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Intrinsic and Extrinsic Factors in the Maintenance of Long Lived Plasma Cells

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Intrinsic and Extrinsic Factors in the Maintenance of Long Lived Plasma Cells

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Arsalan Derakhshan

Advisor: Dr. Rafi Ahmed, PhD

An abstract of

A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Bachelor of Science/Master of Science in Biology

2012

Abstract

Intrinsic and Extrinsic Factors in the Maintenance of Long Lived Plasma Cells

By Arsalan Derakhshan

Some vaccines that are received during childhood have been shown to elicit a humoral response that can be measured for the duration of an individual's life. The mechanism by which long-term humoral immunity can be maintained is not fully understood. Studies have described long-term bone marrow (BM) resident antibody secreting cells (ASCs) that have come to be known as long-lived plasma cells (LLPCs). Great strides have been made in understanding how LLPC populations are maintained and how they differ from plasma cell populations that are short-lived. However, we are far from understanding the complete picture. It is thought that LLPCs have been preprogrammed during B cell development to receive and process environmental signals as to render extended longevity. In this study, we investigate some of the intrinsic and environmental factors that govern how LLPCs are maintained and whether environmental factors differentially affect B lineage subsets. The role of CD59, a gene that is differentially over-expressed in LLPCs, is briefly explored in the context of longevity and affinity maturation. In this study, we also review some of the survival factors that are essential in LLPC maintenance and analyze various bone marrow cells and subsets that provide those survival factors. Through *in vitro* experiments, we identified many BM subsets capable of maintaining LLPCs, which casts doubts on the role of any single BM subset. Furthermore, our *in vivo* experiments suggest that eosinophils may not be required for the maintenance of LLPCs as was recently reported.

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Introduction

Upon encountering antigen, B cells can go through various developmental stages, which are associated with the expression of different genes and markers. Plasma cells represent the terminally differentiated stage of B-cells, which help the humoral branch of the immune system by producing antibodies. Long-lived plasma cells (LLPCs) are thought to be the main component of humoral immunity by secreting antigen-specific antibodies over an extended period of time.[1] A longitudinal study of subjects' response to the smallpox vaccination has shown significant antigen specific-antibody titers up to 75 years after vaccination.[2] Another longitudinal study of antigen-specific antibody titers unambiguously showed the stability of antibody responses by finding estimated half-lives of 200 years for antibody responses to measles and mumps viruses.[3] Because the half-life of secreted immunoglobulins is relatively very short, these antibody titers are known to be produced by persistent numbers of LLPCs.[4]

Although great strides have been made in elucidating its mechanism, the generation and maintenance of LLPCs are not yet fully understood. Depending on many factors including antigen affinity, antigen dose, the presence and quality of T-cell help, and inflammatory cytokines, B-cells can differentiate into short-lived plasma cells (SLPCs), memory B cells, or LLPCs[5]. SLPCs predominantly inhabit secondary lymphoid organs and provide rapid protection that can be observed within 4 days of antigen exposure[6]. SLPCs can be generated in the absence of T cell help and independently of germinal centers (GCs). Without GCs, SLPCs tend to secrete low affinity antibodies.[7] In two commonly used antigens, NP-Hapten and LCMV, the SLPC response is reported to peak by day 8 and to virtually disappear by day 14.[8] As the SLPC population diminishes, LLPC and memory B cells begin to form.[9]

In contrast to SLPCs, the production of memory B cells and LLPCs is T cell and germinal center dependent.[10] Germinal centers are transient and dynamic structures created by the interactions of B cells, T-helper cells, and follicular dendritic cells through which B cells are selected for higher affinity immunoglobulin (Ig).[11] In the germinal center, B-cells can isotype switch and stochastically introduce mutations to the genes of the B cell receptor (BCR), a membrane bound immunoglobulin (Ig) that can bind to antigen.[12] The process in which mutations are introduced in the immunoglobulin variable gene segments at very high rates in response to T- dependent antigen has come to be known as hypermutation.[13] Mutations in the variable genes of the B cell receptors can sometimes lead to higher affinity binding to antigen. B cells that express higher affinity immunoglobulins receive stronger signals for proliferation and thus are selected for. The process in which hypermutation leads to higher affinity Ig, expressed as either BCR on memory B cells or secreted antibody, is known as affinity maturation.[10] LLPCs and memory B cells are both long-lived,[14] have undergone affinity maturation, and have several ontological features in common such as requiring germinal center interactions.

However, these two subsets of B cell development are different in several important ways and differ in their immune function.[15] Due to preferential homing receptors and varying survival factor requirements, memory B cells and LLPCs can be found in different anatomical sites[16]. It is estimated that roughly 80% of LLPC reside in the bone marrow (BM) whereas memory B cells migrate to splenic marginal zone, tonsillar epithelium, or enter blood circulation to distal sites.[17] Furthermore, memory B cells continue to express common B cell markers such as surface Ig and major histocompatibility complex (MHC) class II which LLPCs have down-regulated and ceased to express.[18] These changes in gene expression render LLPC as non-dividing cells[19] that secrete large quantities of antibodies whereas memory B cells do not actively secrete antibodies. Upon stimulation, memory B cells can quickly divide and differentiate into antibody secreting

plasma cells.[15] Due to the parallels that can be drawn between memory B cells and LLPCs, it is easy to see why memory B cells were thought to be responsible for replenishing LLPC populations. However to date, this concept is disputed and is a source of much debate.

There are 3 main models for explaining sustained antibody levels over extended periods of time.[20] These models differ in what they believe the role of memory B cells to be and whether virtually immortalized plasma cells can exist. The first model contends that long-term humoral immunity is conferred through continual production of SLPC by activation of memory B cells[21]. This model requires the existence of persistent antigen and is viewed as a more outdated model as antigen has been shown to be cleared rapidly.[22] Another model explains humoral immunity as conferred by LLPCs with fixed lifespan, which are replenished by memory B cells. This model does not require the persistence of antigen but rather contends that memory B cells are stimulated by activated T cells, cytokines, and toll-like receptors through polyclonal or bystander activation. [23,24] In support of this model, it was reported that a correlation exists between serum IgG antibody and the frequency of antigen-specific memory B cells. However, this correlation was found in a sample size containing 5 subjects and no such correlation was found in a 26-year study of 45 subjects in response to 8 vaccines.[3]

The last model differs from the previous two models principally in rejecting that memory B cells replenish LLPCs. This model contends that plasma cells can achieve virtual immortality and confer lifelong immunity.[3, 19,25,26] Given accumulating evidence for the latter model, more research has begun to focus on the factors that allow LLPC longevity. Plasma cells are not inherently long-lived given that rapid apoptosis ensues when they are cultured alone. However if co-cultured with bone marrow cells, plasma cell life can be extended significantly.[6] Various types of BM cells have been found to secrete survival factors that can help maintain LLPCs. By knowing

which survival factors are required for LLPC survival, we can begin to analyze which cell types would be best in supporting LLPCs based on the production of these survival factors. In fact, many bone marrow cell types have been claimed to play an essential role in LLPC survival including monocytes,[27] osteoclasts,[28] megakaryocytes,[29] basophils[30], and eosinophils.[31]

Although some studies have shown that the importance of survival factors in LLPC longevity cannot be disputed, other studies have shown that access to survival factors do not sufficiently explain the differential lifespan in SLPC and LLPC populations. SLPCs and LLPCs can be found inhabiting the same anatomic sites, presumably with equal access to survival factors in the environment, but the two subpopulations still exhibit a differential lifespan.[32] Further, experiments in mice with defective germinal centers show a normal antibody response and normal populations of plasma cells at day 8 post-infection, but these mice show a near complete absence of LLPCs.[33,34] The plasma cells generated in germinal center defective mice do not become long-lived, despite there being no environmental differences in the bone marrow of these mice in comparison to control mice. These studies suggest intrinsic differences in LLPCs and SLPCs, which cannot be explained by environmental factors. The Ahmed lab conducted microarray analysis and found many genes to be preferentially expressed in long-lived populations such as memory plasmablasts and LLPCs in comparison to short-lived populations such as naïve B cells and SLPCs. Altogether, these findings suggest that plasma cell longevity is not only contingent on environmental survival factors but also the ability to process these signals which may be preprogrammed prior to becoming a plasma cell.[5,35] In this study, we explored some of the intrinsic and environmental parameters behind the maintenance of humoral immunity by investigating the survivability of LLPCs. In doing so, we investigated the role of a preferentially expressed gene in LLPCs and co-cultured LLPCs with various BM subsets.

Materials and Methods

Harvesting and Prepping Bone Marrow Cells

Mice were anesthetized using isoflurane and sacrificed according to IRB approved protocol. Both femurs and both tibias were harvested from each mouse. RPMI 1640 (Cellgro) media that had been chilled sufficiently on ice was used to flush out the bone marrow into a 50 mL conical using a 23-gauge needle (BD Biosciences) and a 5 mL syringe (BD Biosciences). The cells were kept on ice or at 4° C throughout both the harvesting and prepping the bone marrow step. The cells were spun down, the supernatant was discarded into the waste, and the rim of the 50 mL conical was gently blotted on a paper towel (this was done by inverting the conical only once in order to prevent disturbance of the pellet). In order to remove the erythrocytes, the bone marrow cells were suspended in 1mL of ACK lysing buffer (Lonza) for 1 minute and the lysing process was quenched with the addition of 10 mL of RPMI 1640 (Cellgro). The solution was then pipetted through a cell strainer into another 50 mL conical in order to remove the lysed cell membrane.

Staining Cells for Flowcytometry

The harvested cells were counted and resuspended at 1 x 10^7 cells/mL in RPMI 1640 (Cellgro) and 10% Fetal Calf Serum (Hyclone). 100 μL of cell suspension was added to a well of a round bottom 96 well plate. 200 μL of FACS buffer (described below) was added to the well and the plate was spun down for 2 minutes at 2000 RPM. The liquid was flicked out leaving the pellet of cells in tact at the bottom of the well. The cells were resuspended in 100 μL of live/dead stain (BD, Amcyan) at 1:1000 in FACS buffer and were allowed to incubate for 30 minutes at 4° C. 1 μL of FC block antibodies (BD Biosciences) was added to the wells (making a 1:100 concentration in FACS Buffer) and allowed to incubate for 5 minutes at 4° C. 200 μL of FACS buffer was added to the well and the plate was spun down for 2 minutes at 2000 RPM. The liquid was flicked out leaving the pellet of cells in tact at the bottom of the well. The cells were resuspended in 100 μL of the staining

panel (described below). The cells were allowed to incubate for 1 hour at 4° C. 200 μL of FACS buffer was added to each well and the plate was spun down for 2 minutes at 2000 RPM. The cells were washed 3sx with 300 μL of FACS buffer by spinning cells down, flicking out the liquid, and adding new FACS buffer. This was done to remove excess staining antibodies. After the third wash, the cells were spun down, the liquid flicked off, and resuspended in 100 μL of fixative. The cells were allowed to incubate for 10 minutes at 4° C. 200 μL of FACS buffer was added, the cells were spun down, and the liquid was flicked out. The cells were resuspended in 200 μL of FACS buffer. Due to the fixative, cells can be read out by flow cytometry for up to a week.

All Antibodies were ordered from BD Biosciences Company.

Eosinophil Stain Marker	**Fluorophore**	**Concentration**	**Plasma Cell Stain Marker**	**Fluorophore**	**Concentration**
Siglec F	PE	1:100	CD44	Pac Blue	1:250
GR-1	Pac Blue	1:250	CD93	PE-Cy7	1:250
CD11b	APC-Cy7	1:250	CD138	APC	1:100
F-480	APC	1:100	B220	Alexa 700	1:100
CD11c	FITC	1:250	CD11b	APC-Cy7	1:250
			Ly6C	Percp-Cy5.5	1:250

FACS Buffer:

PBS +1% FCS +0.1% Sodium Azide

In Vivo Depletion of Eosinophils

In the first *in vivo* eosinophil depletion experiment, 4 B6 naïve mice (NCI) were injected intraperitoneally with $2x10^5$ PFU of LCMV Armstrong. After at least 80 days post infection (to allow enough time for only long lived antigen-specific antibody secreting cells to be maintained), 2 of the mice were injected intraperitoneally 1x, every other day, for three days with 20 μg of soluble anti-mouse Siglec-F (BD Biosciences) in 200 μL of PBS. The other 2 mice were injected intraperitoneally with 200 μL of PBS (Hyclone) 1x, every other day, for three days as a control group. At day 4 after the final injection, the bone marrows of the 4 mice were harvested and the

eosinophils were analyzed through flow cytometry. Plasma cells were also analyzed in all 4 mice using both flow cytometry and ELISPOT assays (ELISPOT assays were conducted before cells were stained for flow cytometry).

The *in vivo* eosinophil depletion experiment was repeated using 3 mice in the Siglec-F treated group and 2 mice in the control group.

Gata-1 KO Vs WT Serial Antibody Titers

5 dbl-Gata 1 knockout mice (Jax, 05653 C.cg- gata1tm6sho/J) and 5 BalbC mice, age and gender matched, were injected intraperitoneally with $2x10^5$ PFU of LCMV Armstrong in 200 μL of PBS. On day 7, 14, 21, 35, 49, 63 post infection, blood was collected from the mice through submandibular bleeding by using disposable lancets (Fischer scientific). In efforts to reduce blood coagulation, 10 μL of EDTA (ethylenediaminetetraacetic acid) was pipetted into 2 mL Safe-Lock micro test tubes (Eppendorf) in which the blood was collected. The samples were spun down at 14000 rpm at 4° C for 10 minutes. The serum was collected from the top as to avoid disturbing the precipitate and frozen at negative 80° C. Enzyme-linked Immunosorbent assays were then used to measure antibody titers between dbl-Gata 1 knockout mice and Balb C control group.

Quantifying Antibody Titers- Enzyme Linked Immunosorbent Assay (ELISA)

Coating Plates:

The ELISA plates were coated with either an antigen or goat anti-mouse total capture antibodies. In order to capture LCMV specific antibodies, LCMV Nucleoprotein was diluted to 1μg/1μL in PBS and 100μL was pipetted into each well. Plates were covered with parafilm and incubated up to 4 days at 4°C

Blocking Plates:

On the day when ELISA was performed, the antigen solution was dumped into the waste and the plate washed 3 times with PBS + 0.5% Tween- 20. The residual liquid was tapped out and dry blotted on paper towels. 100 μL of blocking solution, composing of PBS (Hyclone), 0.2% Tween-20, 10% Fetal Calf Serum (Hyclone), was added to each well and allowed to incubate for 2 hours at room temperature.

Adding Serum to Plates:

The plate was emptied by flicking off residual liquid and dry blotting the plate on paper towels. 100 μL of new blocking solution was added to each well once again. The serum was diluted 1:30 in blocking solution and 50 μL was added to the 100 μL of blocking solution in the first column of the ELISA plate (such that the first column ends up with a serum dilution factor of 1:90). Three-fold serial dilutions were made by transferring 50 μL of solution down the columns of the ELISA plate and pipetting up and down 10 times after each transfer. The 50 μL from the last column was discarded, leaving 100 μL of solution in each well. The serum was allowed to incubate for 90 minutes at room temperature

Adding Horseradish Peroxidase Antibody:

The plate was washed 3 times with PBS + 0.5% Tween-20. The plate was emptied by flicking off the residual liquid and dry blotting the plate on paper towels. 100 μL of goat anti-mouse IgG-HRP (SouthernBiothech, 1030-05) was added to each well at a concentration of 1:5000 in blocking solution. The plate was allowed to incubate for 90 minutes at room temperature.

Reacting with Substrate:

The plate was washed 3 times with PBS. The plate was emptied by flicking off the residual liquid and dry blotting the plate on paper towels. A substrate was prepared by dissolving 4mg of OPD (Sigma, P8787) in 10 ml of citrate buffer. The OPD tablets were not touched prior to use and 33

μL of 3% hydrogen peroxide (Fisher, H324500) was added to 10 ml of substrate immediately prior to use. 100 μL of substrate was added to each well and allowed to incubate for exactly 7 minutes. The reaction was quenched by adding 100 μL of 1M HCl. The optical density (OD) of the wells was read at 490nm using Microplate Manager (Bio Rad).

Reagents:

PBST: PBS + 0.5% Tween-20: Add 10 ml of Tween-20 to 200 ml of 10X PBS and make 2 liters of solution by adding milliQ water.

Citrate Buffer (pH 5.0):

Add 4.8 g anhydrous citric acid (Sigma) to 7.0 g Na_2HPO_4 anhydrous and make 500 mL of solution by adding miliQ water. Filter sterilize and store and 4° C.

Quantifying Affinity Maturation Using ELISA in NP-Hapten System

Preparing NP-2 Conjugated Ovalbumin and NP-20 Conjugated Ovalbumin:

In order to prepare NP-2 conjugated ovalbumin (NP-2 Ova), 0.6 μL NP-OSU stock (Sigma Aldrich) was added to 450 μL ovalbumin (10mg/mL). In order to prepare NP-20 conjugated ovalbumin (NP-20 Ova), 6 μL NP-OSU stock (Sigma Aldrich) was added to 450 μL ovalbumin (10mg/mL). The solutions were spun on a rotator overnight. The solutions were spun down in centrifuge at 14000 RPM for 2 minutes in order to remove the insoluble substance. Size exclusion chromatography was used to collect conjugated protein and to remove unconjugated protein.

Coating Plates:

Half of the wells of the ELISA plates were coated with NP-2 Ova and the other half were coated with NP-20 Ova at 100ng/well in 100 μL. Plates were covered with parafilm and incubated up to 4 days in 4 degree Celsius refrigerator.

Blocking Plates:

This procedure was conducted according the ELISA protocol above.

Adding Serum to Plates:

The plate was emptied by flicking off residual liquid and dry blotting the plate on paper towels. 100 μL of new blocking solution was added to each well once again. The serum was diluted 1:30 in blocking solution and 50 μL was added to the 100 μL of blocking solution in the first column that was coated with NP-2 ova and 50 μL was added to the 100 μL of blocking solution in the first column that was coated with NP-20 (such that the first column ends up with a serum dilution factor of 1:90). Three-fold serial dilutions were made by transferring 50 μL of solution down the columns of the ELISA plate and pipetting up and down 10 times after each transfer. The 50 μL from the last column was discarded, leaving 100 μL of solution in each well. The serum was allowed to incubate for 90 minutes at room temperature

Adding Horseradish Peroxidase Antibody:

This procedure was conducted according the ELISA protocol above.

Reacting with Substrate:

This procedure was conducted according the ELISA protocol above.

CD55/CD59 Double Knockout Vs WT Serial Antibody Response to NP-Hapten & Alum

4 CD55/CD59 double knockout mice and 4 B6 WT mice, age and gender matched, were injected intraperitoneally with 100ng NP-KLH in 200μL of Alum solution. On day 8, 18, 25, 45, 61 post-immunization, blood was collected from the mice through submandibular bleeding by using disposable lancets (Fischer scientific). In efforts to reduce blood coagulation, 10 μL of EDTA (ethylenediaminetetraacetic acid) was pipetted into 2 mL Safe-Lock micro test tubes (Eppendorf)

in which the blood was collected. The samples were spun down at 14000 rpm at 4° C for 10 minutes. The serum was collected from the top as to avoid disturbing the precipitate and frozen at negative 80° C. Enzyme-linked Immunosorbent assays were then used to measure antibody titers and to quantify affinity maturation between the CD55/CD59 double knockout mice and B6 WT control group.

CD55/CD59 Double Knockout Vs WT Serial Antibody Response to NP-Hapten & CpG

6 CD55/CD59 double knockout mice and 6 B6 WT mice, age and gender matched, were injected intraperitoneally with 100ng NP-KLH and 10μL of CpG in 200μL volume PBS solution. On day 7, 14, 28, 45, 63 post-immunization, blood was collected from the mice through submandibular bleeding by using disposable lancets (Fischer scientific). In efforts to reduce blood coagulation, 10 μL of EDTA (ethylenediaminetetraacetic acid) was pipetted into 2 mL Safe-Lock micro test tubes (Eppendorf) in which the blood was collected. The samples were spun down at 14000 rpm at 4° C for 10 minutes. The serum was collected from the top as to avoid disturbing the precipitate and frozen at negative 80° C. Enzyme-linked Immunosorbent assays were then used to measure antibody titers and to quantify affinity maturation between the CD55/CD59 double knockout mice and B6 WT control group.

Antibody Response to NP-Hapten & Alum Vs NP-Hapten & CpG in CD55/CD59 Double Knockout Mice

3 CD55/CD59 double knockout mice were injected intraperitoneally with 100ng NP-KLH in 200μL of Alum. 2 CD55/CD59 double knockout mice were injected intraperitoneally with 100ng NP-KLH and 10μL of CpG in 200μL volume PBS solution. On day 7, 21, 28, 42 post-immunization, blood was collected from the mice through submandibular bleeding by using disposable lancets (Fischer scientific). In efforts to reduce blood coagulation, 10 μL of EDTA (ethylenediaminetetraacetic acid)

was pipetted into 2 mL Safe-Lock micro test tubes (Eppendorf) in which the blood was collected. The samples were spun down at 14000 rpm at 4° C for 10 minutes. The serum was collected from the top as to avoid disturbing the precipitate and frozen at negative 80° C. Enzyme-linked Immunosorbent assays were then used to measure antibody titers and to quantify affinity maturation in response to NP-Hapten & Alum Vs NP-Hapten & CpG in CD55/CD59 double knockout mice.

Enumeration of Direct Ex-vivo Antibody Secreting Cells (ASC) From Mouse Tissue

Coating Plate:

The wells of the ELISPOT plate were coated with the antigen of interest. In order to detect LCMV specific ASC, the wells were coated with 100 μg LCMV Nucleoprotein in 100 μL PBS. The plates were allowed to incubate overnight at 4° C up to 7 days under sterile conditions.

Blocking Plate:

Plates were washed 3 x with 200 μL/ well of PBS- 0.05% Tween-20. Residual liquid was flicked off and the plate was dry blotted. Wells were blocked with 100 μL of RPMI + 10% FCS for 2 hours at room temperature in order to prevent non-specific binding.

Adding ASCs to the Wells:

Cells harvested from mice tissue were suspended at $1X10^7$ cells/mL and 50 μL of cell suspension was added to the first row (already comprising of 100 μL of RPMI + 10% FCS from blocking phase). Serial three-fold dilutions were made by transferring 50 μL to the next row and pipetting up and down 10x. The 50 μL solution from the last dilution was discarded such that the final volume in all the wells were 100μL. The plates were incubated in 37° C for 8 hours making sure to not disturb the plates as double spots may result from the disturbance.

Adding Biotinylated antibody:

100 μL of biotinylated goat anti-mouse IgG was added in a dilution of 1:1000 in PBS-T-FCS. The plate was allowed to incubate overnight at 4° C

Adding Horseradish peroxidase conjugated avidin-D:

Plates were washed 3 x with 200 μL/ well of PBS- 0.05% Tween-20. Residual liquid was flicked off and the plate was dry blotted. 100 μL of HRP-conjugated avidin-D (Vector, 5 μg/ml) was added to each well at a dilution 1:1000 in PBS-T-FCS. The plate was allowed to incubate for precisely 60 minutes at room temperature.

Adding the Substrate:

The wells were washed 3x with PBS-T followed by 3x with PBS. The residual liquid was flicked off and the plate was dry blotted. 100 μL of enzyme chromogen substrate (see below) was added to each well and allowed to incubate for approximately 8 minutes (until red spots become visible and background coloring is starting to become significant). The reaction was stopped by thoroughly washing the plate by running tap water over it and by removing the plastic base from the plate and running water over its backside. The wells were blotted dry and stored in the dark over night.

Acquiring Images:

The software program Image Acquisition was used to take pictures of each individual well and to compile a composite picture of the ELISPOT plate. The close-up pictures were then used to analyze the number of spots in each well.

Reagents:

PBS-T: PBS + 0.05% Tween-20

PBS-T-FCS: PBS+.05% Tween-20 + 1% FCS

0.1M sodium acetate solution:

13.61g of sodium acetate trihydrate was dissolved in milliQ water to make 1 liter of solution. HCl was used to lower the pH of the solution until pH meter indicates a ph of 4.8-5.0

AEC Stock Solution:

20mg/ml stock solution of AEC, 3 amino-9-ethyl-carbozole, was made in dimethylformamide (N,N-dimethylformamide, Sigma). This solution can be stored at 4° C in the dark for up to 1 month.

Enzyme Chromogen Substrate:

The AEC stock solution was diluted 1:67 into 0.1M Sodium acetate buffer (pH 4.8-5.0) giving a final concentration of 0.3mg/ml (150μL stock AEC per 10 ml acetate buffer). This solution was filtered through a 0.22μm membrane (the solution will go from being yellow to becoming clear). Immediately before use, right after the last wash, 100 μL of 3% hydrogen peroxide was added per 10 ml of substrate.

Co-culture Experiment 1: *Establish an in vitro culture of stromal cells and plasma cells:*

Acquiring Stromal Cells:

The bone marrow was harvested and prepped from one naïve naïve B6 IgHa mouse as described in the "Harvesting and Prepping Bone Marrow Cells" protocol above. The BM cells were cultured in B-cell media for 3 weeks at 37° C. The media of the cells was changed twice per week. After 3 weeks, the cells were stained with Alexa-488 labeled acetylated LDL for 3 hrs. The cells were washed, trypsinized, and sorted for SSC^{hi} and $Alexa\text{-}488^{lo}$. The cells were seeded in 24 well of a flat-bottom 96 well plate at 1×10^4 stromal cells in 150μL of B-cell medium.

Acquiring primary plasmablasts, memory plasmablasts, and LLPCs:

1) The bone marrow from 4 LCMV memory mouse (B6 IgHb) that had been infected with LCMV (Armstrong strain) several months before was used to extract the LLPC. 2) The spleen from a

naïve B6 IgHb mouse that had been infected with LCMV (Armstrong strain) 8 days previous to the extraction was used to extract the (splenic) extrafollicular plasmablasts. 3) The spleen from a naïve B6 IgHb mouse that had been infected with LCMV (Armstrong strain) several months before extraction and then again boosted with LCMV (clone 13 strain) 5 days prior to the extraction was used to harvest the memory plasmablasts.

After lysing the erythrocytes, the bone marrow was stained with B220, CD11b, and CD138 and the extractions from the spleen were stained with CD11b, and CD138. From the bone marrow of 4 LCMV memory mice that were not boosted, LLPCs were sorted based on $B220^{lo}$ $CD11b^{lo}$ $CD138^{hi}$ expression at 5 x 10^3 cells/ well. From the spleens of day 8 post-infection mice and day 5 post infection memory mouse, extrafollicular plasmablasts and memory plasma blasts were sorted based on $CD11b^{lo}$ $CD138^{hi}$ respectively, and seeded at 5 x 10^3 cells/ well.

8 replicates for each type of plasma cell were made with the following two conditions:

1) Plasma cells were seeded on top of stromal cells
2) Plasma cells were seeded alone (without stromal cells)

The supernatant of the cells was collected 2, 5, 8, 12, and 16 post-culture and IgG titers were quantified through ELISA.

Co-culture Experiment 2

Acquiring bone marrow stromal cells:

The bone marrow was harvested and prepped from naïve Balb C mice as described in the "Harvesting and Prepping Bone Marrow Cells" protocol above. The cells were stained with PE-CD11b (BD Biosciences) and –Alexa 700-B220 (BD Biosciences) at a 1:100 concentration and allowed to incubate for 30 minutes at 4° C in the dark. The cells were washed with MACS buffer and sent to Emory School of Medicine for sorting. The following subpopulations of cells were collected and plated at 1x10^5 cells per well in 100μL of B-cell media in a 96-well plate. Five replicates were plated for each co-culture condition.

No Cells (Media Only)	Mock Sort	CD11b- B220-	CD11b+ B220-	CD11b- B220+	Unsorted (Whole BM)

Acquiring Plasma Cells:

Plasma cells were harvested and prepped from the bone marrow of LCMV memory mice (Balb C mice that were injected with $2x10^5$ PFU LCMV Armstrong 60+ days prior to harvesting the bone marrow) as described in the "Harvesting and Prepping Bone Marrow Cells" protocol above. Due to the high density of cells ($3x10^7$ cells/ mL), the cells were stained with Pac Blue- CD44, PE-CD11b, Alexa 700-B220 at a concentration of 1:100 and APC-CD138 at a concentration of 1:50. The cells were allowed to incubate for 30 minutes at 4° C in the dark. Because plasma cells comprise a relatively small proportion of the bone marrow, the bone marrow cells were enriched with Anti-APC MicroBeads (Miltenyi Biotec, 130-090-855) and positive selection LS column (Miltenyi Biotec) to increase the proportion of plasma cells. After the incubating for 30 minutes, the cells were washed with MACS buffer and resuspended in 80μL of buffer per $1x10^7$ cells. 20μL of Anti-APC MicroBeads were added per $1x10^7$ cells and allowed to incubate for 15 minutes at 4° C. Cells were washed with approximately 2mL of MACS buffer per $1x10^7$ cells, spun down, and resuspended at $1x10^8$ cells in 500μL of MACS buffer. The LS column was placed in the magnetic field of a MACS Separator (SuperMACS) and 3mL of MACS buffer was allowed to run through and rinse the column. The cells suspension was added to the column and allowed to go into the column such that no visible liquid remained in the column reservoir. The column was washed with 3mL of MACS buffer 3x, only adding new buffer when the reservoir was empty. The column was removed from the magnetic separator and placed on a 15mL conical tube in order to collect the enriched cells. 5mL of MACS buffer was added to the column cells were flushed out by firmly pushing the plunger into the column. The cells were sent to Emory School of Medicine for sorting. CD11b low, B220 low, CD44 high, CD138 high were collected, evenly distributed, and plated on

top of the stromal cells cultures that were sorted the previous day. A day 0 Elispot was conducted and 5 days after plating the plasma cells, the co-culture replicates were pooled together and a day 5 Elispot was conducted.

Co-culture Experiment 3

Acquiring bone marrow stromal cells:

Bone marrow stromal cells were harvested, prepped, and sorted in the same way as described in the "Co-Culture Experiment 1" protocol the only difference being that the stromal cells were further divided into subpopulations. After the bone marrow stromal cells were prepped, half of them were stained an eosinophil panel: Pac Blue- CD11b (1:500), Alexa 700- B220 (1:500), PE-Siglec-F (1:1000) and the other half were stained with a megakaryocyte panel: Pac Blue- CD11b (1:500), Alexa 700- B220 (1:500), PE-CD41 (1:1000). The following subpopulations of cells were collected and plated at $1x10^5$ cells per well in 100μL of B-cell media in a 96-well plate. Five replicates were plated for each co-culture condition with the exception of the CD11b+ SiglecF+ eosinophil subpopulation and the CD11b- B220- CD41+ megakaryocyte subpopulation because the number of cells that were collected for these rare subpopulations was a limiting factor. Three replicates of the eosinophil population and one replicate of the megakaryocyte population were plated. These numbers were accounted for in the bar graph.

Media	Unsorted (Whole BM)	CD11b- B220+	CD11b+ B220-	CD11b- B220-	CD11b+ SiglecF+	CD11b+ SiglecF-	CD11b- B220- CD41+	CD11b- B220- CD41-

Acquiring Plasma Cells:

The same protocol was used to collect plasma cells as described in "Acquiring Plasma Cells" above.

Results

In order to assess genes that are specific for PC longevity, we conducted RNA microarray analysis of plasma cell subsets and focused on genes that were over-expressed in both LLPCs from the bone marrow and PCs isolated after recall challenge of mice with LCMV. The latter PC subset is derived from memory B cells and thus GC- derived. Since germinal centers are required for B lineage longevity, genes expressed in GC- derived PC subsets may mediate longevity. These PCs were compared to day 8 spleen PCs which are GC-independent. The microarray data revealed CD59 as a good candidate gene as it was over-expressed in both LLPCs and recall PCs (Figure 1). CD59 is a complement inhibitor that prevents inappropriate perforation of host cells through complement activation. We believed that the over-expression of CD59 could contribute to the longevity of LLPCs through its protection against complement mediated lysis.

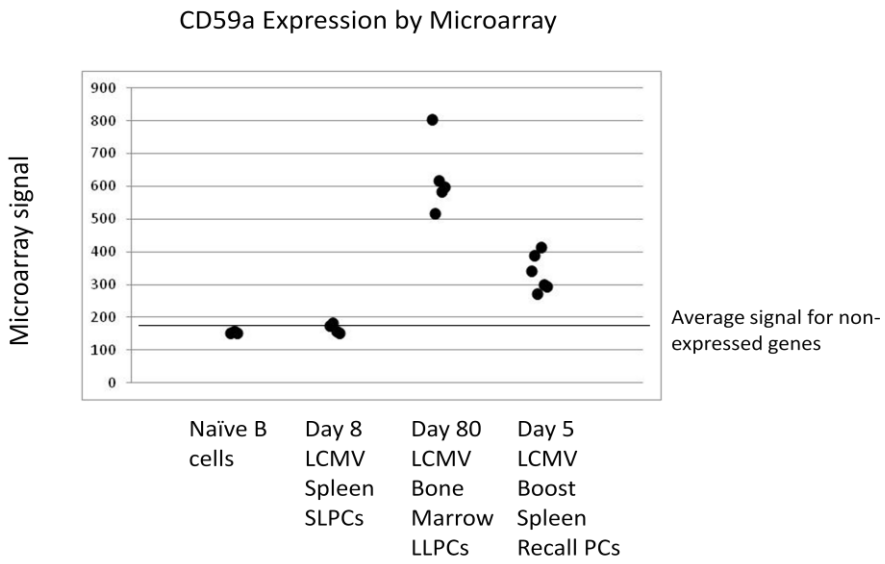


Figure 1. Naïve B cells, day 8 LCMV spleen SLPCs, day 80 LCMV bone marrow LLPCs, and day 5 LCMV boost spleen recall PCs were sorted with high purity and sent for Illumina microarray expression analysis.

We obtained CD59 knockout (KO) mice from Wenchao Song Lab (University of Pennsylvania). In our preliminary experiments, CD59 KO mice showed reduced LCMV specific antibody titers over time (data not shown). Technical observations made through enzyme-linked immunosorbent assays (ELISA) suggested that LCMV antibody responses were low affinity in CD59 KO mice. We used a 4-hydroxy-3-nitrophenyl (NP) acetyl-hapten system to assess possible defects in affinity maturation in CD59 KO mice. By using NP-2 ova capture and NP-20 ova capture in enzyme-linked immunosorbent assay, we were able to distinguish low affinity antibodies that could only bind to highly conjugated NP-20 ova from high affinity antibodies that could bind to NP-2. By conducting this assay over time, we could track affinity maturation of the serum response in the CD59 KO mice. Day 8 post-immunized WT mice generated a better antibody response as captured by both NP-2 ova and NP-20 ova (Figure 2A). By day 18, WT and CD59 KO mice show a similar antibody response as captured by NP-20 ova, but WT mice have a higher IgG response that is captured by NP-2 (Figure 2B). Similarly, the time points after day 18 reveal high levels of affinity maturation in WT mice as indicated by almost equivalent levels of NP-2 to NP-20 IgG response while CD59 KO mice continue to show a defect in affinity maturation as indicated by low levels of NP-2 to NP-20 IgG response (Figure 2C-E). This trend can be directly observed through the graph of NP-2 to NP-20 IgG response over time (Figure 2F). The ratio of NP-2 to NP-20 capture is significantly greater in the WT group than the CD59 KO group at every time point after Day 8.

Throughout the course of this study and in efforts to characterize other complement inhibitors, we inadvertently discovered that the CD59 KO mice we were conducting experiments on were in reality CD59/CD55 double KO mice due to a mix up in the lab they were ordered from. However before realizing the mix up in the mice genotype, this experiment was repeated using CpG in place of alum (Figure 3). We made this decision in an effort to reduce complement

CD55/CD59 KO Vs WT Mice Immunized with NP-KLH & Alum

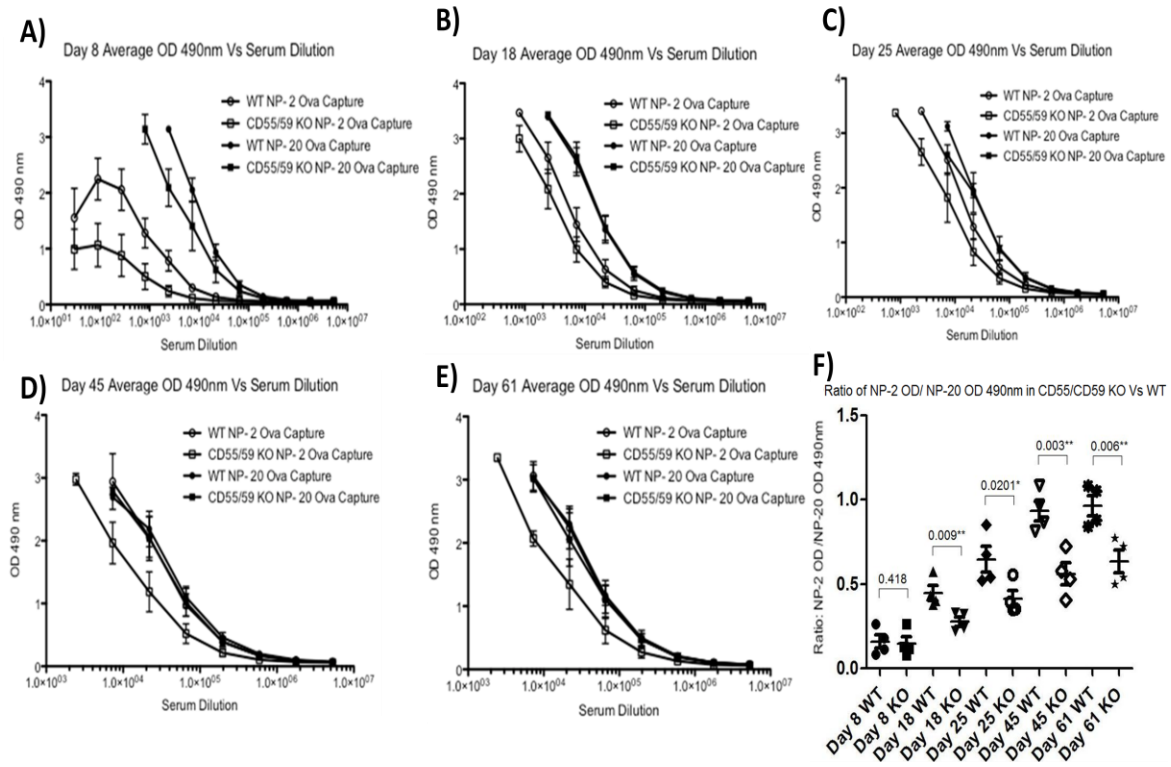


Figure *2. IgG Response to NP-Hapten & Alum in CD55/CD59 KO and Control Mice*

ELISA reactivity of NP-2 and NP-20 was assessed at **(A)** day 8 **(B)** day 18 **(C)** day 25 **(D)** day 45 and **(E)** day 61. **(F)** Shows the ratio of NP-2 to NP-20 capture at a serum dilution of 1:270 in WT and CD55/CD59 KO mice at Day 8, 18, 25, 45, and 61 post-immunization. *P* values were calculated through one-way, unpaired t-tests. N = 4 per group.

activation after learning that alum could independently activate complement.[39] The repeat experiment using NP-Hapten and CpG (figure 3) did not generate similar results as before. Initially, the NP-2 response in both WT and KO started very low and took longer to develop than compared to the previous NP-Hapten experiment (Figures 2 and 3). Furthermore, at no time point does there appear to be an affinity maturation defect in the KO mice. (Figure 3A-E). This is mirrored by the fact the ratio of NP-2 to NP-20 is not significantly different in WT and CD55/CD59 at any time point ($P > 0.05$) (Figure 3F). It was at this time that we discovered the mix up in the KO mice genotype and decided to investigate the environmental factors of LLPC maintenance while we backcrossed the CD59/CD55 double KO mice to its B6 background to obtain CD59 KO mice.

CD55/CD59 KO Vs WT Mice Immunized with NP-KLH & CpG

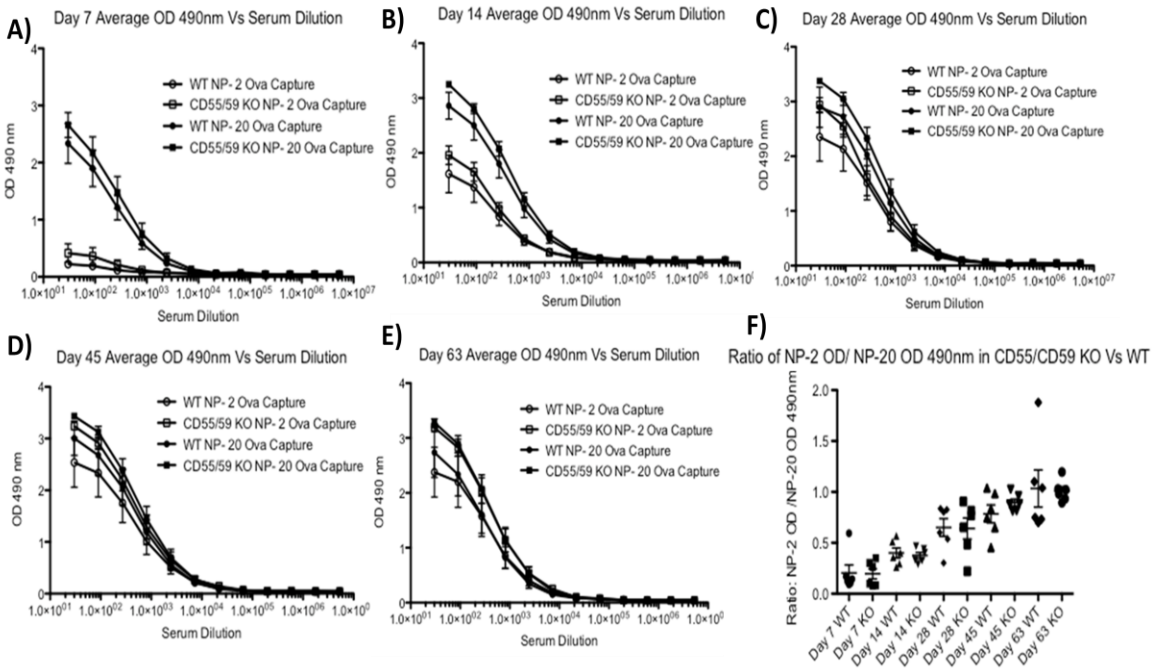


Figure 3. ***IgG Response to NP-Hapten & Alum in CD55/CD59 KO and Control Mice***

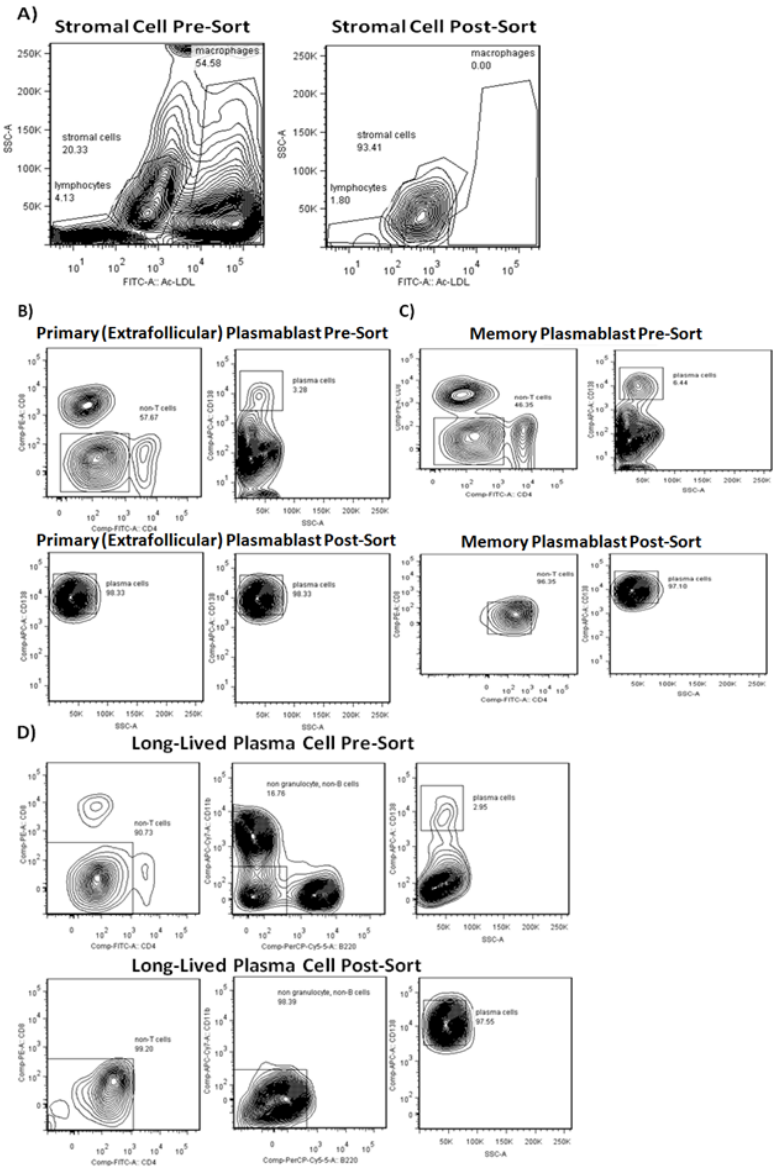
Figure 3 A-E) Respectively, Day 7, 14, 28, 45, and 63 post-immunization IgG response captured by NP-2 Ova and NP-20 Ova in CD55/CD59 KO Vs WT. **F)** Shows the ratio of NP-2 to NP-20 capture in WT and CD55/CD59 KO mice at Day 7, 14, 28, 45, and 63 post-immunization. *P* values were calculated by one-way, unpaired t-tests.

In order to analyze the effect of environmental factors of various B lineage subsets, we established an *in vitro* culture with primary plasmablasts, memory plasmablasts, and LLPCs in the presence and absence of bone marrow stromal cells (Figure 4). This protocol was taken from a study by Minges Wols et al which describes these stromal cells as bone marrow derived non-hematopoietic cells.[6] The aim of this experiment was to analyze whether, BM stromal cells can affect PC longevity *in vitro,* and if so, whether they have differential affects on B lineage subsets. Stromal cells alone (SC Alone) show no production of Ig throughout the culture (Figure 4E). The Ig titers of plasma cells cultured alone (without stromal cells) are higher than plasma cells cultured with stromal cells at the early time points, but the Ig titers of PCs alone decrease more rapidly than the Ig titers of PCs co-cultured with SCs. Primary plasmablasts that were co-cultured with SC produced antibodies 3 days longer than primary plasmablasts that were cultured alone while this effect was not seen in memory plasmablasts. LLPCs were most affected by SCs, which helped maintain LLPC Ig titers with relative stability up to 16 days. This *in vitro* assay helped answer our questions by showing that 1) bone marrow stromal cells can extend PC longevity and 2) bone marrow stromal cells had differential effects on the longevity of different PC subsets.

This *in vitro* assay proved to be useful in developing our understanding of environmental factors and the intrinsic and varied abilities of PC subsets in processing such environmental factors. However because this assay was an indirect measurement of PC survival through Ig production, we switched to an ELISPOT assay that could more directly measure PC survival. To further investigate the role of PC supporting cells in the bone marrow and in an effort to further characterize them, we sorted the bone marrow in broad subsets on the basis of CD11b and B220 expression, CD11b being a myeloid-specific marker and B220 being a B cell marker (Figure 5). CD11b was chosen because it divides the BM cell population into broad yet important subsets.

Some CD11b^{+} positive populations include, monocytes/macrophages,[36] myeloid dendritic cells,[37] eosinophils,[31] and some CD11b^{-} populations include bone marrow-derived stromal cells,[6] plasmacytoid dendritic cells,[41] and megakaryocytes.[29] Furthermore, B220 was chosen as a marker to primarily as a control to see if B cells could confound the results through activation, and they did not.

The Effect of Stromal Cells on Primary Plasmablasts, Memory Plasmablasts and LLPCs *In Vitro*



E)

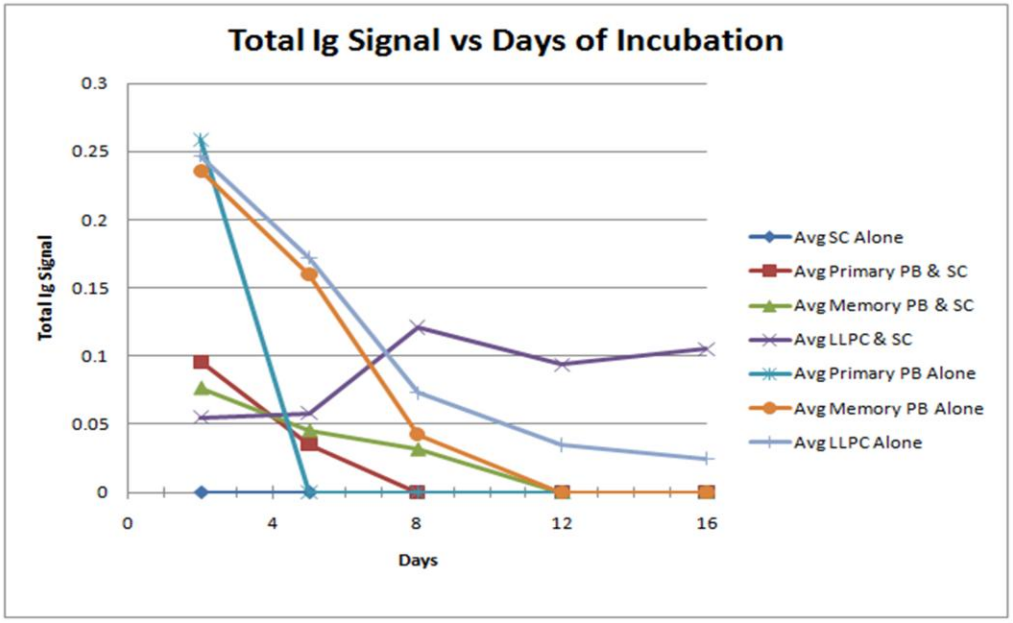


Figure 4. Stromal Cells Differentially Affect B Lineage Subsets **A)** Stromal cells were sorted on the basis of SSC^{hi} and acetylated-LDL^{lo} expression. **B-C)** Primarily plasmablasts and memory plasmablasts were both sorted on the basis of $CD4^{neg}$ $CD8^{neg}$ SSC^{lo} and $CD138^{hi}$ the former was harvested from the spleen of a day 8 post-infection mouse and the latter was harvested from the spleen of a day 5 post-boost memory mouse. **D)** Long-lived plasma cells were harvested from the bone marrow of memory mice and sorted on the basis of $CD4^{neg}$ $CD8^{neg}$ $B220^{neg}$ $CD11b^{neg}$ SSC^{lo} $CD138^{hi}$. **E)** Graph of enzyme-linked immunosorbent assay measuring total immunoglobulin (Ig) production from the supernatant of cell cultures on day 2, 5, 8, 12, and 16.

This *in* vitro co-culture and assay revealed CD11b$^-$ B220$^-$ cells as the only BM subtype that maintained PCs better than the never sorted BM cells (Figure 5B). All the other cells maintained PCs better than media alone condition except for CD11b$^-$ B220$^+$, B-cells. In efforts to further characterize BM subsets that have the capacity to maintain LLPCs, this *in vitro* co-culture experiment was repeated and expanded to specifically look at megakaryocytes and eosinophils (Figure 6C). Both megakaryocytes and eosinophils have been described in the literature as cells that directly provide survival factors for PCs and that the absence of either megakaryocytes or eosinophils results in reduced PC maintenance.[41, 31]

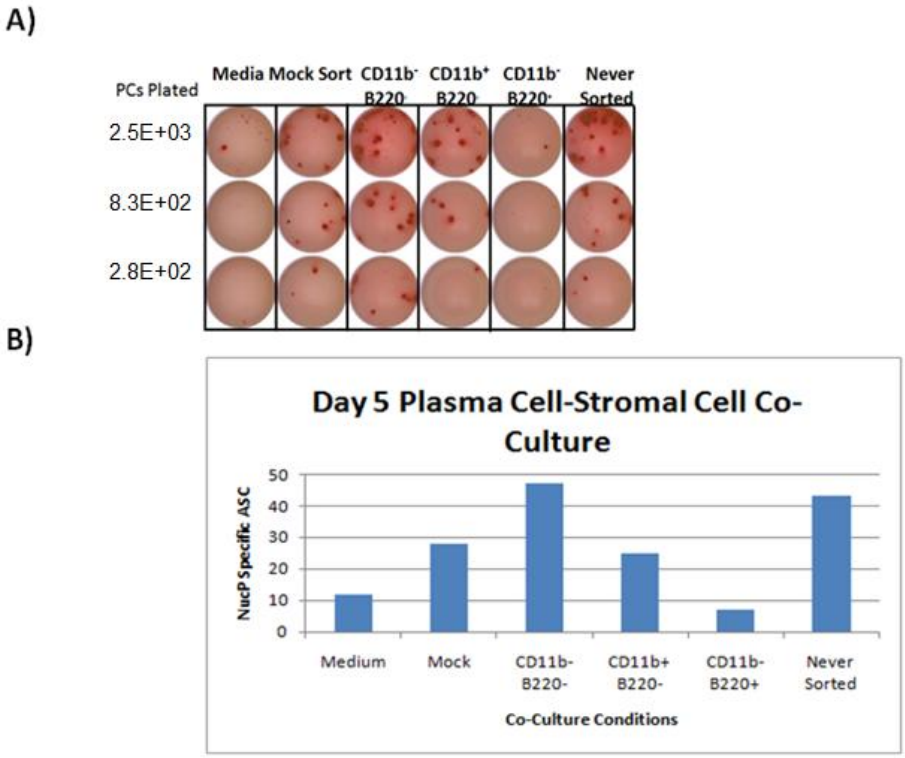


Figure 5. A) Enzyme-linked immunospot analysis of IgG ASC specific for LCMV nucleoprotein on day 5 of *in vitro* culture. **B)** Graph of enumerated spots quantified through ELISPOT. Medium represents PCs in culture with no BM cells. Mock represents PCs in culture with whole BM cells stained with antibodies that were not sorted with. CD11b$^-$ B220$^-$ represents PCs in culture with CD11b$^-$ B220$^-$ BM cell population. CD11b$^+$ B220$^-$ represents PCs in culture with CD11b$^+$ B220$^-$ BM cell population. CD11b$^-$ B220$^+$ represents PCs in culture with CD11b$^-$ B220$^+$ BM cell population. Never sorted represents PCs in culture with unstained whole bone marrow. Plasma cells were cultured with 1x10^5 BM cells.

An ELISPOT was performed on plasma cell-BM cell co-culture as well as on BM cell cultures alone as a control that BM cells do not confound the number of enumerated spots. These results indicate that the BM cells do not contribute to the number of spots (Figure 6B). Furthermore, $CD11b^{-}$ $B220^{-}$ cells, $CD11b^{+}$ $SiglecF^{+}$ cells (eosinophils), $CD11b^{+}$ $SiglecF^{-}$ cells (granulocytes), $CD11b^{-}$ $B220^{-}$ $CD41^{+}$ cells (megakaryocytes), and $CD11b^{-}$ $B220^{-}$ $CD41^{-}$ cells all helped maintain LLPCs equally or better than the never sorted BM cells.

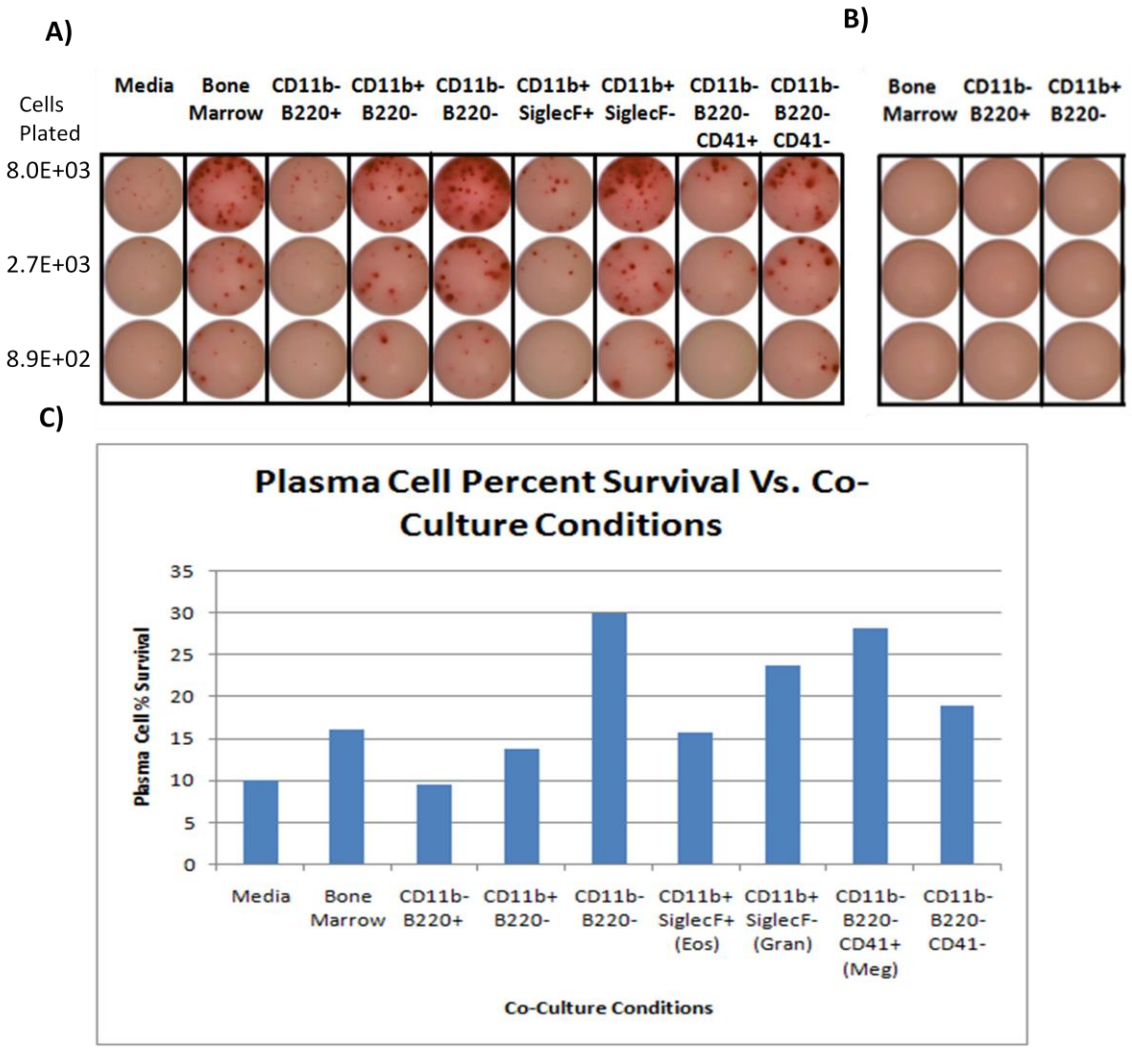


Figure 6. A) Enzyme-linked immunospot analysis of IgG ASC specific for LCMV nucleoprotein on day 5 of *in vitro* co-culture of BM cells and LLPCs. **B)** Enzyme-linked immunospot analysis of IgG ASC specific for LCMV nucleoprotein on day 5 of *in vitro* culture of BM cells alone (no PCs). **C)** Graph of plasma cell survival rate as quantified by the ratio of enumerated spots on a day 5 ELISPOT to a day 0 ELISPOT.

$CD11b^+$ $SiglecF^+$ cells (eosinophils) had a plasma cell survival rate of nearly 16% while the $CD11b^+$ $SiglecF^-$ subset excluding eosinophils had a plasma cell survival rate of nearly 24% (Figure 6C). $CD11b^-$ $B220^-$ $CD41^+$ cells (megakaryocytes) had a plasma cell survival rate of over 28% while the $CD11b^-$ $B220^-$ $CD41^+$ subset excluding megakaryocytes had a plasma cell survival rate of nearly 19%. $CD11b^-$ $B220^-$ cells helped maintain LLPCs better than all the other BM subsets by keeping 30% of LLPCs alive. All the BM subpopulations maintained PCs better than the media alone condition except for $CD11b^-$ $B220^+$, B cells.

Although eosinophils were not the best at maintaining LLPCs in our *in vitro* data, the paper that reported the role of eosinophils in maintaining PCs paper drew us to further investigate this BM subset by reporting that eosinophils not only help sustain LLPCs but that they are actually required for the maintenance of LLPCs.[31] We started by replicating the eosinophil *in vivo* depletion experiment that was described in the paper. Anti-mouse SiglecF antibodies were used to deplete the eosinophils 80 days after LCMV infection and the frequency of eosinophils was quantified using flowcytometry in eosinophil depleted and control mice (Figure 7). This flow cytometry data was highly representative of the repeat experiment, and thus, the flowcytometry data for the second *in vivo* eosinophil depletion is not shown here. The frequency of plasma cells was quantified using both flow cytometry and ELISPOT in eosinophil depleted and control mice (Figure 8 and 9). Furthermore, absolute plasma cell numbers were calculated for each mouse by multiplying the frequency of plasma cells, as quantified by ELISPOT, by the total BM cell count (Figure 9 and 10). The last calculation was done in order to account for any changes in the total number of BM cells as a result of anti-mouse SiglecF antibodies that could change the frequency of existing plasma cells but not necessarily the total count of plasma cells. In both *in vivo* eosinophil depletion experiments, there was never a significant difference in the absolute count of PCs between EOS depleted and control mice both in NUCP- specific ASCs or IgG^+ ASCs.

Furthermore, there was no difference in the frequency of NUCP specific plasma cells in EOS depleted mice and control mice (in both experiments); however, there was a significant reduction in the IgG^{+} PC frequency in the eosinophil depleted group of the first (but not second) experiment.

***In Vivo* Eosinophil Depletion**

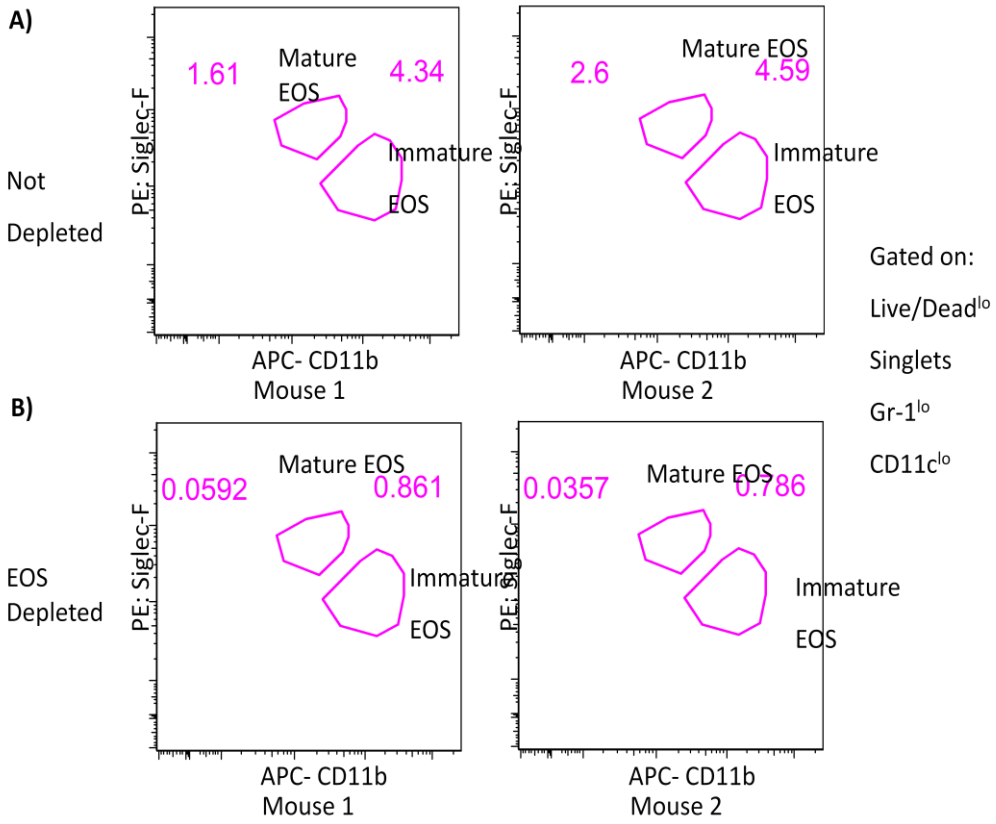


Figure 7. Anti-SiglecF antibodies effectively deplete eosinophils

Flow cytometry analysis of the frequency of mature and immature eosinophils in the bone barrow of BALB/c mice. **A)** Control mice were injected intraperitoneally with 200 µL of PBS 1x, every other day, for three days the bone marrow was analyzed for GR-1^{lo} $CD11c^{lo}$ Siglec-F^{hi} $CD11b^{int}$ eosinophils and GR-1^{lo} $CD11c^{lo}$ Siglec-F^{int} $CD11b^{hi}$ immature eosinophils **B)** EOS depleted mice were injected intraperitoneally with 20 µg of anti-mouse Siglec-F antibodies 1x, every other day, for three days the bone marrow was analyzed for GR-1^{lo} $CD11c^{lo}$ Siglec-F^{hi} $CD11b^{int}$ eosinophils and GR-1^{lo} $CD11c^{lo}$ Siglec-F^{int} $CD11b^{hi}$ immature eosinophils**.** N = 2 per group in the first experiment. N = 2 for control group, N = 3 for eosinophil depleted group in the second experiment.

Flowcytometry Analysis of the Frequency of Plasma Cells in Eosinophil Depleted and Control Mice

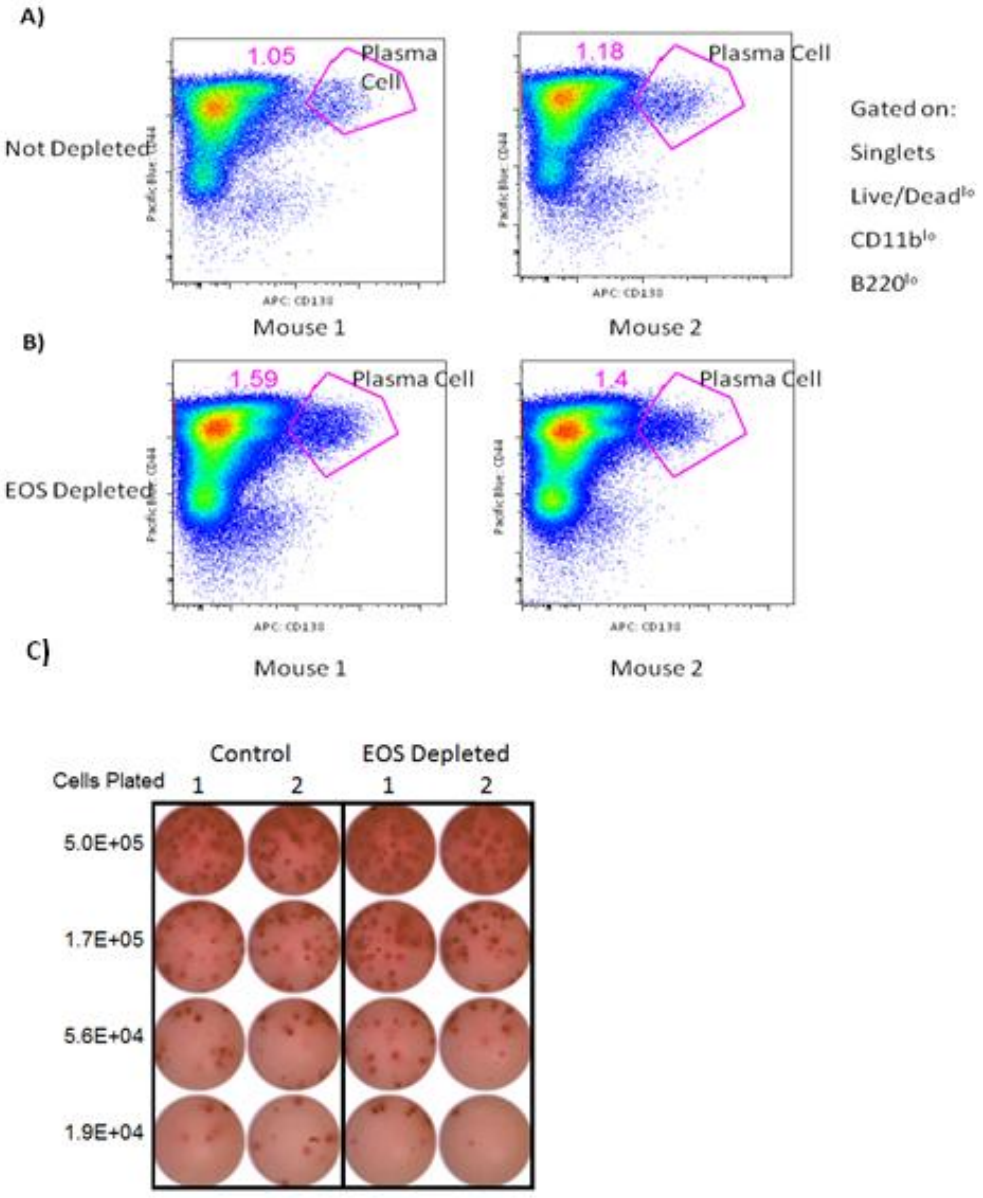


Figure 8. Similar *Plasma cell Frequencies in Eosinophil Depleted Mice and Control Mice*

Flow cytometry analysis of the frequency of plasma cells the bone barrow of BALB/c mice. **A)** Control mice were injected intraperitoneally with 200 μL of PBS 1x, every other day, for three days the bone marrow was analyzed for CD11b^{lo}B220^{lo}CD44^{hi}CD138^{hi} plasma cells **B)** EOS depleted mice were injected intraperitoneally with 20μg of anti-mouse Siglec-F antibodies 1x, every other day, for three days the bone marrow was analyzed for CD11b^{lo}B220^{lo}CD44^{hi}CD138^{hi} plasma cells (n = 2 per group). **C)** Enzyme-linked immunospot analysis of IgG ASC specific for LCMV nucleoprotein.

ELISPOT Analysis of the Frequency and Absolute Count of Both LCMV-Specific and Total IgG Plasma Cells in Eosinophil Depleted and Control Mice

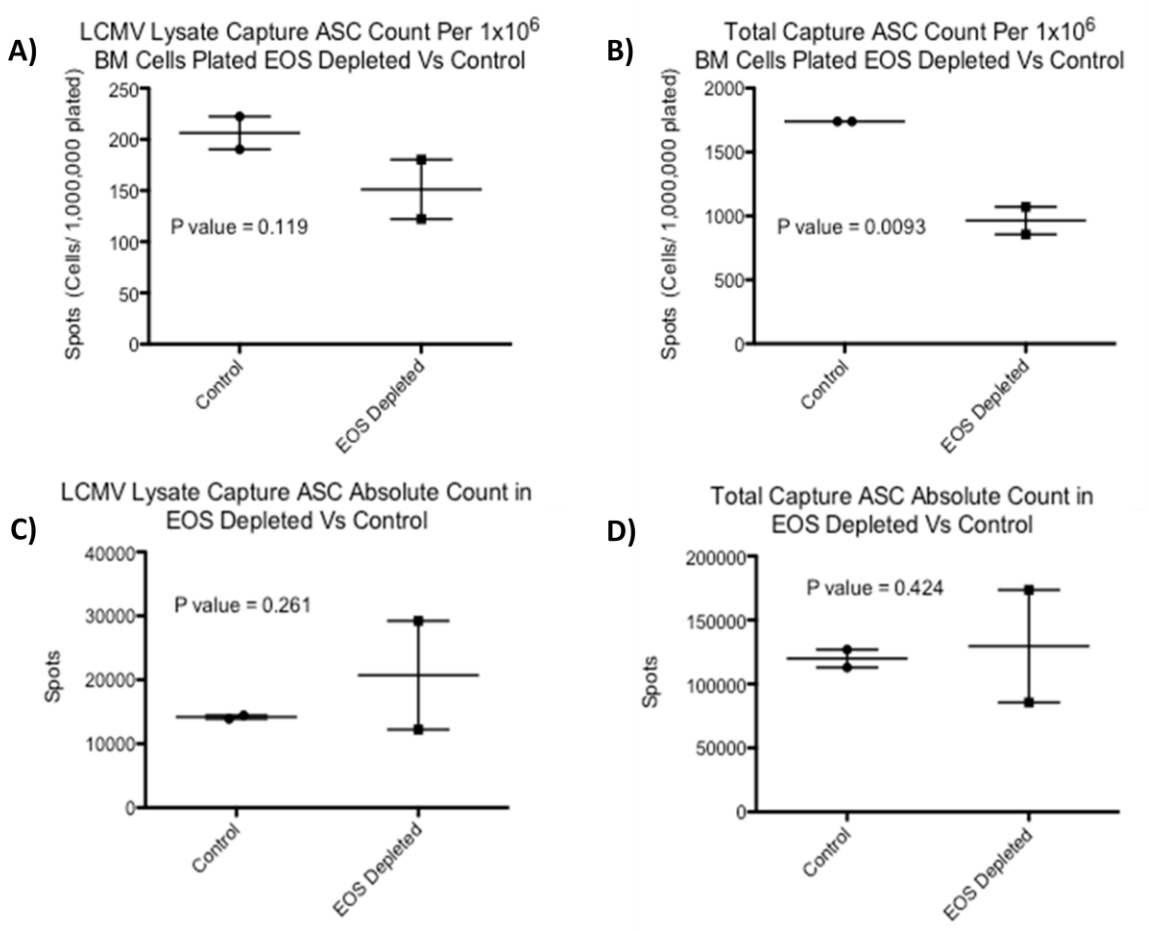


Figure 9. Enzyme-linked immunospot analysis of Antibody Secreting Cells (ASCs)

A) LCMV lysate specific ASCs normalized to spots counted per $1x10^6$ BM cells plated in EOS depleted versus control. There is no significant difference in LCMV lysate specific ASC frequency between EOS depleted and control mice **B)** Total IgG ASCs normalized to spots counted per $1x10^6$ BM cells plated in EOS depleted versus control. **C)** Absolute count of LCMV Lysate specific ASCs per four bones (2 femurs, 2 tibias) in EOS depleted versus control. **D)** Absolute count of total IgG ASCs per four bones (2 femurs, 2 tibias) in EOS depleted versus control. n = 2 per group)

ELISPOT Analysis of the Frequency and Absolute Count of Both LCMV-Specific and Total IgG Plasma Cells in Eosinophil Depleted and Control Mice

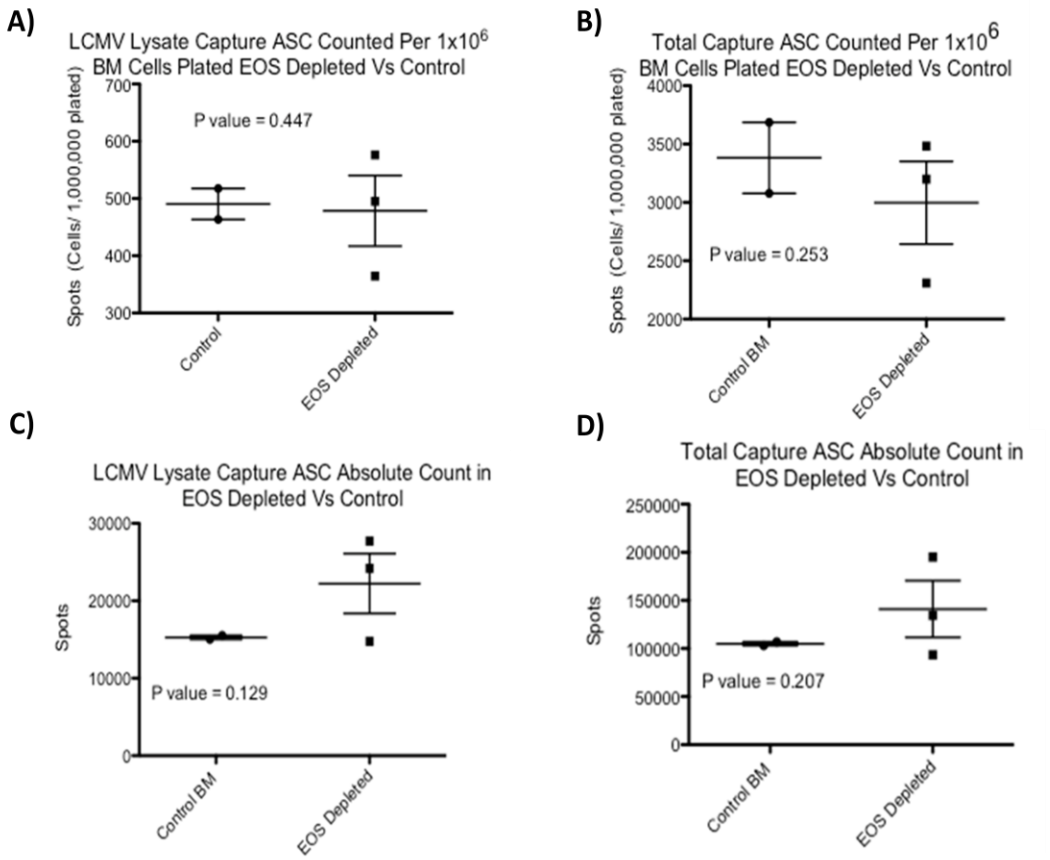


Figure 10. Enzyme-linked immunospot analysis of IgG ASC

A) LCMV lysate specific ASCs normalized to spots counted per $1x10^6$ BM cells plated in EOS depleted versus control. **B)** Total IgG ASCs normalized to spots counted per $1x10^6$ BM cells plated in EOS depleted versus control. **C)** Absolute count of LCMV Lysate specific ASCs per four bones (2 femurs, 2 tibias) in EOS depleted versus control. **D)** Absolute count of total IgG ASCs per four bones (2 femurs, 2 tibias) in EOS depleted versus control. (n = 2 in control group, n = 3 in EOS depleted group)

To further characterize the role eosinophils in the maintenance of LLPCs and to distinguish LLPC maintenance from PC generation, we conducted ELISA to measure LCMV NUCP over 63 days. The IgG response in WT and KO are not statistically different at day 7 post-infection. On day 14 post-infection, IgG response in WT is significantly greater than IgG response in dbl-Gata KO with a P value of 0.006. At day 28 post infection, the difference in IgG response between WT and dbl-GATA1 KO is lowered but still significantly greater in WT with a P value of 0.044. By day 49, the significance in the difference of IgG production between WT and dbl-GATA mice are continue to be lost at day 63 post infection. Measuring antibody titers over a period 60+ days is a good way to indirectly gauge the kinetics of plasma cell generation. Because the IgG response between WT and dbl-GATA1 KO is only significantly different on day 14 and 28 post-infection, it suggest potential defects in the maintenance of SLPCs or more likely a delayed generation of LLPCs in dbl-GATA1 KO mice.

LCMV NUCP Specific IgG Titer in dbl-GATA KO Vs Balb C

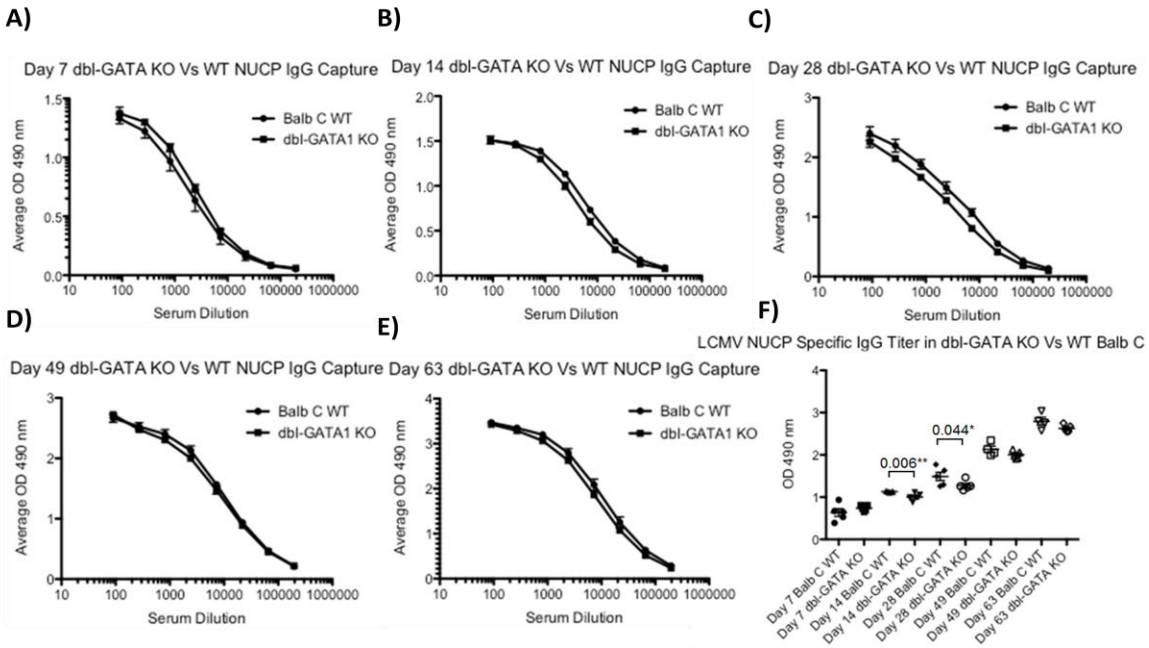


Figure 11. ELISA quantified IgG response in dbl-GATA KO versus WT to LCMV NUCP (A) day 7 (B) day 14 (C) day 28 (D) day 49 and (E) day 63 post-infection. N = 5 per group

Discussion

With increasing evidence supporting LLPCs as the main component of lasting humoral immunity and the potential of creating highly efficacious vaccines, more efforts should be placed in elucidating the mechanism of plasma cell longevity. How are LLPCs generated and how are they maintained that such that they are distinguished from SLPC and recall plasmablasts? In answering this question through a study of gene expression patterns within B-lineage development, CD59, was identified as a good candidate gene to investigate. Because CD59 was expressed in only B cell subsets that required germinal centers and because germinal centers are required for longevity in B cell subsets, CD59 was implicated in the longevity of B cell subsets. As a complement inhibitor, the theoretical mechanism through which CD59 could confer longevity was not difficult to postulate. The complement system, in which CD59 operates, is a chain of serum protein reactions which culminates with the formation of a membrane attack complex (MAC) which can perforate a cell.[38] Because CD59 effectively inhibits the MAC from perforating the host cells, the mechanism through which an over-expression of CD59 could confer longevity is rather straight forward.

In preliminary experiments, CD59 KO mice showed decreased antibody titers over time in response to LCMV. It was confounded whether this phenotype was due to 1) decreased survivability in the number of plasma cells 2) decreased antibody production per plasma cell 3) production of lower affinity antibody allowing a greater percentage of antibody to be washed off during enzyme-linked immunosorbent assays. Before comparing mutation frequencies of the variable immunoglobulin genes of WT and KO mice, enzyme-linked immunosorbent assays (ELISAs) were modified to more easily measure molecular affinity differences as opposed to genetic differences through sequencing. Through comparing high affinity NP-2 capture antibodies and low affinity NP-20 antibodies, CD59 KO mice, that were immunized with NP-KLH &

alum, were shown to have a defect in affinity maturation (Figure 2) On average, 93% of antibodies produced in WT mice were high affinity by day 45 whereas only 56% of the antibodies produced in CD59 KO mice were high affinity by day 45.

We found came across a study reporting alum to be an intrinsic activator of the complement system that was not necessarily dependent on the classical and alternative pathways.[39, 40] In an effort to minimize complement activation and to reduce a phenotype due to unspecific complement-mediated destruction, we repeated the experiment using CpG as adjuvant, which does not activate complement. To our surprise, the results that were observed in the NP-Hapten & alum experiment did not repeat with CpG (Figure 3). There was no measurable difference between the affinity maturation of WT mice and CD59 KO mice when immunized with NP-KLH and CpG. Tail snip genotyping was conducted on the KO mice as a control in characterizing other complement inhibitors in WT mice, and it was inadvertently discovered that the CD59 KO mice were in reality CD55/CD59 double knockout mice. CD55 inhibits the deposition of a complement protein, C3, to similar effects effect as CD59.[40] Because CD59/CD55 DKO are prone to many severe complications due to complement activation which could confound the gathered results, we decided to backcross CD59/CD55 DKO with their B6 WT background until we have a population of CD59 single knockout mice before continuing with these experiments. This hurdle shifted our focus from investigating intrinsic factors in LLPC longevity to extrinsic, environmental factors in LLPC longevity.

These results gathered from *in vitro* culture of primary plasmablasts, memory plasmablasts, and LLPCs in the presence and absence of bone marrow-derived stromal cells provided evidence for both a genetic and environmental component of LLPC longevity (Figure 4). This experiment suggested 1) environmental components such as bone marrow-derived stromal cells can increase the lifespan of PCs and 2) there are intrinsic differences among primary

plasmablasts, memory plasmablasts, and LLPCs which cause these B cell subsets to process environmental factors differently. Interestingly, the subset that was most affected by the presence of stromal cells were LLPC, which are naturally found in the BM, suggesting intrinsic differences that confer increased survivability in the BM. Furthermore, studies that have neutralized the survival factors, BAFF and APRIL, have reported a near complete loss of mature B cells, GC B cells, LLPCs but an untouched population of memory B cells and a normal generation of memory plasmablasts.[50] This evidence supports our data in suggesting intrinsic differences between memory plasmablasts and LLPCs that render different survival requirements. Before discussing the roles of various BM subsets in maintaining PCs, it is critical to discuss what is known in the literature about the survival factors of B cell subsets.

BAFF (B-cell activating factor belonging to the tumor necrosis factor (TNF) Family) and APRIL (a proliferation-inducing ligand) have been identified as survival factors that mediate B-lineage survival and longevity.[49] Both BAFF and APRIL are members of the TNF family and share two receptors, B-cell maturation antigen (BCMA) receptor and transmembrane activator and calcium modulating ligand interactor (TACI) receptor.[5] Additionally, BAFF can bind BAFF-R (BAFF receptor) whereas APRIL cannot[41] Identifying which ligands bind to which receptor has shown to be a very difficult task which continues to evolve.[5] The exact expression of these receptors has not been completely elucidated within the B-lineage as different studies have had varying results. One study described TACI as the receptor first expressed in B cell development followed by a gradual upregulation of BAFFR, followed by BCMA expression upon PC differentiation.[5] Another study found TACI to be absent on naïve B cells but expressed on germinal centers and activated B cells.[42] Despite the uncertainty in the signaling pathway, BAFF and APRIL have been shown to be critical in B-lineage development and maintenance.

Studies of BAFF and BAFFR knockout mice show almost a complete loss of mature B cells excluding memory B cells which appear to have different survival requirements.[43] Studies of APRIL knockout mice show that APRIL is needed for the generation and maintenance of plasmablasts,[5,44] but LLPCs accumulated to normal levels over time, suggesting redundancy in APRIL and BAFF signaling. [45, 46] Evidence of signaling redundancy was mirrored in the findings that *in vivo* neutralization of BAFF and APRIL, but neither one independently, depleted antigen specific bone marrow plasma cells[47] Furthermore, BCMA deficient mice were shown to have 80% less LLPCs than wildtype mice.[48] Interleukin-6 (IL-6) is also required in LLPC maintenance *in vitro* but its absence has no effect *in vivo*.[49] With these specifications of B cell and PC survival factors, we can predict that PC-supporting cells in the bone marrow secrete APRIL and possibly BAFF and in vitro studies will skew the data towards BM populations that also secrete IL-6. This understanding of PC survival factors will allow for the reconciliation of some potentially disparate results. Furthermore, BAFF and APRIL can exist in either bound or soluble form and how that affects signaling is not fully understood. Not only can this caveat help explain whether PC-supporting BM cells are required to co-localize with PC but it also renders certain approaches in this area of research more effective than others.

In this study, we investigated the capacity of myeloid-derived and non-myeloid-derived BM subsets in maintaining LLPCs by sorting for $CD11b^+$ and $CD11b^-$ BM cells. Furthermore, we expanded this study in a subsequent experiment in order to specifically investigate the capacity of eosinophils and megakaryocytes in maintaining LLPCs. Although there is strong evidence that BM cells can help maintain LLPCs (Figures 3, 5 & 6), it is clear that no single cell type is the sole provider of survival factors for PCs. This observation is supported in the literature through copious studies reporting the critical contributions of various BM cell types in maintaining LLPC. Monocytes, characterized as Ly-$6G^{neg}$ Ly-$6C^+$ $CD11b^+$ cells in the bone marrow, were found to co-

localize with LLPCs at the highest frequency in comparison to megakaryocytes, neutrophils, and eosinophils. One study also found monocytes to be the main producers of APRIL followed by eosinophils and neutrophils precursors.[39] Other studies have also reported that eosinophils and megakaryocytes are major sources of APRIL and IL-6. Furthermore, in an altogether different pathway, osteoclasts were shown to support plasma cells *in vitro* in a cell-cell contact dependent, BCMA independent fashion suggesting BAFF and APRIL were not involved.[40] These studies all provide evidence supporting our data that many BM subsets can maintain LLPCs. However, our *in vitro* data suggests that some BM subsets may be better in maintaining LLPCs than other BM subsets. In fact, $CD11b^-$ $B220^-$ cells (in both experiments), $CD11b^+$ $SiglecF^+$ (eosinophils), $CD11b^+$ $SiglecF^-$ (granulocytes), $CD11b^-$ $B220^-$ $CD41^+$ (megakaryocytes), and $CD11b^-$ $B220^-$ $CD41^-$ cells all helped maintain LLPCs equally or better than the never sorted BM cells. An interesting observation from our study was that $CD11b^+$ $SiglecF^+$ cells (eosinophils) did worse in maintaining than $CD11b^+$ $SiglecF^-$ subset which excludes eosinophils. In contrast, $CD11b^-$ $B220^-$ $CD41^+$ cells (megakaryocytes) did better in maintaining LLPCs than the $CD11b^-$ $B220^-$ $CD41^-$ subset which excludes megakaryocytes. Although these *in vitro* results suggest that megakaryocytes have increased importance in maintaining LLPCs, two stipulations may render these findings a purely *in vitro* phenomenon that may not be accurate of physiological processes. As mentioned above, *in vitro* data of plasma cell maintenance will be skewed towards BM subsets that secrete IL-6, which is known to be redundant in the maintenance of LLPCs *in vivo*.[50] Megakaryocytes have been reported as one of the primary producers of IL-6.[41] Furthermore, *in vitro* studies typically do not mirror the physiological proportions in which cell types are found. Although megakaryocytes may be effective in maintaining LLPCs on a per cell basis, they are very rare *in vivo.* Other cells, which are less potent at preserving LLPCs *in vitro,* might be more important in vivo due to their

greater numbers. Despite these limitations, our *in vitro* data provides strong evidence that a variety of BM subsets can maintain LLPCs.

However, what remains unclear is how *in vivo* depletion of one BM subset can reduce the maintenance of LLPC where there both redundancies in survival factor signaling and where a variety of BM subsets that provide survival factors. *In vivo* depletion experiments that reduce the maintenance of LLPCs have been reported in depleting basophils,[42] monocytes,[27] megakaryocytes,[41] and eosinophils.[31] If the absence of all these BM subsets individually results in decreased maintenance of LLPCs, it is unlikely to be explained by a reduction in survival factors. In order to explore this idea, we conducted further investigations on eosinophils which Chu et al reported to be required for the maintenance of LLPCs.[31]

Although this group used a protein antigen, phOx-CSA, for most of their immunizations, they reported the same effects in response to lymphocytic choriomeningitis virus (LCMV) suggesting that these effects were not exclusive to a T_H1 response but could also be seen in a T_H2 response. T_H1 and T_H2 are characterized by a differential cytokine profiles, which are associated with allergic anti-inflammatory and viral pro-inflammatory responses.[50] Our data confirms that administration of anti-mouse Siglec-F antibodies, as described by Chu et al, effectively depletes eosinophils yet we did not observe a reduction in LLPC numers5 (Figure 7-10). However after the *in vivo* depletion of eosinophils, we observed a substantial increase in the number of BM cells, presumably from proliferating eosinophil precursors. This may help explain the relative decrease in the frequency of IgG^+ PCs. Our data also showed a relative decrease in the frequency of IgG^+ plasma cells (p value= 0.0093, Figure 9)but expressing PCs as an absolute number by accounting for the increase in BM cells reveals no measurable loss in PC count. This trend, however, was only observed in IgG^+ plasma cells (not in NUCP specific PCs), and it was not observed in the subsequent *in vivo* eosinophil depletion experiment. Furthermore, NUCP-specific IgG antibodies

were measured in dbl-GATA 1 KO mice which are known to be deficient in eosinophils.[51, 31] The day 7 NUCP-specific IgG response was not significantly different in WT and KO mice, indicating that there is no defect in the generation of SLPC in dbl-GATA1 KO mice. However, there is a significantly reduced antibody response in dbl-Gata KO in comparison to WT (P value of 0.006) at day 14 and day 28 (P value of 0.044), but this significance is lost by day 49 and no difference in IgG titers continues to be observed at day 63. Interestingly, this can be interpreted as either a defect in maintaining short-lived plasma cell, a delay in generating LLPCs, or as a defect in homing since mice deficient in the most prominent plasma cell chemokine receptor, CXCR4, have a similar phenotype.[52]

The investigation of some of the intrinsic and environmental factors in LLPC longevity that is presented in this thesis suggest that both the signals that are available environmentally, and the intrinsic programming, that enable the processing of such signals, play an essential role in the maintenance of LLPCs. In elucidating these mechanisms, we can potentially tap into rich clinical resource. There are many medical implications behind understanding the factors that help sustain plasma cells, ranging from creating more efficacious vaccines to better understanding certain autoimmune disorders to potentially combating multiple myeloma. However, as our results suggest, effective therapeutic manipulation will most likely not be result of targeting one BM subset. Rather, we should avoid limiting ourselves and cast the net wide in search of the whole picture. With the clinical targets mentioned above in mind, the future direction of this study is both exciting and multifaceted. After completing the CD59 single KO experiments, we hope to identify other candidate genes that could be implicated in GC-derived B lineage longevity and work towards better understanding the specifications of BAFF and APRIL redundancy through BCMA, TACI, and BAFF-R.

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