Evidence for Renewal and Reconstitution of Marginal Zone Macrophages in Young and Aged Mice

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LOYOLA UNIVERSITY CHICAGO

EVIDENCE FOR RENEWAL AND RECONSTITUTION OF MARGINAL ZONE MACROPHAGES IN YOUNG AND AGED MICE

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN CELL BIOLOGY,
NEUROBIOLOGY, AND ANATOMY

BY
ERIKA FERRO BAHAMON
CHICAGO ILLINOIS
MAY 2013
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER I: LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER II: SPECIFIC AIMS</td>
<td>11</td>
</tr>
<tr>
<td>CHAPTER III: METHODS</td>
<td>12</td>
</tr>
<tr>
<td>Mice</td>
<td>12</td>
</tr>
<tr>
<td>BrdU Treatment</td>
<td>12</td>
</tr>
<tr>
<td>IHC- Tissue Preparation</td>
<td>13</td>
</tr>
<tr>
<td>IHC- Antibody Staining</td>
<td>13</td>
</tr>
<tr>
<td>IHC- Photo Analysis</td>
<td>14</td>
</tr>
<tr>
<td>Flow Cytometry- Staining</td>
<td>14</td>
</tr>
<tr>
<td>Adoptive Transfer</td>
<td>18</td>
</tr>
<tr>
<td>CHAPTER IV: AIM 1 RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>CHAPTER V: AIM 2 RESULTS</td>
<td>40</td>
</tr>
<tr>
<td>CHAPTER VI: DISCUSSION</td>
<td>48</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>56</td>
</tr>
<tr>
<td>VITA</td>
<td>62</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. List of antibodies used in this thesis.</td>
<td>16</td>
</tr>
<tr>
<td>2. List of juvenile and young adult mice used to….</td>
<td>26</td>
</tr>
<tr>
<td>3. Frequency of BrdU+ donor MZM in recipient young mice.</td>
<td>44</td>
</tr>
<tr>
<td>4. Percentage of BrdU+ donor MZM in recipient old mice.</td>
<td>47</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cellular structure of the splenic marginal zone.</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Protocol of preparation of splenocytes…</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Adoptive transfer protocol.</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>Comparison of previous and current staining…</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Criteria used to validate successful staining method.</td>
<td>22</td>
</tr>
<tr>
<td>6AB</td>
<td>Two examples of collages of 100X magnification…</td>
<td>24</td>
</tr>
<tr>
<td>6C</td>
<td>Representative example of a double positive…</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>Previous flow cytometry gating technique used…</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>Flow cytometry gating strategy to enrich MZM.</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>Flow cytometry gating strategy to distinguish BrdU+…</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>Representation of BrdU-labeled bone marrow.</td>
<td>32</td>
</tr>
<tr>
<td>11</td>
<td>Comparison of young and old SIGNR1+BrdU+…</td>
<td>34</td>
</tr>
<tr>
<td>12</td>
<td>Flow cytometry comparison of BrdU+MZM in young…</td>
<td>35</td>
</tr>
<tr>
<td>13</td>
<td>Comparison of uptake of BrdU by MZM of young…</td>
<td>37</td>
</tr>
<tr>
<td>14</td>
<td>Comparison of young and old SIGNR1+ BrdU+ mice…</td>
<td>38</td>
</tr>
<tr>
<td>15</td>
<td>Pooled data from both drinking water and i.p…</td>
<td>39</td>
</tr>
<tr>
<td>16</td>
<td>Flow cytometry profiles of young recipient and control…</td>
<td>43</td>
</tr>
<tr>
<td>17</td>
<td>Flow cytometry profile of an old recipient and control…</td>
<td>46</td>
</tr>
<tr>
<td>18</td>
<td>Comparison of the efficiency of transfer of donor MZM…</td>
<td>47</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>FO B</td>
<td>Follicular B cell</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LT-α</td>
<td>Lymphotoxin-α</td>
</tr>
<tr>
<td>MARCO</td>
<td>MAcrophage Receptor with COllagenous domain</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal Zone</td>
</tr>
<tr>
<td>MZ B cell</td>
<td>Marginal Zone B cell</td>
</tr>
<tr>
<td>MZM</td>
<td>Marginal Zone Macrophage</td>
</tr>
<tr>
<td>RPM</td>
<td>Red Pulp Macrophage</td>
</tr>
<tr>
<td>SIGN-R1</td>
<td>Specific Intracellular Adhesion Molecule-3 Grabbing Nonintegrin Homolog-Related-1</td>
</tr>
</tbody>
</table>
Aging of the adaptive immune system in the elderly human population

According to current estimates, about 13% of the US is over the age of 65 [1]. Aging in humans is associated with a noticeable decline in health, particularly immune health [2]. Aging has negative effects on both innate and adaptive immunity resulting in a chronic inflammatory state referred to as “inflammaging”, that refers to constitutive levels of elevated inflammatory cytokines; [2][3]. The elderly also have a delayed and more difficult time mounting an immune response [3].

The effects of aging on adaptive immunity include involution of the thymus, resulting in a decrease in production of naïve T cells and expansion of longer lived T memory cells, through a decrease in IL-2 [3]. Decrease in the naïve to memory T cell ratio is thought to constrict the available T cell repertoire and make the aged animal more susceptible to infection[3]. Although the total number of circulating mature B cells stays the same, there is a decline in B cell production that may also result in decreased adaptive immunity [4].

Elements of the innate immune system important to my thesis include monocytes and macrophages, which are particularly important for clearing T-independent pathogens.
Macrophages in aged mice have also been shown to have decreased MHC class II expression, which is important for antigen presentation to adaptive immune cells such as cytotoxic T cells [5, 6]. There is a decrease of ability of wound-healing resident skin macrophages and anti-angiogenic ability of ocular macrophages [7, 8]. Macrophages that primary occupy a tissue and serve a specific purpose are called resident macrophages. This thesis focuses on a particular resident macrophage that is reduced with age, the marginal zone macrophage (MzM)

**The age-associated decline in immune protection to Streptococcus pneumoniae**

The effects of aging on the innate immune system become apparent when the elderly are exposed to *Streptococcus pneumoniae*, a gram-positive bacterial pathogen that elicits a T-independent humoral response from B cells. *S. pneumoniae* causes community-acquired bacterial pneumonia, a leading cause of bacterial meningitis, together which result in 175,000 hospitalizations and over 7000 deaths every year in the United States [9-11]. According to the Center for Disease Control, the very young and elderly are at increased risk of infection from *S. pneumoniae*. Current prevention methods for the elderly include a pneumococcal polysaccharide vaccine, but this has been shown to have only about 50-70% effectiveness[12]. Because *S. pneumoniae* is T-independent, another alternative being utilized in children is a pneumococcal vaccine that is conjugated with immunogenic carrier proteins that elicit a T cell response [12]. A successful treatment in the future will include a thorough understanding of the mechanism of MzM with their relationship to MZ B cells in clearing *S. pneumoniae*. 
The significance of the spleen and the marginal zone

In the case of systemic or blood-borne pneumococcal infection, one primary defense cell is the marginal zone macrophages (MZM), which resides in the marginal zone (MZ) of the spleen. In additional to its role as a secondary lymphoid organ, the spleen is the body’s largest filter of blood [13, 14]. In this way, the spleen is important for capturing blood-borne pathogens [15]. Splenectomized patients, individuals with hyposplenic function, and the elderly are particularly susceptible to blood-borne bacterial infection [16]. The area specific for recognition of *S. pneumonia* and other T1-independent pathogens is the marginal zone, which is the boundary between the red pulp and white pulp. The red pulp consists of red pulp macrophages (RPM) that filter blood and recycle iron. Red pulp cords also contain plasmablasts and plasma cells. The white pulp consists of T cell compartments (periarteriolar lymphoid sheaths) and B cell compartments (follicles) and plays a large role in the adaptive immune system [17]. The lymphocytes in the white pulp enter from the circulation through MAdCAM-1+ fenestrated sinus-lining cells in the MZ [18]. In the MZ sinus, blood flow slows down as arterioles change into venous channels. This allows for efficient capturing of blood-borne antigen by cells in the MZ [13]. The MZ includes MZB cells, dendritic cells, T cells, marginal zone metallophilic macrophages, and MZM (Fig. 1)[19-22]. The MZ B cells have cell-surface IgM and can generate a rapid antibody response to T-independent pathogens [23]. The MZ B cells also are important in the development and maintenance of the MZ, which is absent at birth and begins around the fifth day in mice [21, 24-27].
The proper architecture of the MZ is important and cells normally present in the MZ are co-dependent on each other. Nolte et al. showed that when all B cells are deficient via a B cell receptor knockout, the MZ fails to establish [24]. Expression of CD19 is needed to form MZ B cells, but not follicular B cells [28]. In these KO mice, MZ B cells and MZM are absent [28]. Tumor necrosis factor family members such as lymphotxin-α (LTα) and NF-κB regulate the development of the MZ, and NF-κB deficient mice do not form an MZ but still develop MZM [29-31]. LT-α is important in setting up the architecture of the MZ, as shown through LT-α deficient mice which lack a marginal sinus [30]. These findings help us to understand the importance of the correct structuring of the MZ, starting at a young age and maintenance throughout life.
Figure 1. Cellular composition of the splenic marginal zone. This picture illustrates the many cell types in the marginal zone and their location respective to one another.
Marginal Zone Macrophages

MZM are the sentinels in the body which combat bacterial pneumococcal infection [32]. They are found as a thick layer in the MZ on the side of the red pulp. MZM are identified by antibodies against “specific intracellular adhesion molecule-grabbing nonintegrin” (SIGN-R1) and “macrophage receptor with collagenase structure” (MARCO) [23, 33]. MZM establish residence by migrating out of the circulation in response to chemokines CCL19 and CCL21 expressed by endothelial cells in the MZ [34]. Lack of functional CCL19 and CCL21 results in absence of MZM at the MZ [34]. MZM are distinguished from RPM in that MZM are larger in size, display Fc and C3 complement receptors, and lack MHC Class II [15]. MZM are unique from other resident macrophages in that MZM are larger in size, display Fc and C3 complement receptors, and lack MHC Class II [16].

Loss of MZM has negative effects in the immune system, as shown in mice that have depleted MZM by using clodronate liposomes, which specifically kill MZM [35]. In the absence of MZM, the spleen is unable to clear S. pneumoniae from the circulation and this causes the animal to become more susceptible to bacterial infection [32, 36]. In addition to clearance of S. pneumonia, MZM are also important for clearing *Leishmania donovani*, Lymphocytic Choriomeningitic Virus, and *Listeria monocytogenes* [35, 37, 38]. Negative effects also include decreased cell trafficking of apoptotic cells by MZM [39]. This loss of MZM can also lead to autoimmunity triggered by increase of apoptotic cells, such as an acceleration of systemic lupus in mice [39]. In mice, advanced age has recently been shown to have an effect on MZM and MZB. Birjandi *et al.* showed that the tissue in the spleens of old mice a deteriorated MZ as well as a reduction of MZM and
MZB, which may explain the susceptibility of aged mice and humans to pneumococcal infection [40]. In order to fully understand the role of MZM in fighting infection, the following studies have been done on the antigen receptor SIGN-R1 as well as the scavenger receptor MARCO expressed on MZM.

**Macrophage receptor with collagenase structure (MARCO)**

MARCO is a scavenger receptor expressed in mice on the cell surface of MZM, medullary cord macrophages in lymph nodes, peritoneal macrophages, and liver macrophages following infection [41, 42]. MARCO specifically binds prevalent bacteria Staphylococcus aureus and Escherichia coli, but not S. pneumoniae [43]. In an ontogeny study, Chen *et al.* showed that MARCO is expressed before SIGN-R1 following birth [41]. Interestingly, in MARCO knockout mice, there is slow appearance of SIGN-R1 and the MZ does not form properly [41]. This suggests an important role of MARCO by directing the establishment of the MZ after birth, as well as the expression of SIGN-R1. MZM also have a direct relationship with MZB cells via MARCO through an undetermined ligand on MZB cells which, along with SIGN-R1, may help localize the MZB cells to the MZ [43]. In this thesis, MARCO is used as an identification marker in histology to mark MZM.

**Specific intracellular adhesion molecule-grabbing nonintegrin (SIGN-R1)**

SIGN-R1 is the murine homologue to dendritic cell-specific intracellular adhesion molecule-grabbing nonintegrin (DC-SIGN) in humans [44]. In addition to expression by
MZM, SIGN-R1 is also expressed by a subset of peritoneal macrophages, liver sinusoidal endothelial cells, and medullary macrophages in the lymph nodes [44]. However, many macrophage populations do not constitutively express SIGN-R1, including alveolar and splenic red pulp macrophages [44, 45]. SIGN-R1 is the pattern recognition receptor necessary for clearance of *S. pneumoniae* [32, 46]. SIGN-R1 binds to the pathogen and internalizes it to the lysozome [44]. Koppel *et al.* reported that SIGN-R1 signals early IgM responses from MZB cells in response to bacterial infection, but a study by Moens *et al.* did not observe this [47, 48]. Other studies have shown that SIGN-R1 associates with TLR4 on the surface of the MZM to expedite innate immune responses [49]. Kang *et al.* have shown that SIGN-R1 is directly involved in a novel complement pathway where SIGN-R1 assembles C3 convertase [49]. SIGN-R1 may also have a role in facilitating lymphocyte migration through binding to ICAM2 expressed on lymphocytes [44].

Not only do SIGN-R1 knockout (KO) mice fail to clear *S. pneumoniae*, they also fail to produce an early IgM response by the MZB cells [50]. This suggests an important role of MZM with MZB in the clearance of *S. pneumoniae* from the blood. Unlike MARCO, studies involving SIGN-R1 KO mice have shown that it is not necessary for the development of the marginal zone in early development [51].

**Marginal Zone B cells**

MZB cells are specialized B cells located between the white and red pulp areas of the spleen and are unlike typical follicular B cells [20]. They express high levels of IgM and CD1d (a receptor that presents lipid antigen to NKT cells), high levels of CD21 (a
component of the complement pathway), but lack IgD and CD23 [52]. MZB cells capture antigen that is opsonized with complement via CD21 [53, 54]. They are also unique in that they can produce antibody to polysaccharides [55]. The innate immune response that produces rapid IgM is necessary for battling *S. pneumoniae* infection, which occurs within three days of infection [50, 56]. Birjandi *et al.* showed that in aged mice, MZB cell numbers are reduced, correlating with a reduction in MZM [40]. You *et al.* showed that after depletion of MZB cells, MARCO+SIGN-R1+ MZM disappeared [57]. This suggests an intimate relationship between MZM and MZB cells and may explain the reduction of clearance of *S. pneumoniae* in the elderly human population.

**Measuring Cell Turnover and Proliferation by Uptake of BrdU**

It is possible that the decreased prevalence of MZM in advanced age observed by Birjandi *et al.* is due to MZM failing to replenish themselves. There are no current studies on the turnover of MZM and if this turnover is impacted in age. In order to answer this question, 5-bromo-2-deoxyuridine (BrdU) can be used to mark a recently dividing cell. BrdU is a thymidine analog that is incorporated into DNA during the S phase of cell cycle [59]. However, unlike tritiated thymidine, it is not reutilized in DNA synthesis [60]. In order to detect uptake of BrdU into the DNA, the cells must be fixed, permeabilized, and the DNA denatured to allow antibody against BrdU to enter and attach to the exposed DNA strands [60]. BrdU has been used routinely to study proliferating cell populations, mostly to establish precursor relationships in the hematopoietic cell lineages. For
example, BrdU studies on B cells have shown that transitional (immature) and mature B cells in the spleen turnover more slowly in old mice [4].

A few studies have focused on monocyte proliferation and turnover. BrdU has been used to label monocytes in to establish the half-life of monocyte turnover in the marrow (12-24 hours in rabbits, and 17.4 hours in mice) [61-63]. Goto et al. also found that inhalation exposure to \textit{S. pneumoniae} caused an increase of monocyte turnover in the marrow and shortened migration time to the tissues [62]. This is an interesting effect that has not been tested in MZM. In fact, there are no studies on normal turnover of MZM, or studies comparing age.

BrdU has also been used as a way to label highly proliferative cell populations, such as bone marrow, and track the fates of cells transferred to host animals. However, there are limitations to using BrdU. A study that labeled transferred bone marrow stromal cells showed 5\% of labeled cells were taken up nonspecifically by macrophages [64]. Treatment with BrdU may have negative effects as well, such as causing a toxic effect when proper doses are exceeded. Mice also experience stress from repeat injections and handling, which may be reflected in a decline of B and T lymphopoeisis after 3-4 days of administration [60]. Using BrdU as a technique in this thesis, I was able to assess turnover and reconstitution of MZM in young and old mice.
CHAPTER II
SPECIFIC AIMS

Previous research in our lab has shown that with age, there is a deterioration of the MZ as well as a decrease in frequency of MZM. The cause for the decrease of MZM is unknown. The objective of this thesis is to address whether MZM, once established after birth, are maintained by turnover with newly-made cells. Cell proliferation is one measure used to indicate turnover of hematopoietic cell lineages; however, little is known about maintenance or proliferation of MZM. The specific goal of my thesis was to seek evidence for proliferation in young mice and then ask if a decrease in proliferation occurs in the MZM of aged mice. This thesis also tests if adoptive transfer of young bone marrow into old mice would replenish the lost MZM.

Aim 1: To determine if MZM are turning over in young mice, and whether there is a decrease in proliferation of MZM in aged mice.

Aim 2: To determine if MZM can be reconstituted in aged mice with an unselected bone marrow transfer from young mice.
CHAPTER III

METHODS

Mice

Female BALB/c mice between the ages of 4 weeks and 22 months were obtained from Harlan Laboratories through the National Institute of Aging (Indianapolis, IN). Mice were housed under specific pathogen-free conditions at the Animal Research Facility in Loyola University Medical Center (Maywood, IL). All mice were sacrificed by CO2 inhalation. Old animals with visible abnormalities including tumors and enlarged or abnormal spleens were not used.

Bromodeoxyuridine (BrdU) Treatment

BrdU was administered to mice in two ways: via drinking water or through i.p. injections. In experiments with mice treated with BrdU drinking water, 0.08g of BrdU (COMPANY) in 100mL of distilled autoclaved water was given. BrdU drinking water in the cages was covered with foil to prevent exposure to light and was changed every other day. Mice treated with i.p.
injections of BrdU were given only a maximum of 3 injections every 24 hours. 100 uL of BrdU was given intra-peritoneally with a 1mL syringe and 27 ½ gauge needle.

**Immunohistochemistry-Tissue Preparation**

Immediately following sacrifice, the middle third of each mouse spleen was embedded in Tissue Tek® O.C.T. freezing medium (Sakura, Torrance, CA) and flash frozen using dry ice and 95% ethanol. Spleens were stored in a -80°C freezer until time for sectioning. Spleens were cryosectioned at 8 μm onto Superfrost®/Plus slides. Three splenic sections were stained per slide.

**Immunohistochemistry- Antibody Staining**

Splenic sections were removed from the -80°C freezer and air dried for 10 minutes. A BrdU In-Situ Detection Kit by BD Pharmingen™ (cat: 51-75512L) was used for double-staining experiments. The sections were blocked with Fixation Buffer (kit) for 15 minutes. A humidity chamber was used throughout the experiment and between every step, slides were washed with 1x PBS three times for 5 minutes each. Sections were incubated with 0.3% H2O2 in 1x PBS for 10 minutes. To identify MZM, slides then were incubated with unconjugated rat anti-mouse IgG1 anti-MARCO at 0.4 mg/mL at a 1:200 dilution (ED31; BMA Biomedicals, Switzerland) diluted in Diluent Buffer (kit) for 1 hour. Secondary antibody mouse anti-rat IgG1-HRP (G11C5; cat: 3061-05, Southern Biotech) was then used for 30 minutes followed by the purple substrate Vector® VIP (Vector® VIP Substrate Kit for Peroxidase, cat: SK-4600, Burlingame, CA) for 5 minutes.

In order to identify BrdU-positive cells, sections were incubated again with hydrogen peroxide for ten minutes. Then, sections were heated in an 89°C water bath with Antigen Retrieval Solution (kit) for ten minutes, and let cool for 1 hour. The sections are incubated with mouse anti-BrdU-Biotin for two hours, followed by a 20 minute streptavidin-HRP incubation.
The color is revealed with one drop of DAB chromogen (kit) diluted into 1mL DAB buffer (kit) for 5 minutes. The counterstained used was Methyl Green (cat: H-3402, Vector Laboratories). Slides were air dried for ten minutes and covered with mounting medium (Vectamount AQ, Vector Laboratories). They were viewed and photographed using a Leitz Diaplan microscope.

**Immunohistochemistry- Photo analysis**

After immunohistochemical staining, the MZ was viewed at 100X magnification using a Leitz Diaplan microscope. An entire marginal zone picture was composed of a collage of 8-10 photographs. Each 100X magnification picture was scored for double positive MARCO+BrdU+ MZM. All MZM were counted based on a purple (Vector VIP) MARCO cell surface stain. From these MZM, double positive cells were counted if a dark (DAB) BrdU+ nucleus was observed. Frequencies were counted for each marginal zone and averaged.

**Flow cytometry- Staining**

Splenic MZM, MZB, bone marrow B cells, immature/transitional B cells, mature B cells and Red pulp macrophages (RPM) were all identified via flow cytometry analysis. After sacrifice, the spleens were processed by gently smashing between two slides. The cells were centrifuged and treated with 0.8% ammonium chloride solution to lyse red blood cells on ice for four minutes. Bone marrow was flushed with long term bone marrow culture media with a syringe and spun down. All cells were resuspended in FACS Staining Buffer (BD Pharmingen BrdU APC Flow Cytometry Kit), labeled with Trypan Blue, and counted. 1 x 106 cells per sample were pretreated with rat anti-mouse CD16/32 FcR-block (FCR4G8; AbD Serotec) and then incubated with primary antibody for 20 minutes on ice. For MZM staining, primary antibody was anti-Armenian hamster SIGN-R1 purified (0.5 mg/mL at 1:200 dilution; eBioscience) and APC-Cy7
labeled rat anti-mouse CD11b (0.2 mg/mL at 1:100 dilution; M1/70; eBioscience). The isotype control for SIGN-R1 used CD11b antibody plus Armenian hamster IgG purified (0.5 mg/mL at 1:200 dilution; eBioscience). For MZB, mature B cells and immature B cells, primary antibodies were rat anti-mouse CD23-PE-CY7 (0.2 mg/mL at 1:200 dilution; eBioscience), rat anti-mouse IgM-PE-Cy5.5 (0.2 mg/mL at 1:200 dilution; eBioscience), rat anti-mouse B220-FITC (0.1 mg/mL at 1:100 dilution; Southern Biotech), and rat anti-mouse CD21/35-PE (0.2 mg/mL at 1:200 dilution; eBioscience). RPM antibodies were F4/80-PE (0.2 mg/mL at 1:200 dilution; eBioscience). Bone marrow B lineage cells were only stained with B220-FITC. Bone marrow B cells, MZB and RPM staining did not have secondary antibodies. MZM and its isotype control were incubated for 20 minutes with anti-Armenian hamster IgG-biotin (0.5 mg/mL at 1:200 dilution; eBioscience) followed by 20 minutes of SA-PE (0.5 mg/mL at 1:200 dilution; eBioscience). Cells were fixed in Cytofix/Cytoperm (BD Pharmingen) for 30 minutes, and stored in 10% DMSO 90% HI FBS overnight in -80 °C. Antibodies used for both immunohistochemistry and flow cytometry are listed in Table 1.

The following day, cells were thawed for ten minutes then refixed with Cytofix/Cytoperm for five minutes. Cells were treated for ten minutes with DNase (BD Pharmingen) then incubated in 37 °C with anti-BrdU antibody for one hour. Cells were then washed, resuspended in FACS staining buffer (BD Pharmingen) then analyzed using flow cytometry on Canto II (Fig 2).
### Table 1

#### MACROPHAGE STAINING

<table>
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#### B CELL SUBSET STAINING

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**Table 1.** List of antibodies used. The table above shows all antibodies used in the experiments listed in this methods section. This table includes both immunohistochemistry antibodies and flow cytometry antibodies.
**Figure 2.** Protocol of preparation of splenocytes and bone marrow for flow cytometry analysis. This schematic shows the steps of isolating splenocytes and bone marrow from mice in preparation for flow cytometry analysis.
Adoptive transfer

Donor mice were treated with 3 i.p. injections of 100 µL of BrdU (BD Pharmingen) 24 hours apart to label the bone marrow. Mice were sacrificed on the fourth day and bone marrow was isolated as described above. 25-35 million bone marrow cells were suspended in 100 µL of sterile 10% phosphate-buffered saline and injected into either young or old recipient mice using a 1 mL syringe and 27 ½ gauge needle in the tail vein. Cells isolated from a donor mouse were transferred to one recipient mouse. After 7 days, mice were sacrificed and spleens were analyzed via flow cytometry (Fig. 3).

**Figure 3.** Adoptive transfer protocol. Donor mice were treated with 3 i.p. injections of BrdU. These mice were sacrificed and the bone marrow was harvested and transferred via tail vein into a recipient mouse. After 7 days, the recipient mouse was sacrificed and its spleen was harvested and SIGNR1+BrdU+ cells were analyzed with flow cytometry.
CHAPTER IV

TO DETERMINE IF MZM ARE TURNING OVER IN YOUNG MICE, AND WHETHER THERE IS A DECREASE IN PROLIFERATION OF MZM IN AGED MICE

Even in young mice, it is unknown whether MZM proliferate or are constantly replenished from proliferating precursor cells. It is possible that once the marginal zone is established at a young age, MZM are stable for life. If MZM are a static population, loss of cells without regeneration would explain the decrease of MZM in aged mice. Therefore, it was important to first resolve whether MZM do turnover (proliferate) throughout life. If true, I wanted to determine if the turnover is greatly decreased as the marginal zone cellular architecture deteriorates in old age.

Establishment of an immunohistochemistry protocol to test uptake of BrdU

The literature most often defines MZM in the context of their location in the splenic MZ and by expression of MARCO as a defining marker [41]. To begin testing for evidence of proliferation, I first approached the problem by qualitatively testing uptake of BrdU in splenic tissue sections before moving on to a more quantitative flow cytometry approach for detecting uptake of BrdU by MZM. First, I had to find the optimal way to stain and use the best substrate
color scheme. I found that the staining color combination used before in the lab (DAB-BrdU, AEC-MARCO and Nuclear Fast Red-counterstain) (Fig. 4A) was not sufficient to visualize good nuclear BrdU staining in MZM. Vector VIP (purple) gives a more defined reaction deposit that develops quickly. An optimal time and titration for the substrate was tested in order to obtain the best MARCO visualization. Anti-BrdU antibody also was titrated to obtain the best positive staining with DAB chromogen. In all immunohistochemistry experiments, Vector VIP (purple; MZM), DAB (brown; BrdU) and Methyl Green (counterstain) were used (Fig. 4B).

The purpose of next experiment was a trial to identify BrdU+ MZM. Because this has not been done before, I reasoned that (1) juvenile mice, which have a newly-established MZ region, may have proliferating MZM and (2) a longer treatment period with BrdU would maximize chances of detection. Therefore, 3 week old mice were given BrdU in drinking water continuously for 16 days. Frozen splenic sections were stained as described above. Figure 4 shows that overall BrdU labeling revealed the expected staining in the two main areas of the spleen. The white pulp showed little labeling because the mature B and T cells are long-lived [4]. The red pulp showed evidence of BrdU labeling because of the prevalence of neutrophils, a population known for rapid turnover [65] (Figure 5). Figure 5 right panel demonstrates relatively little staining in the gross MZ region; a few scatter brown nuclei were observed at this low power. However, BrdU+ MZM were observed at higher magnification, as described in the next section.
Figure 4. Comparison of previous and current staining method at 40X magnification. (A) Previously, the lab used substrate AEC (red) to stain MARCO and Nuclear Fast Red was used as a counterstain, but when combined with anti-BrdU with DAB chromogen (brown), the brown was too difficult to visualize. (B) I had to find a more optimal color combination. MARCO labeled with the purple Vector VIP, BrdU is brown DAB, and the counterstain is Methyl Green. Here it is possible to distinguish BrdU+ cells (many can be seen in the red pulp and a few in white pulp). MZM at high magnification are shown in Figure 6C. Stars indicate B cell follicles.
Figure 5. Criteria used to validate successful staining method. Vector VIP (purple) stains MZM, shown in rings encircling the white pulp. BrdU is labeled here for fourteen days with the DAB chromogen, as can be more clearly seen in the picture on the right. Scheme is validated because white pulp shows little evidence of BrdU staining, and red pulp shows great evidence, as would be expected.
Evidence of proliferation by MARCO+ MZM using immunohistochemistry in young mice

In order to semi-quantify the amount of BrdU+ MZM, I took 100X magnification photographs of the MZ region and scored MARCO+ cells if the nucleus was visible (Fig. 6A&B). The nucleus of each MARCO+ cell was judged to be BrdU+ or BrdU- yielding the frequency of BrdU+ cells out of total MARCO+ cells (Fig. 6C). Using various lengths of treatment and delivery of BrdU by i.p. injection or by in the drinking water, I found evidence of BrdU+ MARCO MZM, with a frequency as little as 18% in 3 month old mice with two i.p. injections (over 48hr) and as much as 34% in 37 day old mice with 16 days of BrdU drinking water (Table 2). The results in tissue sections provided qualitative evidence of MZM proliferation in young mice.
Figure 6 A and B. Two examples of collages of 100X magnification photos showing representative MZ. 8-13 pictures were taken along each MZ, as shown. From these individual photos, MZM were scored for double labeling with BrdU.
Figure 6 C. Representative example of a double positive MARCO+ BrdU+ and single positive MARCO+BrdU- MZM. Clear nuclei associated with MARCO stain (purple) were assessed as negative for BrdU staining. A dark nucleus associated with purple-stained cytoplasm constituted a double positive MARCO+BrdU+ cell.
Table 2. List of juvenile and young adult mice used to determine double MARCO+BrdU+ immunohistochemical staining. Mouse ages ranged from 37 days to 7 months. Both drinking water and 3 i.p. injections were tested, as well as variable length of treatment. All treatments showed evidence of double staining. The number of nucleated MZM scored per animal ranged from 96 to 416.
Flow cytometry gating strategy to identify BrdU+ MZM

A drawback of the IHC approach was the subjective judgment involved in assessing whether MARCO+ cells were BrdU positive or BrdU negative. In the next experimental approach, flow cytometry was used to quantify the frequency of BrdU+ MZM. Mice aged 3-7 months underwent a 5 day treatment with BrdU in the drinking water, and the mice were sacrificed on the sixth day. One-fourth of each spleen was frozen in OTC for cryostat sectioning, and the remaining spleen was dispersed for FACS staining. Generally, each experiment included 2-3 treated mice and 1 untreated mouse as a negative control for the anti-BrdU antibody. Femoral bone marrow also was harvested as a positive control because most bone marrow cells should be labeled within 3-5 days (see next chapter also).

For analyzing MZM, I adapted the phenotype and gating approach used previously by Birjandi et al. in our lab. SIGN-R1 is another cell surface antigen receptor indicative of MZM. SIGN-R1 is the most commonly used antibody for MZM found in the literature and has been used successfully with flow cytometry by previous members in the lab. MZM are rare in a dispersed preparation of spleen cells, composing less than 1% of total splenocytes. A gating strategy was formulated to enrich MZM within the discriminating gate. Macrophages are larger than lymphocytes, so they would fall in a higher forward scatter. The first gate, forward (FSC) vs side scatter (SSC), included lymphocytes and larger cells that had low to moderate side scatter. This gate eliminated doublet cells, dead cells, and granulocytes. The second gate included cells expressing low levels of CD11b. The CD11b low gate included SIGN-R1+ cells and a positive gate was always compared to cells stained with Armenian Hamster IgG isotype control antibody. Birjandi et al. used a broad forward scatter CD11b gate, and I attempted to narrow the gate in order to enrich SIGNR1+ cells. Figure 7 and 8 contrast my "enriched" gating with the gating
reported by Birjandi et al. Fluorescent staining of BrdU was gated from these cells and compared to spleen cells from a non-BrdU treated control mouse stained in parallel with identical antibodies (Fig. 9). A positive control in these experiments was the BrdU staining of bone marrow (Fig 10).
Figure 7. Previous flow cytometry gating technique used in the lab (Birjandi et al., 2011) to identify SIGN-R1+ MZM among dispersed spleen cells. The forward vs. side gate included all viable cells, and from this a gate of total CD11b lo was chosen. SIGN-R1+ cells are gated compared to the Armenian Hamster IgG isotype control. The following figure shows my gating strategy to enrich SIGN-R1+ MZM. Fluorochromes: CD11b-efluor 780, SIGN-R1 or Armenian Hamster IgG-PE.
Figure 8A and B. Flow cytometry gating strategy to enrich MZM. (A) A lymphocyte gate plus larger forward scatter cells was taken. From this gate, cells were gated again in a CD11b low scatter population. An enriched SIGN-R1+ population fell within a high forward scatter. These cells were then gated on CD11b and SIGNR1 to enrich observation of MZM. SIGNR1 cells were deemed positive when gated against an Armenian Hamster IgG isotype control. (B) CD11b low cells with low forward scatter do not show enriched SIGN-R1+ MZM. Fluorochromes: CD11b-eFlour 780, SIGN-R1 or Armenian Hamster IgG-PE.
Figure 9. Flow cytometry gating strategy to distinguish BrdU+ SIGNR1+ MZM. From the positive SIGN-R1 population, cells were analyzed against BrdU. The cells from treated mice were compared to negative control cells that were not exposed to any BrdU but were processed and treated identical to the treated samples. Mice were given 5 days of BrdU drinking water.
Figure 10. Representation of BrdU-labeled bone marrow. After 3 i.p. injections of BrdU, about 80 percent of the bone marrow is labeled. This is verified by comparing to a non-BrdU treated control whose cells are treated the same as the samples.
Confirmation of MZM proliferation in young mice using flow cytometry analysis after treatment with BrdU in drinking water

Like the IHC analysis, I found evidence of proliferation (BrdU+ SIGNR1+ MZM) after 5 days of BrdU drinking water (Fig. 11). Every young mouse tested showed some evidence of positive labeling (average = 6.7 +/- 3.98% BrdU+ MZM of total MZM). However, I did observe a decrease in prevalence of BrdU+ MZM as compared to the results using the IHC technique. This could be due to SIGN-R1 possibly being a subset of MARCO, as one paper has stated [28]. Or it could be due to the IHC technique being so subjective. Flow cytometry provides a more quantifiable means to obtain cell counts and frequencies but has the limitation that localization of the SIGN-R1+ cells to the MZ cannot be made.

Comparison with BrdU uptake by MZM from old mice using flow cytometry analysis after treatment with 5 days of BrdU drinking water

After obtaining positive data in young mice, old mice ages 18-22 months given the same treatment were tested. There was a reduction in amount of proliferation compared to young mice (Fig. 12). Overall, there was a statistically significant decrease in proliferation between young versus old mice (p=0.02), although the mouse to mouse variation was high, surprisingly more so in the young age group than in the aged as is usually seen (Fig. 11).
Figure 11. Comparison of young and old SIGNR1+BrdU+ MZM after treatment with five days of drinking water. Young mice (n=7) were compared with old mice (n=7) after BrdU treatment for evidence of BrdU uptake by MZM. There is a significant difference of p=0.02 after analyzed with a Student's T test.
Figure 12. Flow cytometry comparison of BrdU+MZM in young and old mice from 4 experiments.
Using 3 days of i.p. injections as a different delivery method of BrdU

It is challenging to try to control or monitor exactly how much drinking water is ingested by mice, especially with more than one mouse per cage. It is also possible that old mice drink less water. Also, as previously noted, there was a difference in the frequency of BrdU+ MZM detected by flow cytometry versus IHC, even though both techniques showed positive data. In order to be certain that all mice received equivalent amounts of BrdU, both young and old mice were given three i.p. injections over three days (every 24hr), and sacrificed on the fourth day. In young mice, data showed a similar average and wide variation of BrdU uptake comparing five days of drinking water with 3 i.p. injections (Fig.13A). Thus, the delivery method of BrdU did not make a difference in the outcome.

When given 3 i.p. injections of BrdU, aged mice showed a similar range of proliferation when compared with the drinking water route, suggesting that aged mice took in as much drug as did young mice (Fig.13B). In this experiment, significance was not reached (p=0.15) when old was compared to young, but there was the same downward trend observed with the BrdU drinking water method (Fig.14). This further proves that there is likely a reduction in proliferation as the mice age.

Because of the close similarity of results with both delivery methods, I pooled all the data and reached significance (p=0.004) with an unpaired T test. Here the difference is even more visually apparent (Fig. 15).
Figure 13. Comparison of uptake of BrdU by MZM of young mice with two BrdU delivery methods. Young (A) and old (B) mice given 5 days of drinking water compared to mice given 3 i.p. injections showed no difference (p=.93 and p=0.12, respectively) in amount of double positive cells MARCO+BrdU+ observed.
Figure 14. Comparison of young and old SIGNR1+ BrdU+ mice after 3 i.p. injection treatment. There is no statistical significance between the two groups (p=0.15), but a downward trend is apparent.
Figure 15. Pooled data from both drinking water and i.p. treatments comparing BrdU uptake by MZM of young and old mice. Pooling all of the data reaches significance (p=0.004) when comparing young versus old double positive MARCO+BrdU+ MZM.
CHAPTER V

TO DETERMINE IF MZM CAN BE RECONSTITUTED IN AGED MICE WITH AN UNSELECTED BONE MARROW TRANSFER FROM YOUNG MICE

The studies in this chapter represent preliminary trials to replenish MZM in aged mice with young donor cells. Assumptions were made to support the rationale for the experimental approach, but there is limited information in the literature. Some assumptions may need to be addressed in more detail in the future. First, my results in Chapter IV indicated that the MZM population in young mice does turnover by a proliferating cell population and this appears to be reduced in aged mice. Second, I assumed that MZM are derived from monocyte precursor cells in the bone marrow. Presumably, these precursors would be proliferating and rapidly label with BrdU. Therefore, BrdU could be used as a labeled to mark donor MZM or B-lineage cells derived from a transplant of bone marrow cells. Third, I assumed that MZM potentially could be established if depleted based on two previous studies. An older study showed that when all macrophages are depleted in a young mouse, MZM do repopulate the MZ (albeit, later than other macrophage subsets) [66]. Recently, You et al. showed that in a CD19 knockout mouse that lacks MZB cells, the MZM were also absent. When they adoptively transferred WT splenocytes, they observed rapid return of MZB cells, followed by MZM after 7 days. In aged mice, which are decreased in both MZB cells and MZM, rescue of MZB cells via adoptive transfer of bone marrow precursor B-cells could possibly encourage reconstitution MZM in spleens of old mice.
Establishment of adoptive transfer protocol

My protocol was loosely based on Minges Wols/Johnson et al. and You et al. The former paper showed that BrdU could be used to track donor bone marrow-derived immature B-cells in the host spleen. The latter paper suggested the 7 days post transplant timepoint that I decided to use in my experiments. Bone marrow is a highly proliferative population and about 70-90% of the bone marrow labels with 3 i.p. injections of BrdU given over 72hr, as is verified by flow cytometry compared with bone marrow from an untreated mouse. Initially, only normal young mice (2 months) were used as donors and recipients to establish a protocol and determine if BrdU-labeled donor cells were detected 7 days post transfer (Fig. 3, Methods). Approximately thirty million unselected donor bone marrow cells were injected i.v. into the tail veins of recipient mice. After sacrifice 7 days later, splenocytes were harvested and analyzed with flow cytometry for double staining of BrdU+ SIGNR1+ MZM or BrdU+ B-lineage subsets, identical to previous methods.

Evidence of BrdU-labeled donor MZM in young recipient mice after transfer

Of the 7 mice that had transfer, only 4 mice showed evidence of transferred MZM (Fig. 16 and Table 3). Although the data are highly variable, I concluded that the bone marrow cell transfers yielded evidence that some donor cells became incorporated into the host MZM population. Because the negative control for BrdU stain used the anti-BrdU antibody on spleens from untreated mice, frequencies below 1% were deemed as credible. Low frequency of donor cells in most young mice could be due to limited "space", insufficient time for MZM to differentiate from a precursor cell, or low frequency of precursor cells. Irradiation or other host conditioning strategies were not used in my experiments. Irradiation of host or pre-enrichment
Presence of donor cells within the splenic B-cell subsets was also analyzed for 4 recipient mice. The unselected donor marrow pool would include pro-, pre-, and immature stage B-cells, all of which are dividing or derived from dividing cells and would be labeled within 72 hr [4]. Mature B-cells in the bone marrow inoculum would not be labeled. It is known that immature B cells home immediately to the MZ area of the spleen, and there, must undergo further differentiation into FOB cells or MZB cells [67]. Therefore, I expected to see some evidence of BrdU+ B cells in the recipient. Of the 4 mice sampled, 3 mice showed some evidence of transfer of B cells precursors (immature B cells, Table 3). Only one mouse showed slight reconstitution (0.65%) of donor MZB cells and another mouse showed similar low reconstitution (0.54%) of FOB cells. The low reconstitution is likely to be due to using unselected bone marrow for transfer. However, these pilot bone marrow transfers into young mice met the goal of finding evidence for incorporation of donor MZM, and I next moved on to asking if MZM could be repopulated in aged mice following a bone marrow transplant.
Figure 16. Flow cytometry profiles of young recipient and control mice from two experiments. Young recipient mice received 30 million BrdU-labeled bone marrow cells i.v., 7 days prior to sacrifice.
Table 3. Frequency of BrdU+ donor MZM in recipient young mice. Bone marrow was harvested from 2 month old mice and transferred into 2 month old recipient mice. Out of the 7 transfers, only 4 mice showed evidence of MZM transfer. The percentage reflects frequency of BrdU+ MZM cells out of all MZM.

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Table 3. Frequency of BrdU+ donor MZM in recipient young mice. Bone marrow was harvested from 2 month old mice and transferred into 2 month old recipient mice. Out of the 7 transfers, only 4 mice showed evidence of MZM transfer. The percentage reflects frequency of BrdU+ MZM cells out of all MZM.
Evidence of transfer in aged mice

After seeing evidence of positive transfer in the young to young experimental group, I tested my hypothesis that MZM can be reestablished in aged mice with a young bone marrow transplant. The same protocol was followed as described above. For each experiment, 2-3 old mice and 1 young mouse were given approximately 1 million bone marrow cells from the same pool. About 1/3 of the old mice had to be discarded at sacrifice due to observable tumors or enlarged spleen.

Frequencies of BrdU+ cells were always assessed against an untreated splenic control using the identical anti-BrdU antibody. Figure 17 is a representative example of positive transfer and Figure 18 graphs the outcome and indicates evidence of transfer of donor MZM in four of the six recipient old mice. The transfer appeared to be more consistent than that seen in the young recipient group (Table 4).

Evidence of MZB cell transfer was important to address in the aged mice, which may have diminished MZB cells. Donor MZB cells were observed in three of five mice tested (Table 4). The same three mice were positive for donor-derived immature B cells, and two of these mice were positive for donor-derived mature B cells (Table 4). A trend toward correlation between transplant of donor MZB cells with repopulation of MZM was noted, but further experiments would be needed to confirm or deny this premise.
Figure 17. Flow cytometry profile of an old recipient and control mouse. A recipient old mouse received 30 million BrdU-labeled bone marrow cells i.v., 7 days prior to sacrifice.
Figure 18. Comparison of the efficiency of transfer of donor MZM between young and old recipients. More old mice are observed to have received donor MZM than young mice, even though the mean (1.7 versus 1.2) is lower.

Table 4. Percentage of BrdU+ donor MZM in recipient old mice. Bone marrow was harvested from 2 month old mice and transferred into 18 month old recipient mice. Out of the 6 transfers, 5 mice showed evidence of MZM transfer. 3 mice showed evidence of MZB transfer and immature B cell transfer, and two mice showed mature B cell transfer.
CHAPTER VI
DISCUSSION

This study tested the hypothesis that the reduction of MZM observed in aged mice is due to decreased turnover of the MZM. To approach this, first, a reliable method to detect evidence of MZM proliferation was needed. BrdU was chosen because of its success in previous kinetics experiments in the lab. However, using BrdU as a label for proliferating MZM in tissue sections and flow cytometry had not been done previously. Much of this thesis focused on optimizing techniques and establishing preliminary findings as a foundation for future detailed studies about the homeostasis of MZM throughout the lifespan.

Using BrdU over other methods has advantages and disadvantages. BrdU is not radioactive, which makes it less dangerous than tritiated thymidine (TdR). Also, unlike TdR, BrdU is not re-used during DNA synthesis, which makes it useful in pulse-chase experiments[60]. In order to effectively use BrdU as a marker for proliferation in MZM, techniques had to be optimized in both immunohistochemistry and flow cytometry.

My studies began by testing for uptake of BrdU by MZM with immunohistochemical staining. Although this technique is limited by only providing a qualitative analysis, the advantage is that MZM have been most often characterized in splenic tissue sections [28, 32, 40, 52]. Initially based on previous experience of the lab, I tried a double staining system to look at co-localization of MZM and BrdU by staining MZM with anti-MARCO with AEC substrate (reddish-purple), anti-BrdU with DAB substrate (brown), and counterstaining with Nuclear Fast
Red. The double staining system was refined on spleen sections of a mouse given BrdU injections. My objective here was not to look for proliferating macrophages, but instead to determine if proliferation can be detected where expected—in the red pulp which contains many highly proliferative neutrophils. I also assessed non-specific binding by looking for negative staining in the white pulp, because mature lymphocytes should have low proliferation rates (BrdU negative). With this color scheme (AEC + DAB), evidence of BrdU+ granulocytes in the red pulp was not easily detected, so a different substrate color scheme was chosen: Vector VIP (purple) for MZM, DAB (brown) for BrdU, and the counterstain Methyl Green. The purple staining reaction is more localized and darker. Using this two-color scheme (VIP + DAB), it was easy to visualize proliferating granulocytes, as well as a clearly defined MARCO+ MZM.

After choosing a scheme to identify BrdU+ cells and MARCO+ cells, a method had to be formulated to visualize co-localization. Because this had not been done before on MZM, a treatment schedule had to be chosen that would yield the best chance for visualization of double-positive MZM and BrdU staining. The MZ establishes after birth. Juvenile mice have a newly established MZ [24] and, therefore, should have the highest level of proliferation. A relatively long exposure to BrdU in drinking water was given for two weeks to ensure an adequate labeling time in case the MZM population turned over slowly. The combination of young age and lengthy treatment showed qualitative evidence of double-positive labeling of MZM with BrdU in the MZ.

Once double-positive MZM were detected in frozen sections of spleens from juvenile mice, my investigation was expanded to include young adult mice 3-7 months of age. The BrdU treatment times were varied in order to (1) maximize detection of BrdU+ MZM and (2) not be
toxic to the mouse. Osmond et al. have shown that more than 3 intra-peritoneal injections of BrdU have a negative effect on B and T lymphopoiesis [60]. I chose 4 different treatment regimes: 2 i.p. injections for 48 hours; 2 i.p. injections for 48 hr and then BrdU drinking water for 7 days; 2 i.p. injections for 48 hr and drinking water for 9 days; and drinking water only for 9 days.

I also had to formulate qualitative system to score BrdU-positive and BrdU-negative MZM. 100X magnification photographs of the MZ region of a splenic section from each mouse were used. First, MARCO+ cells were scored if the nucleus was visible. Then, from those cells, each was judged to be BrdU+ or BrdU- and a frequency was recorded of BrdU+ MZM out of total MZM per section. About 8-13 photographs were scored per mouse. As a collage, the photos formed a complete MZ encircling a cross-section of white pulp. Each adult mouse tested, regardless of treatment given, showed evidence of uptake of BrdU suggesting that MZM do turnover with newly-made cells. Based on these positive results, my studies then focused on testing the hypothesis that the lack of MZM in aged mice reflects a decrease in MZM proliferation. In order to investigate this hypothesis, I developed detection of BrdU uptake in MZM by flow cytometry, which was more quantitative.

In the literature, MZM have been analyzed by flow cytometry using the SIGN-R1 cell surface marker; yet, MARCO is used often to stain MZM in tissue [32][41][39]. Attempts by me and others in our lab to use MARCO in flow cytometry were without success. One possibility is that MARCO is expressed intracellularly in greater abundance than on the cell surface; thus, the molecule might be weakly detected in flow cytometry analysis without cell permeabilization.

To analyze BrdU+ MZM by flow cytometry, a gating strategy to enrich MZM was first explored. The previous gating strategy established by S. Birjandi determined that SIGNR1+ MZM are CD11b-lo and have greater forward scatter (FSC, larger) than lymphocytes with side
scatter (SSC) intermediate between lymphocytes and granulocytes. The previous method was modified by narrowing the CD11b-lo and FSC vs SSC gates in order to determine where most of the SIGN-R1 cells fell. I gated SIGN-R1 against CD11b, and demarcated the positive MZM against its isotype control antibodies. Isotype controls are essential because MZM have more background staining than do lymphocytes. These SIGN-R1 positive cells were then displayed versus BrdU and were compared to spleen cells (from a non-treated control mouse) stained with identical antibodies. Once this gating strategy was established, it was then possible to quantitatively analyze BrdU+ MZM using flow cytometry. In order to verify in adult mice that some MZM are indeed proliferating, young adult mice aged 3-7 months were treated for 5 days with BrdU in drinking water only. FACS analysis showed evidence of proliferating MZM.

There was a difference in frequency of BrdU-positive MZM observed by FACS analysis compared to that predicted by scoring in tissue with immunohistochemistry. In immunohistochemistry, 18-34% of MZM were judged to be BrdU+ after 2-14 days of BrdU treatment. In flow cytometry, performed after 5 days of BrdU treatment, only an average of 6.4% of MZM were detected as BrdU+. The most likely explanation for this discrepancy is that the scoring in immunohistochemistry is subjective and not blinded, in contrast to flow cytometry, which is more quantifiable if appropriately controlled. A separate, unresolved issue is the recent report that SIGN-R1+ MZM are actually a subset of MARCO+ MZM [28]. Their findings were from immunofluorescence of splenic sections; to date, our lab has not confirmed the extent of You's observation (unpublished). However, existence of MZM subsets could explain the decrease seen the number of proliferating MZM (SIGNR1+) in flow cytometry compared to MARCO+ MZM in tissue sections. Nonetheless, a positive proliferative population was observed in mice 3-7 months of age by flow cytometry.

Old mice ages 18-22 months were compared to young mice. According to Birjandi et al., there is a decrease in the MZM population in most aged mice, which is seen both in tissue
section and quantified as a frequency via flow cytometry. I sought to answer if this decline is due to a decrease in turnover, which would be reflected by reduced proliferation. After giving the old mice identical treatment to young mice (5 days of BrdU-drinking water), I found that there indeed was evidence of a decrease in the proliferation of SIGN-R1+ MZM from the spleens of old mice, with a range of 0-3.1% that was statistically significant when compared to that in young mice (p=0.02). However, there is much variability in old mice and in order to have more confident results, more mice should be tested in the future. A possible explanation for the decrease in uptake of BrdU in old mice is that old mice may drink less BrdU-water or that the five day treatment could be more toxic to the MZM in aged spleens. To be assured that young and old mice received equivalent amounts of BrdU, the experiment was repeated on young and old mice using 3 i.p. injections, 24 hours apart. Using the i.p. method of delivery showed similar results to drinking water. Young mice had a range of proliferating MZM of 1.9-13.7% and old mice 1.3-4.9%. Therefore, it is unlikely that the drinking water affected the old mice in a negative way. When all data was pooled, the differences in old vs. young reached significance (p=.004), indicating that the MZM in aged mice proliferate (turnover) less than in young mice.

These studies have limitations in that the immunohistochemistry technique is qualitative and the scoring is subjective, and the flow cytometry technique is quantitative, but not visual. Since MZM are defined by their localization in the MZ, flow cytometry is limited by the accuracy phenotype being restricted to MZM only. This is why both techniques were used to observe proliferation. However, time did not permit do a full analysis double-stain in tissue for all old and young mice in these comparison studies. This can be done in the future since samples of tissues are stored.

My results show two novel findings: (1) MZM proliferate in juvenile and young adult mice, and (2) proliferation is decreased in advanced age. The decrease in proliferation could
explain the reduction in MZM seen in aged mice. Possible causative factors may include reduction in chemokine signals or the decrease of MZB observed with age. Regarding CCL21 chemokine, preliminary data showed no change in mRNA expression between old and young splenic cells [68]. It is known that MZB cells and MZM have an intimate relationship with antigen presentation[47]. It is also known that when the MZB cells are depleted and subsequently re-established, the MZM will also return several days later[28]. The source of returning MZM is unknown. It is therefore possible that a decline in the presence of MZB cells affects the number and the turnover of MZM. MZB cells may provide a signal to MZM to proliferate; therefore, the absence of MZB with age may negatively affect proliferation of MZM.

Another cell type may begin the downward spiral. Previous research has shown that NKT cells are localized in the MZ and are increased with age [69]. NKT cells regulate MZB cells by decreasing their numbers [70]. Therefore, I hypothesize that in old age the increase of NKT cells reduces the number of MZB cells, which subsequently affects MZM cell number and proliferation. In order to test this hypothesis, studies can be done to deplete NKT cells in old mice and then test for an increase in MZB prevalence and MZM regeneration and proliferation.

Experimental evidence suggests that, in young mice, MZM can repopulate the MZ after depletion. In 1989, van Rooijen et al. found that after liposomal depletion of splenic macrophages, MZM return after a month [66]. More recently, You et al. showed that in CD19 KO mice, which lack MZB cells and MZM but retain FOB cells, the MZB cells are replenished when wild type splenocytes are transferred into the KO host. MZM also return a week later after MZB cells, suggesting an important relationship between MZB and MZM - it is necessary to have MZB in order to have MZM. I speculated that transfer of young bone marrow may have a similar effect in aged animals; by replenishing the MZB, subsequently the MZM would be replenished from BrdU-labeled precursors in the donor bone marrow. In order to test this, I
transferred BrdU-labeled bone marrow from young mice into old mice. I had to establish a FACS protocol to detect transfer of young donor cells old or young into host mice. Initially, I attempted to transfer cells between young mice. The protocol chosen was loosely based on previous adoptive transfer protocols in the lab. I used a transfer time of 7 days, similar to You et al., in order to detect the presence of MZM phenotype donor cells. Flow cytometry gating was performed as previously stated. Out of 7 young mice, 4 showed evidence of MZM transfer. This variable, low level of reconstitution may be due the unselected heterogeneous populations in whole bone marrow transferred. I assumed that the precursor of the MZM is in the monocyte lineage, but whether monocyte precursors can differentiate directly into MZM or if an intermediate stage exists is unknown. Interestingly, no young host mice showed evidence of MZB transfer from donor BrdU labeled cells. This may be due to MZB already being established and prevalent in the MZ in young host mice.

When the young, BrdU-labeled bone marrow cells were transferred into old mice, 4 out of 6 mice showed evidence of donor-derived MZM establishment and 3 mice showed evidence of donor MZB cells. The frequency of MZM replenished was low, but my experiments indicate that re-establishment of MZM in the aged spleen may be possible. In future experiments, the bone marrow lineage-specific precursor subsets should be separated in order to enrich and identify the subpopulation that results in the re-establishment of MZM. Also, future experiments should address the possibility that in old mice the microenvironment cannot support more MZM and MZB cells, due to physical deterioration of structural components, positive chemotactic signals, or inhibitory cytokines. It is also possible that in old mice, 7 days is not long enough to replenish MZM from precursor cells. However, in all, my results show that it is possible to restore at least a low number of MZM, and this has important implications.

If a protocol can be optimized to fully reestablish the MZ with MZM in old mice, these mice can then be challenged with *S. pneumoniae* and compared to young mice. It would be
interesting to see if these mice respond better to a pneumococcal challenge.

In summary, my data show that young mice have MZM that do proliferate or are derived from recently proliferating precursor cells, and there is a quantifiable decrease in proliferation of MZM from aged mice. Also, these data show that transplantation of a young bone marrow can reconstitute some MZM and MZB cells in old mice. Taken together, my thesis observes two aspects of the nature of MZM in old mice: its ability to proliferate and its ability to be re-established. These new pieces of information can be used to paint a better picture of the nature of MZM, and to find new ways to keep the MZ intact. A properly-functioning MZ is integral to the clearance of \textit{s. pneumonia}, and the more information we can gather about the MZ, the closer we will be to eradicating the prevalence of this disease in old age.
REFERENCES


VITA

Erika Ferro Bahamon was born on May 24, 1988 in Maywood, Illinois at Loyola University Medical Center to Maria Teresa Ferro and Juan Bahamon. Erika grew up in Corpus Christi, Texas and attended Richard King High School. She graduated in 2010 from Iowa State University with a Bachelors of Science in Biology and a minor in Psychology. She was also an avid member of Sigma Lambda Gamma sorority where she served as president.

Erika entered the Cell Biology, Neurobiology and Anatomy graduate program in 2010 following her undergraduate education. She joined Dr. Pamela Witte's lab in January 2011 to begin working on her Master’s thesis. Her research focuses on the homeostasis of marginal zone macrophages in young and old mice.

Upon completion of her master’s degree, Erika will be moving to Lubbock, Texas to attend Texas Tech University Health Sciences Center School of Medicine. She plans to become a family practice physician and serve in the Latino population.