) and marginal zone (MZ). In spleens from young mice, MAdCAM-1+ MZ sinus lining cells formed a continuous, thin ring of cells; however in spleens from old mice, MAdCAM-1+ MZ sinus lining cells appeared as diffused or multilayered. The right panel shows that the extent of this diffused formation was quantified by measuring the relative linear portion affected in the line of MAdCAM-1+ cells in old (92.0 ± 3.3%) versus young (12.2 ± 1.4%) spleens, as detailed in Materials and Methods. Each symbol indicates an individual animal. The horizontal bars indicate the average and the SEM for each group. p = 0.0001 determined by unpaired t test; n = 5 for each age group. Filled circles indicate young mice (2–3 mo); open squares indicate old mice (18–23 mo. B, Sections were costained for MAdCAM-1+ MZ sinus lining cells (red, left panels; original magnification ×25) and MOMA+ macrophages (MMM; green, middle panels; original magnification ×25). High magnification showed that MMMs were tightly aligned with MAdCAM-1+ cells in young tissue. In contrast, MMMs appeared diffuse and infiltrated into the white pulp in old tissue. Far right panels show representative isotype controls. C, Staining for MZMs indicated by costaining for MARCO (red) and SIGN-R1 (green). MZM showed a continuous line encircling the white pulp in young animals in contrast to a patchy, discontinuous distribution in many spleens from old mice (original magnification ×25). Far right panels show isotype controls.
MARCO Ab (Fig. 5A). Spleen sections were stained with anti-MAdCAM-1 to distinguish the MZ sinus lining cells as a landmark. Confocal microscopy revealed a clear ring of bound dextran-FITC adjacent to the MAdCAM-1+ cells in the MZs of young spleens (Fig. 5B, upper panels). In contrast, deposition of dextran-FITC was less and in a patchy array in the MZ areas in old spleens, similar to tissue staining patterns for MARCO (Figs. 2, 3A, 5B, lower panels).

**FIGURE 3.** Semiquantitative assessment of MZM disruption in spleens from old mice. A, Immunocytochemistry of frozen sections (6–8 μm) stained to reveal MAdCAM-1+ MZ sinus lining cells (blue) and MARCO+ MZMs (brown). MZMs were semiquantified for the abundance of MARCO+ stain present and the continuity of MARCO+ MZM positioning around the MZ sinus (original magnification ×10 and ×25). Four separate white pulp areas were graded blind per animal and given a score from 1 to 3 (worst to best). Representatives from each grade are shown. Top panel represents a grade of 3, showing the typical abundant and continuous stain for MARCO+ MZMs surrounding the MZ sinuses. The middle panel corresponds to a grade of 2, showing moderate abundance of MARCO+ MZMs that moderately encircled the MZ sinus with patches. The lower panel corresponds to a score of 1, showing low or virtual absence of MARCO+ MZMs that, if present, were found in patches along the MZ sinus. B, Semiquantitative scoring of MZMs for the criteria above. Each symbol represents an average of the summation of the four scores for each animal. Horizontal bars indicate the average and SEM for each group. *p* = 0.0012 as determined Mann–Whitney *U* test; *n* = 13 young mice (2–3 mo, filled circles); *n* = 15 old mice (18–23 mo, open squares).

**FIGURE 4.** Quantitative assessment of the frequencies of MZMs in spleens from old versus young mice by flow cytometry. A, Identification of SIGN-R1+ cells (MZMs) in the CD11b low splenic fraction. Contours shown are representative of young spleen cells. B, Comparison of the frequencies of MZMs present in spleens from young and old mice. Each symbol represents an individual animal. Horizontal bars indicate the average and SEM for each age group. Old mice have on average an ~36% reduction in MZMs compared with young mice, *p* = 0.0163 determined by Mann–Whitney *U* test; *n* = 14 young mice (2–3 mo, filled circles); *n* = 15 old mice (18–23 mo, open squares). C, Comparison of the MFIs of SIGN-R1 of MZMs from young and old mice (*p* = 0.0088). D, Comparison of the forward scatter (relative cell size) of MZMs from young and old mice (*p* = 0.0006). Data presented in C and D are represented using the ratio of sample value over the average value of all young obtained within each experimental group. Data are expressed as mean ± SEM and are representative of four separate experiments. *n* = 10 young mice (2–3 mo); *n* = 10 old mice (19–23 mo).
Clearance of pathogens requires phagocytosis and binding to MZMs. MZMs are known for their highly phagocytic properties (10), and a decrease in functional MZMs could amplify reduced clearance caused by demised cell numbers in the aged spleen. Therefore, we next compared the phagocytic function of MZMs isolated from young and old mice. MZMs comprise 1–2% of total splenocytes, and isolating this relatively small population of splenocytes is difficult. We took advantage of the rapid in vivo binding of FITC-labeled dextran to SIGN-R1 on MZMs to visualize these cells after harvest and culture. Adherent splenocytes obtained from dextran-FITC injected mice were incubated with pHrodo to measure phagocytic ability of MZMs.

S. aureus has been shown to bind the MARCO receptor on MZMs (16). The pHrodos are pH sensitive and emit fluorescence only after phagocytosis in the acidic conditions of the endosomal and phagolysosome compartments. Live confocal microscopy was used to visualize these cells after harvest and culture. Adherent splenocytes obtained from dextran-FITC injected mice were incubated with pHrodo to measure phagocytic ability of MZMs. MZMs from old mice showed no difference in the amount of pHrodo phagocytized when compared with MZMs from young mice (Fig. 6B; n = 10 young mice, n = 9 old mice, p = 0.2689). Similarly, no differences were found between the age groups in the number of phagocytic compartments within individual cells (Fig. 6C; n = 8 young mice, n = 7 old mice, p = 0.5989). The MFI of dextran bound by young and old MZMs revealed no significant difference between age groups (Fig. 6D; n = 9 young mice, n = 9 old mice, p = 0.4256). To rule out differences in cell size of adherent MZMs in young versus old mice, which may affect the MFI, the sizes (area) of the MZMs from young and old mice were also determined, but were not significantly different between age groups (Fig. 6E; n = 10 young mice, n = 9 old mice, p = 0.1317). Notably, after imaging numerous fields from both young and old adherent splenocytes, we found that the young mice had ~40% more dextran+ pHrodo+ MZMs compared with cultures from old mice, consistent with the flow cytometry and immunocytochemical staining showing that the frequency of MZMs declined with age. However, the MZMs remaining in aged spleens appeared to have normal phagocytic function.

Decrease in the frequency of MZ B cells in old mice correlates with the decrease in MZM

It is well established that MZMs directly interact with MZ B cells via the scavenger receptor MARCO located on the surface of MZMs with an undetermined cell surface ligand on MZ B cells (22–24). Nolte et al. (36) found that in young adult mice, loss of the B cell population corresponded with loss of MZMs. Furthermore, Frasca et al. (37) reported diminished frequency of MZ B cells in old mice. Therefore, we predicted that spleens from old mice with decreased MZMs may also show a decline of the MZ B cell population in the spleen. For each animal, two thirds of the spleen was prepared for flow cytometric analysis in which both MZM and MZ B cell frequencies were determined. The remaining

FIGURE 5. Comparison of the ability of MZs of old versus young mice to clear blood-borne dextran particles. Confocal images of frozen splenic sections harvested 45 min after dextran-FITC (green) was given i.v. As reported by Ato et al. (31) for young mice, dextran binds to MZMs, appearing as a continuous ring on the outer edge of the MZ sinus. A, Colocalization of dextran on MARCO+ MZM (red) is observed in yellow after staining dextran positive tissue from a representative young mouse and old mouse with young-like display of MZM. B, Typical deposition, represented by two of six young (top panels) and two of six old (bottom panels) mice. In the spleens of most old mice, uptake of dextran appeared in discontinuous patches along the outer edge of the MZ sinus. MAdCAM-1+ MZ sinus lining cells (purple) are stained as a landmark. Young (2–3 mo) and old (18–23 mo) female BALB/c mice. C, Spleens from control, PBS-injected mice showing absence of green deposition around the MAdCAM-1+ MZ sinus. Flow cytometry was used to confirm the frequency of MZMs for each spleen (four of six old spleens had MZM frequency less than all the young spleens).
one third of the spleen was frozen for immunocytochemistry staining to assess the MZM qualities described in Fig. 3. Flow cytometry gating on MZ B cells was adapted from Won et al. (32) (Fig. 7B; spleen cells were initially gated on the B220+, CD23+ population followed by gating on the CD21/35high and IgMhigh fraction). Fig. 7A shows that the analysis confirmed a significant reduction in average frequency of MZ B cells in old versus young mice (n = 15 young mice, n = 17 old mice, p = 0.0002). Although the average reduction was statistically significant, a wide variation was again apparent in individual old mice, with 65% of the old mice below that of all the young mice. Some spleens showed extremely reduced MZ B cell frequencies equivalent to the midrange of the young group. In an independent study to determine absolute cell number per spleen, we describe age-related changes in the overall architecture of the MZ compartment and specific reductions in MZMs and MZ B cells. The MZs of young adult mice have a characteristic, uniform, dense appearance around the white pulp and follicles. In contrast, the MZs of old mice have a statistically significant cellular disruption when compared with young mice. Alterations to the MZ included 1) reduction in abundance of MZMs, but no differences in the phagocytic function of individual MZM, 2) reduction in the frequency of MZ B cells that correlated with the reduction observed with MZMs, and 3) unusual staining patterns for MAdCAM-1 sinus lining cells and MMMs that may indicate positional changes.

Discussion

The MZ of the spleen is well known to be the site where blood-borne Ags first enter the spleen at the MZ sinus. In this study, we describe age-related changes in the overall architecture of the MZ compartment and specific reductions in MZMs and MZ B cells. Alterations to the MZ included 1) reduction in abundance of MZMs, but no differences in the phagocytic function of individual MZM, 2) reduction in the frequency of MZ B cells that correlated with the reduction observed with MZMs, and 3) unusual staining patterns for MAdCAM-1 sinus lining cells and MMMs that may indicate positional changes.

An age-related decrease in MZMs may have consequences on the immunity in aged individuals. If the spleen architecture in humans suffers a similar decline with advanced age, one conse-
FIGURE 7. Comparison of frequencies of MZ B cells in young and old mice and correlation with abundance of MZM. A. Frequency of MZ B cells within the B220^+ population. Symbols represent individual mice, and the horizontal bars indicate the average and SEM for each age group. On average, old mice have ~40% decrease in the frequency of MZ B cells compared with young mice. p = 0.0002, determined by Mann–Whitney U test (n = 15 young and 17 old mice). B. The MZ B cell population was determined by gating on the B220^+CD23^+ fraction followed by gating on the CD21/35^{high}/IgM^{high} fraction. C. Pearson correlation analysis was performed on 25 spleens from both age groups shown in A that were also sectioned, stained with anti-MARCO, and scored for MZM criteria. r = 0.6429; p = 0.0003. D. Pearson correlation analysis was performed in a separate experiment to compare the frequency of MZ B cells versus the frequency of MZMs assessed by flow cytometry; r = 0.5857; p = 0.0278 on 14 additional spleens (not shown in panel A) from both age groups. Symbols in A, C, and D represent young mice (2–3 mo, filled circles) or old mice (18–23 mo, open squares). E. Representative distribution of MZ B cells 5 min after in vivo injection with anti-CD21/PE Abs (30). Left panel, Young female BALB/c mice (2–3 mo); right panel, old female BALB/c mice (18–23 mo).

quence may be less efficient clearing and response to bacteria, as has been reported in patients with asplenia or after splenectomy (38). For example, pneumonia caused by S. pneumoniae is one of the leading causes of mortality in advanced age (1, 2). SIGN-R1 [the murine homolog of human DC-SIGN (17)] on MZM binds the capsular polysaccharide of S. pneumoniae (19, 20). It is possible that a decrease in the SIGN-R1^{+} MZM pool may be a factor for increased susceptibility to S. pneumoniae reported in older patients. To determine whether reduced frequency of MZMs affected Ag clearance in the MZ, fluorescently labeled polysaccharide dextran, which binds SIGN-R1 (9, 18), was administered via the tail vein into young and old mice. On tissue sections, less dextran was observed in the MZs of most old mice compared with young mice (Fig. 5B). When we examined the phagocytic function of individual MZMs, we found no differences between young and old mice, implying that it is the reduction in the MZM population rather than loss of cell function with age that may contribute to the age-related risk for certain pathogenic infections.

Little is known about the homeostasis of the MZM compartment in the adult. Postnatally, maturation of the MZ compartment, including establishment of the MZM layer, is delayed compared with other lymphoid areas of the spleen (39, 40). This delay is thought to be the underlying cause for increased susceptibility of infants to pneumococcal disease (2, 41). Thus, both the very young and the old share two elements: compromised anatomy of the MZ and higher risk for bacterial and viral diseases. In advanced age, three possible mechanisms can contribute to the reductions in MZMs: decreased MZ B cells, decreased chemotactic factors important for MZM homing to the MZ, or decreased MZM turnover. Evidence in this study supports the first mechanism, because decreases in MZ B cells in old mice, when present, correlate with decreased abundance of MZM. Similar correlations have been noted in other studies demonstrating that gradual loss of B cells in young mice with a conditional knockout of CD79 led to subsequent loss of MZMs (36). In addition, a report by Kraal et al. (42) observed that recovery of MZM correlated with full restoration of the B cell population in the MZs of mice after in vivo liposome treatment that led to depletion of all splenic macrophages and most MZ B cells. It is also possible that the disruptions in the microarchitecture of the spleen in old tissue are caused by decreases in the chemotactic factors important for the proper homing of MZMs to the MZ. CCL21 is a chemokine secreted by gp38^+ stromal cells that is largely responsible for MZM homing to the MZ (31). Preliminary work from our laboratory found no differences in total splenic expression of CCL21 mRNA in old versus young mice; however, examination of CCL21 using anti-CCL21 Ab on splenic sections revealed reduced chemokine present along the outer border of the MZ in old tissue compared with young tissue (data not shown). Finally, it is possible that MZMs may turnover slowly, die, and not be replaced. Kraal et al. (42) showed that new MZMs will replenish a macrophage-depleted spleen by 30 d, implying that MZM precursors exist in young animals. Therefore, it is possible that with advanced age, there is a decrease in MZM replenishment, precursors, or both. It is also conceivable that a lifetime of recurring exposure and uptake of viruses and bacteria causes repeated production of proinflammatory cytokines and chemokines, leading to overall damage to the microenvironment and decrease in immune cells such as

Downloaded from http://www.jimmunol.org/ by guest on July 25, 2017
MZMs. For example, Khanna et al. (43) recently found that TNF-α is responsible for the decline in MZMs that they observed during response to heat-killed *Listeria monocytogenes*.

The decrease in MZ B cell frequency in old mice is puzzling because an age-related increase toward maintenance of existing MZ B cells has been predicted by Allman and Miller (44). Moreover, certain conditions in old mice are more aligned with MZ B cell development than with follicular B cell development, such as low levels of E2A and IL-7 (12, 44–51). A shift toward expanded MZ B cell differentiation at the expense of follicular B cell production with age is an attractive hypothesis, and increased MZ B cell numbers have been reported in aged B10.D2 mice (52). However, our findings confirm a report that that MZ B cells are decreased in most old mice (37). Furthermore, it may be possible that MZ B cells are being produced at expanded levels in the old mice; however, because of the collapse in the MZ microarchitecture, the newly formed MZ B cells might not home effectively to the aged MZ compartment. In earlier work, young MZ B cells transferred to old mice appeared compromised in homing to the spleen (27). Another consideration is suggested by recent studies showing that MZ B cells are lost during chronic inflammation (53, 54). Traggiai et al. (53) reported changes in B-lineage subpopulations that appear similar to those reported in old mice (55, 56). Chronic inflammation has been viewed by many investigators as part of immunosenescence (57, 58), raising the possibility that increased inflammation with age promotes gradual loss of MZ B cells and perhaps subsequent decline of MZMs.

The MZs in old mice also showed alterations in the MadCAM-1+ MZ sinus lining cells, which are part of the reticular family of cells (40, 59). Moreover, these cells act as a polarized barrier to overt penetration of Ag from the MZ sinus into the follicles, whereas Ag readily moves from the sinus outward toward the MZM and MZ B cell layer (30, 60). In aged spleens, we found that the expression of MadCAM-1 was diffused into the white pulp and less organized in old mice compared with young mice (MMMs were found to be similarly disrupted). Strikingly similar morphologic disruptions in the MadCAM-1+ sinus lining cells and MMMs were reported by Girkontaite et al. (61) in sphingosine-1-phosphate receptor 3 (SIP3) deficient mice, leading to the conclusion that SIP3 is necessary for the positioning of MadCAM-1+ sinus lining cells and MMMs along the continuity of the MZ sinus (61). SIP has also been implicated in the induction of proteins found in junctional complexes, and at least one receptor (SIP1) was found on the surface of reticular stromal cells, as well as endothelial cells, in lymph nodes (62). Recently, Kataki et al. (40) provided evidence that the MadCAM-1+ sinus lining cells derive from a type of reticular stromal cell that requires constant LTßR stimulation for survival and production of CXCL13. These observations, with the previous finding that MZ sinus lining cells of aged spleens had less CXCL13 (27), could indicate a problem in LTßR stimulation. It is possible that either S1P and its receptors or LT/LTßR provide a link between reduced MZM-MZ B cells and disposition of MadCAM-1+ cells.

Understanding the alterations that occur with age in lymphoid tissues such as the spleen is important for developing more efficient therapies for preventing diseases, such as bacterial pneumonia, that have shown to be highly detrimental in the elderly (1, 2). The cells that contribute to the framework of the MZ of the spleen are largely responsible for initiating an effective immune response to *S. pneumoniae* and other TI Ags. In most old mice, MZs appeared disrupted by reduction or positional changes in the cellular components that would be expected to provide a front-line defense to pathogens. If viable markers could be found to allow predeter-

### Acknowledgments

We thank Dr. Sherri Yong (Department of Pathology, Loyola University Chicago Medical Center) for assistance with tissue analysis, Patricia Simms and the Loyola FACS Core Facility for assistance with flow cytometry, Dr. James Sinacore for statistical help, Dr. Seth Robia and Daniel Blackwell for assistance with phagocytosis imaging, and Dr. Heather Minges Wols and Dr. Phong Le for discussions.

### Disclosures

The authors have no financial conflicts of interest.

### References


