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Inhibition of B Lymphopoiesis by Adipocytes and Myeloid-Derived Suppressor Cells

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INHIBITION OF B LYMPHOPOIESIS

BY ADIPOCYTES AND

MYELOID-DERIVED SUPPRESSOR CELLS

A DISSERTATION SUBMITTED TO

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DOMENICK EDWARD KENNEDY

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For my loving wife Justine Kennedy, our son Niccolo, and our daughter Colette

I am still learning… Michelangelo

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5-FU	5-fluorouracil
μg	microgram
ABC	aged B cell
ACM	adipocyte conditioned medium
ADI	adipocyte differentiation medium
alphaMEM	minimum essential medium alpha
Ang-1	angiopoietin-1
ANOVA	analysis of variance
Arg1	Arginase 1
ATRA	all-trans-retinoic acid
BCR	B cell receptor
BM	bone marrow
BM fat-CM	bone marrow fat-conditioned medium
BREC	B cell recombination excision circle
C5L2	C5-like receptor 2
$CaCl_2$	calcium chloride
CAR	CXCL12^{hi} reticular cells
CFSE	carboxyfluorescein succinimidyl ester
CFU-F	CFU-Fibroblasts

CLP	common lymphoid progenitor
cMAT	constitutive marrow adipose tissue
CpG	C-phosphate-G
CVID	common variable immune deficiency
CXCL12	C-X-C motif ligand 12
CXCR4	C-X-C motif receptor 4
D	diversity
DAMP	danger associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
D-NMMA	N^G-monomethyl-D-arginine
EB	early B lineage progenitor
EBF	early B cell factor
FcRγ	Fc receptor gamma
FCS	fetal calf serum
FcγRIIB	Fc receptor two B
Flt3L	Flt3 ligand
FPLC	fast protein liquid chromatography
GALT	gut associated lymphoid tissues
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage stimulating factor

GPCR	g-protein coupled receptor
H and E	hematoxylin and eosin
HGPRT	Hypoxanthine guanine phosphoribosyltransferase
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell populations
IFN-γ	interferon gamma
Igκ	immunoglobulin kappa
Igμ	immunoglobulin mu
IGF-1	insulin-like growth factor
IgH	immunoglobulin heavy chain
Igλ	immunoglobulin lambda
IL-10	interleukin-10
IL-12(p40)	interleukin-12 40kDa subunit
IL-12(p70)	interleukin-12 70kDa subunit
IL-13	interleukin-13
IL-17	interleukin-17
IL-1RA	interleukin-1 receptor antagonist
IL-1α	interleukin-1alpha
IL-1β	interleukin-1beta
IL-2	interleukin-2
IL-3	interleukin-3
IL-4	interleukin-4

IL-5	interleukin-5
IL-6	interleukin-6
IL-7	interleukin-7
IL-7R	interleukin-7 receptor
IL-9	interleukin-9
iNos	inducible nitric oxide synthase
IVIG	intravenous immunoglobulin G
J	joining
kDa	kilo Dalton
L-NMMA	N^G-monomethyl-L-arginine
LPMPS	lymphoid primed multipotent progenitors
M	molar
MCP	macrophage chemoattractant protein
MDI	isobutylmethylxanthine, dexamethasone, insulin medium
MDS	myelodysplastic syndrome
MDSC	myeloid-derived suppressor cells
MDSC-CM	myeloid-derived suppressor cell-conditioned medium
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1 beta
ml	milliliter
MPP	multipotent progenitor
MSC	mesenchymal stem cell

NaCl	sodium cloride
NK	natural killer
NLRP3	nod-like receptor pyrin domain containing 3
Nor-NOHA	N^{ω}-hydroxy-nor-arginine
Nos2	nitric oxide synthase 2
OCT	optimal cutting temperature support medium
Pax5	paired-box 5
PBSF	and pre B cell growth stimulating factor
PCR	polymerase chain reaction
PFAS	performic acid Schiff reagent
PPAR-γ	peroxisome proliferator-activated receptor gamma
PRR	PTH/PTH peptide receptor
qPCR	quantitative polymerase chain reaction
RANTES	regulated on activation, normal T cell expressed and secreted
RBC	red blood cell
RDA	representational difference analysis
rLP	rabbit lymphoid progenitor
rMAT	regulated marrow adipose tissue
ROS	reactive oxygen species
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9

SCF	stem cell factor
SD	standard deviation
SDF	stromal derived factor
SDS	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sIgM	surface immunoglobulin M
siRNA	small interfering RNA
SNO	N-Cadherin$^+$CD45$^-$ osteoblastic cell
STAT3	signal transducer and activator of transcription 3
SVF	stromal vascular fraction
Tdt	Terminal deoxynucleotidyl transferase
TLR	toll-like receptor
TLR9	toll-like receptor 9
TNFα	tumor necrosis factor alpha
UPC1	uncoupling protein1
V	variable
WT	wild-type
XLA	X-linked agammaglobulinemia

ABSTRACT

B lymphopoiesis declines with age in humans, mice, and rabbits. Impaired B lymphopoiesis correlates with increased fat in the bone marrow (BM), suggesting that adipocytes negatively regulate this process. In fact, adipocyte factors were found to inhibit B cell development in BM cultures.

Our goal was to understand the mechanism by which adipocytes inhibit B cell development. Through culturing mouse BM cells on OP9 stromal cells in the presence of adipocyte-conditioned medium (ACM), we found that adipocytes promote the accumulation of CD11b$^+$Gr1$^+$ myeloid-derived suppressor cells (MDSCs). These cells were not simply bystanders, as we report for the first time that MDSCs potently inhibit B cell development.

ACM-generated MDSCs express high levels of arginase and iNos, which are important for suppressing T cells. However, these effector molecules did not mediate the loss of B lymphopoiesis. By cytokine array analysis of MDSC-CM, we found that ACM-generated MDSCs produce IL-1. Further, neutralization of IL-1 in BM cultures containing MDSCs restored B lymphopoiesis, suggesting that MDSCs inhibit via IL-1. Inhibition by IL-1 did not directly block B lineage development, but instead acted at the MPP stage of hematopoietic development to drive myelopoiesis at the expense of B lymphopoiesis.

In contrast to humans and mice, where B lymphopoiesis declines in mid-to-late life, B lymphopoiesis arrests at two-to-four months of age in rabbits. Characterization of rabbit BM showed an increased number of adipocytes, an expanded myeloid compartment, and increased expression of inflammatory factors when B lymphopoiesis is arrested. This reduction in B lymphopoiesis and increase in myeloid cells was recapitulated in BM cultures treated with BM fat-CM. These data coupled with the identification of an inhibitory myeloid population, suggest that the BM microenvironment is responsible for the arrest of B lymphopoiesis in rabbits.

Our study has uncovered potential targets for therapies aimed at boosting B lymphopoiesis in scenarios with fatty BM, such as aging and obesity. For example, blocking the NLRP3 inflammasome in ACM-treated BM cultures prevented MDSC accumulation and enhanced B lymphopoiesis. We envision this observation; along with our other findings will provide insight into mechanisms that negatively regulate B cell development in fatty BM.

CHAPTER I

LITERATURE REVIEW

SECTION 1: B CELL DEVELOPMENT

Introduction

Production of B cells and antibodies are essential for effective immune responses against infectious agents, and for robust immune responses to vaccines. Genetic mutations impairing the generation of B cells (eg. X-linked agammaglobulinemia (XLA)) or antibodies (eg. XLA and common variable immune deficiency (CVID)) result in a severe immunocompromised state (Bonilla and Geha, 2009, Bruton, 1952, Durandy et al., 2013). Patients with XLA and CVID routinely receive intravenous IgG (IVIG) therapy to protect against infections, highlighting the importance of antibodies in immune protection.

In otherwise healthy individuals, aging imposes changes to hematopoietic progenitors and the BM microenvironment that result in reduced production of new B cells (Crane et al., 1996, Jasper et al., 2003, McKenna et al., 2001, Miller and Allman, 2005, Scholz et al., 2013). Decreased B lymphopoiesis contributes to higher susceptibility to infection and poor immune responses to vaccines in aged individuals. Therefore, an understanding of mechanisms that negatively regulate B cell development will be valuable in developing therapeutics to boost naïve B cell production in the elderly.

B cell development

B cell development takes place in the bone marrow (BM) of many mammals, including humans, mice, and rabbits. The BM consists of hematopoietic lineage cells that will differentiate into immune effector cells, as well as stromal cells which support the development of immune cells. The normal progression of B cell development begins with the hematopoietic stem cell (HSC). The HSC has the potential to differentiate into all cell types of the immune system, while cues from the microenvironment influence this differentiation potential promoting one lineage over the other.

To generate B cells, the HSC differentiates through a series of multi-potent progenitors, which can then differentiate into the common lymphoid progenitor (CLP), pre-proB cell, proB cell, preB cell, and finally the immature B cell that will exit the BM. With each subsequent differentiation step, the genetic and epigenetic landscape changes while these cells gradually lose the potential to differentiate into other lineages (eg. myeloid and T lineage) resulting in total commitment to the B lineage (Hu et al., 1997, Maes et al., 2008, Ramirez et al., 2010, Weishaupt et al., 2010).

Mouse B cell development. B lymphopoiesis is best characterized in mice, due to an abundance of genetic models and reagents generated to study this species. This process begins with the HSC, which differentiates through the successive developmental stages of MPP, CLP (Lin$^-$Sca1$^+$c-kit$^+$IL-7R$^+$), pre-proB cell (B220$^+$c-kit$^-$CD19$^-$Flt3$^+$CD24$^{low/-}$CD43$^+$IgM$^-$), proB cell (B220$^+$c-kit$^+$CD19$^+$Flt3$^-$CD24$^+$CD43$^+$IgM$^-$), large preB cell (B220$^+$c-kit$^-$CD19$^+$Flt3$^-$CD24$^+$CD43$^-$IgM$^-$), and

small preB cell (B220⁺c-kit⁻CD19⁺Flt3⁻CD24⁺CD43⁻IgM⁻FSCˡᵒʷ) before becoming

the immature B cell (B220⁺c-kit⁻CD19⁺Flt3⁻CD24⁺CD43⁻IgM⁺) that leaves the BM

and enters the periphery (Nagasawa, 2006) (Figure1). The timing of specific

differentiation or proliferative events at each of these developmental stages is

critical for successful B cell development. Alterations to these developmental

processes results in immune deficiencies such as XLA (Bruton, 1952,

Cunningham-Rundles and Ponda, 2005) or B cell cancers (Hamel et al., 2014).

VDJ gene recombination . Generation of a B cell pool with a diverse

antibody repertoire is necessary to protect a host from an array of pathogens.

The way many vertebrates generate this antibody diversity is through

combinatorial joining of various variable (V), diversity (D), and joining (J)

segments of the antibody loci. Because surface antibody or the B cell receptor

(BCR) is critical to B cell identity and function, successful rearrangement of the

heavy chain (*Igμ*) and light chain (*Igκ* or *Igλ*) loci are mandatory for successful

maturation to the immature B cell stage. Rearrangement of the *Igμ* locus begins

at the pre-proB cell stage with recombination of the diversity (D) and joining (J)

regions of this locus (Gellert, 2002, Jung et al., 2006). Upon successful DJ

rearrangement, this process continues in late proB cells with the joining of

variable (V) gene regions to the already formed DJ segment through

recombination. Successful heavy chain VDJ gene recombination allows for

expression of the *Igμ* locus which then complexes with surrogate light chain

(consisting of VpreB and λ5) to form the pre-BCR (Nishimoto et al., 1991). The

pre-BCR mediates expansion of large preB cells in conjunction with IL-7

signaling. Attenuation of IL-7 signaling and active pre-BCR signaling transitions development to the small preB cell stage, where proliferation is halted and the *Igκ* (light chain) locus becomes accessible for VJ recombination (Mandal et al., 2015). Successful recombination and expression of the *Igκ* or Igλ loci allows for functional BCRs present on the surface of immature B cells (Clark et al., 2014) (Figure 1.1).

							↗ BCR
							↗ Pre-BCR
			DJ	V-DJ	VDJ	VDJ V-J	VDJ VJ
HSC	MPP	CLP	Pre-pro B	Pro-B	Large Pre-B	Small Pre-B	B cell
Lin⁻	Lin⁻	Lin⁻	B220⁺	B220⁺	B220⁺	B220⁺	B220⁺

Figure 1.1 B cell developmental stages in mice. B cell development begins with the HSC which progresses through various developmental stages before reaching the immature B cell stage (B cell). VDJ gene recombination begins in pre-proB cells with *Igµ* DJ rearrangement, followed by V-DJ recombination in proB cells. The *Igµ* and surrogate light chain are expressed on the surface of preB cells as the pre-BCR, followed by rearrangement of Igκ light chain. Successful Igκ rearrangement and surface expression of the BCR complete differentiation at the immature B cell stage. These cells then leave the BM and enter the periphery to participate in immune responses. Each developmental

signaling. Attenuation of IL-7 signaling and active pre-BCR signaling transitions development to the small preB cell stage, where proliferation is halted and the *Igκ* (light chain) locus becomes accessible for VJ recombination (Mandal et al., 2015). Successful recombination and expression of the *Igκ* or Igλ loci allows for functional BCRs present on the surface of immature B cells (Clark et al., 2014) (Figure 1.1).

Figure key: ↗ BCR; ↗ Pre-BCR

Cell stage labels (left to right): DJ | V-DJ | VDJ | VDJ V-J | VDJ VJ

HSC	MPP	CLP	Pre-pro B	Pro-B	Large Pre-B	Small Pre-B	B cell
Lin⁻	Lin⁻	Lin⁻	B220⁺	B220⁺	B220⁺	B220⁺	B220⁺
Sca1⁺	Sca1⁺	Sca1⁺	c-kit⁻	c-kit⁺	c-kit⁻	c-kit⁻	c-kit⁻
c-kit⁺	c-kit⁺	c-kit^lo	CD19-	CD19⁺	CD19⁺	CD19⁺	CD19⁺
Flt3⁻	Flt3⁺	IL-7R⁺	Flt3⁺	Flt3⁻	Flt3⁻	Flt3⁻	Flt3⁻
			CD24^low/-	CD24⁺	CD24⁺	CD24⁺	CD24⁺
			CD43⁺	CD43⁺	CD43⁻	CD43⁻	CD43-
			IgM⁻	IgM⁻	IgM⁻	IgM⁻	IgM⁺
						FSC^low	

Figure 1.1 B cell developmental stages in mice. B cell development begins with the HSC which progresses through various developmental stages before reaching the immature B cell stage (B cell). VDJ gene recombination begins in pre-proB cells with *Igµ* DJ rearrangement, followed by V-DJ recombination in proB cells. The *Igµ* and surrogate light chain are expressed on the surface of preB cells as the pre-BCR, followed by rearrangement of Igκ light chain. Successful Igκ rearrangement and surface expression of the BCR complete differentiation at the immature B cell stage. These cells then leave the BM and enter the periphery to participate in immune responses. Each developmental

stage can be identified with the presence or absence of the indicated cell markers.

Human B cell development. The B cell developmental process in humans is similar to that found in mice, although a different set of surface markers is used to define each progenitor stage (Figure 1.2). Human B lineage progenitors are defined as CLP (CD34$^+$CD10$^+$CD19$^-$), Early B lineage progenitor (EB) (CD34^{hi}CD10$^+$CD19$^-$), ProB (CD34$^+$CD10$^+$CD19$^+$), large preB (CD34$^-$CD19$^+$), small preB (CD34$^-$CD19$^+$FSC^{lo}), and immature B cell (CD34$^-$CD19$^+$sIgM$^+$) (Blom and Spits, 2006, Galy et al., 1995, LeBien, 2000). VDJ gene recombination occurs in successive developmental stages as occurs in mice (LeBien, 2000), where recombination of the heavy chain locus occurs at the EB cell stage (Jung et al., 2006). Upon successful IgH recombination, the heavy chain pairs with surrogate light chain (consisting of VpreB and λ14.1) to form the pre-BCR found on preB cells. Pre-BCR signaling is needed to induce *Igκ* light chain VJ recombination in preB cells. Successful in-frame VJ rearrangements and expression of *Igκ* then allow for formation of the mature BCR and localization to the cell surface in immature B cells (Hystad et al., 2007, Schiff et al., 1990).
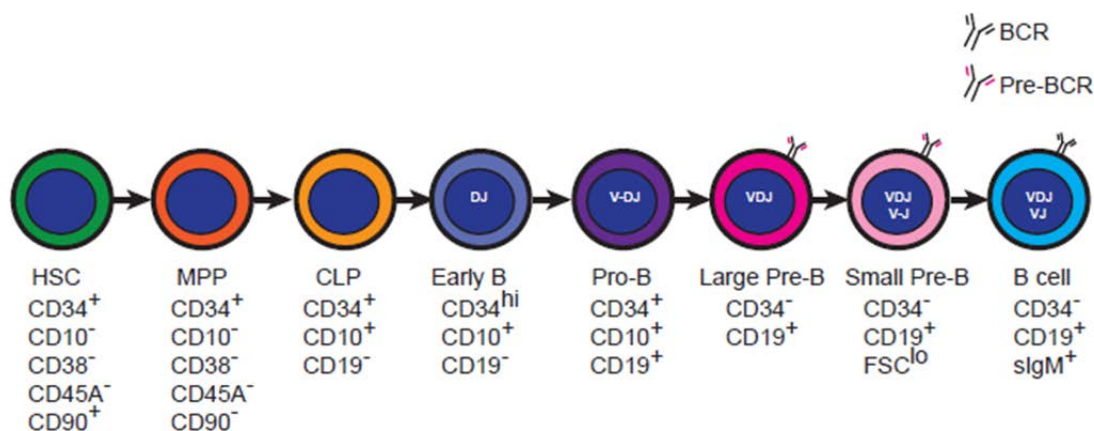
Figure 1.2 Human B cell developmental stages. Human B cell development begins with the HSC and MPP as defined above (Pieper et al., 2013, van Galen et al., 2014). Early progenitors are followed by the CLP and Early B progenitors. VDJ gene recombination begins in the Early B stage and is completed as indicated in the subsequent developmental stages. Successful rearrangement of the *Igμ* and Igκ loci result in sIgM expression on the surface of B cells that leave the BM and enter the periphery. Each developmental stage is defined as indicated above.

Rabbit B cell development. Many fundamental discoveries in the area of B cell biology were performed using rabbits. These studies contributed to our current understanding of allotypes and allelic exclusion, the antibody genetic locus, antibody structure, and the localization of antibody on the cell surface (Cebra et al., 1966, Feinstein, 1963, Fleischman et al., 1963, Gilman-Sachs et al., 1969, Oudin, 1956, Pernis et al., 1965, Pernis et al., 1970, Sell and Gell,

1965, Todd, 1963). Despite many early studies of B cells in rabbits, the number

of studies has declined over the years. While the B cell developmental process

appears to resemble that seen in humans and mice, not all of the developmental

stages have been resolved phenotypically. It is believed that this process starts

with the HSC (undefined) which can differentiate into the earliest described rabbit

B lineage progenitor, the rabbit lymphoid progenitor (rLP) (Kalis et al., 2007).

This population is thought to contain the equivalent of a CLP population seen in

human and mouse, and is defined as MHCII$^-$ IL7R$^+$. Further analysis showed that

rLPs express *Tdt*, *EBF*, and *Pax5*, genes commonly expressed by B lineage

cells. Development continues with proB cells defined as CD79a$^+$ cytoplasmic μ$^-$

surface μ$^-$ (CD79a$^+$Cμ$^-$ Sμ$^-$) (Jasper et al., 2003), preB cells (CD79a$^+$Cμ$^+$ Sμ$^-$)

(Hayward et al., 1978, Jasper et al., 2003, McElroy et al., 1981), followed by B

cells (CD79a$^+$Cμ$^+$ Sμ$^+$) (Jasper et al., 2003, Pernis et al., 1965, Sell and Gell,

1965) (Figure 1.3).

Rabbit B lineage progenitors also undergo VDJ gene recombination while

developing in the BM (Figure 1.3). Heavy chain DJ rearrangements are found in

proB cells, and successful VDJ gene rearrangements are found in preB cells,

showing a similar stage-specific process of recombination as seen in humans

and mice (Jasper et al., 2003). Unique to rabbits is the extent to which VDJ gene

recombination contributes to the diversity of the rabbit antibody repertoire.

Compared to humans and mice, which rely heavily on VDJ gene recombination

for antibody diversity, rabbits chiefly rearrange only one V_H, V_H1, the 3'-most V_H

gene segment of the IgH locus, limiting the amount of diversity generated

through this process (Becker et al., 1990, Friedman et al., 1994, Knight and

Becker, 1990, Raman et al., 1994, Tunyaplin and Knight, 1995). Alternatively,

rabbits do utilize many V and J regions during κ light chain rearrangement

(Sehgal et al., 1999). Future studies will be needed understand how light chain

diversity affects the overall diversity of the rabbit antibody repertoire.
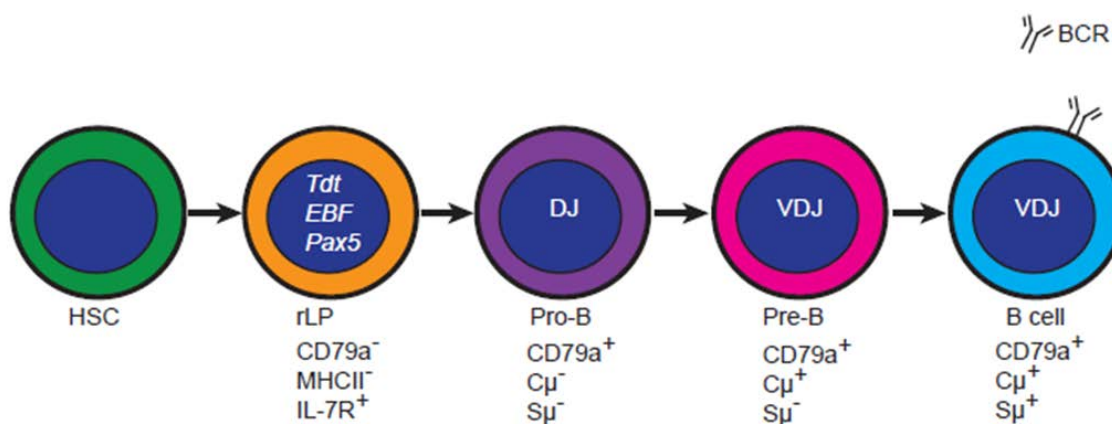
Figure 1.3 Schematic of rabbit B lineage development stages. Rabbit B cell

development is thought to begin with a yet-to-be defined HSC population. The

earliest progenitor population defined is the rLP, followed by the proB cell, preB

cell, and B cell. VDJ gene recombination occurs in rabbit BM beginning in the

proB cell. Each stage is identified by the combination of markers indicated above.

Generation of antibody diversity in rabbit GALT

Limited usage of V regions during VDJ gene recombination does not result

in a restricted rabbit antibody repertoire. This is because rabbits use somatic

diversification in gut associated lymphoid tissues (GALT) to further diversity their

BCRs. In fact, naïve B cells, resulting from successful B lymphopoiesis in the

BM, leave the BM and home primarily to the appendix where they diversify the

BCRs through gene conversion and somatic hypermutation (Lanning et al., 2000,

Mage et al., 2006). This method of diversification is similar to that in chickens and

sheep, which also utilize GALT for B cell development and maturation (Alitheen

et al., 2010, Ratcliffe, 2006, Reynaud et al., 1987, Reynaud et al., 1995).

Therefore, rabbit B lymphopoiesis occurs in two phases; 1. early B cell

development that mirrors human and mouse generation of naïve B cells; 2.

Further maturation and diversification in GALT. The focus of this dissertation is

on B cell development in the BM, but the utilization of GALT for further

maturation of naïve B cells is important to note in generating additional antibody

diversity in rabbits.
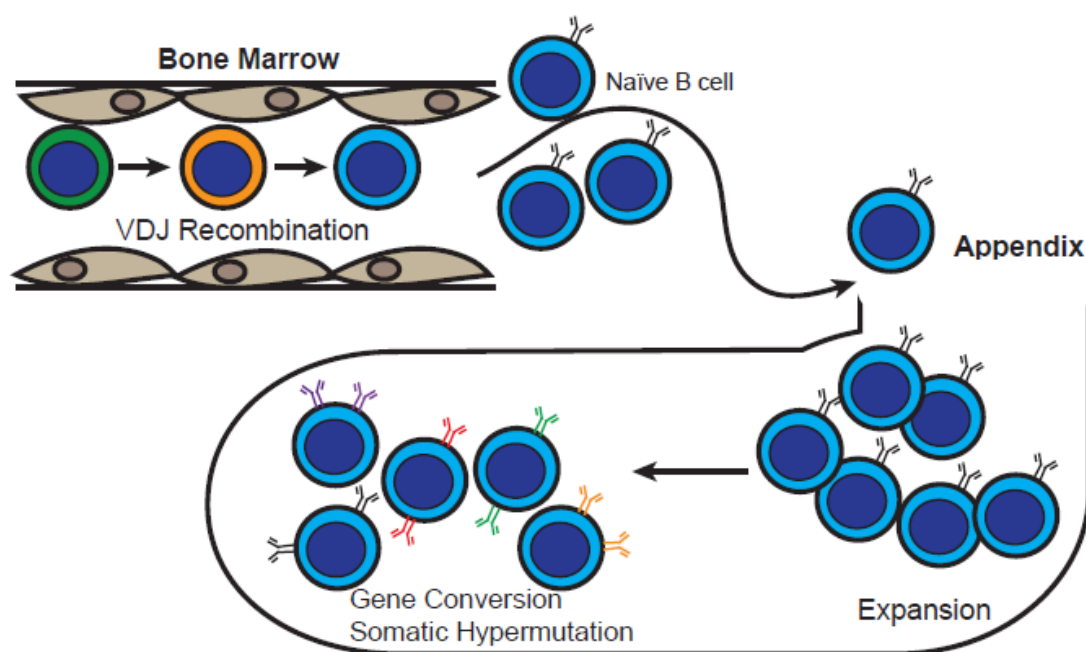
Figure 1.4 Rabbit B cell development and maturation in the BM and

appendix. B lineage cells develop from hematopoietic stem and progenitors cells

in the BM. VDJ gene recombination occurs in the BM, but to a limited extent (as described in the text). Development in the BM serves as a source of naïve B cells which travel to the appendix (and other GALT). In the appendix, naïve B cells undergo expansion and somatic diversification. The primary mechanisms by which rabbit B cells generate a diverse primary antibody repertoire is through gene conversion and somatic hypermutation, occurring in GALT. (Adapted from Kennedy et al., 2016).

The bone marrow microenvironment

As described above for mice, humans, and rabbits, B cell development is an orchestrated process where any given differentiation (eg. *Igµ* VDJ gene recombination, *Igκ* VJ gene recombination) or proliferation (expansion of large preB cell) event must occur at the appropriate time. While these events appear to occur intrinsically within B lineage progenitors, the timing of each step is mediated by signals provided by supporting cells in the BM microenvironment (Nagasawa, 2006). For example, mouse large preB cells expand before transiting to the small preB cell stage to undergo κ light chain recombination. Proliferation cannot occur simultaneously with DNA recombination, as this may lead to DNA translocations that result in cell death or transformation (Hamel et al., 2014). To separate expansion and recombination preB cells integrate signals from the BM microenvironment. Large preB cells require IL-7 from the BM microenvironment to proliferate, but must lose the IL-7 signal and gain pre-BCR signaling to rearrange the *Igκ* locus (Johnson et al., 2008, Mandal et al., 2009,

Ochiai et al., 2012). Therefore different stromal cell types form niches that support successive stages of B lymphopoiesis.

The use of primary BM stromal cells and BM stromal cells lines (such as OP9 cells) to support B lymphopoiesis *in vitro* (Collins and Dorshkind, 1987, Holmes and Zuniga-Pflucker, 2009, Hunt et al., 1987, Whitlock and Witte, 1982, Whitlock et al., 1987) further suggests that BM stromal cells produce molecules important for this process. Additional studies identified several BM stromal cell types that support B cell development including osteoblasts, reticular cells, and endothelial cells (Calvi et al., 2003, Jacobsen and Osmond, 1990, Kiel et al., 2005, Lichtman, 1981, Taichman et al., 1996, Tokoyoda et al., 2004, Weiss, 1976, Zhu et al., 2007).

Osteoblasts. Osteoblasts are derived from mesenchymal stem cells (MSC) and can be identified by markers that include alkaline phosphatase, type I collagen, and osterix (Fakhry et al., 2013, Murshed et al., 2005, Nakashima et al., 2002). Aside from their function in bone building, they also serve an important role during hematopoiesis. Mature osteoblasts are typically found along the endosteal wall of cortical bone and have been visualized in close proximity with hematopoietic stem and progenitor cells HSPCs) (Calvi et al., 2003, Zhang et al., 2003). *In vitro* cultures suggest osteoblasts support B lymphopoiesis, and *in vivo* ablation of these cells results in impaired B lineage development (Visnjic et al., 2004). Osteoblasts support general hematopoiesis by providing a niche for HSCs, and support B lymphopoiesis by producing factors such as CXCL12 and IL-7.

One example of osteoblast-mediated support came from a study of mice with a genetic deletion that blocked signaling downstream of the PTH/PTH peptide receptor (PRR) specifically in osteoblasts. Signaling through PRR on osteoblasts is known to enhance support for B lineage development (Zhu et al., 2007); therefore you would expect blockade of this pathway will result in altered B cell development. In fact these mice displayed a decreased amount of trabecular bone and impaired development from the proB to preB stage (Wu et al., 2008). Interestingly, osteoblasts in these mice exhibited decreased expression of IL-7, a critical factor for proB cell development.

Reticular Cells. Reticular cells are abundant in the BM microenvironment and have been implicated in providing niches for B cell development (Tokoyoda et al., 2004). CXCL12^{hi} reticular cells support HSCs and early hematopoietic progenitors. These cells often surround endothelial cells and are an important component of the vascular HSC niche (described below). CXCL12^{hi} reticular cells were found to be distinct from IL-7 expressing stromal cells suggesting CXCL12 and IL-7 expressing stromal cells make up distinctive niches. Overall, these reticular stromal cells and osteoblasts provide critical molecules for developing B lineage progenitors.

Adipocytes. Adipocytes are a major contributor to the state of the BM microenvironment. These cells, and how they contribute to hematopoiesis, will be covered in Section 3, and are a key focus of this dissertation.

Stromal cell-derived supportive factors

CXCL12. CXCL12 is a 10kDa chemokine that interacts with its receptor CXCR4. CXCR4 is a seven transmembrane spanning G-protein coupled receptor (GPCR). CXCL12 has the alternative names stromal derived factor 1 (SDF-1) and preB cell growth stimulating factor (PBSF) alluding to its production by stromal cells and its ability to stimulate B lineage progenitors. CXCL12$^{-/-}$ and CXCR4$^{-/-}$ mice are embryonic lethal due to improper development of the heart, nervous system, and vasculature. Additionally these mice have impaired B lymphopoiesis and myelopoiesis resulting in the inability to recruit hematopoietic progenitors to their niche (Nagasawa et al., 1994, Nagasawa et al., 1996, Peled et al., 1999, Tachibana et al., 1998, Zou et al., 1998). To assess the requirement of CXCL12-CXCR4 signaling during adult B cell development, fetal liver CXCR4$^{-/-}$ hematopoietic progenitors were adoptively transferred into lethally irradiated WT mice. Mice reconstituted with CXCR4$^{-/-}$ hematopoietic progenitors had defects in B lineage development (Kawabata et al., 1999, Ma et al., 1999). A similar result was seen in adult mice genetically engineered to remove the CXCR4 gene upon poly(I)-poly(C) administration(Sugiyama et al., 2006), suggesting CXCL12-CXCR4 signaling is required for normal B lymphopoiesis.

IL-7.This BM stromal cell-derived factor is required for B cell development. In fact, von-Freeden-Jeffry reported in 1995 that IL-7$^{-/-}$ mice were the first identified single cytokine knockout mouse with severe defects in lymphocyte development (von Freeden-Jeffry et al., 1995). Together, studies of IL-7$^{-/-}$ and IL-7R$^{-/-}$ mice identified IL-7 as a critical factor early in development as these mice

exhibit a loss of B lymphopoiesis at the proB cell to preB cell stage (Peschon et al., 1994, von Freeden-Jeffry et al., 1995). IL-7 promotes the expansion of proB cells and large preB cells (Clark et al., 2005, Clark et al., 2014, Hardy et al., 1991, Herzog et al., 2009), while later stages do not rely on this cytokine. IL-7R signaling is lost in downstream B lineage progenitors, which is a requirement for successful transition into the small preB cell stage and *Igκ* light chain rearrangement (Clark et al., 2014, Johnson et al., 2008, Mandal et al., 2009, Ochiai et al., 2012).

SCF, Flt3L, and IGF-1. Additional supportive molecules produced by the BM microenvironment include stem cell factor (SCF), Flt3-Ligand (Flt3L), and insulin-like growth factor (IGF-1). SCF binds hematopoietic cells through its receptor c-kit. Evidence that the SCF-c-kit interaction supports B cell development came from the study of mice with mutant c-kit, which exhibited impaired B lymphopoiesis (Waskow et al., 2002). Additionally, Driessen et al. found membrane bound SCF to be important for HSC attachment to the endosteal HSC niche (Driessen et al., 2003). Similar to c-kit mutant mice, Flt3L$^{-/-}$ mice (McKenna et al., 2000) and IL-7R$^{-/-}$Flt3L$^{-/-}$ (Jensen et al., 2008, Sitnicka et al., 2003) also have defects in B lineage development. For IGF-1, studies suggest that stromal cell-derived IGF-1 is important for enhancing the proliferative signal provided to proB cells by IL-7 (Gibson et al., 1993, Landreth et al., 1992). These and other microenvironment-derived factors are important to support B cell development, but successful development is only achieved when these signals are provided at the correct time. Therefore different niches exist in

the BM to support the development of each progenitor stage ranging from the

HSC to immature B cells which leave the BM.

Development in the context of different niches

B lineage progenitors require different signals from the BM

microenvironment to progress through successive stages of development. This

notion is supported by studies that visualized various B lineage progenitors in

close contact with stromal cells expressing cytokines required to support B cell

development in a stage dependent manner (Tokoyoda et al., 2004). Together,

these observations give rise to a model where micro-niches, made up of a subset

of BM stromal cells, usher B lineage progenitors through a series of

differentiation steps.

HSCs in the endosteal versus vascular niches. There are two primary

sites that serve as niches for HSCs in the BM; the endosteal niche and the

vascular niche (Figure 1.5). Calvi et al. and Zhang et al. (2003) described the

endosteal niche where HSCs are supported by osteoblasts lining the endosteal

bone surface (Calvi et al., 2003, Zhang et al., 2003). These osteoblasts are the

source of secreted, as well as membrane bound factors that promote HSC

maintenance. For example, osteoblasts produce the molecules Ang1,

osteopontin, and SCF. Ang1 and osteopontin help maintain HSC quiescence

(Arai et al., 2004, Guerrouahen et al., 2011, Nilsson et al., 2005), while SCF

through interactions with c-kit on HSCs promotes adherence of HSCs to this

niche (Driessen et al., 2003). Further, a specialized form of osteoblast in the

endosteal niche called the N-Cadherin$^+$CD45$^-$ osteoblastic cell (SNO) was found

to be in close contact with HSCs through an N-cadherin mediated mechanism (Guerrouahen et al., 2011).

The visualization of HSCs located away from the bone surface, in close contact with endothelial cells forming sinuses, provided evidence of what is now known as the vascular niche (Kiel et al., 2005). Similar to the endosteal niche, Sugiyama et al. found that the HSC vascular niche is dependent on CXCL12-CXCR4 signaling (Sugiyama et al., 2006). In contrast, the source of CXCL12 was believed to come from CXCL12^{hi} reticular cells (CAR) (Tokoyoda et al., 2004) that surround endothelial cells and are critical for support.

Studies of the endosteal and vascular HSC niches suggest these locations have different roles in the maintenance of HSC quiescence. As described above, the endosteal niche provides molecules, such as Ang-1, osteopontin, and SCF that promote quiescence. Low oxygen levels (hypoxia) characterized in the endosteal niche is thought to further promote quiescence. In contrast, the less hypoxic vascular niche provides more oxygen for proliferation/differentiation and therefore contains HSCs in a less quiescent state (Hermitte et al., 2006, Shima et al., 2010, Suda et al., 2011, Wilson et al., 2008). In line with this idea, studies of the vascular niche suggest it provides signals promoting proliferation/differentiation. One such signal was found by Winkler et al., who identified E-selectin as a critical factor expressed by endothelial cells in the vascular niche. E-selectin promoted proliferation in HSCs, and when blocked resulted in HSC quiescence (Winkler et al., 2012). Therefore, the endosteal and vascular niches may exist to provide a balance between maintaining a long term

HSC pool and repopulating the peripheral compartments in response to various stimuli.

Progression through the BM microenvironment during B cell development. Tokayoda et al. visualized various stages of B lineage progenitors in the BM of mice, to understand their relationship with BM stromal cells (Tokoyoda et al., 2004). Hematopoietic multipotent progenitors (MPP) and pre-proB cells were visualized in close contact with CXCL12^{hi} reticular cells (CAR) dispersed throughout the BM. While MPPs were localized with the processes of CAR cells, pre-proB cells were found near the cell body. The next B lineage progenitor, the proB cell was not associated with CXCL12^{hi} CAR cells, but instead localized with IL-7^{+} stromal cells. Furthermore, preB cells were visualized away from both CXCX12 and IL-7 expressing cells. Instead, preB cells are localized with galectin-1^{+} stromal cells. Galectin-1 is a stromal cell-derived ligand for the pre-BCR, expressed on preB cells (Espeli et al., 2009, Gauthier et al., 2002, Mourcin et al., 2011). Upon successful differentiation to the immature B cell stage, these cells leave the BM and enter the periphery. In the periphery, the B cells participate in immune responses, and some eventually mature to plasmablasts. Plasmablasts express CXCR4 and home to the BM, where terminally differentiated plasma cells are found localized to CXCL12^{hi} reticular cells (Tokoyoda et al., 2004) (Figure 1.5).

The BM is a complex space where many processes take place. In addition to the cell types described above, the BM is also home to additional stromal cells in different stages of maturity (eg. immature stages of osteoblasts and

adipocytes). Alternatively, hematopoietic lineage cells, such as osteoclasts and

progenitors differentiating into lineages other than the B lineage also contribute to

the overall BM microenvironment. The identification of niches for each stage of

development clarifies how so many processes can occur.
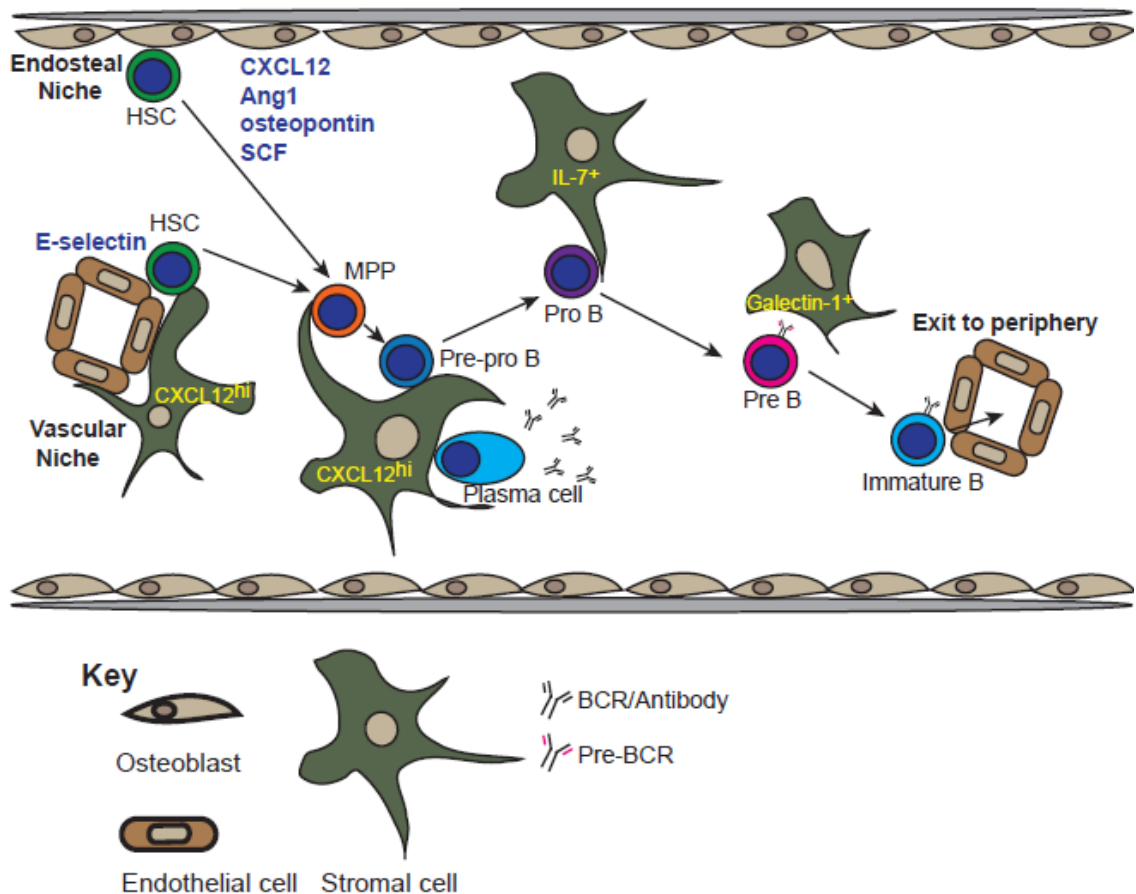
Figure 1.5 BM niches for B cell development. HSCs can be found at the

endosteal and vascular niches. The endosteal niche is formed by osteoblasts

which line the bone surface, and provide important molecules to HSCs (indicated

in blue). The vascular niche is made up of endothelial cells and CXCL12^{hi}

reticular cells which provide support for HSCs through molecules like E-selectin.

MPPs are known to localize to the ends of CXCL12^{hi} reticular cells, while pre-proB cells are found at the cell body. ProB cells are found in a distinct niche in contact with IL-7$^+$ stromal cells. PreB cells migrate away from IL-7 producing stromal cells and undergo pre-BCR activation from galectin-1 producing stromal cells. Once reaching the immature B cell stage, these cells then leave the BM to further mature and partake in antibody responses. Upon successful completion of germinal center reactions in the periphery, plasmablasts traffic back to the BM where terminally differentiated plasma cells are found in contact with CXCL12^{hi} reticular cells. (Adapted from Nagasawa, 2006).

SECTION 2: DECLINE OF B LYMPHOPOIESIS IN MAMMALS

B lymphopoiesis is first found in the fetal liver before moving to the BM in humans, mice, and rabbits. The BM serves as the primary site of B cell development post birth, producing naïve B cells which can then enter the periphery. As these mammals age, changes to hematopoietic progenitors and the BM microenvironment results in reduced production of new B cells. While this process wanes on a different timeline in each species, many studies in humans, mice, and rabbits have contributed to our current understanding of how this may occur. Age-related defects to B cells and their development result in increased susceptibility to infection and decreased immune responses to vaccination, as seen in the elderly (Frasca et al., 2011, McElhaney and Effros, 2009). Therefore understanding the mechanisms underlying these changes will identify targets for therapeutics aimed at rejuvenating B cell development in aged individuals.

Decline of B lymphopoiesis in mice

The B cell developmental process and age-related decline is best characterized in mice. Multiple studies using different mouse strains have characterized this decline and have started to uncover various intrinsic and extrinsic mechanisms contributing to reduced B lymphopoiesis in aged mice.

Decreased frequency and production of B lineage precursors with age. Several studies have characterized a reduction in frequency or absolute number of specific B lineage precursors in aged mice. For example Stephan et al. (1996) asked if the number of B lineage precursors in the BM of BALB/c mice varied at 1,4,12, and 24 months of age (Stephan et al., 1996). Looking for pre-proB and proB cell numbers, no difference was found. Alternatively, the number of preB cells was reduced as a consequence of aging. The decline in preB cell number occurred in two steps; an initial reduction was found between 1 and 4 months of age, and further decreased between 12 and 24 months. This study is further supported by others who reported reduced preB cell pools with age (Kirman et al., 1998, Riley et al., 1991, Sherwood et al., 1998).

Consistent with observations of decreased preB cell number and percentage in the BM with age, Johnson et al (2002) found the production rate of preB cells was also reduced in aged mice (Johnson et al., 2002). While the rate of new immature B cells downstream of the preB cells was not found to be different between young and old mice, the authors suggest aged immature B cells have impairments in refilling more mature B cell pools in the periphery.

In addition to reduced preB cell pools in the BM of aged mice, several other studies found reductions in additional B lineage precursor stages with age. These observed differences in characterizing the decline of B lymphopoiesis can be explained by mouse strain differences or by the use of additional markers to define precursors. The studies discussed above characterized B cell development in BALB/c mice, while the following studies performed experiments using C57BL/6 mice and used AA4.1 as an additional B lineage marker. In C57BL/6 mice, Miller and Allman assessed the absolute number and frequency of EBP/CLPs (Lin$^-$ IL-7Rα^+ AA4$^+$ Sca-1^{low}), pre-proB cells (Ly6C$^-$ CD24/HSA$^-$B220$^+$ AA4$^+$), and proB cells (B220$^+$ CD43$^+$ CD19$^+$ AA4$^+$) in mice 2, 7, 10, 14, 20, and 24 months of age (Miller and Allman, 2003). In female and male mice, each of these populations declined as a function of aging. In females, EBP/CLPs made up 0.058% of BM at 2 months and 0.001% at 24 months, pre-proB cells 0.191% (2 month) vs. 0.047% (24 months), and proB cells 0.673% (2 months) vs. 0.084% (24 months). Assessing male mice, EBP/CLPs made up 0.042% of BM at 2 months and 0.007% at 18 months, pre-proB cells 0.189% (2 month) vs. 0.051% (18 months), and proB cells 0.564% (2 months) vs. 0.078% (18 months). Overall these early B lineage precursors were found at lower frequency and absolute number in aged mouse BM, leading the authors to conclude that the decreased preB cell pool, often seen in aged mice by other studies, could be in part due to diminished populations of earlier B lineage precursors that occur with aging.

A study by Min et al. also assessed the effect of senescence on B lineage development (Min et al., 2006). The authors expected that exposing the hematopoietic system to stress could uncover subtle impairments that normally occur during aging. To this end, young and old mice were characterized for age-related changes affecting early B lineage progenitors during homeostasis or after 5-fluorouracil (5-FU) treatment. In addition to confirming the observations by Miller and Allman during normal aging (Miller and Allman, 2003), 5-FU treatment uncovered proliferative defects in CLPs and pre-proB cells, as well as an impaired differentiation capacity in CLPs from aged mice. The cumulative data produced through all the aforementioned studies define the outcome of age-related changes and how they affect B cell development. Upon establishing this phenomenon, additional studies continue to expand our mechanistic understanding of this decline.

Mechanisms that contribute to declining B lymphopoiesis. Successful development from the HSC to immature B cell stage requires healthy hematopoietic progenitors, as well as the appropriate BM stromal cell support. Intrinsic changes in hematopoietic progenitors and extrinsic changes to the BM microenvironment are both known to contribute to the decline of B lymphopoiesis in aged mice. This becomes evident when comparing several studies that performed adoptive transfers of aged HSCs/BM cells into young irradiated recipients, and obtained conflicting results. The expectation is if intrinsic defects in BM progenitors drive impaired B lymphopoiesis, then transfer of old BM progenitors into a young BM microenvironment will not restore B lineage

development. If the microenvironment is responsible for the decline, then transfer of aged BM progenitors into a young BM microenvironment will restore B lineage development from aged BM donors. In fact, several studies found normal B cell development of aged BM progenitors when transferred into young recipients (Chen et al., 1999, Miller and Allman, 2005, Morrison et al., 1996). Alternatively, transfer experiments into young hosts by Sudo et al. did not yield B lineage cells, while myeloid cells did develop (Sudo et al., 2000); suggesting age-related intrinsic changes affect hematopoietic progenitor potential. Because both intrinsic and extrinsic mechanisms result in declining B cell development, it is important to understand how these changes affect different stages of development.

Aging affects even the earliest hematopoietic progenitors. HSC numbers are increased in old mice compared to young mice, but their quality is reduced (Geiger et al., 2013). A major characteristic of aging hematopoiesis is unbalanced production of fewer lymphocytes and increased myeloid cells. This may be due to intrinsic changes in HSCs, as gene expression profiling found aged HSCs to have upregulated myeloid lineage and decreased expression of lymphoid lineage genes (Rossi et al., 2005). This may also be due to intrinsic differences in lymphoid and myeloid biased HSCs resulting in more myeloid biased HSCs and fewer lymphoid biased HSCs maintained through aging (Muller-Sieburg and Sieburg, 2008).

The reduced preB cell pool during aging observed by Stephan et al. (1996) can be explained by changes in B lineage precursors, as well as the environment. The authors found that preB cells from aged mice had a decreased

ability to proliferate when cultured on BM stromal cells, compared to preB cells

from young mice (Stephan et al., 1996). Impaired responsiveness to IL-7 was

also found in earlier B lineage progenitors, such as proB cells in both BALB/c and

C57BL/6 mice (Miller and Allman, 2003, Stephan et al., 1998). Another difference

found is that preB cells from aged mice exhibit decreased expression of the SLC

components VpreB and λ5, which are necessary for pre-BCR surface expression

in preB cells (Sherwood et al., 1998, Sherwood et al., 2000). Whether due to a

lack of pre-BCR signaling or not, aged preB cells were found to be more

apoptotic upon isolation from BM and after *in vitro* culture (Kirman et al., 1998).

Multiple B lineage precursors have impaired responsiveness to IL-7 with

age, but BM stromal cells were also found to change with age. In fact, primary

BM stromal cells isolated from aged mice could not stimulate IL-7 dependent B

lineage cell lines as efficiently as BM stromal cells isolated from young mice

(Stephan et al., 1998). This suggests BM stromal cells from aged mice have a

lower supportive capacity for B cell development. BM stromal cells isolated from

young and old mice were actually found to have similar IL-7 protein levels, but

the defect in aged BM stromal cells appeared to be in IL-7 release.

One of the most powerful examples linking the aged microenvironment to

declining B lymphopoiesis came from Labrie et al (Labrie et al., 2004) In this

study, it was established that aged proB cells exhibit decreased V(D)J

recombinase activity. To demonstrate this impairment was due to the aged BM

microenvironment, the authors performed a series of transfer experiments. Aged

BM hematopoietic progenitors were transferred into young recipients, which

resulted in restored V(D)J recombinase activity in proB cells. To complement this experiment, young BM hematopoietic progenitors were transferred into aged recipient mice, leading to impaired V(D)J recombination in developing proB cells. Together, these results suggest altered V(D)J recombinase activity in proB cells is dependent on changes to the BM microenvironment that accumulate during aging.

   The systemic environment and the decline of B cell development. In addition to changes in hematopoietic progenitors and the BM microenvironment, one study provides evidence that the peripheral B cell pool that accumulates with age negatively regulates B lymphopoiesis. Keren et al. used three approaches to deplete peripheral B cells in an attempt to boost B cell development (Keren et al., 2011). By generating conditional BAFF receptor deficient mice, or using various strategies to deplete peripheral B cells with anti-CD19, anti-B220, anti-CD22 and anti-CD20 the authors were able to enhance B cell development. In aged mice, depletion of B cells also resulted in increased B lymphopoiesis and an enhanced antibody response. Although increased, B cell depleted aged mice did not completely recover B lymphopoiesis and antibody responses to levels seen in young mice. This suggests that while the peripheral B cell pool may contribute to decreased B lineage development, intrinsic and BM extrinsic factors likely also contribute.

   The study by Keren et al. brings to light the idea that systemic factors, whether generated locally or across the body, can regulate processes occuring in the BM. Also, further studies will be needed to see if peripheral B cells negatively

regulate B cell development through soluble factors, or if they traffic to the BM to exert their effect. Consistent with this study, Montaudouin et al. found that the amount of IgG in circulation can regulate the number of IgM producing B cells in the periphery through an interaction with the inhibitory Fc receptor, FcγRIIB (Montaudouin et al., 2013). It will be interesting to see if B lineage progenitors also have a similar ability to quorum sense and regulate new B cell output based on demand. Alternatively, aged B cells (ABC) which accumulate with age were also found to inhibit B cell development through TNFα production (Ratliff et al., 2013). Future studies will also be needed to determine if the depletion strategies used by Keren et al. altered a quorum sensing system and/or depleted inflammatory B cells to boost new B cell generation.

Decline of B lymphopoiesis in humans

Evidence of early B lymphopoiesis can be found in the yolk sac of the human embryo, and appears in the fetal liver at day 8. At week 12, the BM becomes the primary site of B cell production through 80 years of life (Nunez et al., 1996). Several studies suggest that B lineage development declines with age in humans. Although there are some conflicting data between studies, the majority find reductions in B lineage progenitors and ability to differentiate to the B lineage.

Decreased Frequency of B lineage cells with aging. Difficulty in collection of BM from healthy donors often limits the number of extensive studies performed on this population in humans. In spite of this restriction, several investigators were able to characterize the frequency of B lineage progenitors in

BM as a function of age. Rego et al. examined the frequency of $CD10^+CD19^+$ B

lineage (proB cells) present in sternums from individuals of various ages (Rego

et al., 1998). Of the age groups analyzed, this study found the highest frequency

of $CD10^+CD19^+$ cells in the first four years of life. The frequency then decreased

with increased age being the lowest in individuals categorized as being >15

years of age.

Another study analyzed early B lineage cells from human rib sections

ranging in age from fetal development to 80 years of age (Nunez et al., 1996).

Several B lineage progenitor cell types were analyzed, including: $CD19^+sIgM^-$,

$CD10^+sIgM^-$, and $CD24^+sIgM^-$. Looking at $CD19^+sIgM^-$ pro/preB cell frequencies

in human BM samples, the authors found an age-related decrease. The BM

mononuclear cells of a 19 week fetus and 3 year old contained 55% and 44%

$CD19^+sIgM^-$ preB cells, whereas this population was reduced to 15% in the BM of

a 56 year old individual. Similarly identifying B lineage precursors as $CD10^+sIgM^-$

or $CD24^+sIgM^-$, there was a reduced frequency in the 56 year old BM sample.

A study by McKenna et al., also supports the above studies (McKenna et

al., 2001), finding a decreased frequency of B lineage precursors in BM samples

from aged individuals. However, a study by Rossi et al is inconsistent with the

other studies cited. These authors did not find significant age-related differences

in B lineage precursor frequencies in BM taken from hip surgeries (Rossi et al.,

2003). Overall, there are some inconsistencies between studies focused on

human B lymphopoiesis and aging. Obtaining enough healthy BM samples of

various ages is a limitation that affects the statistical power of these studies.

Additionally, differences in BM collection methods and BM samples from different types of bone likely contribute to inconsistencies between studies. Access to tissues (including BM) from young and old healthy donors is often a limitation to aging studies in humans. Establishment of a bank for such tissues was recently discussed this past January 2016 at the "Effects of aging on hematopoiesis" symposium at the National Institutes of Health (sponsored by the NIDDK and NIA).

Reduced B lineage potential in aged HSCs. The reduction in B lineage progenitors may be the result of a reduced capacity for HSCs to differentiate to the B lineage. In fact, Pang et al. found this to be true (Pang et al., 2011). HSCs isolated from human donors 20-35 years or >65 years were compared for their potential to differentiate into the B lineage vs. myeloid lineage *in vitro*. Compared to young HSCs, HSCs from individuals >65 years exhibited reduced B lineage potential while maintaining myeloid potential. In addition, xenotransplantation of human HSCs into immunodeficient mice resulted in a greater production of myeloid cells compared to B lineage cells from aged human HSCs. These data, in conjunction with aged HSCs showing increased expression of myeloid lineage genes, provide mechanistic evidence for declining B lymphopoiesis in individuals >65 years of age (Pang et al., 2011).

Changes to the BM microenvironment. In addition to reduced B lineage potential by HSCs, changes to the BM microenvironment may also contribute to the loss of B cell development. Alterations to mesenchymal stem cells (MSC) or their progeny; osteoblasts and adipocytes may affect hematopoiesis. Osteoblasts

are known to support B cell development, while adipocytes have a negative impact on this process (Bilwani and Knight, 2012, Naveiras et al., 2009, Zhu et al., 2007). Therefore decreases in osteoblasts and/or increases in BM adipocytes could contribute to decreased B lineage development.

   MSCs and aging. Several studies characterized the frequency of MSCs found in human BM during aging. One study saw a decrease in frequency of MSCs in BM occurring at 30 years of age compared to newborns (Caplan, 2007). In contrast with this study, studies by Justesen et al., and Stenderup et al. did not find decreases in MSC frequency between young and old (Justesen et al., 2002, Stenderup et al., 2001). However, young was defined as approximately 20-40 years and old was defined as approximately 65-70 years in these later studies. Additionally, variations in methods to identify MSCs may have also contributed to these inconsistencies.

   Whether or not MSC frequency changes with age in human BM, evidence suggests these cells are different in aged individuals. One study found MSCs taken from individuals >50 years old were more prone to undergo apoptosis compared to MSCs from young donors. Aged MSCs also showed increased senescence-associated β-galactosidase, required more time to divide, and had reduced capacity to differentiate into osteoblasts (Zhou et al., 2008).

Arrest of B lymphopoiesis in rabbits

   Consistent with human and mice, B cell development in rabbits is found in the fetal liver, where preB cells can be found as early as 25 days into fetal development (Hayward et al., 1978, McElroy et al., 1981). After birth, the BM is

the primary site of hematopoiesis. In the first two weeks of life, preB cells peak, comprising 9-19% of nucleated BM cells. The frequency of preB and proB cells then drops dramatically by 2 months of age and these cells are undetectable by 4 months of age (Jasper et al., 2003).

Characterization of declining B cell development. Rabbit B cell development arrests at 2 months of age (Jasper et al., 2003, Kennedy et al., 2016), making the rabbit an accelerated model for declining B lymphopoiesis, which usually occurs in the mid to late stages of life in humans and mice (McKenna et al., 2001, Scholz et al., 2013). The early loss of B cell development is well characterized in rabbits, as evidenced by allotype suppression experiments, flow cytometry analysis of B lineage progenitor frequencies, and B cell recombination excision circle (BREC) analyses.

Allotype suppression experiments. The idea that B lymphopoiesis is short lived in rabbits came in the 1960s, when Dray performed allotype suppression experiments. Antibodies specific for a particular paternal IgH allotype were injected into neonate rabbits that were heterozygous for that paternal IgH allotype. This treatment effectively depleted the target allotype antibody in these rabbits (Dray, 1962). Looking as late as 2 years later, the suppressed allotype did not reappear. It is not likely that this specific allotype is being actively suppressed through the life of the rabbits, therefore these data suggest that B cell development is arrested early in life (Eskinazi et al., 1979). In fact, the paternal allotype never recovered even though a low frequency of paternal allotype containing progenitors could be found (Simons et al., 1979).

Therefore, it was concluded that there must be a block in B cell development.

Because mouse B lymphopoiesis does not decline until late in life, you would

expect allotype suppression experiments would not produce a lasting effect. This

is the case, as one study performing allotype suppression experiments in young

mice found the suppressed allotype reappeared 6 weeks after suppression (Lalor

et al., 1989).

      B lineage progenitor frequencies. Several studies have tracked various

B lineage progenitor frequencies as a function of time by flow cytometry. Jasper

et al., found that proB cells reach peak levels in the first few weeks following birth

(Jasper et al., 2003). Several other studies found that preB cells also reach their

highest frequencies shortly after birth (Gathings et al., 1981, Gathings et al.,

1982, Hayward et al., 1978, McElroy et al., 1981). Within the first month of life

proB and preB cells each comprise approximately 7% of hematopoietic cells in

the BM. By 2 months these populations were reduced to 1% of the BM, and

undetectable in the BM of 4 month old rabbits. These date indicate that active B

lineage development arrests by 2-4 months of age. Interestingly, Jasper et al.

(2003) noticed that as B lineage progenitors were lost, increased frequencies of

mature B cells could be found in the BM. This finding is consistent with that of

Nunez et al., who saw a similar phenomenon in human BM (Nunez et al., 1996).

      B cell recombination excision circle analysis. The early decline of

rabbit B lymphopoiesis was further confirmed on the molecular level through

BREC analysis of rabbit BM. BRECs form in B lineage progenitors during VDJ

gene recombination and can be used as a measure of ongoing B cell

development. Crane et al. utilized this method looking for BRECs formed from VD and DJ rearrangements (Crane et al., 1996). As expected, BRECs were found at high levels in newborn rabbits and reduced in adult rabbits. Consistent with the percentages of B lineage progenitors found in rabbit BM, the highest levels of BRECs could be detected in BM shortly after birth. Additionally, BREC detection was reduced at 2 months of age, and was barely detectable at 4 months of age (Jasper et al., 2003). In control experiments, young (1-2 week) and adult (4 month) mouse BM was analyzed for BRECs. Young and adult mouse BM contained robust levels of BRECs, consistent with ongoing B lymphopoiesis at this time in mice. Overall, these molecular and cellular studies of B cell development lead us to conclude that rabbit B lymphopoiesis is lost by 2-4 months of age.

Mechanisms that negatively regulate B cell development. As reviewed earlier, the decline in mouse B lymphopoiesis can be attributed to both intrinsic defects in hematopoietic progenitors and extrinsic changes to the microenvironment. To address whether intrinsic or extrinsic mechanisms contribute to the arrest in rabbit B lymphopoiesis, Kalis et al. asked if rLPs from the BM of >2 month old rabbits are capable of differentiating into B lineage cells *in vitro* (Kalis et al., 2007). The authors cultured isolated MHCII⁻IL-7R⁺ rLPs with OP9 BM stromal cells (Holmes and Zuniga-Pflucker, 2009), then assessed the number of B lineage cells resulting from these cultures. Interestingly, rLPs isolated from the BM of >2 month old rabbits were capable of differentiating into B lineage cells. To complement this finding, the authors also adoptively

transferred BM progenitors from >2 month old rabbits into young (<2 month old) irradiated rabbits. Consistent with *in vitro* culture of rLPs, BM progenitors derived from >2 month old rabbits were able to differentiate into B lineage cells after transferred into young irradiated recipients. The ability of BM cells from >2 month old rabbits to differentiate into B lineage cells suggests that the loss of B cell development in rabbits is not due to intrinsic defects in hematopoietic progenitors, and instead suggests changes to the BM microenvironment are responsible for this arrest.

B lymphopoiesis is an orchestrated process, where even subtle changes to the microenvironment can alter the output of naïve B cells. Changes in the BM microenvironment fall into two major categories; the loss of supportive factors or an increase in negative regulators. Several studies have assessed how each of these categories contributes to the decline of B lymphopoiesis in rabbits. Together, the studies reviewed next provide the basis for which B cell development is lost.

Loss of supportive microenvironment. IL-7 is one of the most critical supportive factors provided by the BM microenvironment. IL-7$^{-/-}$ mice have impaired B cell development (Tsapogas et al., 2011, Wei et al., 2000), and Stephan et al (1998) found that BM stromal cells from aged mice are defective in their ability to supply hematopoietic progenitors with IL-7 (Stephan et al., 1998). Further, high fat diet was found to impair B lineage development in mice, at least in part through decreased expression of IL-7 in the microenvironment (Adler et al., 2014). In rabbits, IL-7 was also found to be critical for B lymphopoiesis (Kalis

et al., 2007). Therefore it is plausible that a reduction of IL-7 expression in BM from >2 month old rabbits could result in impaired B cell development. To test this, Kalis et al. (2007) analyzed IL-7 expression in the BM of <2 month and >2 month old rabbits by northern blot. Surprisingly, IL-7 was found to increase in the older rabbits, suggesting decreased IL-7 is not responsible for the impairment. In a separate study, an additional isoform of IL-7 identified as IL-7II, also increased in the BM of older rabbits (Siewe et al., 2010). It was thought that this isoform could possibly have a negative impact on B lymphopoiesis. However further study of IL-7II, found that it bound IL-7R and acted similarly to canonical IL-7. From these studies, we can conclude that changes in the supportive cytokine IL-7 do not contribute to the decline of B lymphopoiesis in rabbits.

Siewe et al. set out to characterize BM stromal cells to identify supportive factors that are altered with age (Siewe et al., 2011). The authors performed a representational difference analysis (RDA), comparing MSCs isolated from a newborn rabbit and a 2 year old rabbit. Several factors were identified as downregulated in the 2 year old rabbit, with periostin (extracellular matrix protein) being the lowest expressed. The authors hypothesized that the decrease in periostin could contribute to the decline in B cell development. *In vitro*, the authors found periostin to be required for rabbit B lineage development, as siRNA knockdown of periostin in OP9 stromal cells inhibited their ability to support development. Further gene expression analysis showed that in OP9 with siRNA knock down, *IL-7* and *CXCL12* were also decreased. Periostin^{-/-} mice are readily available, and the authors found that B lymphopoiesis was not impaired in

these mice, suggesting that *in vivo* loss of periostin may be masked by other redundant supportive factors.

Expression of fibronectin, collagen type I, thrombospondin, the tumor suppressor FAT, and frizzled 4 were also found to be decreased in BM stromal cells from the 2 year old rabbit. To date, the decrease in these or other factors have not been linked to the loss of B cell development in rabbits. Additional studies assessed whether changes in support on the cellular level, as well as increases in negative regulators contribute to decreased B cell development.

Age-related changes to BM stromal cells. To identify changes in BM stromal cells at 2 months of age, Bilwani and Knight focused on MSCs which give rise to osteoblasts and adipocytes (Bilwani and Knight, 2012). The authors first examined if MSC number changes between young rabbits and older rabbits (>2 months of age). Through analysis of the number of CFU-Fibroblasts (CFU-F) (Mareschi et al., 2012), Bilwani and Knight found that MSC number was reduced 10-fold shortly after birth. In addition to decreased number, MSCs from rabbits >2 month old were more likely to differentiate into adipocytes rather than osteoblasts. This study suggests that fewer osteoblasts in the BM could contribute to decreased B lymphopoiesis, as osteoblasts support this process.

Adipocytes fill the bone marrow with age. The finding that rabbit MSCs had increased potential toward the adipocyte lineage raises some questions; do adipocytes increase in rabbit BM with age, and do adipocytes contribute to the arrest of B cell development? Interestingly, adult rabbit BM has been reported to contain significant amounts of fat (Bigelow and Tavassoli, 1984). In fact, the

accumulation of adipose tissue in the BM of rabbits mimics that of aged humans, where 50% of the femur and 70% of the tibia fills with adipose tissue (Li et al., 2013). It should also be noted that while adipose tissue increases with age in humans, mice, and rabbits (Chinn et al., 2012, Justesen et al., 2001, Lecka-Czernik et al., 2010, Rosen et al., 2009, Tuljapurkar et al., 2011), the timing correlates with declining B lymphopoiesis.

In the past, the prevailing view of BM fat was that it is inert and simply filling unused space. Recent studies have now established adipocytes as major producers of local and systemic factors that influence many processes. Because adipocytes accumulate as B lymphopoiesis declines, Bilwani and Knight tested if adipocytes influence B lineage development (Bilwani and Knight, 2012). Through culture of rabbit BM progenitors with OP9 stromal cells in the presence or absence of adipocyte-conditioned medium (ACM), it was found that cultures containing ACM had fewer CD79a^{+} B lineage cells develop. This result suggests that adipocytes produce molecules that actively inhibit B lymphopoiesis. Therefore, the loss of B cell development in aged rabbits is likely due to having decreased support from fewer osteoblasts and an increase in negative regulators produced by adipocytes. While adipocytes inhibit B lymphopoiesis, the mechanism by which this occurs is unknown.

SECTION 3: THE IMPACT OF BONE MARROW FAT AND INFLAMMATION ON LYMPHOPOIESIS

Early studies of BM fat were performed in rabbits by Tavassoli and colleagues during the 1970s. In recent years there has been renewed interest in

understanding BM fat in humans, mice, and rabbits. Adipose tissue can be described in multiple ways, such as by color (brown vs. beige vs. white), inflammatory state (pro-inflammatory vs. anti-inflammatory), and in terms of its ability to fuel hematopoiesis (regulated vs. constitutive). Here I will review critical studies which have shaped our understanding of the relationship between adipose tissue, hematopoiesis, aging, and obesity.

In addition to aging, adipocytes commonly accumulate in the BM of patients following chemotherapy and irradiation. Therefore, it is important to understand how adipocytes impact neighboring red marrow. Several studies suggest that BM fat has a negative effect on hematopoiesis (Bilwani and Knight, 2012, Naveiras et al., 2009). Naveiras et al. compared BM from mouse thoracic vertebrae (adipocyte free) and tail vertebrae (adipocyte rich) to understand if adipocytes influence hematopoietic activity. Upon analysis of hematopoietic stem and progenitor cell populations (HSPC), the frequency and number of progenitors was significantly lower in adipocyte rich tail vertebrae compared to thoracic vertebrae. A similar analysis of thoracic and tail vertebrae in fatless mice (A-ZIP/F1) showed no difference in adipocyte accumulation and similar numbers of progenitors in these locations, suggesting that the presence of adipocytes negatively affects hematopoietic progenitor numbers. Fat accumulates in the BM after irradiation and is thought to negatively impact engraftment of newly transplanted BM. To test this notion, the authors performed BM transplants in WT or fatless mice with the expectation that fatless mice would have better recovery of hematopoietic cells compared to WT. As expected, fatless mice had

significantly less BM fat than WT mice after irradiation and exhibited better recovery of leukocytes after BM transplantation. These results suggest that the presence of adipocytes negatively impacts hematopoietic activity. Coupled with a study by Bilwani and Knight that found B lymphopoiesis to be particularly sensitive to adipocyte factors in human and rabbit cultures (Bilwani and Knight, 2012), these studies suggest strategies to limit adipocytes in BM would enhance hematopoietic activity.

White, brown, and beige adipocytes

Adipocytes are typically referred to as white, brown, or beige. White adipose tissue is best known for its role in energy storage, expanding in states of nutrient excess and contracting with a negative energy balance. Recent advances in adipose tissue biology have also identified white adipose tissue as an endocrine organ, able to integrate metabolic signals and produce adipokines that influence other organs (Ouchi et al., 2011). Consistent with its ability to respond to nutrient state, adipose tissue is thought to become a source of pro-inflammatory cytokines in scenarios of high fat diet (Chatterjee et al., 2009, Ouchi et al., 2011). The inflammatory state of adipose tissue will be discussed further below.

Brown adipocytes. Brown adipocytes produce heat through the process of thermogenesis. While brown adipocytes also accumulate lipids, they can be distinguished from white adipocytes through their abundant numbers of mitochondria and high levels of uncoupling protein1 (UPC1), which are critical components for heat production (Cannon and Nedergaard, 2004).

Thermogenesis from brown adipocytes is important to maintain body temperature and is typically induced by cold stress (Peirce et al., 2014). This process requires large amounts of energy, therefore making brown adipose tissue is an important regulator of energy balance. In fact, Rothwell and Stock (1979) discovered diet induced thermogenesis in rats, where heat production was activated by brown adipose tissue in response to overeating (Rothwell and Stock, 1979). The authors further suggested that brown adipose tissue may counteract obesity.

Lowell et al. tested if brown adipose tissue counteracts obesity by generating two transgenic mouse lines that are deficient in brown adipocytes (Lowell et al., 1993). Both mouse lines developed obesity, identifying a role for brown adipose tissue in the prevention of obesity. Consistent with this result, one of the transgenic mouse strains was able to recover brown adipose tissue over time, which was coupled with the resolution of obesity in these mice. Similar results were seen in a study by Feldman et al. while studying UCP1 deficient mice (Feldmann et al., 2009). As expected the enormous amount of energy utilized by brown adipocytes during thermogenesis is key to the anti-obesity effect, as Cannon and Nedergaard found that mice kept in cooler temperatures needed to eat more calories to maintain the same body weight as mice kept at warmer temperatures. Mice kept at room temperature need to eat two thirds more than mice kept at 30° to stay at a consistent body weight (Cannon and Nedergaard, 2009, Peirce et al., 2014).

Beige and white adipocytes. Beige adipocytes are similar to brown adipocytes in that they are capable of producing heat through thermogenesis

(Vitali et al., 2012). Beige adipocytes are induced in white adipose tissue suggesting that they share a precursor with white adipocytes, but they behave like brown adipocytes which come from a distinct precursor cell (Seale et al., 2008). In other terms, brown adipose tissue consists of UCP1$^+$ brown adipocytes capable of producing heat. White adipose tissue contains UCP1$^-$ white adipocytes that store excess energy and produce various adipokines, while also containing UCP1$^+$ beige adipocytes which are induced to undergo thermogenesis by various activators (Petrovic et al., 2010, Wu et al., 2013). Because beige adipocytes expend large amounts of energy, they may also contribute to the prevention of obesity (Harms and Seale, 2013). Therefore it is logical that changes in adipose tissue leading to less brown/beige fat and increased white fat, may contribute to the pro-inflammatory state seen during aging and obesity.

Age-related change in BM adipose tissue phenotype. Krings et al. characterized BM fat in young/adult (5 months) and old mice (24-26 months) for brown and white adipocyte characteristics (Krings et al., 2012). While BM adipocytes were increased in aged mice, there were also specific changes in the type of adipocytes present with increased age. BM adipose tissue was analyzed for the expression of six genes characteristic of brown adipose tissue including UCP1, as well as leptin and adiponectin which are characteristic of white adipose tissue. Young/adult BM adipose tissue expressed genes characteristic of both brown and white adipose tissue, suggesting BM fat has a brown and white, or beige phenotype. This phenotype, however, changed with age. Compared to young/adult mice, old BM adipose tissue lost features of brown adipose tissue,

and more closely resembled white adipose tissue. The resemblance of aged BM

fat to white adipose tissue is also supported by an observation by Bainton et al.

who observed that yellow marrow stromal cells in adult rabbits had

characteristics of 3T3.L1 white adipocytes (Bainton et al., 1986). The changes in

BM adipose tissue phenotype with age, suggests there are also functional

consequences. Krings et al. found the age-related changes in BM adipose tissue

to be consistent with the phenotype of BM adipose tissue in yellow agouti

diabetic mice. Together these results led the authors to suggest the changes in

BM adipose tissue during aging and diabetes likely contribute to the negative

effect on hematopoiesis seen in these conditions.

Regulated versus constitutive marrow fat

Several studies suggest that fat has a negative effect on

hematopoiesis/lymphopoiesis (Bilwani and Knight, 2012, Naveiras et al., 2009).

In light of the studies described above (in this section), it is now clear that

adipose tissue and the types of adipocytes that comprise adipose tissue are very

complex. Therefore, it is likely that certain types of adipocytes support

hematopoiesis in young healthy mammals, but age and obesity related changes

to BM adipose tissue results in a negative impact on hematopoiesis.

In support of this idea, several studies have categorized marrow fat as

regulated or constitutive. This categorization refers to marrow fat and its

relationship to hematopoiesis. In the 1970s, Tavassoli observed two types of

adipocytes in rabbit BM differentiated by their fatty acid composition through

staining with performic acid Schiff reagent (PFAS) (Tavassoli, 1976). PFAS[+]

adipocytes are found in red marrow and are thought to fuel hematopoiesis, as treatment of rabbits with hemolysis agents stimulated hematopoiesis and depleted PFAS$^+$ adipocytes. Therefore PFAS$^+$ adipocytes in red marrow are now referred to as regulated marrow fat rMAT (Scheller and Rosen, 2014). In contrast to rMAT, PFAS$^-$ adipocytes are found in yellow marrow and are not deleted by hematopoiesis. In fact, PFAS$^-$ marrow fat appears to be unresponsive to changes in diet, as 10 days of starvation did not reduce the volume of fat in the distal tibia of rabbits (Tavassoli, 1974). Therefore, this type of marrow fat was termed constitutive marrow fat (cMAT). Similar to that seen in mice (Naveiras et al., 2009), increased marrow fat was also found to correlate with reduced hematopoiesis in rabbits (Bigelow and Tavassoli, 1984).

While more studies are needed to link the way various studies have described adipocytes (color, inflammatory state, in terms of supporting hematopoiesis), it is likely that the age/obesity related loss of brown characteristics and dysregulation of white adipose tissue resulting in increased danger signals and pro-inflammatory cytokines lead to a negative impact on lymphopoiesis.

Adipocytes, inflammation and the decline of T lymphopoiesis

Inflammatory mediators and lipotoxic danger signals from adipose tissue have been implicated in thymic atrophy and the decline of T cell development during aging and obesity. Similar to the BM, adipose tissue accumulates in the thymus during atrophy with age. Youm et al. hypothesized that increased adipose tissue in the thymus provides pro-inflammatory danger signals that

contribute to thymic decline. By comparing young and old (24 month) WT or

inflammasome deficient (NLRP3$^{-/-}$ or ASC$^{-/-}$) mice, the authors found that blocking

inflammasome activation delayed thymic atrophy (Youm et al., 2012). This study

suggested that inflammatory danger signals that accumulate with age negatively

regulate T lymphopoiesis. Adipose tissue derived products and diet were further

linked to thymic decline as high fat diet accelerated and calorie restriction

delayed thymic atrophy (Yang et al., 2009a, Yang et al., 2009b). It is unknown

whether B lymphopoiesis is negatively regulated through inflammasome

activation. While B cell development is negatively regulated by adipocyte

products, the responsible molecules remain to be identified.

Adipose tissue derived molecules

Adipose tissue is made up of adipocytes, as well as infiltrating immune

cells. As a whole, the pro-inflammatory or anti-inflammatory state of adipose

tissue is representative of the molecules produced by adipocytes and immune

cells. Healthy adipose tissue is characterized by unstressed adipocytes and anti-

inflammatory immune cells (such as M2 suppressive macrophages) (Chawla et

al., 2011, Mraz and Haluzik, 2014). Adipose tissue seen in aging and obesity is

pro-inflammatory in nature, resulting in the production of danger associated

molecular patterns (from stressed adipocytes) and inflammatory cytokines (from

adipocytes and immune cells) (Chawla et al., 2011, Lago et al., 2007, Wen et al.,

2011, Youm et al., 2012, Youm et al., 2013). Pro-inflammatory adipose tissue is

also characterized by an increase in dying adipocytes, pro-inflammatory M1

macrophages, and interferon-γ producing TH1 cells (Chawla et al., 2011, Winer

et al., 2009). While adipose tissue is the source of many molecules, some of the most studied are highlighted below.

Adipocytes secrete many factors that influence processes throughout the body, either acting as an endocrine organ or locally. Recent research has identified adipose tissue as an immune organ. Adipocytes secrete many classes of molecules including: Proteins and lipids.

Proteins. Adipocytes secrete many immune-modulatory proteins, such as, adiponectin, TGFβ, and leptin. Adiponectin and TGFβ are known to inhibit B lymphopoiesis indirectly by acting on stromal cells in mouse co-culture experiments(Tang et al., 1997). Conversely, adipocytes also have the potential to support B lymphopoiesis through the production of leptin(Claycombe et al., 2008).

Lipids. Adipocyte-derived lipids consist of a variety of bioactive molecules classified based on their relative insolubility in water and include steroids, fatty acids, and fatty acids derivatives. For example, adipocytes can produce the steroid estrogen(Bulun and Simpson, 1994, Grodin et al., 1973, Nelson and Bulun, 2001, Yamada and Harada, 1990), which was found to inhibit B lymphopoiesis by acting directly on the CLP and acting indirectly on stromal cells(Kouro et al., 2001, Smithson et al., 1995, Yokota et al., 2008). Prostaglandins, a fatty acid derivative, are also known to inhibit B lymphopoiesis by directly acting on B lymphocyte progenitors(Yokota et al., 2003). Additionally, adipocytes produce fatty acids. It is unclear whether fatty acids positively or negatively regulate B lymphopoiesis, but fatty acids are known to activate TLRs

on macrophages and adipocytes promoting the production of inflammatory

cytokines and reactive oxygen species (ROS)(Han et al., 2012, Huang et al.,

2012, Shi et al., 2006, Wong et al., 2009).

Anti-inflammatory molecules

      Adiponectin. Adiponectin is an anti-inflammatory protein produced

primarily by adipocytes. This protein is at its highest concentration in healthy

individuals and is decreased in obese patients (Ryo et al., 2004). Oxidative

stress and pro-inflammatory cytokines produced in dysregulated adipose tissue

block the production of adiponectin by adipocytes (Berg and Scherer, 2005,

Hosogai et al., 2007, Ouchi et al., 2003). Reduced adiponectin is associated with

increased risk of type II diabetes (Li et al., 2009, Ouchi et al., 2003), while calorie

restriction was found to increase adiponectin production in BM adipose tissue,

making it the systemic source of this anti-inflammatory molecule (Cawthorn et al.,

2014). In terms of B lymphopoiesis, adiponectin was found to have a negative

effect (Yokota et al., 2003). Although, adipocyte factors other than adiponectin

were also found to inhibit B lymphopoiesis (Bilwani and Knight, 2012).

Pro-inflammatory molecules

      IL-6. IL-6 is a pro-inflammatory cytokine produced by adipocytes and other

cells in adipose tissue (Fried et al., 1998, Van Snick, 1990). Increased IL-6 levels

are observed in obese individuals and individuals with type II diabetes.

Production of this cytokine appears to increase with increased adipose tissue

and can be controlled by diet, as IL-6 levels were reduced in patients after losing

weight (Esposito et al., 2003, Fried et al., 1998, Ouchi et al., 2011, Ziccardi et al.,

2002). Similar to obesity, production of IL-6 from adipose tissue also increases during aging (Starr et al., 2009, Tchkonia et al., 2010). This cytokine has many immunomodulatory effects and, if available in niches containing HSPCs, has the potential to inhibit B cell development (Maeda et al., 2005, Maeda et al., 2009). IL-6 can also work with other factors to mediate various outcomes. For example, IL-6 treatment in combination with TNFα was found to promote BM macrophage differentiation into osteoclast-like cells (Yokota et al., 2014).

TNFα. TNFα production increases during obesity as adipocytes increase in size and ultimately contributes to insulin resistance (Feingold et al., 1992, Hotamisligil et al., 1994, Ouchi et al., 2011, Spiegelman et al., 1993). Myeloid lineage cells are the main source of TNFα in adipose tissue. Similar to IL-6, adipose tissue production of TNFα increases with obesity and type II diabetes (Hotamisligil et al., 1993), and can be decreased by weight loss (Kern et al., 1995, Ziccardi et al., 2002). If present in the BM, TNFα induces an inflammatory state that promotes granulopoiesis (Ueda et al., 2004).

S100A8, S100A9, and S100A8/A9. Adipose tissue is the source of S100A8 and S100A9. These proteins can form S100A8/A8 or S100A9/A9 homodimers in addition to S100A8/A9 heterodimers. S100A8 and S100A9 proteins have multiple functions intracellularly and extracellularly, which include anti-microbial activity, intracellular calcium binding, and alarmin/pro-inflammatory effects (Vogl et al., 2012). Activated and dying myeloid cells are the primary source of extracellular S100A8 and S100A9 leading to a potent inflammatory response (Ehrchen et al., 2009, Nacken et al., 2003). It was initially thought that

S100A8/A9 was produced by adipocytes in adipose tissue, but recent evidence

suggests that S100A8 is primarily produced by adipocytes and S100A9 is

produced by macrophages within the adipose tissue (Sekimoto et al., 2012). The

expression of these molecules is upregulated in many tissues during aging and

obesity (Nagareddy et al., 2014, Schiopu and Cotoi, 2013, Sekimoto et al., 2012,

Swindell et al., 2013), however the impact these proteins have on B

lymphopoiesis is unknown.

Complement factors . Adipose tissue is a source of many complement

proteins, including C3a, C5a, factor B, and factor D (adipsin) (Pattrick et al.,

2009, Vlaicu et al., 2016). Complement proteins have important functions in the

maintenance of adipose tissue, but can also contribute to adipose tissue

inflammation. For example, a cleavage product of C3a, called C3adesArg is

critical to promote triglyceride synthesis in normal adipocytes and promote

differentiation of pre-adipocytes into mature adipocytes (MacLaren et al., 2008,

Maslowska et al., 2005, Saleh et al., 2011, Yasruel et al., 1991). The generation

of C3adesArg from C3a is mediated by the enzyme carboxypeptidase N.

C3adesArg is unable to bind the C3 receptor but instead binds the receptor C5-

like receptor 2 (C5L2), which can also be bound by C5a (Vlaicu et al., 2016). This

process contributes to the normal maintenance of healthy adipose tissue.

Alternatively, dysregulation of normal homeostasis in adipose tissue leads to

complement mediated inflammation, where both C3R and C5R have been

implicated in adipose tissue inflammation during obesity (Lim et al., 2013,

Mamane et al., 2009, Phieler et al., 2013). Consistent with IL-6 and TNFα serum

levels (as reviewed above), C3a serum concentration is increased in obese

individuals and can be lowered through weight reduction (Nestvold et al., 2015,

Nilsson et al., 2014, Oberbach et al., 2011, Sleddering et al., 2014).

Danger associated molecular patterns (DAMPS). In inflammatory

adipose tissue, adipose tissue macrophages form crown-like structures that

surround dying adipocytes. These adipocytes become the source of additional

danger signals such as lipids and reactive oxygen species that trigger an

inflammatory profile in the macrophages. Adipose tissue macrophages are

commonly seen phagocytosing lipids and becoming lipid laden cells (Chawla et

al., 2011, Strissel et al., 2007). Free cholesterol, lipid crystals, ceramides, and

fatty acids derived from adipocytes and taken up by adipose tissue macrophages

induce inflammation through inflammasome activation (Coppack, 2001, Lago et

al., 2007, Vandanmagsar et al., 2011, Wen et al., 2011, Youm et al., 2012).

These danger signals are sensed by the nod-like receptor, NLRP3, triggering

inflammasome activation. Nod-like receptor activation results in association of

the receptor, adaptor protein ASC, and inactive caspase 1; leading to the

activation of caspase 1. Active caspase 1 then cleaves pro-IL-1β into active IL-

1β, which is a potent mediator of inflammation (Garlanda et al., 2013, Latz et al.,

2013).

Adipocyte mediated inhibition of B lymphopoiesis: direct block or change

in lineage potential?

Adipocyte soluble factors were shown to inhibit B lymphopoiesis in human

and rabbit BM cultures (Bilwani and Knight, 2012), but the mechanism for this is

unknown. While B lineage cells were decreased in cultures treated with ACM, this could be due to a block at a specific stage of B lineage development (eg. proB cell) or due to a redirection of hematopoiesis away from the B lineage and toward another (eg. myeloid, NK, T lineage). Given the large number of molecules produced by adipose tissue and the potential for a synergistic effect on hematopoiesis, it is reasonable to hypothesize that B lymphopoiesis is not simply blocked. In fact, hematopoietic progenitors are able to sense changes in their niche, which may lead to changes in differentiation potential. For example, pathogen-derived TLR ligands can influence HSCs to differentiate into myeloid lineage cells and can induce CLPs to differentiate into dendritic cells (DCs) (Nagai et al., 2006, Welner et al., 2008a, Welner et al., 2008b) (Figure 1.6). Since adipocytes produce fatty acids that can also activate TLRs, it is possible that B cell development is rerouted rather than blocked by adipocyte-derived fatty acids.

Can cells other than adipocytes in the BM negatively regulate B cell development?

Hematopoietic lineage compartments are known to change in the BM in response to aging, obesity, infection, etc., but how does this affect B lineage development? The degree to which cells in the BM other than adipocytes (such as other hematopoietic cells) contribute to declining B lymphopoiesis is not well characterized. During aging, aged B cells (ABC) were shown to accumulate in aged BM negatively regulating new B lineage development (Ratliff et al., 2013), suggesting an altered BM hematopoietic compartment could incluence B cell

development. However, additional studies will be needed to understand whether other hematopoietic lineage cells can contribute to declining B cell development in fatty BM. For example, Enioutina et al. found that myeloid derived suppressor cells (MDSCs) accumulate in the BM of aged mice (Enioutina et al., 2011). MDSCs are a heterogenous popluation of CD11b$^+$Gr1$^+$ immature myeloid cells well known for their ability to suppress T cell responses in cancers (Gabrilovich and Nagaraj, 2009). While suppressive to T cell responses, it is unknown whether these cells can also affect B cell development. Further study will be needed to understand how changes in stromal cells and neighboring hematopoietic cells interact with developing B lineage cells.
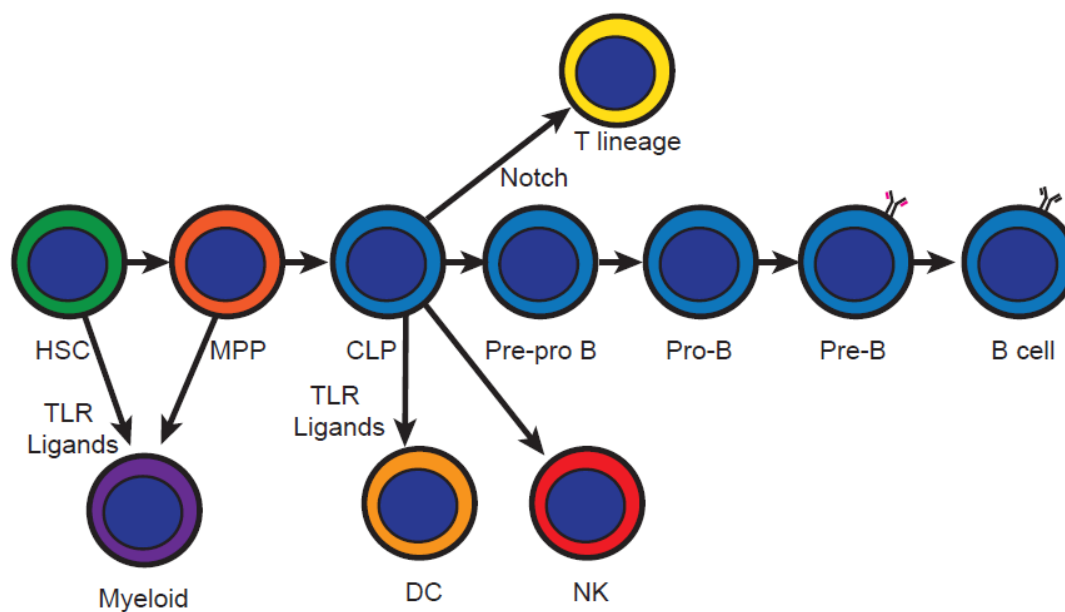
Figure 1.6 Skewing of hematopoietic lineage potenial. Hematopoietic potential can be re-routed away from the B lineage at different stages of development. Progenitors sense different molecules in the microenvironment

which may result in diversion from the B lineage, to any of the lineages diagrammed above. Both TLR ligands and notch ligands have been described to act on different stages of development, resulting in altered hematopoietic potential. (Adapted from Welner et al., 2008)

SIGNIFICANCE

Increased susceptibility to infections and poor immune responses to vaccination put elderly individuals at an increased risk of mortality. Microenvironmental changes in the aged BM and thymus result in decreased output of new lymphocytes into the periphery, limiting the immune system's ability to respond and clear new infections. Accumulation of adipose tissue in aged BM was previously considered to be inert. But recent understanding of adipocytes as immune regulators has allowed the scientific community to realize that adipose tissue is actively influencing hematopoiesis. Multiple studies(Naveiras et al., 2009, Yang et al., 2009a, Youm et al., 2009, Youm et al., 2010) have now shown that adipose tissue negatively regulates the development of new immune cells in the BM and thymus. But detailed study of the mechanisms by which adipocyte-derived factors negatively regulate hematopoiesis is imperative, in order to develop therapeutics aimed at rejuvenating immune cell development in aged individuals.

GOAL OF DISSERTATION

The goal of this dissertation is to **understand the mechanism by which adipocyte factors inhibit B cell development**. We hypothesize that different

adipocyte-derived factors inhibit/reroute B lymphopoiesis by: 1.) Acting directly

on hematopoietic B lineage progenitors and/or 2.) Acting indirectly, altering BM

stromal cells or neighboring hematopoietic cells. These studies were performed

with the intention of identifying targets for therapies designed to boost B

lymphopoiesis in aged and obese individuals with fatty BM.

An additional goal is to **relate the identified mechanisms to the arrest**

of B cell development that occurs at 2 months of age in the BM of rabbits.

The rabbit appears to be a model of accelerated BM aging, therefore further

characterization of rabbit BM will be useful in understanding the changes that

occur later in life of humans and mice.

CHAPTER II

EXPERIMENTAL METHODS

Mice

C57BL/6 breeding pairs were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were used in compliance with protocols approved by the Loyola University Chicago Institutional Animal Care and Use Committee.

Rabbits

New Zealand White rabbits were maintained at Loyola University Chicago. All rabbits were used in compliance with protocols approved by the Loyola University Chicago Institutional Animal Care and Use Committee.

Tissue/cell culture reagents

AlphaMEM and RPMI base medium were purchased from Life Technologies (Grand Island, NY), along with all tissue culture supplements. Recombinant murine IL-7, SCF, Flt3L, IL-1α, IL-1β, IL-13, G-CSF, KC, MCP-1, MIP-1α, MIP-1β, RANTES and human IL-7, SCF, Flt3L were purchased from PeproTech (Rocky Hill, NJ). Recombinant murine S100A8 was purchased from Abcam (Cambridge, MA) and murine S100A9 was purchased from R&D systems (Minneapolis, MN).

Flow cytometry

Antibodies used to stain mouse cells in this study are listed in table 2.1.

Available antibodies for staining rabbit cells are listed in table 2.2. Dead cells

were excluded from flow cytometry analyses using BD Horizon fixable viability

stain (BD Biosciences, San Jose, CA) or Fixable Viability Dye eFluor 450

(eBioscience, San Diego, CA). FACS sorting was performed using a FACSAria

cell sorter (BD Biosciences). All stained cells were analyzed by flow cytometry

using a FACSCanto II or LSRFortessa flow cytometer (BD Biosciences). Flow

cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Antibody reactivity	Clone	Company
B220	RA3-6B2	Biolegend
CD19	6D5	Biolegend
CD11b	M1/70	Biolegend
Gr1	RB6-8C5	Biolegend
CD14	Sa14-2	Biolegend
CD3ε	145-2C11	Biolegend
CD4	GK1.5	Biolegend
CD8a	53-6.7	Biolegend
TER-119	TER-119	Biolegend
CD49b	DX5	Biolegend
CD16/32 –Fc Block	93	Biolegend
CD28	37.51	Biolegend
Ly6C	HK1.4	Biolegend
Ly6G	1A8	Biolegend
Sca1	D7	eBioscience
CD117	2B8	Biolegend
CD127	SB/199	Biolegend
CD135	A2F10	Biolegend
IL-1α	BLa-89	Biolegend
IL-1β	B122	Biolegend

Table 2.1 Mouse antibodies used in this study.

Antibody reactivity	Clone	Specificity
CD1b	LAT3	Rabbit
CD3	PC3/188A	Rabbit
CD4	Ken4	Rabbit
CD9	MM2	Rabbit
CD10	CD-CALLA	Human, cross reacts with rabbit
CD11b	198	Rabbit
CD11b	M1/70	Human, mouse, cross reacts with rabbit
CD11c	3/22	Rabbit
CD14	K4	Rabbit
CD14	TÜK4	Human, cross reacts with rabbit
CD20	B9E9	Human, cross reacts with rabbit
CD21	BL13	Human, cross reacts with rabbit
CD23	9P25	Human, cross reacts with rabbit
CD24	M1/169	Mouse, cross reacts with rabbit
CD25	Kei-α1	Rabbit
CD27	LT27	Human, cross reacts with rabbit
CD38	IB6	Human, cross reacts with rabbit
CD43	L11/43	Rabbit
CD44	W4/86	Rabbit
CD62L	LAM-1	Human, cross reacts with rabbit
CD79a	HM47	Human, cross reacts with rabbit
CD90	5E10	Human, cross reacts with rabbit
BAFF	Polyclonal	Human, cross reacts with rabbit
BCL6	BL6.02	Human, cross reacts with rabbit
BR3	Polyclonal	Human, cross reacts with rabbit
Complement C3	Polyclonal	Rabbit
Caspase 3	C92-605	Human, mouse, cross reacts with rabbit
Ki67	B56	Human, cross reacts with rabbit
MHC II	2C4	Rabbit
Anti-Macrophage	RAM11	Rabbit
IgM	367	Rabbit
IgA	102	Rabbit
IgG	359	Rabbit
Ig Light Chain	Polyclonal	Rabbit
S100A8/S100A9	MAC387	Rabbit, mouse, human, cow, dog, pig, monkey

Table 2.2 Antibodies available for the detection of rabbit immune cell antigens.

Microscopy

Microscopy experiments were visualized on a Leica DM IRB microscope (Leica Microsystems, Buffalo Grove, IL). Image collection was performed using a Magnafire 2.1C camera system and software.

Preparation of bone marrow cells

Mouse. Mouse femurs and tibias were cleaned with 70% ethanol, and then rinsed with alphaMEM medium. The bones were flushed with medium (15ml per 2 femurs and 2 tibias) using a 27 gauge needle, followed by red blood cell (RBC) lysis using ammonium-chloride-potassium lysis buffer. Cells were then washed with medium and used in downstream assays.

Rabbit. Femurs and tibia were washed with 70% ethanol and rinsed with medium. Bones were broken open and the marrow was flushed with 20-40ml of medium using a 16 gauge needle. RBCs were lysed using 0.85% ammonium chloride, washed in medium, and used in downstream cultures or analyses.

B lymphopoiesis assay (BM cultures)

All B lymphopoiesis culture assays were performed in alphaMEM with supplements at the following final concentrations: 10% FCS, 0.5% penicillin/streptomycin, 30 mg/ml gentamicin, and 0.5 mg/ml fungizone.

Mouse. On day 0, 1,000 OP9 BM stromal cells were plated per well in 96 well plates. Day 1, mouse BM cells were depleted of $B220^+$ B lineage cells by magnetic bead separation using an autoMACS Pro separator (Miltenyi Biotec, Auburn, CA). Then, 40,000 $B220^-$ BM cells were plated in 96 well plates (containing OP9 cells) in the presence of murine IL-7 (10ng/ml), SCF (10ng/ml),

and Flt3L (10ng/ml). Cultures were performed with or without the indicated treatments. ACM treatment was typically performed at a 1:6 dilution and MDSC-CM was typically used at a 1:3 dilution. Four days later fresh cytokines were added to BM cultures, followed by termination of cultures between day 7 and day 10. At the end of culture, cells were collected, counted, and prepared for flow cytometry analysis.

Arginase and iNos studies. For experiments assessing the contribution of arginase and iNos, 0.3mM N$^{\omega}$-hydroxy-nor-arginine (Nor-NOHA) and/or 0.3mM N^{G}-monomethyl-L-arginine (L-NMMA) were used to block arginase and iNos, respectively. Additionally, the control compound N^{G}-monomethyl-D-arginine (D-NMMA) was used to assess off-target effects of L-NMMA.

IL-1 studies. For experiments assessing IL-1, 1µg/ml anti-IL-1α and 1µg/ml anti-IL-1β antibodies (or isotype control antibodies) were added at the initiation of BM cultures. Similar results were obtained using these antibodies in combination with 1µg/ml human IL-1R antagonist.

Hematopoietic progenitor studies. For experiments with purified progenitor cells, 500-1,000 HSCs, MPPs, or CLPs were seeded onto OP9 cells in the presence of murine IL-7 (10ng/ml), SCF (10ng/ml), Flt3L (10ng/ml), and the indicated treatment. Cultures were given fresh cytokines (as described previously), followed by flow cytometry analysis 5-10 days after the cultures were initiated. Lineage negative cells (Lin^{-}) were defined as B220^{-}CD3ε^{-}CD8a^{-}CD11b^{-}TER-119^{-}Gr1^{-}CD49b^{-}.

Inflammasome inhibitor studies. The NLRP3 inflammasome inhibitor glybenclamide was purchased from Invivogen (San Diego, CA). Glybenclamide was added at the start of cultures at the concentrations indicated in the figure.

24 well plate format. For assays performed in 24 well plates, 5,000 OP9 cells were plated. 24 hours later 120,000-500,000 B220⁻ BM cells were used as a source of progenitor cells.

Transwell cultures. Transwell cultures were performed in the 24 well plate format, where ACM-derived MDSCs were cultured in contact (below transwell insert) with BM progenitors or separated by transwell (cultured above transwell insert).

Rabbit. 1,000 OP9 cells per well were plated in 96 well plates on day 0. On day 1, 20,000 total BM cells/well (isolated from >2 month old rabbits) were used as a source of BM progenitors. These cultures were performed in the presence of rabbit IL-7 (10ng/ml), SCF (10ng/ml), and Flt3L (10ng/ml). Fresh cytokines were added to cultures 4 days after culture start, followed by termination of the cultures between day 7 and day 10.

T cell proliferation assays

96 well plates were coated with anti-CD3 and anti-CD28 antibodies in 0.1M borate buffer pH 8.0. The next day, mouse splenocytes were stained with 5µM CellTrace violet or Carboxyfluorescein succinimidyl ester (CFSE) (ThermoFisher Scientific, Waltham, MA), followed by resuspension in modified RPMI 1640 containing 10% FCS. Labeled solenocytes were plated in anti-CD3 and anti-CD28 coated wells at the concentrations of 250,000 cells/well or

300,000 cells/well with or without effector cells (ACM-generated CD11b^{hi}Gr1$^+$ cells or CD19$^+$ control cells) ranging from 12,500-100,000 cells/well. Four days later, cultures were stained for flow cytometry analysis with anti-CD4 and anti-CD8 antibodies and the dilution of CFSE was assessed.

For experiments assessing arginase and iNos activity, 0.3mM Nor-NOHA (arginase inhibitor) and 0.3mM L-NMMA (iNos inhibitor) were added to cultures.

Adipocyte differentiation and conditioned medium generation

Mouse Adipocytes. 3T3.L1 pre-adipocytes were used to generate adipocytes (provided by Dr. Neil Clipstone – Loyola University Chicago). 80,000 pre-adipocytes were plated per well in a 6 well plate. Cells were cultured until reaching confluency (designated as Day 0). On day 2, MDI adipocyte differentiation medium was added. MDI medium consists of Dublecco's Modified Eagle Medium (DMEM) containing 10% FCS, dexamethasone (1µM), insulin (10µg/ml), and 3-isobutyl-1-methylxanthine (0.5mM). After 48 hours of culture in MDI (Day 4), the medium was aspirated and replaced with DMEM 10% FCS containing insulin (10µg/ml). On day 6, the medium was again aspirated and replaced with DMEM 10% FCS only. At this point, the cells were cultured four more days, replacing with fresh DMEM 10% FCS every two days until the adipocytes were fully differentiated.

To generate conditioned medium, the adipocytes were washed with serum free DMEM (to remove trace amounts of differentiation supplements), and then cultured for 3 days in 1ml of serum free DMEM. Adipocyte conditioned medium (ACM) was then collected and any cell debris was removed.

Rabbit Adipocytes. To generate rabbit adipocytes, total BM was isolated from rabbits of any age and RBCs were lysed. For BM from rabbits <2 months of age, 380,000 BM cells were plated per well in 12 well plates. When using BM from >2 month old rabbits for adipocyte generation 3,800,000 cells were plated per well in 12 well plates. BM cells were cultured for two days in alphaMEM containing 20% FCS and supplemented with penicillin (100 units/L)/streptomycin (100µg/L), and fungizone (250µg/L). On day 2, non-adherent cells were removed and fresh medium was added. On day 3, the medium was replaced with adipocyte differentiation medium (ADI) consisting of alphaMEM with 15% FCS, L-glutamine (2mM), penicillin (200 units/L)/streptomycin (200µg/L), dexamethasone (0.4µM), indomethacin (40µM), and 3-isobutyl-1-methylxanthine (0.4µM). Every three to four days, the medium was replaced with fresh ADI, aspirating and replacing only half the medium in the well. On day 20, cultures were screened for wells containing 85-100% mature adipocytes. Wells containing a high percentage of adipocytes were then washed with serum free alphaMEM (to remove differentiation supplements), and then ACM was generated by culturing for three days in 700µl-1ml of serum free of alphaMEM. Finally, the supernatant was collected, cell debris was removed, and ACM was then used in downstream assays.

MDSC generation

MDSCs were generated in 24 well plates. 5,000 OP9 BM stromal cells were plated in each well. The next day, 120,000-500,000 B220⁻ mouse BM cells were plated in OP9 containing wells in the presence of murine IL-7, SCF, and

Flt3L. Key to generating MDSCs is the addition of mouse ACM at the start of cultures (typically at a 1:6 dilution). On day 4, the cultures were given fresh cytokines, and on day 7 the cultures were collected for MDSC isolation. Cells resulting from ACM-treated cultures were pooled and stained with anti-B220, anti-CD19, anti-CD11b, and anti-Gr1 and sorted for B220⁻CD19⁻CD11b⁺Gr1⁺ cells by flow cytometry. Sorted cells were then used for downstream culture or analysis.

MDSC-CM

MDSC-CM was generated by culturing 100,000 purified MDSCs per well in 48 well plates. MDSCs were cultured in serum free alphaMEM for 3 days, and then collected for downstream assays.

BM fat conditioned medium

BM fat conditioned medium (BM fat-CM) was generated in two steps; isolation of BM fat from >2 month old rabbits, followed by conditioned medium generation.

Isolation of BM fat. Greater than 2 month old rabbits were used to isolate BM fat because younger rabbits do not have enough BM fat to isolate by this method. BM was flushed from rabbit femurs and/or tibia as described previously. Next, cells were spun down at 1,200RPM for 5-10min at 4 degrees Celsius. Spinning the BM results in two noticeable BM fractions; 1. The BM pellet at the bottom of the tube. This fraction contains most BM cell types (eg. RBCs, white blood cells, stromal cells), but is largely depleted of mature adipocytes. This fraction is also referred to as the stromal vascular fraction (SVF). In this

dissertation, we refer to the pellet that forms after spinning down total BM as total

BM or the SVF. In either terminology, these are depleted of mature adipocytes by

spinning total BM; 2. In addition to the BM pellet, this method also results in the

formation of a mature adipocyte fraction at the top of the tube of cells. In this

dissertation, we refer to this floating fraction as the adipocyte layer or as the BM

fat layer. The BM fat layer contains mature adipocytes, but we have also isolated

hematopoietic lineage cells (many CD11b$^+$ cells) that are tightly associated with

adipocytes in this fraction. Therefore, conditioned medium from BM fat contains

molecules derived from adipocytes and tightly associated hematopoietic cells.

Generation of conditioned medium. BM fat-CM was generated upon

isolation of the adipocyte layer/BM fat layer from >2 month old rabbits. BM fat

was cultured in serum free alphaMEM for 16-24 hours, supernatant was collected

(no adipocytes or other cells were taken), followed by the removal of any debris.

BM fat-CM was then used in downstream assays.

Isolation of cells from BM fat

BM fat was isolated from femurs and/or tibias of >2 month old rabbits, then

cultured overnight in wells of a 12 well plate. By the next day, many cells will

have fallen out of the BM fat fraction to the bottom of each well. The cells at the

bottom of each well were collected and combined with cells isolated through

disruption of the BM fat fraction (pipetting up and down the BM fat). This is the

gentlest way to isolate cells from BM fat. Alternatively, cells were isolated from

BM fat after it was digested with 4mg/ml collagenase II in PBS supplemented

with 0.5% BSA and 10 mM CaCl$_2$, which releases cells from the tissue. Both

methods appeared to work well for isolating hematopoietic cells from this fat fraction.

After isolation of cells from BM fat, CD11b$^+$ and CD11b$^-$ cells were separated by magnetic bead sorting using an autoMACS Pro separator. These cell populations were then used in rabbit BM cultures.

Bone marrow sections

BM sections were performed on femurs and/or tibias taken from rabbits of various age. The upper bone surface was removed very carefully using a new surgical blade (many gentle strokes of the blade on the desired area of bone surface until the surface is breached). Upon removal of the bone surface, the bone looked like a "half-pipe" (Figure 2.1). Although the surface was removed, care was taken so the BM was not disturbed. If BM was disturbed, it was not used for sectioning. Multiple slices of BM were taken from opened bones (with undisturbed BM) and prepared for cryosectioning sectioning. BM was embedded in optimal cutting temperature (OCT) support medium and frozen. Frozen BM was then used for sectioning. After sectioning, BM sections were stained with Hematoxylin and Eosin, or stained for immunofluorescence analysis.

Rabbit Bone

Remove bone surface without disturbing inner BM

Rabbit Bone (after surface removal)

Bone Surface

Bone Marrow

Bone should look like a "half pipe" with undisturbed BM extending out of bone

Rabbit BM (to be sectioned)

- Remove multiple slices of BM.
- Embed in OCT
- Freeze
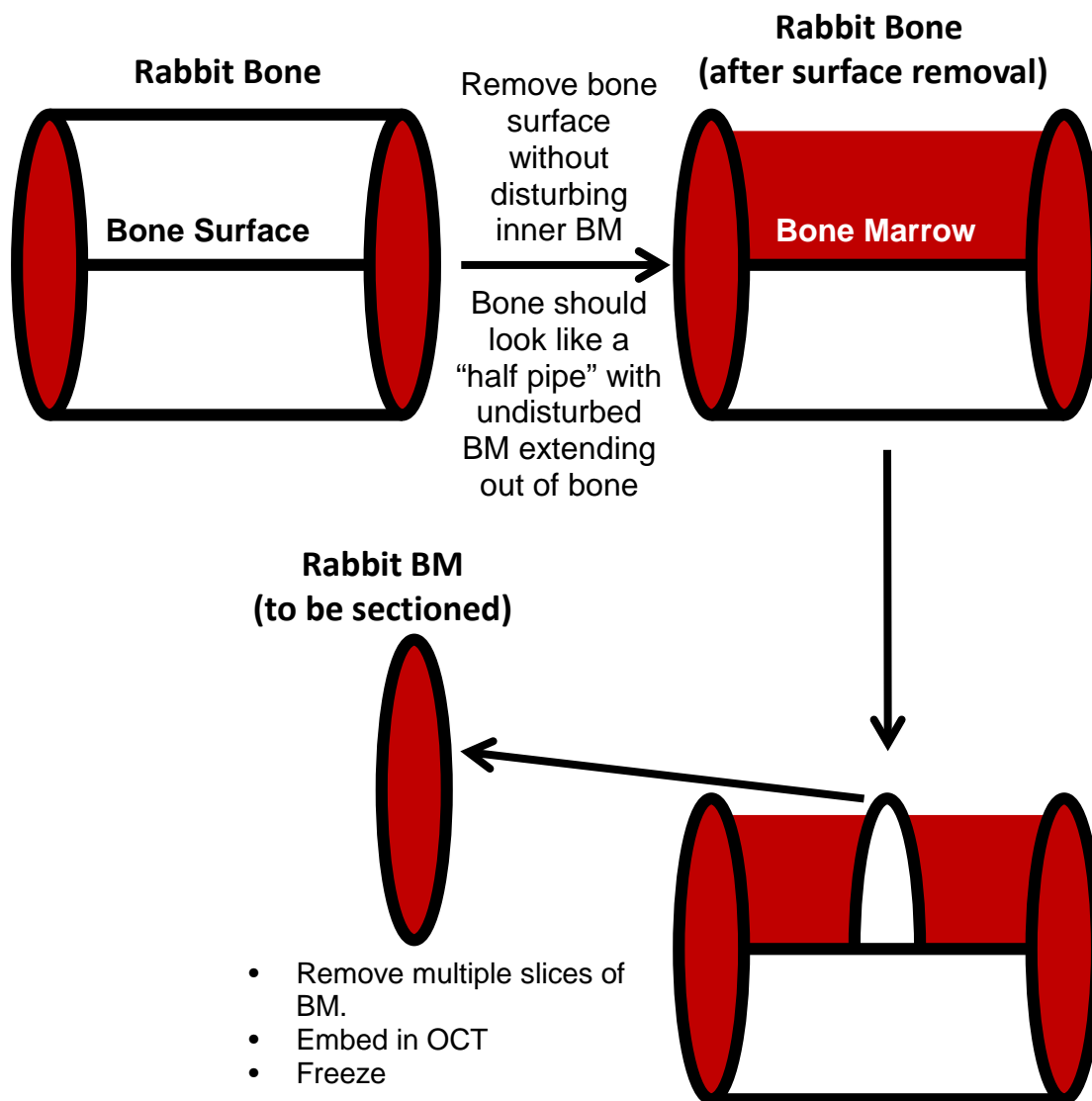
Figure 2.1 Preparation of rabbit bone marrow for sectioning. Rabbit BM was prepared as described in the text and indicated in this figure.

Anion exchange chromatography

Anion exchange chromatography was performed using a 1ml volume HiTrap Q Sepharose High Performance strong anion exchange column (GE Healthcare Life Sciences, Pittsburgh, PA). Chromatography experiments were

performed with an ÄKTA fast protein liquid chromatography (FPLC) system running UNICORN control software (GE Healthcare Life Sciences, Pittsburgh, PA).

Mouse ACM samples were prepared by filtering 8ml of total ACM through a 10kDa filter (Amplicon ultra – EMD Millipore, Darmstadt, Germany) until 100µl of retenate remained above the filter. Greater than 10kDa ACM was diluted 1:10 in FPLC buffer A (running buffer), to a final volume of 1ml which was then loaded on the column. Proteins bound to the column were eluted using a linear increasing salt concentration performed by adding increasing concentrations of FPLC Buffer B (elution buffer).

The protein concentration of each fraction was measured by a UV reader within the FPLC system, and was confirmed using a nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Multiple fractions collected from the column were pooled into the following samples and tested for inhibitory activity in rabbit B lymphopoiesis assays.

Sample in B lymphopoiesis assay	Fractions in each sample
SAMPLE 1	A1,A2,A3
SAMPLE 4	B9,B8,B7,B6,B5,B4
SAMPLE 5	B3,B2,B1,C1,C2
SAMPLE 6	C3,C4,C5,C6,C7
SAMPLE 7	C9,C10,C11,C12,D12,D11,D10,D9,D8,D7,D6,D5,D4,D3,D2,D1,E1
SAMPLE 8	E2,E3,E4,E5,E6,E7,E8,E9,E10,E11,E12

FPLC Buffer A (running buffer) - 10mM TRIS-HCL pH 8.2

FPLC Buffer B (elution buffer) - 10mM TRIS-HCL + 1M NaCl pH 8.2

System/column storage buffer - 20% Ethanol in water

Quantitative RT-PCR

Cells were suspended in TRIzol reagent (Invitrogen, Carlsbad, CA), and RNA was isolated. cDNA was made from isolated RNA and used in qPCR reactions. PCR experiments were performed using a C1000 thermal cycler with CFX96 real-time detection system (Bio-Rad, Hercules, CA). Expression of target genes was normalized to *β-actin* (mouse experiments) or *HGPRT* (rabbit experiments). Tables 2.3 and 2.4 display the primer sets used in qPCR experiments.

Gene	Forward Primer (5'-3')	(Reverse Primer 5'-3')
Actb (β-actin)	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Arg1	AGACCACAGTCTGGCAGTTG	CCACCCAAATGACACATAGG
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG

Table 2.3 Mouse qPCR primers.

Gene	Forward Primer (5'-3')	(Reverse Primer 5'-3')
HGPRT	AGCCCCAGCGTTGTGATTAGT	CATCTCGAGCAAGCCTTTCAG
IL-1β	AGGTCTTGTCAGTCGTTGTG	GTAGTCATCCCAGTGTGCAG
S100A8	TCTACCACAAGTACTCCCTGG	TCCAGCTCTTTGAACCAAGTG
S100A9	ATCATCAACGTCTTCCACCAG	TTATGGCTTTCTCATCCCTCG

Table 2.4 Rabbit qPCR primers.

Cytokine array

The concentration of 23 cytokines was assessed in ACM, MDSC-CM, and control CD11b⁺Gr1⁺ conditioned medium (control-CM) samples using a Bio-Plex Pro mouse cytokine 23-plex (Bio-Rad, Hercules, CA). The cytokines analyzed are as follows: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, G-CSF, GM-CSF, IFN-γ, KC, MCP, MIP-1α, MIP-1β, RANTES, eotaxin, TNF-α. Three ACMs, three MDSC-CMs, and two control-CMs were all generated in independent experiments before being analyzed by cytokine array.

Statistical analysis

Data were obtained and displayed as indicated in the figure legends. Statistical analysis of all experiments was performed using Prism software (GraphPad Software; La Jolla, Ca). Statistical tests used in this dissertation are indicated in the figure legends, and include unpaired two-tailed Student's *t* test or analysis of variance (ANOVA) in combination with Dunnet's or Bonferroni's test for multiple comparisons. * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001

CHAPTER III

RESULTS

SECTION 1: ADIPOCYTES AND THE INHIBITION OF B LYMPHOPOIESIS

In aging and obesity, declining B lymphopoiesis correlates with an accumulation of adipocytes in the BM. Adipocytes were once thought to be inert, simply filling empty BM space. However, recent evidence suggests that adipocytes can regulate multiple aspects of the immune system, including hematopoiesis (Bilwani and Knight, 2012, Naveiras et al., 2009). Therefore, a detailed understanding of how adipocytes influence immune cell development will prove beneficial in developing therapeutics to restore healthy hematopoiesis in scenarios where adipocytes accumulate in the BM, such as aging and obesity.

Bilwani and Knight found that adipocyte-derived factors inhibit human and rabbit B cell development in BM cultures (Bilwani and Knight, 2012), but the mechanism by which this occurs is unknown. Due to a greater abundance of reagents available to study mice than rabbits, and increased access to BM from mice than humans; it would be ideal to tease out the mechanism by which adipocyte factors inhibit B cell development in mice. Therefore, we first needed to establish whether or not adipocyte factors inhibit B lymphopoiesis in mice.

To test if adipocyte factors inhibit mouse B cell development, we needed to generate mouse adipocyte factors, and develop an approach to study the

effect of these factors on B lymphopoiesis. Mouse adipocyte factors were generated using the pre-adipocyte cell line, 3T3.L1. Pre-adipocytes were differentiated into mature adipocytes with insulin, isobutylmethylxanthine, and dexamethasone. Upon differentiation into mature adipocytes, the cells were washed to remove trace amounts of differentiation factors, followed by three days of culture and the collection of adipocyte conditioned medium (ACM). ACM was then used to assess the effect of adipocyte-derived factors on mouse B lymphopoiesis.

B lymphopoiesis was studied using the OP9 stromal cell line, originally isolated from mouse BM. OP9 cells are commonly used to study B lymphopoiesis, as they have been found to support the differentiation of BM progenitors into B lineage cells (Holmes and Zuniga-Pflucker, 2009). In this study, we cultured various subsets of hematopoietic progenitors with OP9 cells to answer different questions using this system.

To determine if adipocyte-derived soluble factors inhibit B lymphopoiesis, mouse BM cells were depleted of B220$^+$ B lineage cells. We cultured these B220$^-$ BM cells with OP9 BM stromal cells in the presence of IL-7, SCF, and Flt3L, cytokines that support B lineage development. We expected that if adipocyte factors inhibit mouse B lymphopoiesis, then treatment of B lymphopoiesis cultures with ACM would result in a decreased frequency of B lineage cells (as evidenced by fewer B220$^+$ cells) observed at the end of cultures. As expected, cultures treated with ACM contained fewer B220$^+$ B lineage cells compared to

untreated cultures (Figure 3.1 A & B), suggesting that adipocyte factors inhibit mouse B cell development.

If the adipocyte-mediated inhibition of B lymphopoiesis is conserved among mammals, we also expected that mouse adipocyte factors would inhibit rabbit B lymphopoiesis. To study rabbit B lymphopoiesis we modified the above described culture system. Total BM cells from >2 month old rabbits was used as a source of BM progenitors and cultured with OP9 cells in the presence of IL-7, SCF, and Flt3L. BM cells from rabbits >2 months old was used as a source of early hematopoietic progenitors because B lymphopoiesis has arrested in rabbit BM by this time, and the cultures are not "contaminated" with proB and preB cells.

To understand if mouse adipocyte factors have the potential to inhibit rabbit B cell development, we cultured BM cells from >2 month old rabbits with OP9 cells in the presence or absence of mouse ACM. We expected that if mouse adipocyte factors inhibit rabbit B lymphopoiesis, then ACM-treated cultures will result in the development of fewer B lineage cells. Reagents are not available to detect the B cell marker B220, therefore we identified the development of new rabbit B lineage cells by the appearance of cells expressing the B cell receptor (BCR) signaling component CD79a (first expressed at the proB cell stage). Interestingly, treatment of rabbit BM cultures with mouse ACM resulted in fewer CD79a$^+$ B lineage cells (Figure 3.1 C & D). These data coupled with the findings by Bilwani and Knight lead us to conclude that adipocytes negatively regulate B lymphopoiesis in a conserved manner between multiple mammals.
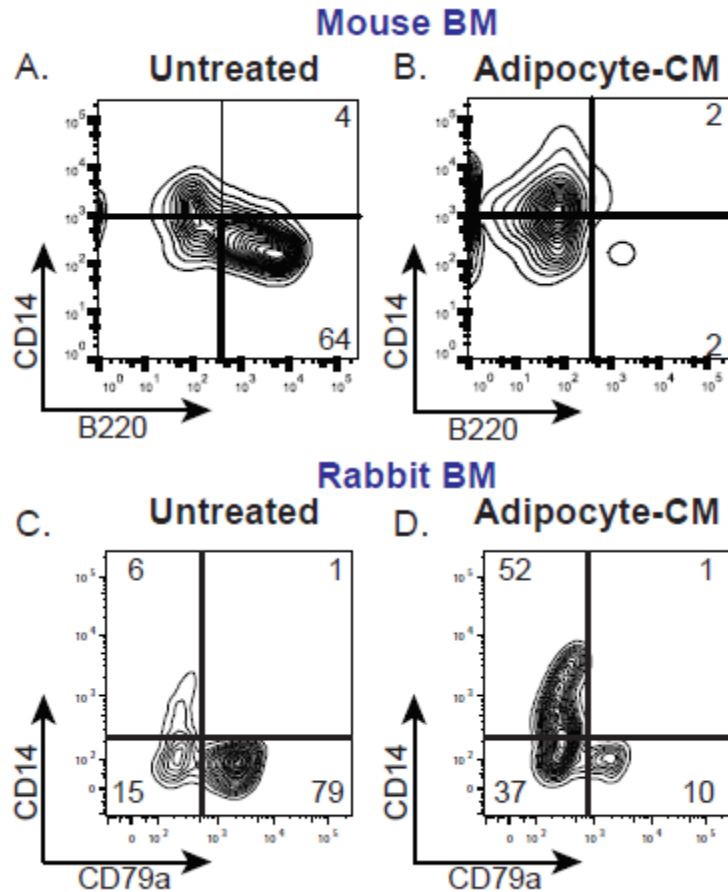
Figure 3.1 Inhibitory potential of mouse adipocyte-derived factors in mouse and rabbit B lymphopoiesis cultures. (A-B) Mouse B220⁻ BM cells were cultured with OP9 stromal cells in the presence of mouse IL-7, SCF, and Flt3L. Flow cytometric analysis at the end of (A) untreated or (B) msACM-treated cultures for the expression of B220 and CD14 on resulting cells. (C-D) BM cells from a >2 month old rabbit were cultured with OP9 stromal cells in the presence of human IL-7, SCF, and Flt3L. Flow cytometric analysis at the end of (C) untreated or (D) msACM-treated cultures for CD79a and CD14 expression on resulting cells. (A-D) Data are representative of at least 3 independent experiments.

Mechanism of adipocyte-mediated inhibition

Adipocyte factors inhibit B lymphopoiesis; although the mechanism by which this occurs is unknown. Because the inhibition of B lymphopoiesis by adipocytes appears conserved among mammals, we expect using mice to understand the mechanism of inhibition will allow us to gain insights applicable to humans, mice, and rabbits.

There are two ways in which adipocyte molecules could inhibit B cell development; directly acting on a B lineage progenitor or indirectly through another cell (such as OP9 stromal cells) in BM cultures. While ACM treatment of cultures containing mouse B220$^-$ BM cells and OP9 stromal cells resulted in the development of very few B220$^+$ B lineage cells (Figure 3.2 A, B&E), these cultures contained a large population of cells that do not express B220. We became interested in identifying which cell type develops after treatment of BM cultures with adipocyte factors. To characterize these B220$^-$ cells, we stained the cells with a panel of lineage specific antibodies (including myeloid [eg. anti-CD11b], NK [eg. anti-NK1.1], T lineage [eg. anti-CD3] specific antibodies) and analyzed the cells by flow cytometry. We expected that if ACM promoted the development of another hematopoietic lineage, then we would visualize the resulting lineage with our panel of antibodies. Alternatively, if adipocyte factors directly block B cell development and hematopoietic progenitors accumulate in ACM-treated cultures, then we would not see staining with our lineage panel. In the latter scenario, additional staining would be needed to identify hematopoietic progenitors. As evidenced by flow cytometric analysis of cells resulting from ACM

treatment, the vast majority of B220⁻ cells were identified with the myeloid

markers CD11b and Gr1 (Figure 3.2 C, D, &F), suggesting that adipocyte factors

promote the accumulation of myeloid lineage cells.

In addition to increased quantity of CD11b⁺Gr1⁺ myeloid cells after ACM

treatment (Figure 3.2 C, D, &F), the quality also appeared to differ from the

CD11b⁺Gr1⁺ cells generated in untreated cultures. Flow cytometric analysis

showed that CD11b⁺Gr1⁺ cells from ACM-treated cultures had higher expression

of CD11b, were larger (FSC), and more granular (SSC) than myeloid cells from

untreated cultures (Figure 3.3). Although yet to be determined, these phenotypic

differences suggest CD11bʰⁱGr1⁺ cells generated in the presence of adipocyte

factors might also have different functional properties as compared to

CD11b⁺Gr1⁺ cells from untreated cultures.
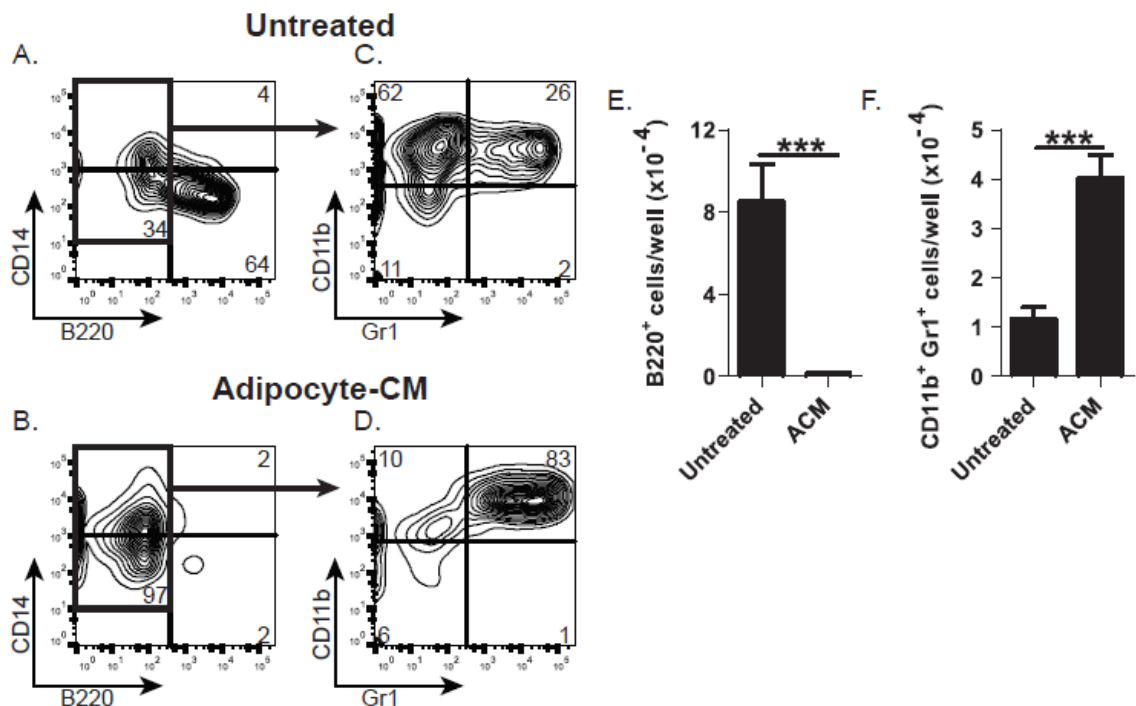
Figure 3.2 Characterization of cells resulting after treatment with adipocyte factors. Flow cytometric analysis and absolute cell numbers after mouse B lymphopoiesis cultures were treated with ACM or left untreated. Cells were stained and analyzed for (A&B) B220 and CD14 expression, followed by (C&D) CD11b and Gr1 expression in the B220$^-$ gate (C&D). Number of (E) B220$^+$ and (F) CD11b$^+$Gr1$^+$ cells present after culture. Data are representative of 3 independent experiments. For (E&F), Student's t test was used to determine significance. Error bars represent the average of triplicate wells +/- SD.

CD11b$^+$Gr1$^+$ cells
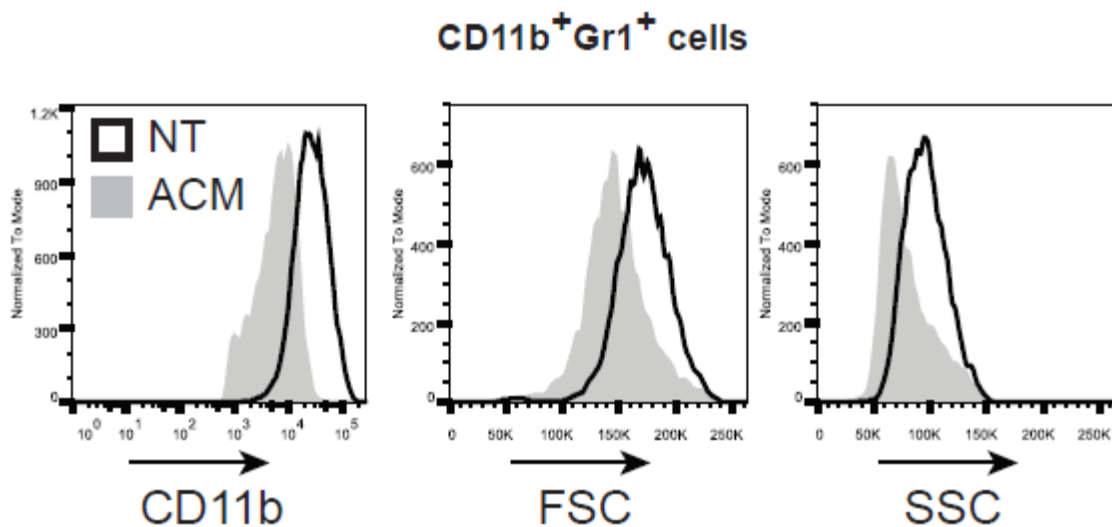
Figure 3.3 Phenotypic analysis of myeloid populations in ACM-treated and untreated cultures. Flow cytometric analysis of mouse B lymphopoiesis cultures 7 days post initiation. Cells were stained for B220, CD14, CD11b, and Gr1 expression to identify B220$^-$CD11b$^+$Gr1$^+$ cells. CD11b$^+$Gr1$^+$ cells resulting from ACM-treated or untreated (NT) cultures were analyzed for mean fluorescence intensity (MFI) of (left) CD11b, (middle) forward scatter (FSC), and (right) side scatter (SSC). Data are representative of at least 3 independent expedriments.

In young healthy BM, cells defined as CD11b$^+$Gr1$^+$ are considered myeloid progenitors, which will quickly differentiate into cells such as monocytes, macrophages, and neutrophils. Although in pathologies such as cancer, MDSCs (also defined as CD11b$^+$Gr1$^+$) accumulate (Gabrilovich and Nagaraj, 2009). To test if the CD11b^{hi}Gr1$^+$ cells that develop in the presence of ACM are MDSCs, we asked if they possess classical characteristics of MDSCs. We assessed if ACM-derived CD11b^{hi}Gr1$^+$ cells have the functional ability to suppress T cell proliferation, as well as co-express *Arg1* and *Nos2* which encode for the enzymes arginase and iNos; important effector molecules for MDSC-mediated suppression of T cell responses.

To understand whether ACM-generated CD11b^{hi}Gr1$^+$ cells express *Arg1* and *Nos2*, we FACS-sorted CD11b^{hi}Gr1$^+$ cells resulting from ACM-treated BM cultures and performed qPCR. We expected if ACM-generated CD11b^{hi}Gr1$^+$ cells are MDSCs, they should co-express higher levels of *Arg1* and *Nos2* compared to CD11b$^+$Gr$^+$ myeloid cells isolated from control untreated cultures and CD19$^+$ B lineage cells. As expected, compared to CD11b$^+$Gr$^+$ myeloid cells (-ACM) and CD19$^+$ cells (CD19$^+$) from untreated BM cultures, the myeloid cells isolated from ACM-treated cultures (+ACM) co-expressed large amounts of *Arg1* and *Nos2* (Figure 3.4 A).

To determine if myeloid cells isolated from ACM-treated cultures actively suppress T cell proliferation, we analyzed their ability to suppress in T cell proliferation assays. In these assays, splenocytes were CFSE-labeled and stimulated with plate-bound anti-CD3 and anti-CD28. Stimulation of splenocytes

activates CD4$^+$ T cells to proliferate, which can be visualized via flow cytometry as the dilution of CFSE in this splenocyte population. The reason this can be visualized is because CFSE is cell permeable and covalently binds to intracellular molecules, and as each division occurs the CFSE labeled components of a parent cell become shared by the resulting daughter cells (diluting the amount of stain per cell).

To assess the suppressive activity of ACM-generated CD11b^{hi}Gr1$^+$ myeloid cells, these cells were FACS-sorted and cultured with stimulated CFSE-labeled splenocytes. If ACM-generated myeloid cells suppress T cell proliferation, then culturing CD4$^+$ T cells with CD11b^{hi}Gr1$^+$ myeloid cells will prevent CFSE dilution (proliferation) in CD4$^+$ T cells. Compared to cultures containing an equal number of control effector cells (Figure 3.4 B dashed line), CD4$^+$ cells in cultures containing ACM-generated myeloid cells proliferated less (Figure 3.4 B solid line). These data coupled with the co-expression of *Arg1* and *Nos2* lead us to conclude that adipocyte soluble factors promote the accumulation of MDSCs.
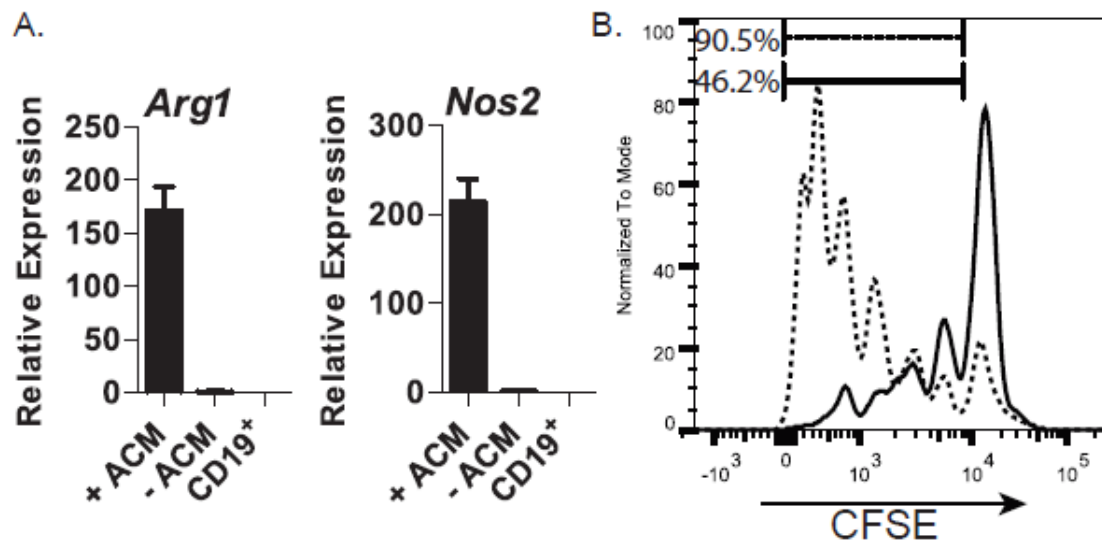
Figure 3.4 Phenotypic and functional characterization of ACM-generated CD11b^{hi}Gr1^{+} cells. (A) qPCR analysis of *Arg1* and *Nos2* expression comparing CD11b^{+}Gr1^{+} cells isolated by FACS from ACM-treated (+ACM), untreated (-ACM) BM cultures, and CD19^{+} control cells . (B) Flow cytometric plot of anti-CD3 and anti-CD28 stimulated CD4^{+} splenocytes (CFSE-labeled) cultured with ACM-generated CD11b^{hi}Gr1^{+} cells (solid line) or CD19^{+} control cells (dashed line). Data in (A) are representative of two independent experiments and in (B) are representative of three independent experiments.

There are two major subsets of MDSCs; monocytic and granulocytic. These subsets can be differentiated based on staining for the molecules Ly6C and Ly6G, where monocytic MDSCs are defined as Ly6C^{+}Ly6G^{-} and granulocytic MDSCs are defined as Ly6C^{+}Ly6G^{+} (Gabrilovich and Nagaraj, 2009, Youn et al., 2008). While both types of MDSCs suppress T cell responses, the mechanisms

regulating their induction and suppressive activity can vary. Understanding the type of MDSC induced by adipocyte factors will be help us identify critical molecules for MDSC induction and suppressive activity.

By flow cytometric analysis of $CD11b^+$ cells resulting from ACM-treated BM cultures, we found that 65% of $CD11b^+$ cells were $Ly6C^+Ly6G^-$ monocytic, while 21% exhibited the $Ly6C^+Ly6G^+$ granulocytic MDSC phenotype (Figure 3.5 A). To complement this result, we also visualized the nuclei of ACM-generated MDSCs, as monocytic MDSCs have nuclei resembling immature monocytes and granulocytic MDSCs have polymorphic nuclei similar to neutrophils. We FACS-sorted ACM-generated $CD11b^+Gr1^{hi}$ MDSCs, plated the MDSCs in tissue culture wells, and then stained with Diff-Quick^{TM} stain to visualize nuclei by light microscopy. Based on our flow cytometry data, we expected that the majority of the MDSCs would have nuclei that resemble monocytic MDSCs. In fact, microscopy analysis of sorted MDSCs confirmed that this population is primarily of the monocytic subset (Figure 3.5 B), as very few MDSCs had a polymorphonuclear appearance similar to neutrophils. These data lead us to conclude that adipocyte factors mainly promote the generation of monocytic MDSCs *in vitro*. Further, the above experiments indicate that the use of adipocyte factors is an efficient means to generate a pure population of MDSCs. This culture system will be a useful tool to generate MDSCs for further study of these cells, as well as for the use of MDSCs as a therapeutic agent.
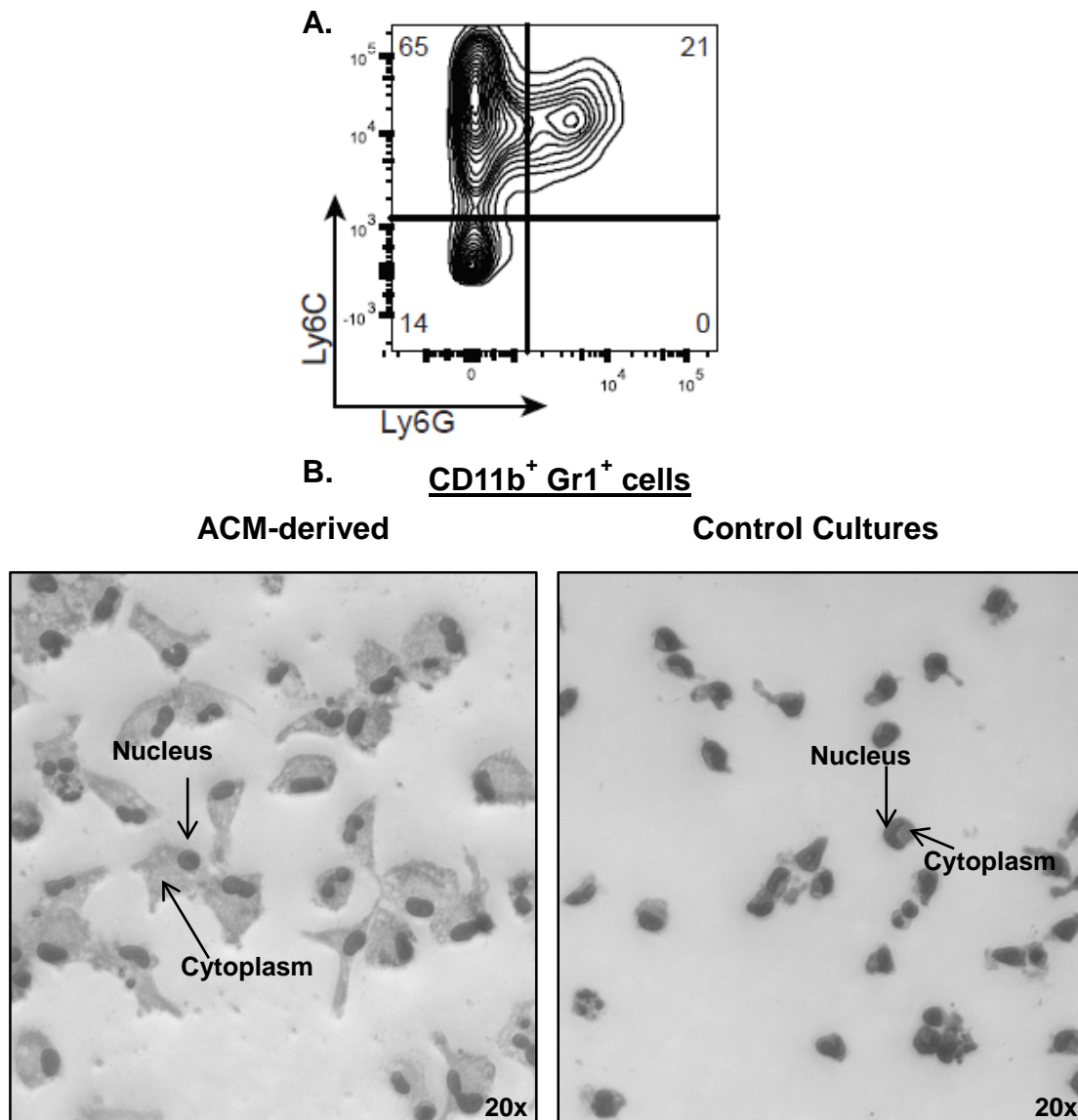
Figure 3.5 Ly6C vs Ly6G expression and morphology of CD11b+Gr1+ cells isolated from ACM-treated or control BM cultures. (A) Flow cytometric analysis of CD11b+ cells from ACM-treated or untreated cultures for the expression of Ly6C and Ly6G. (B) FACS-sorted CD11b+Gr1+ cells were plated in wells and stained with Diff Quick (Siemens, USA).

SECTION 2: MDSCs AND THE INHIBITION OF B LYMPHOPOIESIS

MDSCs are well characterized for their ability to suppress T cells, but it is not known whether these cells alter hematopoiesis. The generation of MDSCs by adipocyte soluble factors could be the result of skewed hematopoiesis, where MDSCs are simply bystanders. Alternatively, because MDSCs are suppressive in nature we hypothesized that ACM-generated MDSCs might contribute to the inhibition of B lymphopoiesis. To test if MDSCs have the functional capacity to inhibit B cell development, we added FACS-sorted MDSCs from ACM-treated cultures or CD11b$^+$Gr1$^+$ myeloid cells from untreated cultures directly to new B lymphopoiesis cultures (no ACM added). If MDSCs contribute to the inhibition of B lymphopoiesis, then we expect BM cultures containing MDSCs will result in a decreased number of B220$^+$ B lineage cells compared to control cultures. Strikingly, wells with ACM-generated MDSCs contained very few B220$^+$ B lineage cells; whereas cultures treated with control myeloid cells exhibited a similar number of B lineage cells to untreated cultures (Figure 3.6). We conclude that MDSCs actively inhibit B lymphopoiesis.
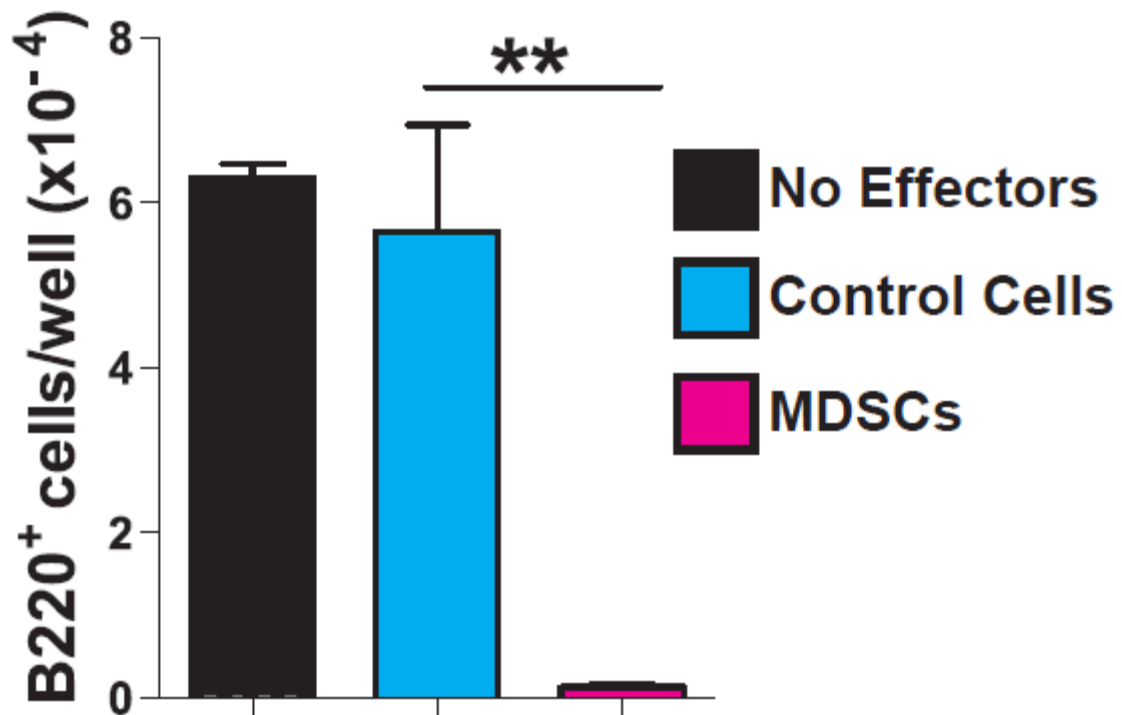
Figure 3.6 Effect of MDSCs on B lymphopoiesis cultures. Absolute number

of B220$^+$ cells resulting from BM cultures treated with the following FACS isolated

effector cells: 5,000 CD11b^{hi}Gr1$^+$ MDSCs from ACM-treated cultures (pink),

5,000 CD11b$^+$Gr1$^+$ control cells from untreated cultures (blue), or no effectors

(black). Data are representative of 3 independent experiments. Student's *t* test

was used to determine significance. Error bars represent the average of triplicate

wells +/- SD.

Mechanism of MDSC-mediated inhibition of B lymphopoiesis

The finding that MDSCs potently inhibit B lymphopoiesis is novel.

Therefore, we asked by what mechanism these MDSCs mediate their inhibitory

effect. Further understanding how adipocyte soluble factor generated MDSCs

inhibit B cell development will help us identify the active molecules produced by adipocytes to trigger this MDSC-mediated inhibition.

Since MDSC effector mechanisms are well-known for the suppression of T lymphocytes, we asked if B cell development is also sensitive to one of these mechanisms. The most well-known way MDSCs suppress T cell responses is through the effector molecules arginase and iNos. These enzymes both use L-arginine as a substrate and can suppress through L-arginine depletion. In addition, nitric oxide produced by iNos has suppressive activity (Gabrilovich and Nagaraj, 2009, Rodriguez and Ochoa, 2008). The idea that these effector molecules could alter B lymphopoiesis is plausible, as one study found arginine deficient mice have impaired B cell development (de Jonge et al., 2002).

To test if arginase and/or iNos are responsible for MDSC-mediated inhibition of B lymphopoiesis, we cultured BM progenitors, OP9 cells, and MDSCs with or without nor-NOHA (arginase inhibitor) and L-NMMA (iNos inhibitor). We expected that if arginase and/or iNos mediate the MDSC inhibitory activity, then blocking these effectors will restore B lymphopoiesis in cultures containing MDSCs. Surprisingly, B lymphopoiesis was not restored when blocking arginase and iNos in cultures containing MDSCs. BM cultures with MDSCs in the absence of any inhibitors contained significantly fewer B220$^+$ B lineage cells compared to control wells (Figure 3.7 A). Consistently, the number of B lineage cells in all wells containing MDSCs was significantly lower than untreated wells and cultures containing control cells, regardless of the addition of

arginase and iNos inhibitors. This result suggests that MDSCs do not inhibit B lymphopoiesis through arginase/iNos.

To ensure the nor-NOHA and L-NMMA inhibitors were active, we tested them in the context of T cell proliferation assays. If ACM-generated MDSCs behave like classical MDSCs, we expected they would inhibit T cell proliferation via arginase and iNos. Therefore using these inhibitors in T cell proliferation assays should restore T cell proliferation in wells containing MDSCs. To test this, CFSE labeled splenocytes were stimulated with anti-CD3 and anti-CD28, and then T cell proliferation (CD4$^+$ and CD8$^+$) was assessed comparing cultures with MDSCs in the presence or absence of nor-NOHA and L-NMMA. If ACM-generated MDSCs inhibit T cell proliferation in an arginase and iNos dependent manner, and if the nor-NOHA and L-NMMA inhibitors are active, then T cell (CD4$^+$ and CD8$^+$) proliferation will decrease in the presence of MDSCs, but will be restored to untreated levels when arginase and iNos are inhibited. Flow cytometric analysis of the CD4$^+$ and CD8$^+$ splenocyte populations showed similar results. CD4$^+$ and CD8$^+$ splenocytes from cultures containing MDSCs exhibited decreased CFSE dilution (proliferation) compared to when no effector cells were added. Further, CFSE dilution (proliferation) was significantly increased in cultures with MDSCs in which arginase and iNos were blocked (Figure 3.7 B & C). We conclude that ACM-generated MDSCs inhibit T cell proliferation in an arginase and iNos dependent manner, although this is not the primary mechanism by which MDSCs inhibit B cell development.
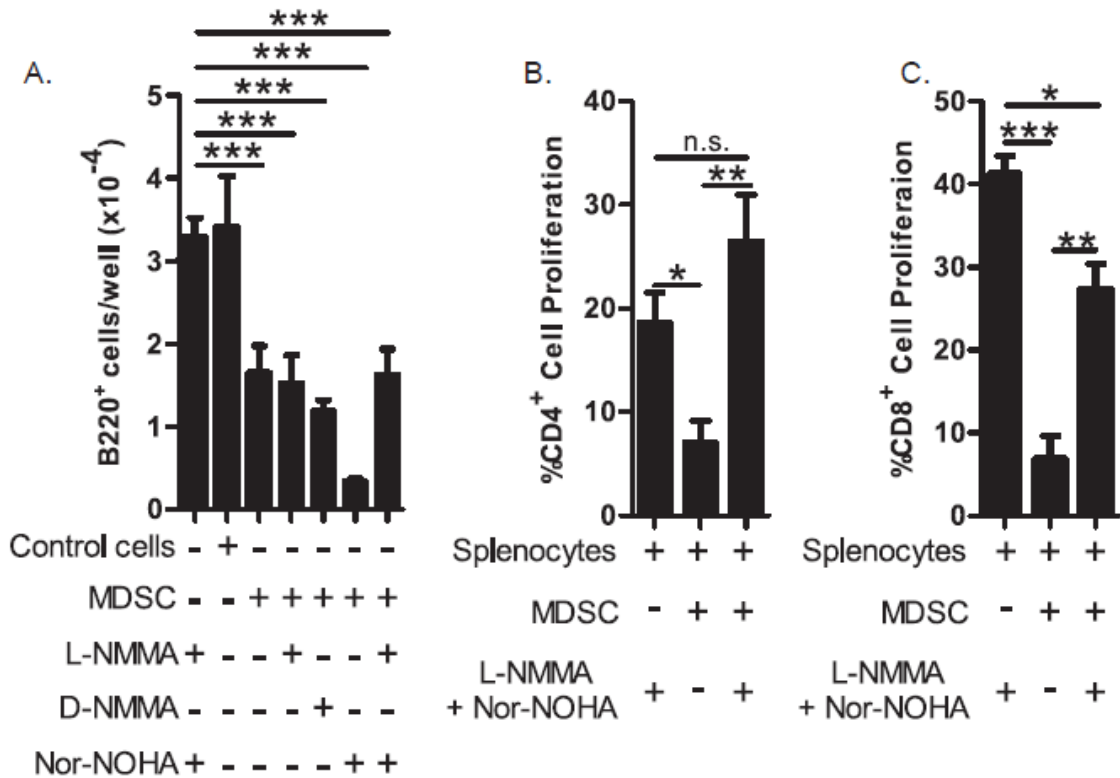
Figure 3.7 MDSC-mediated inhibition of B lymphopoiesis in the absence of arginase and iNos. (A) Number of B220$^+$ cells resulting from B lymphopoiesis cultures containing no effector cells, ACM-generated MDSCs, or non-inhibitory CD11b$^+$Gr1$^+$ control cells. The indicated wells were treated with 0.3mM L-NMMA (iNos inhibitor), 0.3mM D-NMMA (control compound), and/or 0.3mM nor-NOHA (arginase inhibitor). (B-C) Proliferation of (B) CD4$^+$ or (C) CD8$^+$ splenocytes after anti-CD3 and anti-CD28 stimulation in the presence or absence of ACM-generated MDSCs, and treated with 0.3mM L-NMMA and nor-NOHA where indicated. Data in (A-C) are representative of three independent experiments. Error bars represent the average of triplicate wells +/- SD. Data in (A) were analyzed for statistical significance by ANOVA coupled with the Dunnet multiple

comparison test ($p<0.0001$). Data in (B&C) were analyzed for statistical significance by ANOVA coupled with the Bonferroni multiple comparison test ($p=0.001$ and $p=0.003$ respectively).

Do MDSCs require contact with target cells? MDSCs do not use arginase and iNos to inhibit B lymphopoiesis, suggesting MDSCs inhibit via a novel mechanism. To gain a better sense of how MDSCs negatively regulate B cell development, we asked if inhibition required contact with BM progenitors. BM progenitors were cultured with OP9 cells in B lymphopoiesis assays, either with MDSCs in contact with BM progenitors or separated by transwells. We expected that if MDSCs require contact with target cells to mediate inhibition, then MDSCs will not inhibit B lymphopoiesis when cultured in transwells. Interestingly, the number of B220$^+$ B lineage cells was significantly reduced compared to no MDSC controls regardless of whether MDSCs were cultured in contact or away from BM progenitors (Figure 3.8 A). This result suggested that ACM-generated MDSCs do not require contact with target cells to mediate inhibition, and likely inhibit through the production of a soluble factor.

To complement the above finding and to test if MDSC-derived soluble factors inhibit B lymphopoiesis, we asked if conditioned medium from MDSCs (MDSC-CM) contained inhibitory activity. To generate conditioned medium, we isolated MDSCs (by FACS) from ACM-treated BM cultures and plated the isolated cells in wells of a 48 well plate. Several days later, supernatant from MDSC cultures was collected and used as a source of MDSC-derived soluble

factors. The inhibitory activity of MDSC factors was tested by treating mouse BM cultures with MDSC-CM and analyzing the resulting frequency and absolute number of B220$^+$ B lineage cells by flow cytometry. We expected that if MDSCs inhibit via a soluble factor(s), then MDSC-CM treated cultures would exhibit decreased development of B lineage cells compared to untreated cultures. As expected, MDSC-CM inhibited B cell development, where the percentage and number of B220$^+$ cells were significantly reduced in cultures containing MDSC-CM (Figure 3.8 B, D&F). Together these data suggest MDSCs do not require contact with target cells to negatively regulate B lymphopoiesis. We conclude that MDSCs inhibit through the production of a soluble factor(s).

Interestingly, we noticed that BM cultures treated with MDSC-CM exhibited an increased percentage and number of CD11b$^+$Gr1$^+$ myeloid lineage cells (Figure 3.8 C, E&G). These data suggest that MDSCs either produce multiple factors that separately inhibit B lymphopoiesis and promote myelopoiesis, or one factor that has a dual function to inhibit B cell development and promote myeloid cell development and/or survival. Therefore we decided to profile MDSC-derived soluble factors to identify molecules that could recapitulate this phenomenon.
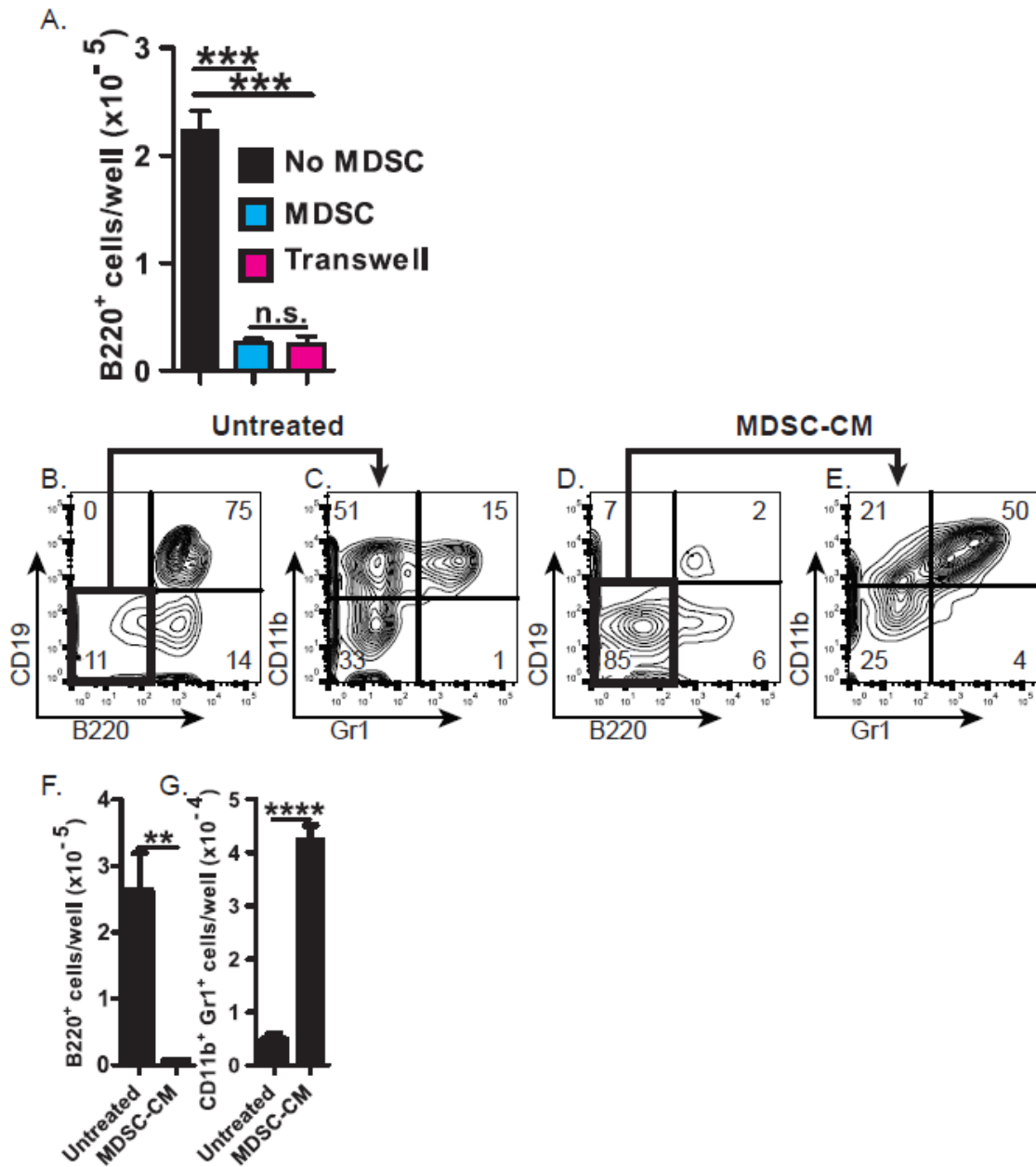
Figure 3.8 Effect of MDSC-derived soluble factors on B lymphopoiesis. (A)

Number of B220$^+$ cells resulting from B lymphopoiesis cultures containing no

effector cells (no MDSC), MDSCs, or MDSCs cultured in a transwell. (B-G) B

lymphopoiesis assays were performed with or without (untreated) MDSC-CM

treatment. (B-E) Flow cytometric analysis of cells stained with antibodies to

(B&D) B220 and CD19, or (C&E) CD11b and Gr1. Number of (F) B220$^+$ cells and (G) CD11b$^+$Gr1$^+$cells resulting from untreated cultures or cultures treated with MDSC-CM. Data in (A) are representative of 2 independent experiments and were analyzed by ANOVA in combination with a Bonferroni multiple comparison test (p<0.0001). Data in (B-G) are representative of 3 independent experiments. Statistical significance in (F&G) was analyzed by Student's t test. Error bars represent the average of triplicate wells +/- SD.

Soluble factors produced by MDSCs. Since MDSCs inhibit through soluble factors, we performed a cytokine array to profile MDSC-derived molecules. Conditioned medium was generated from FACS isolated MDSCs (inhibit B cell development) and control CD11b$^+$Gr1$^+$ myeloid cells (do not inhibit B cell development), then profiled for the concentrations of 23 different factors. We expected that potential inhibitory factor(s) would be present at higher concentrations in MDSC-CM than control-CM. Cytokine array analysis showed that MDSCs produced increased concentrations of IL-1α, IL-1β, IL-13, G-CSF, KC, MCP, MIP-1α, MIP-1β, and RANTES (Figure 3.9), many of which are classified as inflammatory cytokines or chemokines known to recruit inflammatory cells. Alternatively, IL-12(p40) and IL-9 concentrations were higher in control-CM; ruling out these molecules as the MDSC-derived inhibitory factors. Additional proteins that were assessed but not detected in MDSC-CM include: IL-2, IL-3, IL-5, and GM-CSF. Further IL-2, IL-4, IL-6, IL-10, IL-12(p70), IL-17, TNFα, and IFNγ were present, but at levels <10pg/ml. For subsequent studies

we focused only on IL-1α, IL-1β, IL-13, G-CSF, KC, MCP, MIP-1α, MIP-1β, and

RANTES, as these were present in MDSC-CM at higher levels than control-CM.
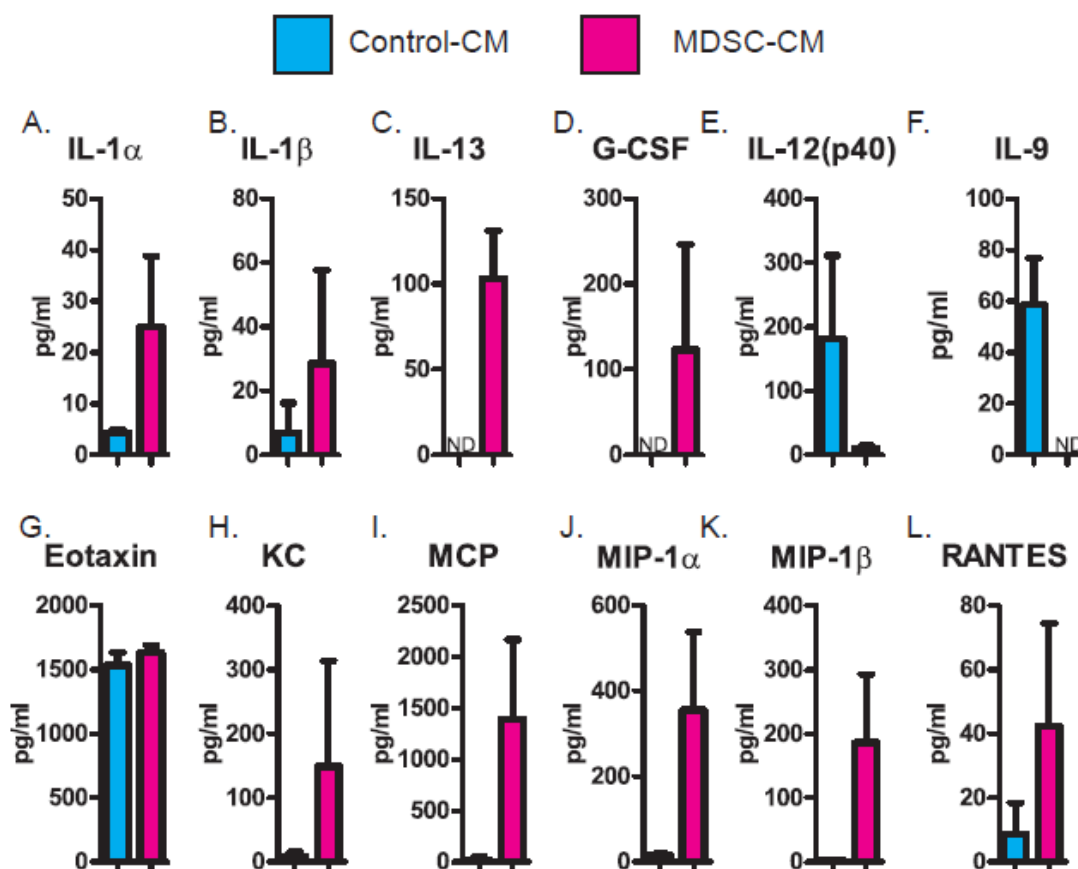
Figure 3.9 Profile of MDSC-derived soluble factors by cytokine array.

Protein concentrations of the indicated factors as detected by cytokine array in

MDSC-CM or control-CM. Data are from three different MDSC-CM and two

different control-CM generated in individual experiments. Error bars show the

average of the two or three different conditioned media +/- SD. ND= not

detected.

We identified nine factors that were upregulated in MDSC-CM and asked

if any of these molecules inhibit B lymphopoiesis. B lymphopoiesis cultures were

performed in the presence or absence of each factor (recombinant) individually, and then the number of B lineage cells was analyzed at the end of culture. We did not think all nine identified factors would inhibit B cell development, but perhaps one or two. We expected if any of these molecules have the potential to inhibit B lymphopoiesis, then BM cultures containing an inhibitory molecule would result in decreased development of B220$^+$ B lineage cells compared to untreated cultures. In fact this experiment drastically narrowed our search for MDSC-derived inhibitory factors because, of all the molecules assessed, only cultures containing IL-1α and IL-1β had significantly lower numbers of B220$^+$ B lineage cells (Figure 3.10 A); suggesting these molecules inhibit B cell development.

Only IL-1α and IL-1β could inhibit when added to B lymphopoiesis cultures individually, but we also sought to determine if the other seven identified factors could inhibit when in combination with the others. To test this, we compared the inhibitory potential of adding all nine factors (IL-1α, IL-1β, IL-13, G-CSF, KC, MCP, MIP-1α, MIP-1β, and RANTES) to BM cultures in combination or without IL-1α/β. We know IL-1α/β can inhibit alone, therefore treatment with all nine factors should result in reduced B lineage development. If the other seven factors are able to inhibit B lymphopoiesis when combined, we expected adding IL-13, G-CSF, KC, MCP, MIP-1α, MIP-1β, and RANTES would also inhibit B lymphopoiesis. However, this did not occur. Cultures containing all nine factors resulted in the development of significantly fewer B220$^+$ cells compared to untreated cultures. But treatment of BM cultures with all factors without IL-1α/β contained a similar number of B220$^+$ cells compared to untreated cultures (Figure

3.10 B). These data suggest that IL-1α and IL-1β are the only molecules identified in MDSC-CM with inhibitory activity to B lymphopoiesis.

Do MDSCs inhibit B lymphopoiesis via IL-1? MDSCs produce IL-1α/β and these cytokines inhibit B lineage development. But is the mechanism by which MDSCs inhibit mediated through IL-1α/β? Our approach to answer this question was to neutralize IL-1α/β in B lymphopoiesis cultures containing MDSCs, and then determine if B lymphopoiesis was restored to untreated levels. We added MDSCs to B lymphopoiesis cultures in the presence or absence of anti-IL-1α and anti-IL-1β to neutralize these cytokines. We expected that if IL-1α/β were the major inhibitory factors produced by MDSCs, then blocking these molecules would enhance B lymphopoiesis in cultures containing MDSCs. Compared to control cultures lacking MDSCs, cultures containing MDSCs exhibited a significant reduction in the number of B220$^+$ B lineage cells. However when IL-1α/β was neutralized in cultures containing MDSCs, the number of B220$^+$ B lineage cells was restored to control levels (Figure 3.10 C). These data lead us to conclude that MDSCs inhibit B lymphopoiesis via IL-1α/β.
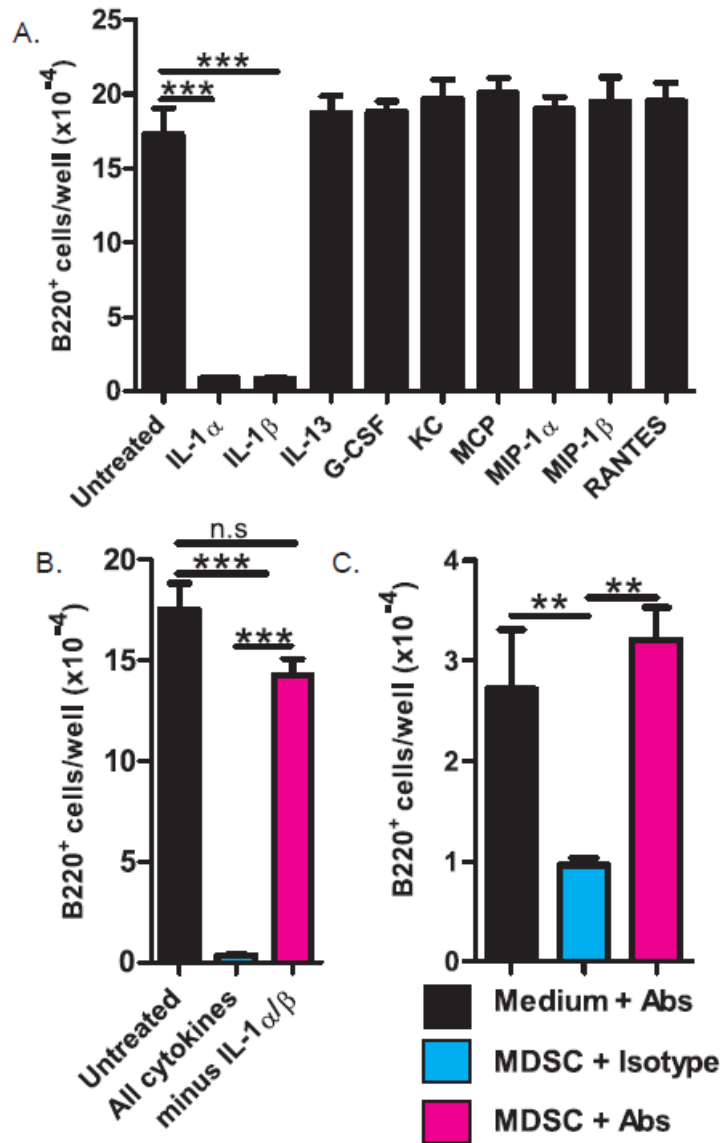
Figure 3.10 Identification of MDSC-derived inhibitory factors. (A&B) Number

of B220+ cells resulting from B lymphopoiesis cultures treated with the indicated

recombinant cytokine(s) (1ng/ml). (C) Number of B220+ cells resulting from B

lymphopoiesis cultures with or without MDSCs and treated with anti-IL-1α

(1μg/ml) and anti-IL-1β (1μg/ml), or isotype control antibodies where indicated.

Data in (A-C) are representative of three independent experiments. Statistical

significance in (A) was determined using ANOVA coupled with a Dunnet multiple

comparison test ($p<0.0001$). Statistical significance in (B & C) were determined

using ANOVA coupled with a Bonferroni multiple comparison test ($p<0.0001$ and

p=0.001 respectively). Error bars represent the average of triplicate wells +/- SD.

Hematopoietic target of IL-1. The identification that MDSCs inhibit B cell

development via IL-1 has uncovered a novel interaction between MDSCs and

hematopoiesis. But how does IL-1act to inhibit B cell development? We know

that IL-1 treatment of BM cultures results in the development of very few (if any)

B220$^+$ cells. Because B220 is first expressed at the pre-proB cell stage, we

hypothesized that the hematopoietic target of IL-1 treatment is a progenitor prior

to the pre-proB cell stage. Therefore IL-1 must act at the HSC to MPP, MPP to

CLP, or CLP to pre-proB transition. To identify the hematopoietic target of IL-1

we FACS-sorted either HSCs (Lin$^-$Sca1$^+$c-kit$^+$Flt3$^-$), MPPs (Lin$^-$Sca1$^+$c-kit$^+$Flt3$^+$),

or CLPs (Lin$^-$Sca1^{lo}c-kit^{lo}Flt3$^+$IL-7R$^+$) (Figure 3.11 A) and assessed the ability of

these progenitors to differentiate into B lineage cells on OP9 stromal cells when

treated with IL-1β. We expected that if IL-1β targets CLPs to inhibit B cell

development, for example, then B lymphopoiesis assays seeded with CLPs will

have fewer B220$^+$ B lineage cells develop in IL-1β treated cultures. Interestingly,

IL-1β treatment did not affect the ability of CLPs to differentiate into B220$^+$ B

lineage cells. Flow cytometric analysis of cultures starting with CLPs showed no

difference in the frequency of B lineage cells comparing untreated and IL-1β

treated wells (Figure 3.11 B). Similarly, there was no significant difference in the

number of B220$^+$ cells resulting from these cultures (Figure 3.11 C). Alternatively,

analysis of cultures seeded with MPPs resulted in a large reduction in B220$^+$ B

lineage cell frequency (Figure 3.11 D) coupled with a significant decrease in the

number of B220$^+$ B lineage cells in IL-1β treated cultures (Figure 3.11 E). Flow

cytometric analysis of cultures beginning with purified HSCs showed a similar

result to cultures seeded with MPPs upon IL-1β treatment (Figure 3.11 F & G).

Because IL-1β inhibited B lymphopoiesis in both cultures seeded with purified

HSCs and MPPs, this could suggest that IL-1β targets both the HSC and the

MPP. Alternatively, the inhibitory effect observed in cultures of HSCs could be

mediated once HSCs differentiate into MPPs. We conclude that IL-1β treatment

targets early hematopoietic progenitors, likely the MPP, to inhibit B cell

development. IL-1α treatment showed similar results, suggesting these MDSC-

derived molecules inhibit through the same mechanism. This was also expected

as IL-1α and IL-1β both act through the same receptor on target cells.
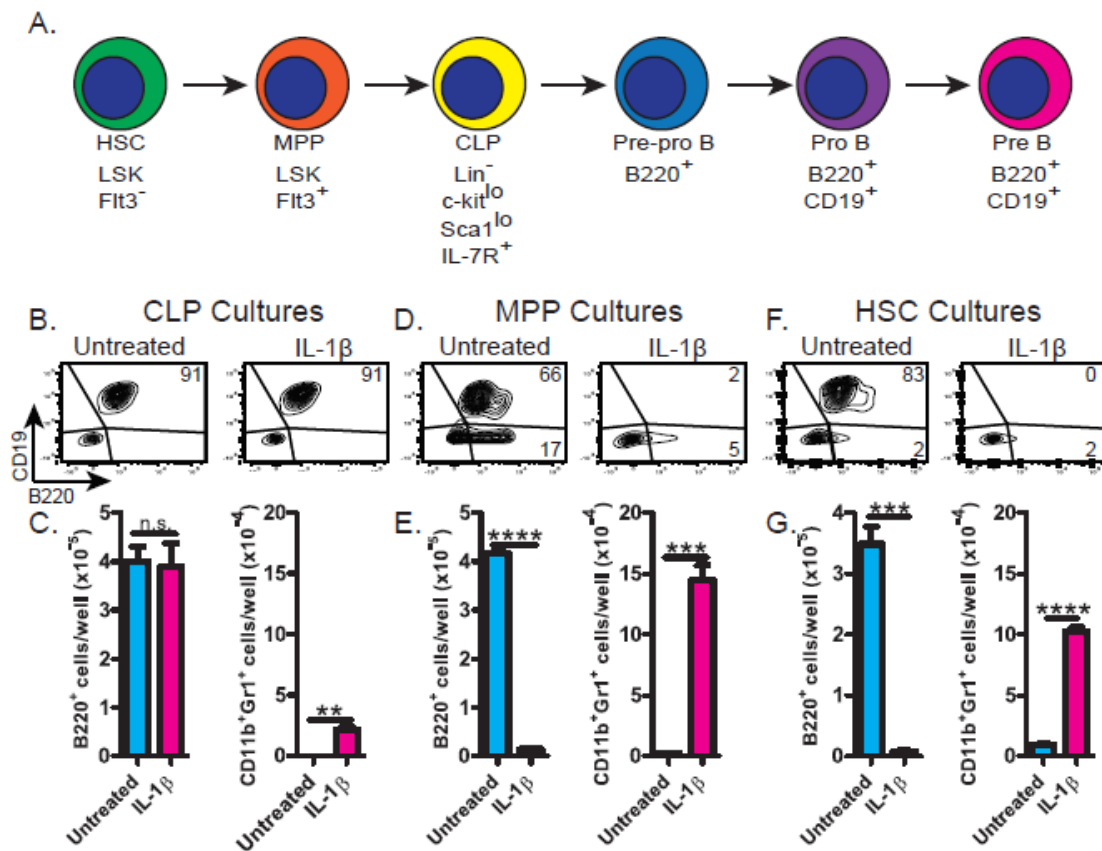
Figure 3.11 Identification of hematopoietic progenitors targeted by IL-1. (A) B lineage developmental stages and key markers for identification. (B, D&F) Flow cytometric analysis of cells resulting from B lymphopoiesis cultures starting with purified (B) CLPs, (D) MPPs, or (F) HSCs. Cultures were performed in the presence or absence (untreated) of IL-1β and stained with anti-B220 and anti-CD19. (C, E, &G) Number of B220+ cells or CD11b+Gr1+ cells resulting from B lymphopoiesis cultures seeded with (C) CLPs, (E) MPPs, or (G) HSCs and treated with or without (untreated) IL-1β. Data in (B-G) are representative of three independent experiments. Statistical significance in (C, E, &G) was determined by Student's *t* test. Error bars represent the average of triplicate wells +/- SD.

IL-1 treatment acts at the MPP stage to inhibit B lymphopoiesis. But is B cell development blocked at the MPP stage, or is hematopoiesis re-routed? The answer to this question came from flow cytometric analysis of myeloid lineage cells resulting from cultures seeded with HSCs, MPPs, or CLPs (Figure 3.11 C, E&G). If B lymphopoiesis is blocked at the MPP stage, we expected to find an accumulation of MPPs in IL-1 treated cultures seeded with HSCs and MPPs. Alternatively, if IL-1 treatment re-routes hematopoiesis to another lineage, then we expected to find the accumulation of cells of another hematopoietic lineage (eg. myeloid lineage) in IL-1 treated cultures. Interestingly, IL-1 treatment of cultures seeded with HSCs or MPPs resulted in a large significant increase in the development of $CD11b^+Gr1^+$ myeloid cell number. While IL-1 treatment of CLPs also resulted in an increase in myeloid cells, this increase was small compared to the number observed in HSC and MPP cultures. Overall, these data suggest that IL-1 treatment acts at the MPP stage to promote myelopoiesis at the expense of B lymphopoiesis. Further, we conclude that IL-1 does not block B cell development, but instead skews hematopoiesis to the myeloid lineage.

In summary, our data suggest that adipocytes inhibit B lymphopoiesis *in vitro* by promoting the accumulation of MDSCs. These MDSCs produce IL-1, which then skews hematopoiesis to the myeloid lineage. While we know that MDSCs produce IL-1, we do not know what adipocyte factor(s) promote MDSCs. We also do not know if adipocytes produce only one factor or many factors that contribute to the loss of B lymphopoiesis, as adipocytes are known to produce many immunomodulatory factors, such as adiponectin and leptin. Further

characterization of ACM is needed to understand if adipocytes produce multiple

inhibitory factors and to identify the most critical factors that induce MDSCs.

SECTION 3: CHARACTERIZATION OF ADIPOCYTE-DERIVED MOLECULES IN THE NEGATIVE REGULATION OF B LYMPHOPOIESIS

The mechanism of MDSC-mediated inhibition of B lymphopoiesis,

elucidated above, is initiated by adipocyte-derived soluble factors. This made us

interested in understanding how adipocyte factors promote MDSC accumulation

and ultimately lead to the loss of B lymphopoiesis. We asked; 1. Do multiple

adipocyte factors contribute to the inhibition of B lymphopoiesis; 2. What

adipocyte-derived molecules are most critical for generating MDSCs; and 3. Can

we target adipocyte factors to prevent MDSC accumulation?

Do multiple adipocyte factors contribute to the inhibition of B lymphopoiesis?

Adipocytes are capable of producing many factors that modulate the

immune system and other processes throughout the body. Adiponectin is one

adipocyte-derived product known to influence hematopoiesis (Yokota et al.,

2003), but we hypothesized that adipocytes produce additional molecules that

can affect immune cell development. To understand how adipocyte-derived

soluble products negatively regulate B lymphopoiesis as a whole, we decided to

characterize different fractions (based on molecular weight) of ACM. We

expected this would give us a better understanding of how adipocyte products

lead to the inhibition of B lymphopoiesis and how they lead to the accumulation of IL-1 producing MDSCs.

To assess the type of inhibitory molecule(s) produced by adipocytes, we fractionated mouse ACM by size and assessed the inhibitory potential of each fraction on B lymphopoiesis. ACM was fractionated using a 10kDa filter to generate ACM <10kDa molecules and ACM >10kD molecules. We reasoned that if ACM contains multiple inhibitory molecules, then we expect that treatment of B lymphopoiesis cultures with ACM <10kDa and ACM >10kDa would result in reduced B lineage development compared to untreated cultures. As expected total ACM-treated BM cultures resulted in a decreased percentage of B220[+] B lineage cells compared to untreated cultures (Figure 3.12 A). In separate experiments, treatment with ACM <10kDa (Figure 3.12 B) and ACM >10kDa (Figure 3.12 C) also resulted in a decreased percentage of B220[+] B lineage cells compared to their respective medium controls. Similar results were obtained when studying the >10kDa and <10kDa fractions of rabbit ACM (data not shown), suggesting that ACM contains multiple inhibitory factors of different size.

The above result only indicates that there is more than one inhibitory factor produced by adipocytes. Naturally, there could be more than one inhibitory factor in the ACM <10kDa, as well as the ACM >10kDa. Our subsequent experiments were aimed to further characterize inhibitory molecules in ACM, to address whether or not they are protein in nature; as this information will guide attempts to identify ACM-derived molecules.
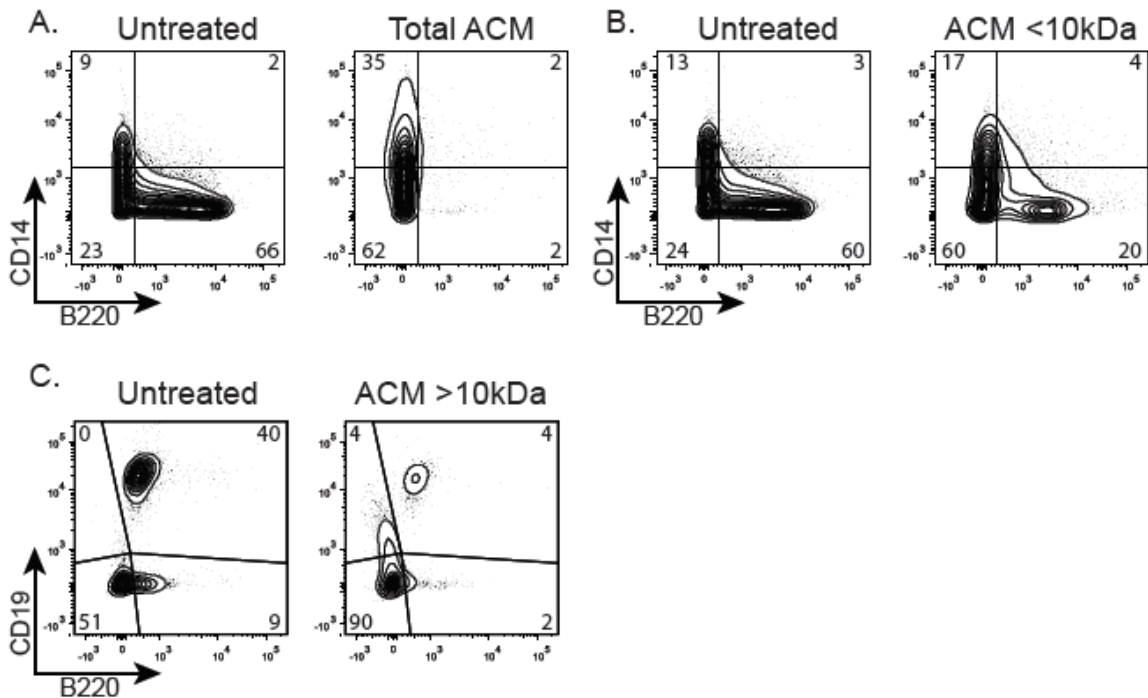
Figure 3.12 Effect of <10kDa and >10kDa adipocyte molecules on B lymphopoiesis. Flow cytometric analysis of B lymphopoiesis cultures performed in the presence or absence (untreated) of (A) total ACM, (B) ACM <10kDa, or (C) ACM >10kDa. (A&B) Flow cytometry plots displaying cells stained with anti-B220 and anti-CD14. (C) Flow cytometry plots displaying cells stained with anti-B220 and anti-CD19. Note the different staining scheme in panel (C). For all panels, total B220$^+$ cells are considered B lineage (49% untreated vs 6% ACM >10kDa in panel C). Data are representative of at least two or three independent experiments per fraction.

Are the inhibitory molecules in ACM protein in nature?

Adipocytes can produce both protein and lipid products. To understand the contribution of these major classes of molecules to the inhibitory effect, we

proteinase K treated the >10kDa and <10kDa fractions of ACM. We expected

that if proteins are responsible for the inhibitory effect in either of these fractions,

then proteinase K treatment would remove the inhibitory activity of ACM >10kDa

or <10kDa. B lymphopoiesis assays performed with mouse ACM and rabbit ACM

both suggest that adipocytes produce inhibitory factors both protein and non-

protein (likely lipid) in nature (data not shown), as proteinase K treatment

removed the inhibitory activity of >10kDa ACM but not smaller molecule fractions.

This information is critical for developing strategies to purify the inhibitory activity

in ACM. Now that we know the active molecules in ACM <10kDa and ACM

>10kDa have different biochemical make up, we tried to exploit this information to

purify inhibitory molecules.

Purification of adipocyte derived-inhibitory factors

Since ACM >10kDa is protein in nature, we asked if we could purify the

inhibitory molecule(s) via anion exchange chromatography. We used mouse

ACM to purify ACM >10kDa molecules because mouse ACM can be generated

in greater abundance and in less time than rabbit and human ACM. By fast

protein liquid chromatography (FPLC), using a strong anion exchange column

and increasing salt concentration to elute, we separated ACM >10kDa based on

charge (Figure 3.13 A). There was a large protein peak in the column flow

through, suggesting not all ACM proteins bound to the column, and two defined

peaks were observed after elution was started. We collected and concentrated

the effluent into five fractions (4-8) as seen in Figure 3.13 A.

To assess whether any of the peaks contained inhibitory activity, we added various column fractions/samples to rabbit B lymphopoiesis assays and analyzed the percentage of CD79a$^+$ B lineage cells resulting after treatment. If this strategy was effective in purifying inhibitory molecules, we expect to find some column samples that contain inhibitory activity and some that do not. Although the flow through contained a large protein concentration peak, this fraction did not contain significant inhibitory activity (Figure 3.13 A&B). In contrast, treatment with samples 6 and 7 resulted in decreased B lineage development similar to unfractionated ACM treatment (positive control for inhibition). Therefore the inhibitory activity was in part purified through this method and could be found in samples 6 and 7.

To see how effective this purification strategy was, we assessed the purity of the inhibitory fractions. We performed SDS PAGE to separate proteins within each column sample and visualized the proteins in each fraction after silver staining. If this strategy effectively purified ACM inhibitory proteins, we expected samples 6 and 7 would only contain several stained protein bands. As expected, unfractionated ACM >10kDa contained many protein bands of various sizes (Figure 3.13 C ACM lane). Although the inhibitory fractions 6 and 7 contained fewer protein bands, there were still many, suggesting that additional purification methods will be needed to purify single protein bands (Figure 3.13 C Sample 6, Sample 7a, 7b, 7c lanes). In sample 6 there was a prominent protein band between the 75kDa and 50kDa size markers, which was less concentrated in the

other samples. Additional experiments will be needed to determine the identity of

the corresponding protein and its inhibitory potential.

As seen in Figure 3.13 C, we further divided sample 7 into 3 smaller

fractions (Sample 7a, 7b, and 7c) and found two of the three fractions contained

an inhibitory factor(s) (as indicated with +). Inhibitory activity was determined

similar to that seen in Figure 3.13 B, where each fraction was dialyzed against

cell culture medium then added to rabbit B lymphopoiesis assays at a 1:2

dilution. Further division of sample 7 did not substantially change the number of

protein bands observed after silver staining (Figure 3.13 C sample 7a, 7b, and 7c

lanes), therefore future studies will be needed to obtain more pure samples to

identify single inhibitory factors.

Our attempts to characterize and purify ACM molecules with inhibitory

activity have focused on the overall inhibition of B lymphopoiesis. Because we

found that adipocyte factors induce inhibitory MDSCs, we focused our search to

identify ACM molecules that promote the accumulation of MDSCs.

A.
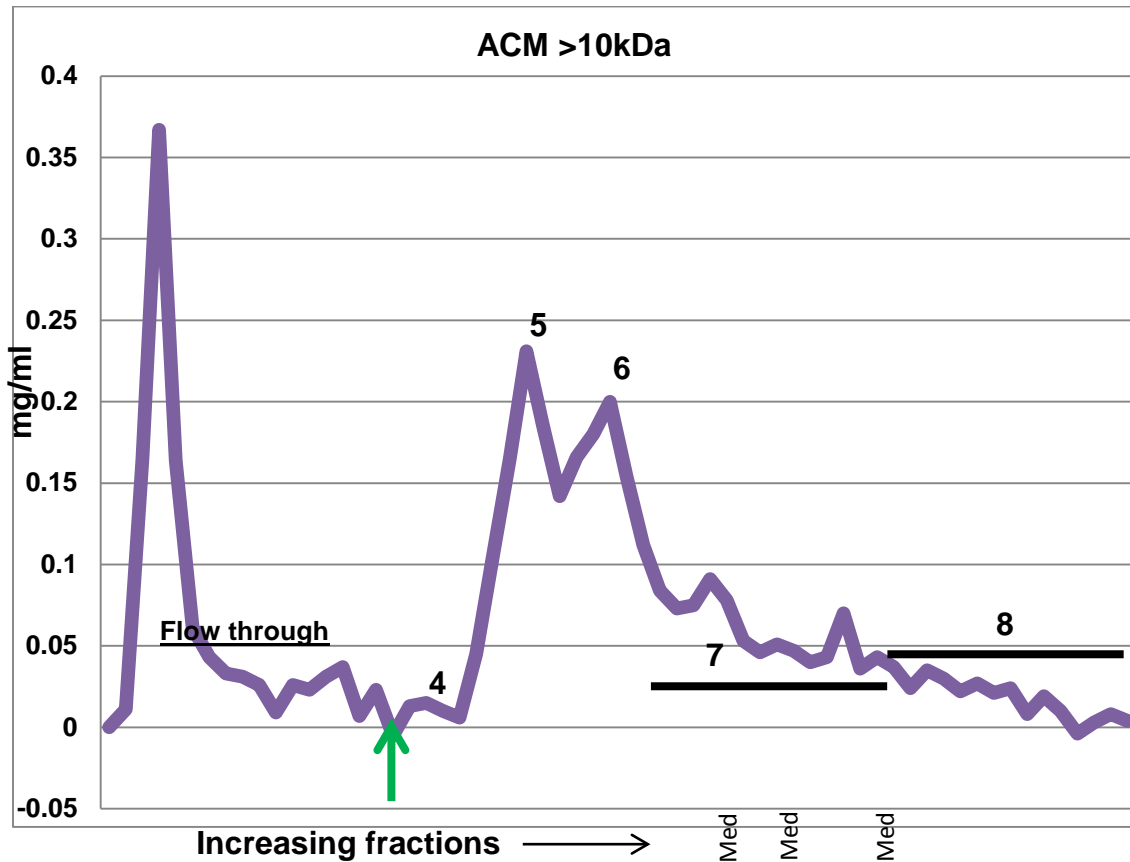
B.
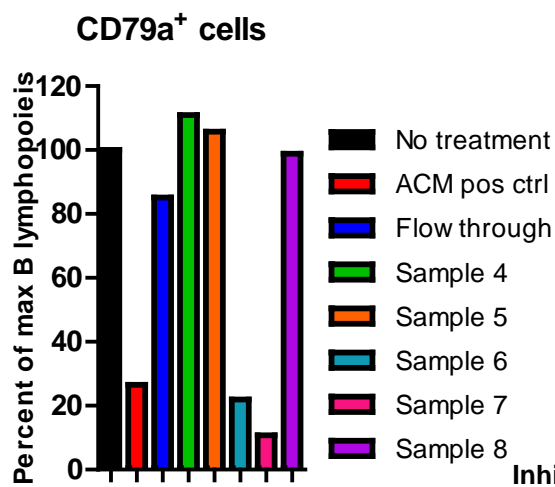
C.
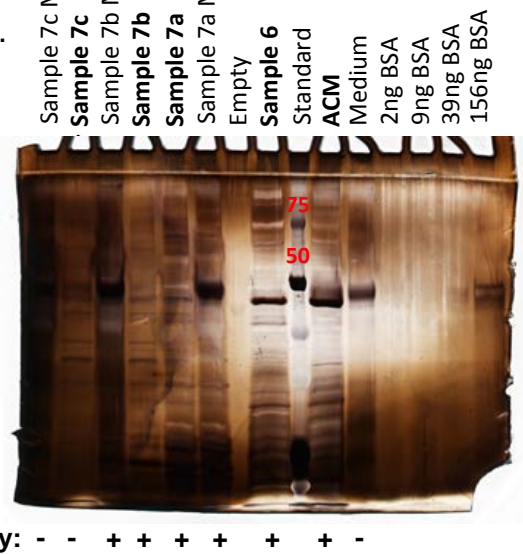
Figure 3.13 Purification of inhibitory molecules in ACM >10kDa. Anion

exchange chromatography was performed on msACM >10kDa with increasing

salt concentration used to elute material from the column. (A) Protein

concentration profile corresponding to fractions collected from the column. Green

arrow marks when linear increasing salt elution (0-1M NaCl) was started.

Collected fractions/samples tested for inhibitory activity in (B) are indicated on

the profile in (A). (B) Percent of maximum B lymphopoiesis resulting from rabbit

BM cultures treated with the indicated samples taken after anion exchange

chromatography of msACM >10kDa. Percent of maximum was calculated by

setting the percentage of CD79a$^+$ cells from "no treatment" cultures to 100%. (C)

Silver stained SDS PAGE gel loaded with the indicated samples. Samples 6, 7a,

7b, and 7c were loaded in anion exchange running buffer. Samples 7a Med, 7b

Med, and 7c Med were dialyzed in alpha mem culture medium, and then loaded

(contain additional medium derived proteins). Inhibitory activity on B

lymphopoiesis is summarized as + (inhibits B lymphopoiesis) or – (does not

inhibit). Precision plus protein standard (BioRad) was used as a size reference

(250kDa, 150kDa, 100kDa, 75kDa (most prominent band mid gel), 50kDa,

37kDa, 25kDa, and 10kDa). Data were obtained only in one experiment but will

provide the basis for future purification strategies.

Which adipocyte-derived soluble factors induce MDSCs?

The above results suggest that adipocytes produce multiple molecules

that negatively regulate B lymphopoiesis. Because ACM promotes the

accumulation of MDSCs that in turn inhibit B lymphopoiesis, we sought to identify

and neutralize the adipocyte-derived molecules responsible for inducing

inhibitory MDSCs. We hypothesized that preventing the accumulation of MDSC, would result in enhanced B lymphopoiesis.

To characterize which ACM molecules promote the accumulation of MDSCs, we used a similar approach as before. We generated ACM <10kDa and >10kDa fractions, and then compared total ACM, ACM <10kDa, and ACM >10kDa for the ability to promote CD11b$^+$Gr1$^+$ MDSCs *in vitro*. Mouse BM progenitors were cultured with OP9 stromal cells and treated with each ACM fraction individually, followed by flow cytometric analysis to determine the number of CD11b$^+$Gr1$^+$ cells resulting after treatment. For example, if ACM >10kDa molecules are sufficient to promote MDSC accumulation, then we expected that treatment with total ACM and ACM >10kDa would result in an increased number of MDSCs compared to untreated cultures. As expected for total ACM-treated cultures, there was a significant increase in the number of CD11b$^+$Gr1$^+$ cells compared to untreated cultures (Figure 3.14). ACM >10kDa treatment also resulted in significantly more CD11b$^+$Gr1$^+$ cells compared to control cultures (Figure 3.14), while ACM <10kDa treatment contained a similar number of CD11b$^+$Gr1$^+$ cells compared to untreated controls (Figure 3.14). These results suggest that an adipocyte factor(s) >10kDa is responsible for generating MDSCs. For the remainder of studies, we focused on adipocyte factors >10kDa.
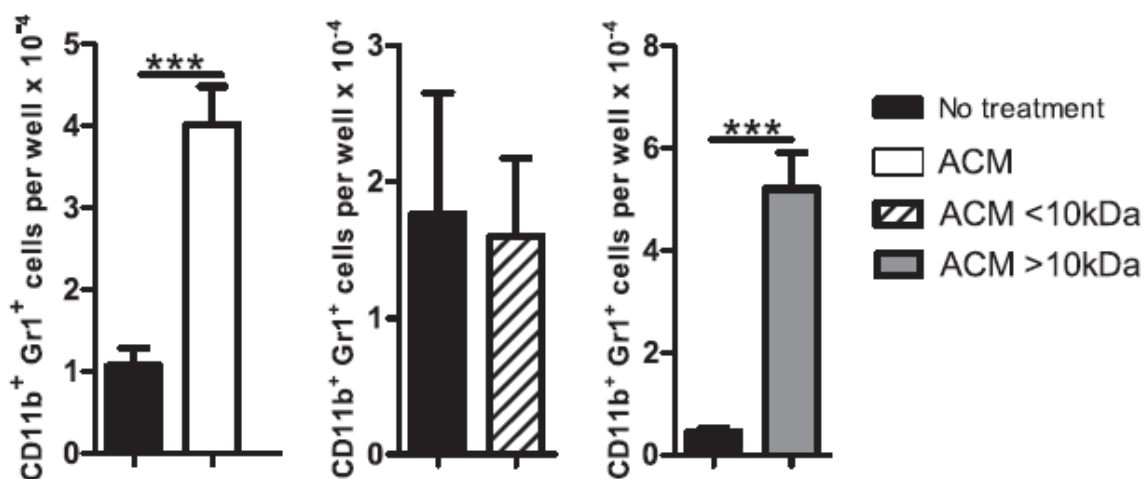
Figure 3.14 Characterization of adipocyte factors for the capacity to induce CD11b⁺Gr1⁺ myeloid cells. Number of CD11b⁺Gr1⁺ cells resulting from B220⁻ BM cells cultured with OP9 stromal cells in the presence or absence of total ACM (left), ACM <10kDa (middle), or ACM >10kDa (right). Data are representative of three independent experiments. Statistical significance was determined using Students *t* test. Error bars represent the average of triplicate wells +/- SD.

To identify potential MDSC-promoting adipocyte molecules, we performed a cytokine array focused on 23 cytokines/chemokines. In this experiment ACM (inhibits B lymphopoiesis/induces MDSCs) was compared to MDSC-CM (inhibits B lymphopoiesis) and CD11b⁺Gr1⁺ control-CM (does not inhibit B lymphopoiesis). We expected that MDSC promoting factors would be increased in ACM as compared to control-CM, because CD11b⁺Gr1⁺ control cells do not induce MDSCs in BM cultures. Interestingly, ACM contained IL-6, IL-12(p40), IL-12(p70), IL-13, G-CSF, KC, MCP, TNFα, Eotaxin, MIP-1α, and RANTES (Figure 3.15). Many of these factors are inflammatory in nature, suggesting that

adipocyte factors produce an inflammatory environment. Additionally, multiple

factors in ACM identified by us (eg. IL-6, IL-13, G-CSF, TNFα, MCP1,

complement C3 [data not shown]) and reported by others to be produced by

adipocytes (eg. S100A8) (Hiuge-Shimizu et al., 2011, Sekimoto et al., 2012) are

known to induce MDSCs (Bunt et al., 2007, Cheng et al., 2008, Drevets et al.,

2004, Gabrilovich and Nagaraj, 2009, Gallina et al., 2006, Hsieh et al., 2013,

Huang et al., 2007, Movahedi et al., 2008, Sawanobori et al., 2008, Sinha et al.,

2008, Terabe et al., 2003, Zhao et al., 2012). Upon identifying that ACM contains

multiple factors known to induce MDSCs, we suggest a combination of these

molecules (rather than one) are likely responsible for the generation of MDSCs

by ACM. This finding is important to consider when developing strategies to

target adipocyte factors.
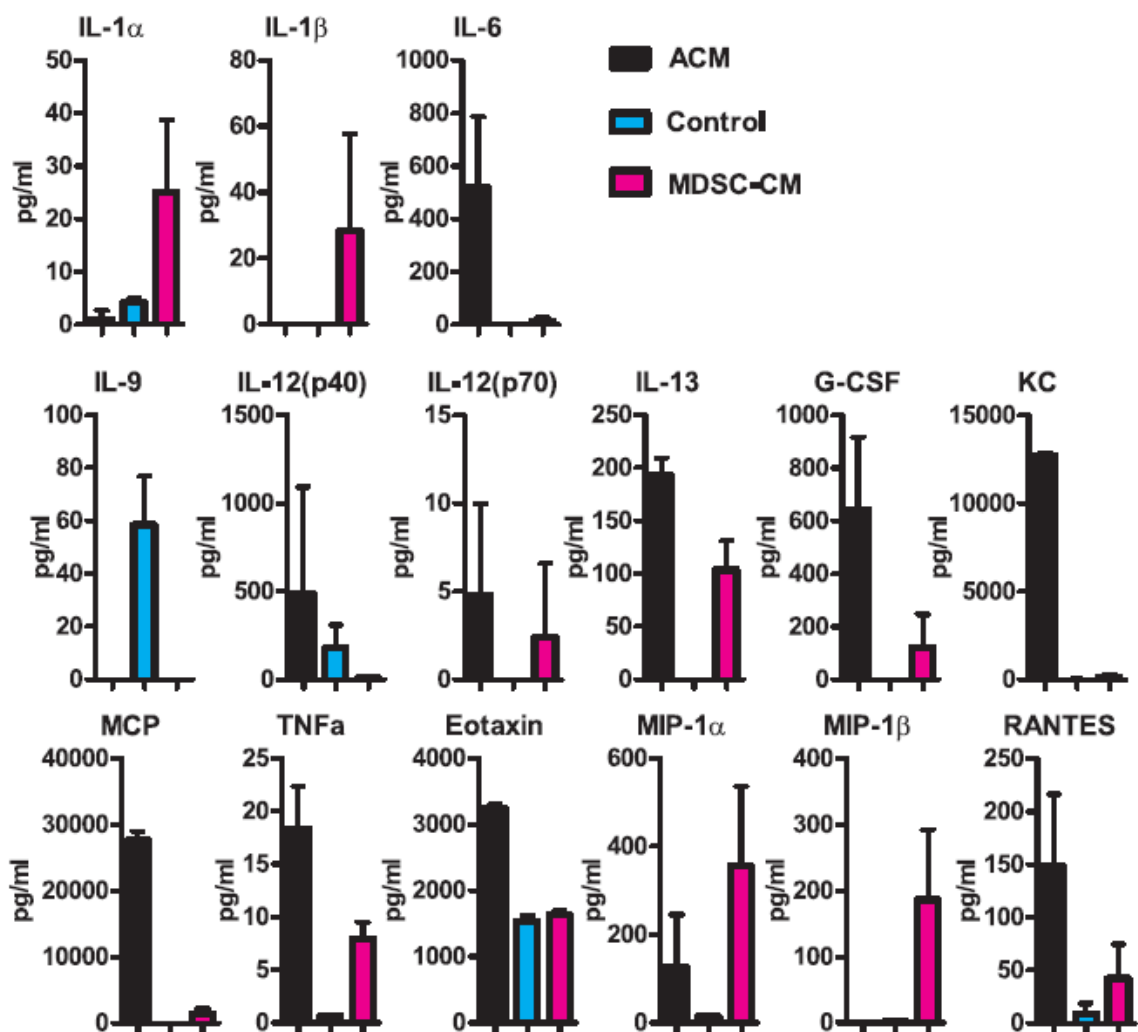
Figure 3.15 Profile of adipocyte-derived soluble factors by cytokine array.

Protein concentrations of the indicated factors as detected by cytokine array in

ACM, MDSC-CM, or control-CM. Data are from three different ACM, three

different MDSC-CM and two different control-CM generated in individual

experiments. Error bars show the average of the two or three different

conditioned media +/- SD.

Can adipocyte factors be targeted to prevent MDSC accumulation?

To develop therapeutics aimed at blocking adipocyte-mediated generation of MDSCs, we must devise a strategy to target the adipocyte, the MDSC, or neutralize adipocyte factors. Since ACM is a source of multiple factors able to induce MDSCs, it would be difficult to identify and block all potential MDSC promoting factors. An approach to identify and block a common pathway used by adipocyte-factors to induce MDSCs might be more successful. Cytokine array analysis of ACM showed that while ACM induces IL-1 producing MDSCs, IL-1 was not detected in the ACM (Figure 3.15), suggesting that a factor (or combination of factors) in ACM other than IL-1, induces this inflammatory molecule. Because IL-1 production is induced through inflammasome activation (Garlanda et al., 2013, Latz et al., 2013) and adipocytes produce NLRP3 inflammasome activators (eg. S100A8, DAMPs) (Coppack, 2001, Nagareddy et al., 2014, Wen et al., 2011, Youm et al., 2012, Youm et al., 2013), we hypothesized that blocking the NLRP3 inflammasome would prevent adipocyte induced MDSC activation/accumulation. To test this we performed BM cultures treated with ACM >10kDa in the presence or absence of glybenclamide, which reportedly blocks NLRP3 inflammasome activation and downstream IL-1 production in myeloid cells (Lamkanfi et al., 2009, Henriksbo et al., 2014, Laliberte et al., 1999, Dostert et al., 2009, Chen et al., 2012, Tavares et al., 2013). We expected that if ACM induces MDSCs through NLRP3 inflammasome activation, then blocking this pathway would prevent MDSC accumulation. As predicted, glybenclamide treatment significantly reduced CD11b$^+$Gr1$^+$ MDSC

generation compared to cultures containing only ACM >10kDa (Figure 3.16 A

and C). Additionally, because MDSCs negatively regulate B lineage

development, we expected that blocking MDSC accumulation would enhance B

lymphopoiesis in cultures containing ACM >10kDa. Strikingly, as seen in Figure

3.16 B and D, blocking MDSC accumulation did increase B cell development as

evidenced by an increase in B220$^+$ cells. These data suggest that blocking the

NLRP3 inflammasome is potentially an effective strategy to prevent MDSC

accumulation and boost B lymphopoiesis in fatty BM.
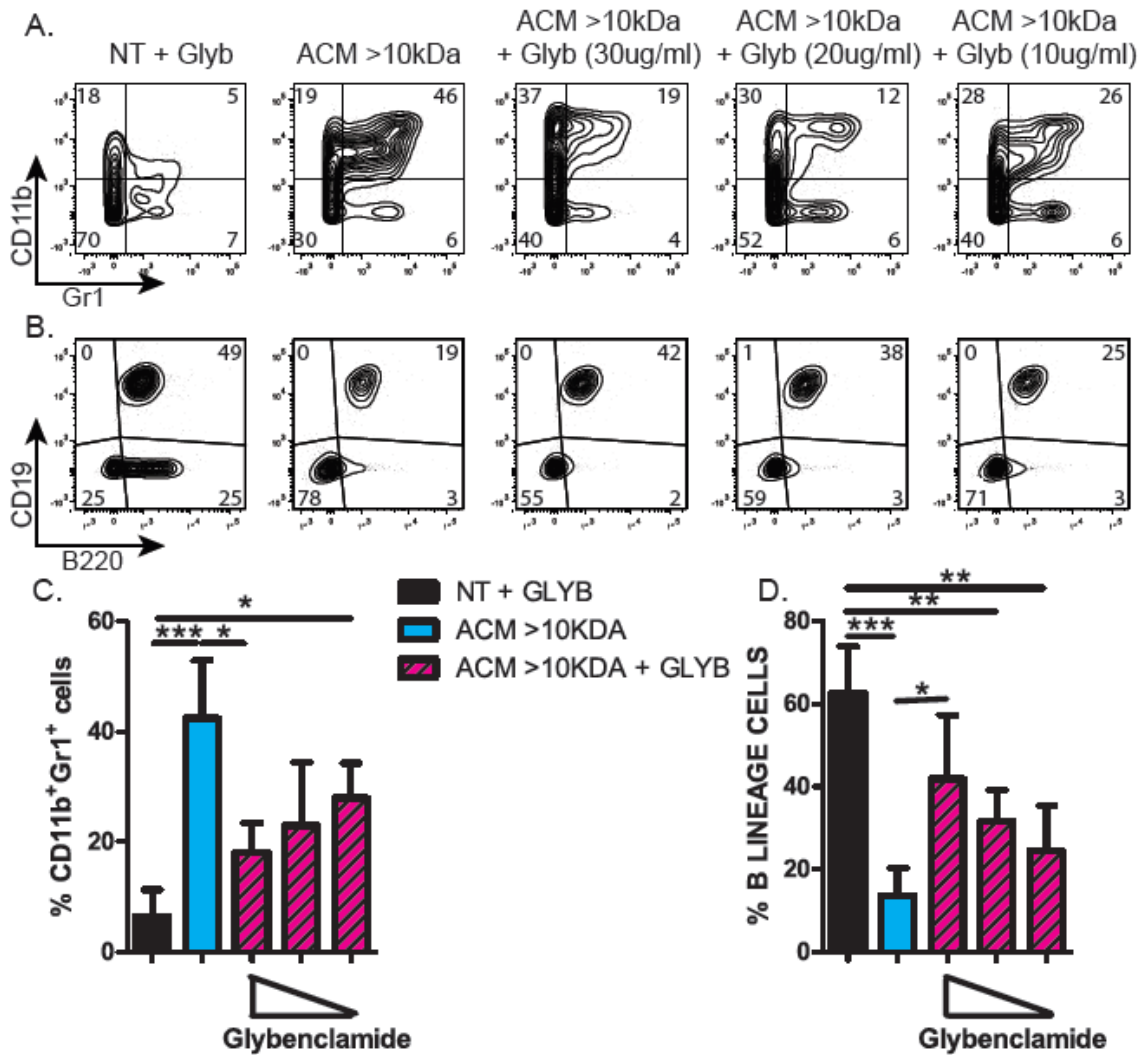
Figure 3.16 Effect of glybenclamide treatment on MDSC accumulation. BM

cultures were performed in the presence of ACM >10kDa with or without

glybenclamide (NLRP3 inflammasome inhibitor). (A&B) Representative flow

cytometric profiles displaying (A) CD11b and Gr1, or (B) B220 and CD19

expression at the end of culture. Percent of (C) CD11b$^+$Gr1$^+$ or (D) B220$^+$ cells

resulting after culture with the indicated treatments. Data are representative of

three to four individual experiments per condition. Significance was determined

by ANOVA in combination with a Bonferroni multiple comparison test (p=0.0005

and p=0.0001 for (C) and (D) respectively). Error bars represent the average of

individual experiments +/- SD.

SECTION 4: CHARACTERIZATION OF RABBIT HEMATOPOIESIS

Through mouse BM cultures, we have uncovered a mechanism where

adipocytes induce inflammatory myeloid cells which inhibit B cell development

through IL-1. This inhibition does not lead to a direct block of B cell development;

rather IL-1 promotes myelopoiesis at the expense of B lymphopoiesis. Because

large amounts of BM fat have been observed in adult rabbit BM, we asked if this

mechanism could be occurring *in vivo* in rabbits at two to four months of age,

when B lymphopoiesis is lost. If true, we expected to find an accumulation of

adipocytes, increased BM myeloid cell compartment, and increased expression

of IL-1 in the BM of rabbits >2 months of age.

In addition to testing whether or not the mechanism of adipocyte-mediated

inhibition of B lymphopoiesis could be occurring in rabbit BM, we also sought to

determine if rabbit BM could be studied as an accelerated model of BM aging.

Our laboratory has established that rabbit B lymphopoiesis arrests early in life,

compared to humans and mice. In this study, we characterized rabbit BM to see

if additional changes occurring at two months of age resemble the BM of two

year old mice; the time when B lymphopoiesis declines (Kirman et al., 1998,

Miller and Allman, 2003, Riley et al., 1991, Sherwood et al., 1998, Stephan et al.,

1996). If the BM of >2 month old rabbits resembles that of aged mice, we would

expect to find increased BM fat and an increase in BM myeloid cells at the time B

lymphopoiesis declines; as mentioned in the previous paragraph, these

characteristics would also be consistent with the elucidated mechanism of

adipocyte-mediated inhibition of B lymphopoiesis.

Several studies allude to the abundance of fat in the BM of adult rabbits

(Bigelow and Tavassoli, 1984, Bilwani and Knight, 2012); however, B

lymphopoiesis arrests in rabbits prior to adulthood. It is unknown if adipocytes

accumulate in the BM at the exact timing B lineage development is lost. Jasper et

al. found that rabbit B lymphopoiesis peaks in the first few weeks of life and

arrests by two to four months of age (Jasper et al., 2003). To visualize the

appearance of BM fat at the time B cell development arrests in rabbits, we

sectioned BM from rabbits of various ages followed by H&E staining. If increased

BM fat correlates with the timing B lymphopoiesis is lost in rabbits, then we

expected to find an increase in fat spaces observed in BM sections taken from

>2-4 month old rabbits compared to <2 month old rabbits. As expected,

representative BM from a 3 month old rabbit (a time after B cell development is

arrested) exhibited an increased number and size of fat spaces compared to

representative BM from a 3 week old rabbit (active B cell development) (Figure

3.17). Interestingly, rabbits older than two to three months (ranging to 13 months

old) also had increased BM fat that appeared similar to that of a two to three

month old rabbit (data not shown). These data suggest that adipocytes

accumulate in rabbit BM at the time B lineage development is arrested. These

data are consistent with increased BM fat found in aged mice (Krings et al.,

2012).

3 week rabbit   **3 month rabbit**

10x
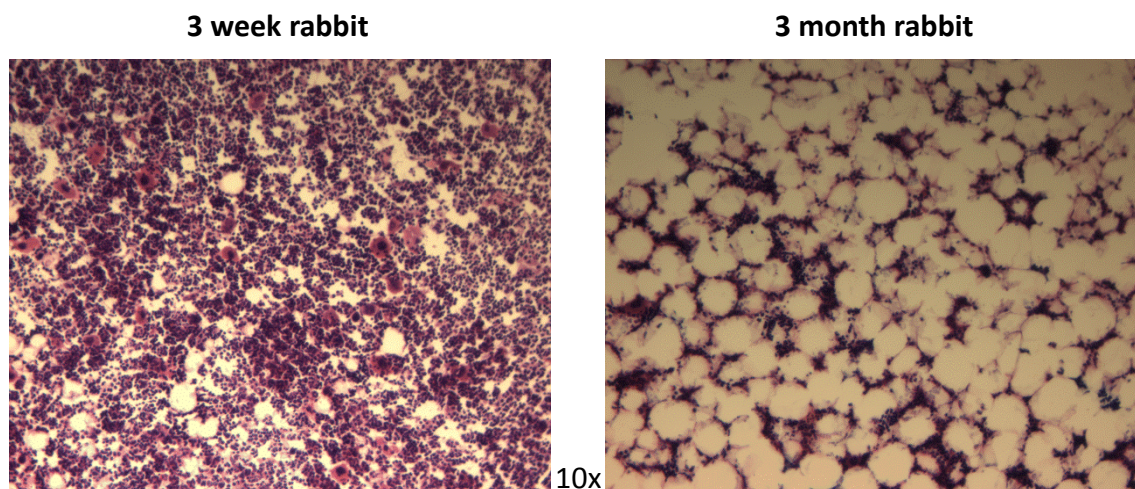
Figure 3.17 Characterization of bone marrow fat in rabbits. Representative

BM sections generated from femurs of a 3 week old rabbit (left) and a 3 month

old rabbit (right). Sections were H&E stained and visualized for the proportion of

fat (white spaces) vs red marrow (pink and purple staining). Multiple sections and

multiple fields of each section were visualized from each rabbit to obtain

representative images. BM sections analyzed from rabbits >3 months of age had

a profile similar to the 3 month (right) section shown above. The displayed

images were taken at 10x magnification.

If the mechanism of adipocyte-induced inhibition of B cell development

(elucidated in this dissertation) occurs in the BM of >2 month old rabbits, we

expect this BM would exhibit a myeloid skew. This would also be consistent with

the myeloid skew observed in two year old mice (Enioutina et al., 2011), when B

lymphopoiesis is declining. To test this, we performed flow cytometric analysis on

BM isolated from rabbits of various ages. If hematopoiesis is skewed toward

myelopoiesis in BM of >2 month old rabbits, we expected to find a greater

frequency of CD11b$^+$ myeloid cells in the BM of >2 month old rabbits as

compared to younger rabbits. Interestingly, the percentage of CD11b$^+$ myeloid

cells was increased in the BM of rabbits >2 months old as seen by flow cytometry

(Figure 3.18 A-C). These data suggest that rabbit BM exhibits a myeloid skew at

2 months of age similar to that seen in two year old mice.

Further flow cytometric analysis of the CD11b$^+$ cells from >2 month old

and <2 month old rabbits showed that myeloid cells from >2 month old rabbits

have higher CD11b expression (Figure 3.18 D). This phenotype is similar to

CD11b^{hi}Gr1$^+$ ACM-generated MDSCs (Figure 3.3), suggesting these cells may

be similar. If CD11b$^+$ cells from aged BM are similar to ACM-generated MDSCs,

we expect they would be influenced by adipocyte-derived molecules. To

characterize the localization of CD11b$^+$ cells relative to BM adipocytes, we

generated frozen OCT embedded BM sections from <2 month old and >2 month

old rabbits, stained for CD11b$^+$ cells and analyzed them by fluorescence microscopy. If BM CD11b$^+$ cells are influenced by BM adipocytes, we expected to find CD11b$^+$ cells near BM adipocytes. Consistent with our flow cytometry data, BM of >2 month old rabbits contained many more cells staining for CD11b expression, compared to BM of <2 month old rabbits (Figure 3.18 E). Further, immunofluorescence analysis of BM sections from >2 month old rabbits sections showed a large proportion of BM CD11b$^+$ cells (Figure 3.18E- green) near large fat spaces (Figure 3.18E- black). These data suggest that CD11b^{hi} rabbit BM cells are likely influenced by adipocyte products.
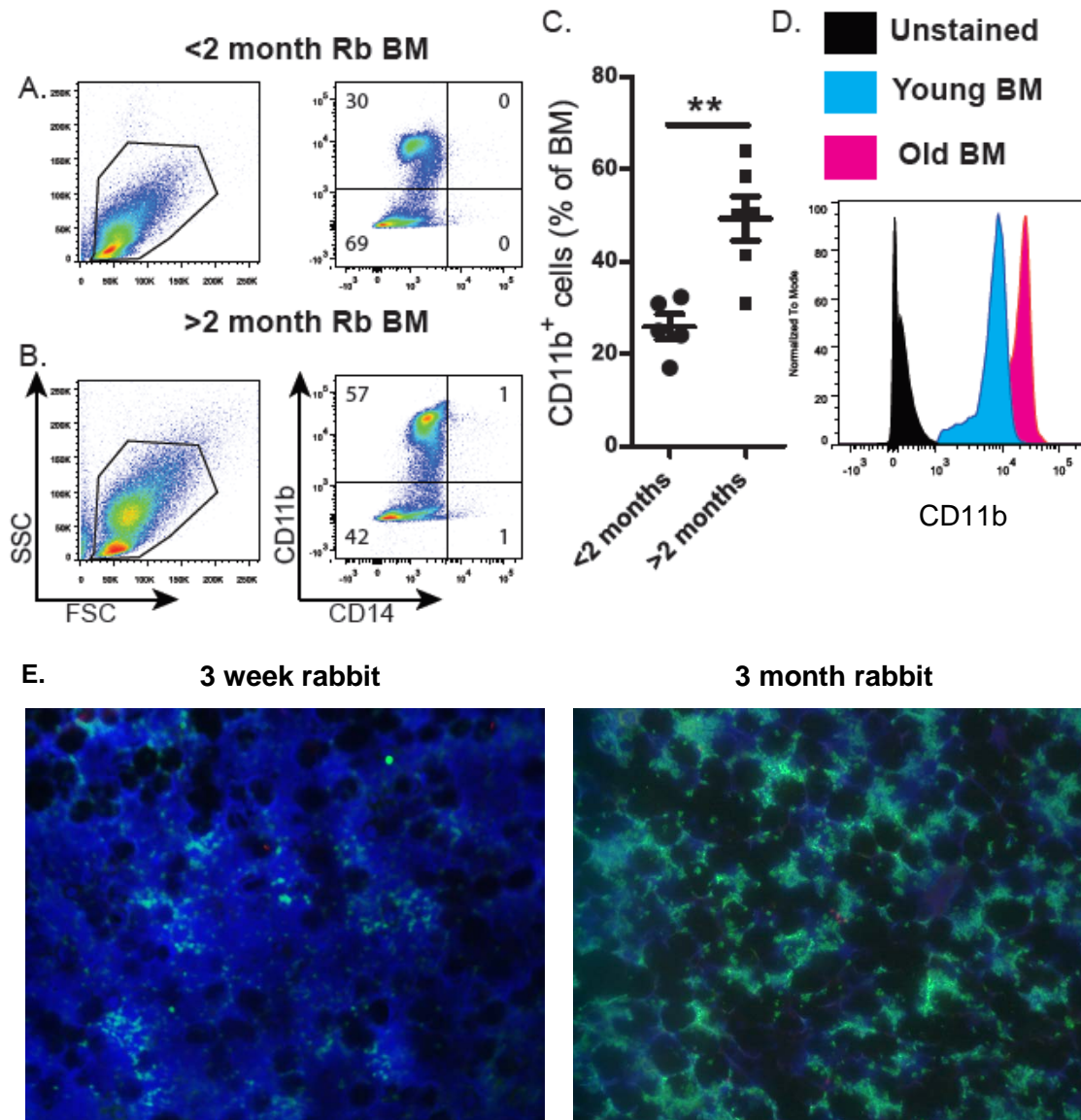
Figure 3.18 Characterization of rabbit bone marrow before and after two months of age. (A&B) Flow cytometry profiles of nucleated cells from (A) BM of <2 month old or (B) >2 month old rabbits. BM cells were analyzed for FSC vs SSC (left), then for CD11b and CD14 (right) staining. (C) Percentage of BM CD11b$^+$ cells in <2 month old (circles) and >2 month old rabbits (squares) (total n=11). (D) Representative flow cytometry profile of CD11b expression in the BM

CD11b$^+$ cell gate comparing a <2 month old rabbit (young) and a >2 month old rabbit (old). (E) Fluorescence microscopy analysis of BM sections stained with Hoechst (blue), anti-CD11b (green), and anti-kappa light chain (red) – very few cells stained with anti-kappa. (A&B) Data are representative of a panel of 11 rabbits. Data in (E) are representative of a panel of 7 rabbits. Significance in (C) was determined by Student's *t* test. Error bars represent the average percentage over multiple rabbits per group +/- SD.

We found that ACM-induced MDSCs inhibit B lymphopoiesis through IL-1 production. To further compare BM of >2 month old rabbits to the mechanism of adipocyte-mediated inhibition of B cell development, we performed qPCR analysis on rabbit BM cells to assess *IL-1* expression. BM was isolated from rabbits <2 months of age and >2 months of age, followed by qPCR analysis for *IL-1β* expression. If IL-1β contributes to the loss of B lymphopoiesis in rabbit BM, we expected to find increased expression of *IL-1β* in BM cells isolated from >2 month old rabbits. In fact, qPCR analysis of total rabbit BM showed that *IL-1β* expression increased with age (Figure 3.19 A).

If IL-1β is produced by an MDSC-like cell in BM of >2 month old rabbits, we expected to find IL-1β expression localized to BM myeloid cells. As anti-CD11b is the best reagent available to identify myeloid cells in rabbits, we used this reagent to isolate CD11b$^+$ and CD11b$^-$ cells from BM of >2 month old rabbits by FACS, then performed qPCR analysis for the expression of *IL-1β*. If IL-1β is expressed by BM myeloid cells, then we expected CD11b$^+$ cells to express

higher amounts of *IL-1β* compared to CD11b⁻ cells. As expected, we found IL-*1β*

was expressed at much higher levels (~30x) in CD11b⁺ cells (Figure 3.19 B).

Together, these data suggest that BM of >2 month old rabbits exhibits a larger

myeloid compartment compared to younger rabbits, and that these myeloid cells

resemble ACM-generated MDSCs based on *CD11b* and *IL-1β* expression.
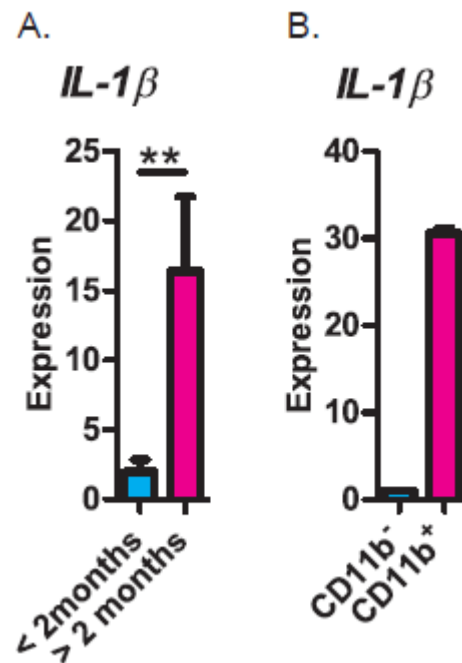
**Figure 3.19 Quantitative PCR anaysis of IL-1β expression in rabbit bone

marrow.** (A) Total BM cells from rabbits <2 months and >2months of age. (B)

FACS-sorted CD11b⁻ and CD11b⁺ populations from BM of >2 month old rabbits.

Each sample was normalized to *HGPRT* housekeeping gene to determine

relative expression. Data in (A) are calculated from a panel of 7 rabbits. Data in

(B) display the average of 3 independent experiments. Statistical significance in

(A) was determined by Student's *t* test. Error bars represent the average
expression of multiple rabbits per group +/- SD.

SECTION 5: CONTRIBUTION OF THE BONE MARROW MICROENVIRONMENT TO ALTERED HEMATOPOIESIS IN RABBITS

The arrest of B lymphopoiesis and the myeloid skew seen at 2 months of
age in rabbits could be due to intrinsic changes to hematopoietic progenitors or
due to changes in the BM microenvironment. Due to the large increase in BM fat
and our findings linking adipocytes to the generation of inhibitory myeloid cells,
we hypothesized that the BM microenvironment of >2 month old rabbits
recapitulates the myeloid skew seen in BM of >2 month old rabbits. To test this,
we isolated the adipocyte layer that forms when preparing BM from >2 month old
rabbits and generated conditioned medium from these fat explants (BM fat-CM)
(Figure 3.20 A). We know that the adipocyte layer also contains CD11b$^+$ myeloid
cells that are tightly associated with adipocytes (data not shown); therefore the
factors in BM fat-CM are the combination of adipocyte factors and myeloid-
derived factors. We expected if this combination of factors is responsible for
decreased B lymphopoiesis and increased myelopoiesis, then rabbit BM cultures
treated with BM fat-CM will have decreased CD79a$^+$ cells and increased CD11b$^+$
cells. As seen in untreated cultures, BM progenitors from >2 month old rabbits
are able to differentiate to the B lineage (Figure 3.20 B), suggesting the
progenitors do not have intrinsic defects. Alternatively, cultures treated with BM
fat-CM had significantly fewer CD79$^+$ B lineage cells and an increase in CD11b$^+$
myeloid cells (Figure 3.20 C-E). These data suggest that the BM

microenvironment, made up of adipocytes and myeloid cells, is a major

contributor to decreased B cell development and increased myeloid development

as seen in BM of >2 month old rabbits.
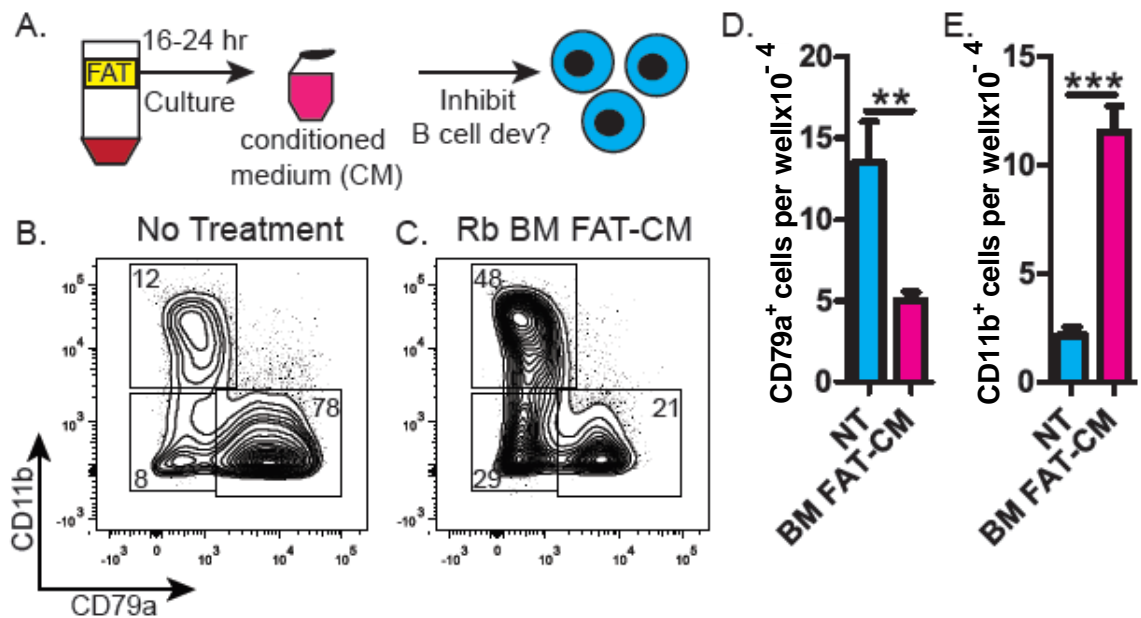
Figure 3.20 Effect of BM fat-CM on rabbit B lymphopoiesis cultures. (A)

Schematic diagram: The adipocyte layer from >2 month old rabbits was isolated

and then cultured 16-24 hours to generate BM fat-CM. BM fat-CM was added to

rabbit B lymphopoiesis cultures and the development of B lineage cells was

assessed. (B&C) Flow cytometry profiles of cells resulting from B lymphopoiesis

cultures with or without (no treatment) BM fat-CM, and analyzed for CD79a and

CD11b expression. (D&E) Number of (D) CD79a$^+$ or (E) CD11b$^+$ cells resulting

from no treatment or BM fat-CM treated cultures. Data are representative of four

independent experiments. Statistical significance in (D&E) was determined using

Student's *t* test. Error bars represent the average of triplicate wells +/- SD.

Do rabbit bone marrow myeloid cells inhibit B cell development?

Through mouse BM cultures treated with ACM, we learned that adipocyte factors activate myeloid cells to inhibit B lymphopoiesis. The previous experiment suggests the combination of adipocyte and myeloid-derived factors also negatively impact B lymphopoiesis in rabbits. We asked if inhibitory myeloid cells could be isolated directly from BM of >2 month old rabbits. We tested this by using magnetic beads to isolate CD11b$^+$ myeloid cells from both the BM adipocyte layer, as well as the adipocyte free BM pellet. The adipocyte-free BM pellet is called the SFV (stromal vascular fraction) and contains all BM cells that are not in the adipocyte layer (corresponds to red BM pellet displayed in Figure 3.20 A). These are the BM cells prepared in previous experiments, referred to as "total BM", and are the only cells isolated from <2 month old rabbits; as an adipocyte layer does not form when preparing younger rabbit BM.

While isolation of CD11b$^+$ cells from the SVF pellet can be performed easily, isolation of hematopoietic cells from the adipocyte layer requires more effort. This was typically done in one of two ways. The first approach is to culture BM fat overnight to allow hematopoietic cells to fall out of the floating adipocyte layer. These hematopoietic cells can then be isolated along with additional cells that are released from the adipocyte layer after repetitive pipetting. The second approach is to digest the adipocyte layer with collagenase, which will release cells that can then be isolated. Both approaches showed similar results in our hands.

Reagents to detect Gr1 in mice, are unavailable for rabbits. Therefore we used CD11b to isolate general BM myeloid cells, reasoning that an inhibitory population would be contained in a smaller percentage of these cells. We isolated CD11b$^+$ cells from the BM adipocyte layer and the BM SVF pellet, then added them to B lymphopoiesis cultures, and assessed the number of B lineage cells that developed. If BM of >2 month rabbits contains an inhibitory myeloid population, we expected treatment of BM cultures with CD11b$^+$ cells isolated from both the BM adipocyte layer and SVF pellet would result in fewer CD79a$^+$ B lineage cells compared to control cultures. In fact, cultures containing CD11b$^+$ cells isolated from the BM fat layer and SVF pellet had a decreased number of B lineage cells develop compared to untreated cultures, and cultures containing an equal number of CD11b$^-$ cells isolated from BM fat (Figure 3.21). These results suggest that a suppressive population within the CD11b$^+$ BM myeloid compartment is capable of inhibiting rabbit B lymphopoiesis *in vitro*. We hypothesize the inhibitory activity in this myeloid population is activated by adipocyte products in the BM of >2 month old rabbits. Generation of additional reagents to study rabbit myeloid cells will be critical to characterizing specific myeloid lineage suppressor cells in BM.
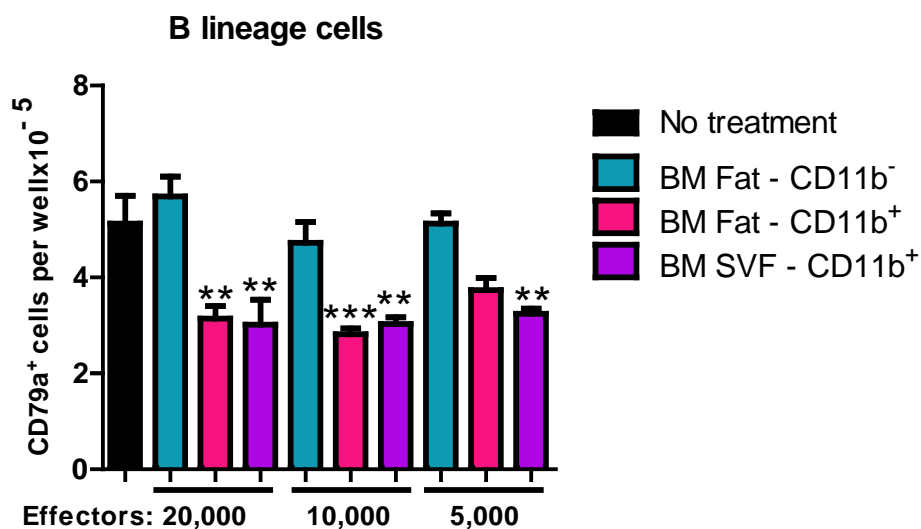
Figure 3.21 Effect of BM myeloid cells from >2 month rabbits on B lymphopoiesis *in vitro*. Rabbit B lymphopoiesis cultures were performed starting with 10,000 BM progenitors with no effectors (no treatment) or the indicated number of CD11b$^-$ cells from BM fat, CD11b$^+$ cells from BM fat, or CD11b$^+$ cells from the SVF fraction of BM. Error bars represent the average of triplicate wells +/- SD. Data were analyzed for statistical significance by ANOVA coupled with the Dunnet multiple comparison test (*p*<0.0001). Data are representative of 3 independent experiments.

Characterization of inflammatory myeloid cells in bone the marrow of >2 month old rabbits

Inflammatory cells that accumulate with age, such as aged B cells, have been shown to negatively regulate B lymphopoiesis (Ratliff et al., 2013). Activation of an inflammatory profile in myeloid cells by adipocytes in adipose tissue has also been reported (Nagareddy et al., 2014, Vandanmagsar et al.,

2011, Wen et al., 2011, Youm et al., 2012). Because BM of >2 month old rabbits has increases in adipocytes and myeloid cells, we looked for the presence of additional inflammatory factors in rabbit BM with age.

Expression of the inflammatory proteins S100A8 and S100A9 is upregulated in many tissues, including adipose tissue during aging and obesity (Schiopu and Cotoi, 2013, Sekimoto et al., 2012, Swindell et al., 2013). Additionally, these factors induce inflammasome activation (Nagareddy et al., 2014, Simard et al., 2013) and promote MDSCs, which can in turn produce more inflammatory S100A8 and/or S100A9 (Sinha et al., 2008). We profiled rabbit BM by flow cytometry using an antibody that recognizes S100A8 and S100A9, to determine if these proteins are increased in the BM of >2 month old rabbits. Interestingly, by flow cytometry, we found a population of S100A8/S100A9 expressing myeloid cells that was increased in the BM of older rabbits (Figure 3.22 A&B), suggesting that inflammatory myeloid cells accumulate in rabbit BM at the time B lymphopoiesis is lost.

The anti-S100A8/S100A9 antibody used to profile rabbit BM by flow cytometry (described above) does not discriminate between S100A8 and S100A9, therefore we do not know if the expression of just one or both *S100A8* and *S100A9* is increased with age. To determine if the expression of *S100A8* and/or *S100A9* is increased in the BM of >2 month old rabbits we performed qPCR analysis on rabbit BM cells (BM SVF pellet – without the adipocyte layer). We expected that both *S100A8* and *S100A9* expression would be increased in the BM of >2 month old rabbits. To our surprise, analysis of *S100A8* expression

was similar between young and older rabbits (Figure 3.22 C), whereas *S100A9*

expression was increased in the BM of rabbits >2 months of age (Figure 3.22 D).

We conclude that S100A9 expressing myeloid cells are increased in the BM of

>2 month old rabbits.
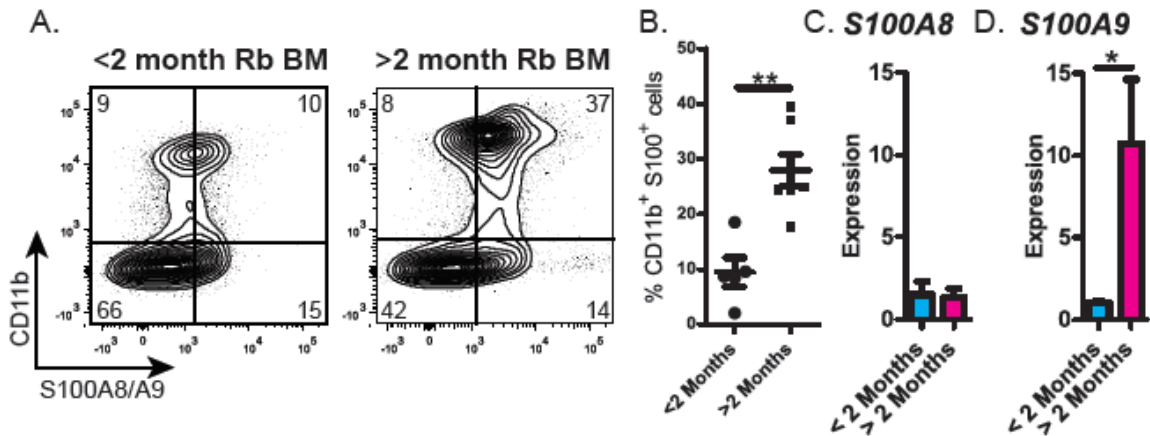
**Figure 3.22 Characterization of S100A9$^+$ myeloid cells in rabbit bone
marrow.** (A) Flow cytometry profile of rabbit BM nucleated cells stained with anti-

CD11b (surface) and anti-S100A8/A9 (intracellular). (B) Percent of

CD11b$^+$S100A8/A9$^+$ cells in nucleated BM cells from <2 month (circles) and >2

month (squares) old rabbits. (C&D) qPCR analysis of total BM nucleated cells

from <2 month and >2 month old rabbits analyzed for the expression of (C)

S100A8 and (D) *S100A9.* Expression was normalized to *HGPRT* housekeeping

gene. For data in (B) n=12 rabbits total. Error bars represent the average of each

population of rabbits +/- SD. (C-D) Data are the average of three independent

experiments. Error bars represent the average of three experiments +/- SD. (B-D)

Statistical significance was determined by Student's *t* test.

S100A9 and the inhibition of B lymphopoiesis

S100A9 is known for its potent inflammatory properties, but it is unknown if this molecule negatively regulates B lymphopoiesis. Because expression of this molecule is increased in BM of >2 month old rabbits (when B lymphopoiesis is arrested), we hypothesized that S100A9 negatively regulates B lymphopoiesis. To test this, we treated mouse B lymphopoiesis cultures with recombinant S100A9, and then assessed the number of B220$^+$ B lineage cells resulting from these cultures. We expected that if S100A9 inhibits B cell development, then cultures containing S100A9 will have fewer B220$^+$ cells develop compared to controls. Flow cytometric analysis of cultures treated with S100A9 exhibited a decreased percentage and number of B220$^+$ B lineage cells compared to untreated cultures (Figure 3.23 A-C). In addition to decreased B lineage development, S100A9 treatment resulted in significantly more CD11b$^+$Gr1$^+$ myeloid cells (Figure 3.23 D-F), reminiscent of cultures treated with IL-1. We conclude that S100A9 inhibits B lymphopoiesis and promotes myeloid cell development/survival.
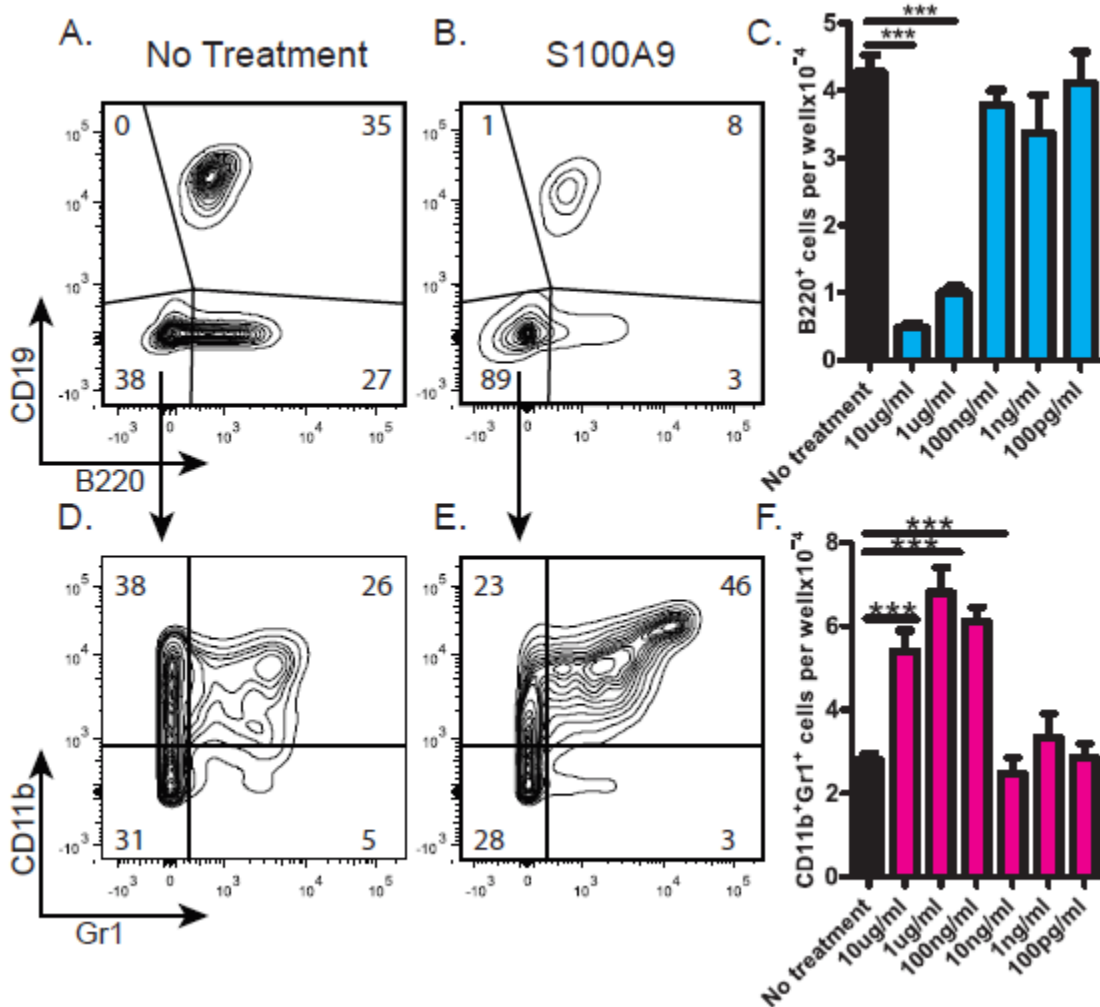
Figure 3.23 Effect of S100A9 on B lymphopoiesis *in vitro*. Mouse B

lymphopoiesis cultures of B220⁻ BM progenitors with OP9 cells were performed

in the presence or absence (no treatment) of recombinant S100A9. Flow

cytometry profiles displaying (A&B) B220 and CD19, (D&E) CD11b and Gr1.

(C&F) Number of (C) B220⁺ cells or (F) CD11b⁺Gr1⁺ cells resulting from cultures

treated with the indicated amount (bar graphs) or 5µg/ml (flow cytometry plots) of

S100A9. Data are representative of 3 independent experiments. Statistical

significance in (C&F) was analyzed by ANOVA coupled with the Dunnet multiple

comparison test (p<0.0001 and p<0.0001 respectively). Error bars represent the average of triplicate wells +/- SD.

Hematopoietic target of S100A9

We showed for the first time that S100A9 negatively regulates B lymphopoiesis. Because S100A9 is an inflammatory molecule, like IL-1, we asked if S100A9 inhibits in a similar manner. We set out to identify the hematopoietic target of S100A9 treatment. If the mechanism of inhibition mirrors that of IL-1 mediated inhibition, we expect S100A9 alters the differentiation potential of MPPs. To test this, we cultured FACS-sorted HSCs, MPPs, or CLPs (Figure 3.24 A) with OP9 cells in the presence or absence of S100A9 treatment. To our surprise, the number of B220$^+$ B lineage cells resulting after S100A9 treatment did not differ from untreated cultures seeded with HSCs, MPPs, or CLPs (Figure 3.24 B). We conclude that neither HSCs, MPPs, nor CLPs are the the direct target of S100A9 treatment.
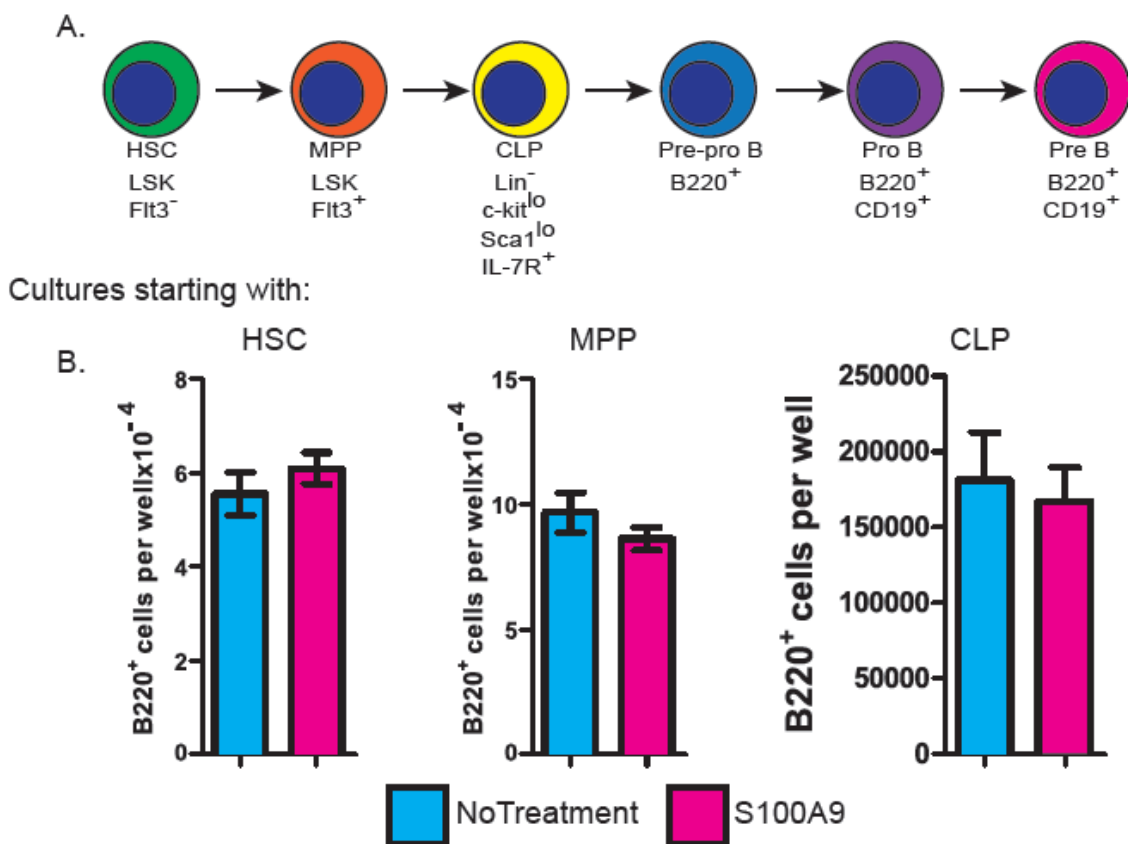
Figure 3.24 Impact of S100A9 treatment on hematopoietic progenitors. (A) B lineage developmental stages and key markers for identification. (B) Number of B220+ cells resulting from B lymphopoiesis cultures seeded with HSCs, MPPs, or CLPs and treated with or without (untreated) S100A9. Data are representative of three independent experiments. Statistical significance was determined by Student's *t* test. Error bars represent the average of triplicate wells +/- SD.

The finding that S100A9 treatment does not act on HSCs, MPPs, or CLPs raised several questions. If early hematopoietic progenitors (with B lineage potential) are not the target of S100A9 mediated inhibition, what cell type is?

Also, if S100A9 does not inhibit B lymphopoiesis in cultures of purified HSCs,

how did inhibition occur in previous B lymphopoiesis assays? We were able to

answer these questions after comparing the methodology used in the experiment

shown in Figure 3.23, and in S100A9 treatment of individually purified

hematopoietic progenitors (Figure 3.24).

In B lymphopoiesis cultures that were inhibited by S100A9 (Figure 3.23),

the starting population of cells was B220$^-$ BM, which contains a mix of non-B

lineage BM progenitors including HSCs, MPPs, etc. In addition to early

progenitors, B220$^-$ BM also contains a population of CD11b$^+$Gr1$^+$ myeloid

progenitors (without suppressive activity) that either do not survive in B

lymphopoiesis promoting conditions or differentiate into normal mature myeloid

lineage cells. This information coupled with a study that found S100A9 treatment

induces pro-inflammatory cytokine production from mature myeloid cells in

human peripheral blood (Simard et al., 2013), led us to ask whether immature

myeloid cells could also respond in a similar manner. To test this, we isolated

CD11b$^+$Gr1$^+$ myeloid cells from healthy mouse BM using magnetic beads (Figure

3.25 A), treated these cells with S100A9 and used qPCR to analyze the

expression of inflammatory mediators known to inhibit B lymphopoiesis (*IL-1β*,

NLRP3, *IL-6*, and *TNFα*) at various time points after treatment. If S100A9 inhibits

B lymphopoiesis by inducing the production of inhibitory molecules from

otherwise normal CD11b$^+$Gr1$^+$ BM myeloid progenitors, we expected S100A9

treatment would induce increased expression of *IL-1β*, *NLRP3*, *IL-6*, and *TNFα* in

these cells. Interestingly, S100A9 treatment induced an early burst in expression

of *IL-1β*, *NLRP3*, *IL-6*, and *TNFα*. Increases were observed as early as 2 hours after treatment, peaking at the 4 hour time point for most of these molecules. Several novel conclusions can be drawn from this experiment. First, BM CD11b⁺Gr1⁺ myeloid progenitors have the appropriate machinery to respond to S100A9 treatment, similar to that seen in mature human peripheral blood myeloid lineage cells. Second, S100A9 treatment induces otherwise normal myeloid progenitors to express inflammatory mediators known to negatively regulate B lymphopoiesis. Finally, this result (induction of *IL-1*) suggests that S100A9-mediated inhibition also feeds into the mechanism previously described for IL-1. This explains why S100A9 treatment (similar to IL-1 treatment) induces myelopoiesis and the loss of B lymphopoiesis.

Overall, our analysis of rabbit BM led/leads us to: 1. Suggest the mechanism of adipocyte-mediated inhibition of B lymphopoiesis (elucidated in mouse BM cultures) contributes to the arrest of B cell development in rabbits; 2. Identify similarities between BM of >2 month old rabbits and two year old mouse BM (to be discussed further); and 3. Identify S100A9 as a negative regulator of B lineage development.
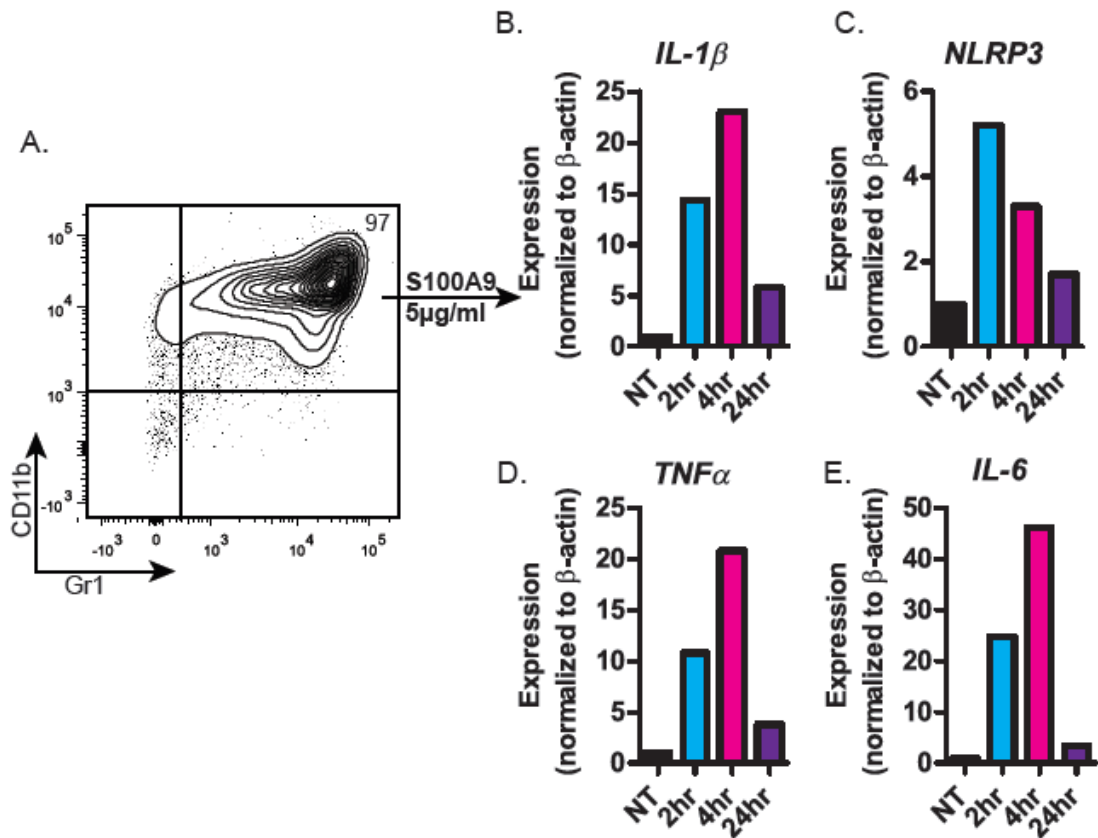
Figure 3.25 Effect of S100A9 treatment on bone marrow myeloid cells. (A) Flow cytometry profile of mouse BM CD11b$^+$Gr1$^+$ myeloid cells after anti-CD11b magnetic bead isolation, and prior to treatment with S100A9 (5µg/ml). (B-E) qPCR analysis of CD11b$^+$Gr1$^+$ BM cells either no treatment, or treated with S100A9 for the indicated amount of time. Following treatment the resulting cells were analyzed for the expression of (B) *IL-1β*, (C) *NLRP3*, (D) *TNFα*, and (E) *IL-6*. Data are representative of three independent experiments.

CHAPTER IV

DISCUSSION

The findings in this study provide mechanistic insight into the negative regulation of B lymphopoiesis by adipocytes. We have found that adipocytes produce soluble factors that promote the accumulation of MDSCs (Figure 4.1). As described for the first time, these MDSCs have the ability to potently inhibit B lymphopoiesis. B cell development does not appear to be directly blocked by MDSCs, instead MDSC-derived IL-1 acts at the MPP stage in development driving myelopoiesis at the expense of B lymphopoiesis.

Our characterization of rabbit BM suggests the above mechanism could contribute to the loss of B lymphopoiesis that occurs at two to four months of age. These findings also lead us to propose the rabbit as an accelerated model to study changes in the BM that result in the decline of B lymphopoiesis occurring in mid to late life in humans and mice. We propose that our observations can be applied to situations that result in adipocyte accumulation in the BM, such as aging and obesity (Adler et al., 2014, Chinn et al., 2012, Justesen et al., 2001, Lecka-Czernik et al., 2010, Luo et al., 2015, Rosen et al., 2009, Tuljapurkar et al., 2011), and will provide the basis for therapeutics aimed at boosting B lymphopoiesis in these scenarios.
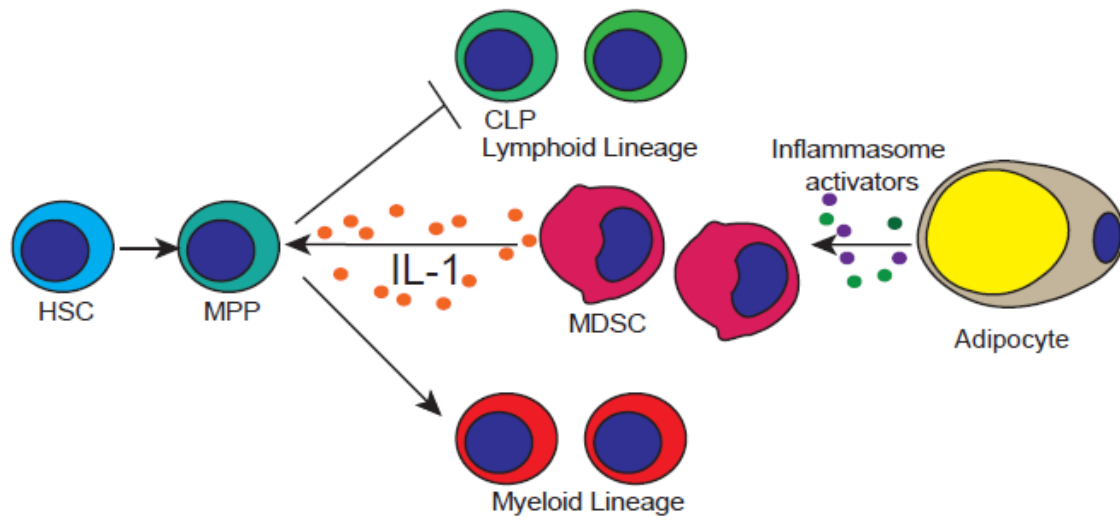
Figure 4.1 Model of Adipocyte-mediated inhibition of B lymphopoiesis.

Hematopoiesis in healthy BM is characterized by a balance of lymphoid and myeloid cell production. This balance is lost with an increased number of adipocytes in the BM. Adipocytes secrete inflammasome activators leading to the accumulation of MDSCs. Adipocyte factor induced-MDSCs produce IL-1, which acts at the MPP stage to promote myelopoiesis resulting in the loss of B lymphopoiesis. Adapted from Kennedy and Knight, 2015.

The bone marrow microenvironment and the arrest of rabbit B lymphopoiesis

The decline of B lymphopoiesis in aging mice has been attributed to both intrinsic changes in hematopoietic progenitors and extrinsic changes to the BM microenvironment (reviewed in chapter I). Our study suggests that the arrest of B

lymphopoiesis in rabbits is primarily mediated through extrinsic changes to the BM microenvironment. Through analysis of BM sections from rabbits of various age, we found that adipocytes accumulate by 3 months of age (Figure 3.17). While it was known that adult rabbits contain significant amounts of BM adipocytes (Bigelow and Tavassoli, 1984), B lymphopoiesis is lost before adulthood. Our observation is the first to suggest that the accumulation of BM adipocytes occurs in the same time frame that B lymphopoiesis arrests in rabbits (by two to four months of age) (Jasper et al., 2003); suggesting adipocyte factors could negatively regulate B lymphopoiesis.

Isolation of BM fat from >2 month old rabbits and the observation that BM fat-CM inhibits B lymphopoiesis in rabbit B lymphopoiesis assays (Figure 3.20) further implicates that the microenvironment negatively regulates B lymphopoiesis in >2 month old rabbits. In fact, adipocyte layers are not found in the BM of <2 month old rabbits (Figure 3.20 A), suggesting the inhibitory component of the microenvironment is only found in older rabbits. Additionally, hematopoietic progenitors from >2 month old rabbits robustly differentiate into B lineage cells in BM cultures, suggesting they are normal. Complementary to our findings, Kalis et al. performed an adoptive transfer study in rabbits to understand if intrinsic or extrinsic mechanisms regulate the loss of B lymphopoiesis. BM from >2 month old GFP[+] rabbits was transferred into young irradiated recipients, and then the generation of B lineage cells from GFP[+] BM was assessed. Consistent with our results, GFP^{+} hematopoietic cells from the BM of older rabbits were able to differentiate into B lineage cells after transfer into young recipients (Kalis et al.,

2007), suggesting that BM progenitors from >2 month old rabbits retain the

potential to differentiate to the B lineage. These data lead us to conclude that B

cell development is inhibited by the BM microenvironment of >2 month old

rabbits.

We described a mechanism in which adipocytes produce negative

regulators to inhibit B lymphopoiesis. But the accumulation of BM adipocytes can

lead to decreased B cell development via other means as well. In addition to

producing negative regulators, the accumulation of adipocytes in the BM of

humans, mice, and rabbits during aging and obesity could impair B

lymphopoiesis by disrupting the normal supportive capacity of the

microenvironment. For example, MSCs from rabbit BM were found to have a

decreased propensity to differentiate into osteoblasts (actively support B cell

development) and increased ability to differentiate into adipocytes (actively inhibit

B cell development) starting at two months of age (Bilwani and Knight, 2012).

While we showed the increase in adipocytes adds negative regulators to the BM

environment, the loss of osteoblasts alone would result in decreased support for

B cell development. Further evidence for this notion comes from a study of mice

fed a high fat diet. These mice exhibited increased BM adipocytes and reduced B

cell development (Adler et al., 2014). Molecules produced by adipocytes were

not addressed in this study, but the authors suggested that an increased number

adipocytes in the BM physically disrupted the normal supportive niche. While the

loss of supportive cells in the BM microenvironment contributes to the loss of B

cell development, this dissertation focused on adipocyte-derived negative

regulators that actively inhibit B cell development, as adipocytes have only recently been appreciated for their ability to modulate the immune system.

Adipocytes and the accumulation of inhibitory myeloid-derived suppressor cells

Through mouse BM cultures, we found that adipocytes produce a combination of molecules that induce MDSC generation. MDSCs are a population of immature myeloid cells identified by the markers CD11b and Gr1, and commonly found to accumulate in cancers (Gabrilovich and Nagaraj, 2009). Most studies of MDSCs highlight their well-known ability to suppress T cell responses (Gabrilovich and Nagaraj, 2009, Talmadge and Gabrilovich, 2013). Important for this suppression, MDSCs co-express the effector molecules arginase and iNos. Expression of these enzymes can be used to differentiate MDSCs from mature inflammatory M1 macrophages and anti-inflammatory M2 macrophages, as M1 macrophages only express iNos and M2 macrophages only express arginase (Gabrilovich and Nagaraj, 2009).

MDSCs come in two varieties; monocytic MDSCs ($CD11b^+Ly6C^+y6G^-$) and granulocytic ($CD11b^+Ly6C^{lo}y6G^+$) MDSCs (Gabrilovich and Nagaraj, 2009, Youn et al., 2008). By flow cytometric and microscopy analysis we found that adipocyte factors primarily promote the accumulation of monocytic MDSCs (Figure 3.5).

The majority of MDSC studies focus on MDSCs in the context of T cell responses. While a few studies of MDSCs interacting with other cell types exist (Green et al., 2013, Green et al., 2015, O'Connor et al., 2015, Zhu et al., 2012), a

thorough understanding of these interactions is lacking. Therefore our finding that

MDSCs inhibit B lymphopoiesis is novel (Figure 3.6). Even more interesting is the

mechanism by which they inhibit B cell development. ACM-generated MDSCs

co-express large levels of arginase and iNos (Figure 3.4) and, similarly to classic

MDSCs, utilize these enzymes to suppress CD4$^+$ and CD8$^+$ T cell proliferation

(Figure 3.7 B&C). Arginase and iNos use L-arginine as a substrate, and as T

cells need L-arginine to proliferate, depletion of this amino acid is one way in

which MDSCs suppress through these enzymes (Gabrilovich and Nagaraj,

2009). Further, over expression of arginase in transgenic mice resulted in

arginine deficiency and impaired B cell development (de Jonge et al., 2002).

Together these data suggest that MDSCs could inhibit B cell development

through arginase and iNos. However we found that ACM-generated MDSCs do

not inhibit B lineage development via these enzymes (Figure 3.7A).

MDSCs and the production of IL-1. MDSCs inhibited B lymphopoiesis

not by arginase or iNos, but by producing IL-1 (Figure 3.10). This finding

suggests that adipocytes induce inhibitory MDSCs with an inflammatory profile,

which was confirmed through cytokine array analysis of MDSC-CM (Figure 3.9).

IL-1 is a potent inflammatory molecule that was previously described to inhibit B

lymphopoiesis (Dorshkind, 1988a, Hirayama et al., 1994), although the

hematopoietic target was unknown until now. In BM cultures we found that IL-1

treatment promoted myelopoiesis, whereas B lymphopoiesis was lost in cultures

starting with HSCs and MPPs. In contrast, B cell development in cultures seeded

with CLPs appeared to be unaffected by IL-1 treatment. Because IL-1 treated

HSC and MPP cultures exhibited this effect, IL-1 treatment likely acts on the MPP to promote myeloid development. IL-1 has been shown to expand HSCs (Ueda et al., 2009), but it is unknown if differentiation potential is affected before the MPP stage. In addition to modulating expansion/differentiation of HSCs and MPPs, IL-1 has been reported to act on additional cells in the BM microenvironment. IL-1 promotes myelopoiesis in myeloid progenitors (Nagareddy et al., 2014), as well as alters the secretion profile of BM stromal cells. IL-1 induces BM stromal cells to produce GM-CSF, G-CSF, and M-CSF which further inhibit B lymphopoiesis and amplify myelopoiesis (Billips et al., 1990, Dorshkind, 1988a, Dorshkind, 1988b, Ueda et al., 2009). We conclude that IL-1 is a master regulator of inflammation with multiple targets in the BM, and as supported by a simple *in vivo* injection of IL-1 into mice (Ueda et al., 2004), this molecule specializes the BM for myeloid development.

Rabbit bone marrow: An accelerated model of bone marrow aging.
Consistent with the above studies and our finding that adipocyte factors promote IL-1 production, rabbit BM also exhibits increased expression of *IL-1* in >2 month old rabbits (Figure 3.19). This timing correlates with an accumulation of BM adipocytes (Figure 3.17), the arrest of B lymphopoiesis (Jasper et al., 2003), and an increased BM myeloid compartment that appears to be the source of *IL-1* expression (Figure 3.19).

The characterization of rabbit BM starting at two months of age appears to share multiple characteristics with BM from aged (2 years old) mice. Consistent with our observations of BM from >2 month old rabbits, 2 year old mice have

increased BM fat (Krings et al., 2012), declining B lymphopoiesis (Kirman et al., 1998, Miller and Allman, 2003, Riley et al., 1991, Sherwood et al., 1998, Stephan et al., 1996), and an expanded myeloid compartment (Enioutina et al., 2011). We conclude that the rabbit is an accelerated model to study how changes in the BM microenvironment affect hematopoiesis, as occurs in aged mice and presumably elderly humans. In fact, the accumulation of BM fat in rabbits appears to mimic that of elderly humans, where 40-50% of the proximal femur and 70% of the tibia fill with adipose tissue (Li et al., 2013); further suggesting that rabbits are a good model to understand these changes that occur later in life for humans and mice.

The mechanisms contributing to the arrest of B lymphopoiesis in rabbits appear to be in contrast to intrinsic defects in hematopoietic progenitors, which have been found, in part, to contribute to the decline of B lymphopoiesis in mice (Rossi et al., 2005, Stephan et al., 1997, Sudo et al., 2000). Because the arrest of rabbit B cell development is primarily due to changes in the BM microenvironment (Figure 3.20 and Kalis et al., 2007), we suggest that the rabbit is a great model to study the extrinsic regulation of B lymphopoiesis in the absence of hematopoietic progenitor defects, which could confound results.

Our characterization of rabbit BM at the time when B lymphopoiesis is lost (increased adipocytes, increased myeloid compartment, increased *IL-1β* expression), implies that the mechanism of adipocyte-mediated inhibition of B lymphopoiesis (elucidated in mouse BM cultures) could be occurring in rabbits by two months of age. Similar to our studies of ACM and inhibitory MDSCs, we found that rabbit BM fat-CM (contains factors from both adipocytes and myeloid

cells) inhibits B cell development and promotes myelopoiesis in rabbit BM

cultures (Figure 3.20). Further, we identified an inhibitory myeloid population in

rabbit BM (Figure 3.21), that we suggest contains rabbit MDSCs. Additional

studies and reagents will be needed to confirm these observations *in vivo* and to

further characterize rabbit BM subpopulations.

Bone marrow suppressor cells and declining B cell development

BM stromal cells are commonly referred to as major component of the BM

microenvironment. Therefore changes to BM stromal cells are usually attributed

to changes in hematopoiesis (eg. decreased osteoblasts and increased

adipocytes). While BM stromal cells are important to the regulation of B

lymphopoiesis, our work highlights the contribution of additional cell types in the

BM microenvironment.

Our work has implicated MDSCs in the inhibition of B lymphopoiesis. We

expect in scenarios where MDSCs are present in the BM, B lymphopoiesis will

be negatively regulated. Interestingly, Enioutina et al. found that MDSCs are

increased in the BM of 22 month old mice (Enioutina et al., 2011), the time when

B lymphopoiesis is impaired (Kirman et al., 1998, Miller and Allman, 2003, Riley

et al., 1991, Sherwood et al., 1998, Stephan et al., 1996), and increased fat is

observed in the BM (Krings et al., 2012). MDSCs from the BM of aged mice were

found to have increased suppressive activity in T cell proliferation assays and

respond to inflammatory stimuli more robustly, compared to CD11b$^+$Gr1$^+$ myeloid

cells isolated from young BM (Enioutina et al., 2011). We suggest it is possible

that inflammatory molecules derived from BM fat (which is increased and has

different properties in aged BM) (Krings et al., 2012) during aging could influence these MDSCs to produce pro-inflammatory molecules that inhibit B cell development.

It is unknown whether MDSCs isolated from the BM of aged mice inhibit B lymphopoiesis, but the identification of an inhibitory myeloid population in the BM of >2 month old rabbits (Figure 3.21) suggests it is possible. The idea that MDSCs inhibit B lymphopoiesis is novel, and it is very interesting to think of how this finding fits into the field as a whole. In fact our work is complemented very well by a series of studies by Soderberg and colleagues, who identified two types of suppressor cells in rabbit BM in the 1980's.

One of the suppressor cells identified in rabbit BM was described as having adherent properties and macrophage-like morphology (Soderberg, 1984a). These cells appear similar to the mouse monocytic MDSCs identified after ACM treatment (Figure 3.5), and could be the suppressive population we found to be contained within the rabbit CD11b$^+$ BM fraction (Figure 3.21); as CD11b is a common marker for macrophages.

Soderberg defined rabbit BM macrophage-like cells as suppressors based on the ability to suppress BM responses to immune complex stimulation (Soderberg, 1984a). While the target of this suppression is unknown, immune complex stimulation has been reported to act on B lineage cells (Morgan and Weigle, 1983). Additional studies and reagents will be needed to further characterize the myeloid lineage suppressor cells identified by both Soderberg

and our study, to determine the exact mechanism(s) of suppression (target cells and effector molecules).

The other suppressor cell identified in rabbit BM was described as non-adherent, FcRγ$^+$, and complement receptor negative (Soderberg, 1984a, Soderberg, 1984b). These cells exhibited the ability to suppress baseline proliferation of BM cells and could suppress T cell responses (Soderberg, 1985). The mechanism in which they suppress T cells is through blockade of IL-2 (Maes et al., 1988). Alternatively, the mechanism by which these cells suppress BM cell proliferation is unknown. The authors suggest these are likely suppressor lymphocytes of the B, T, or NK lineage, ruling them out as potential MDSCs.

Future studies are needed to determine the exact lineage of non-adherent FcRγ$^+$ BM suppressor cells. If they are of the B lineage, these could be IL-10-producing B$_{regs}$ (Tedder, 2015) or possibly inflammatory TNF-producing aged B cells (ABCs) (Ratliff et al., 2013). In aged mice, ABCs were found to inhibit B cell development through the production of TNF (Ratliff et al., 2013), a potent inflammatory molecule known to synergize with IL-1 to induce granulopoiesis (Ueda et al., 2004). In addition to adipocyte factors, it is possible that TNF producing ABCs also promote IL-1 producing MDSCs in aged BM, as TNF is known to promote MDSC accumulation (Zhao et al., 2012). Overall, our study in combination with the referenced studies, highlight the importance of hematopoietic lineage suppressor populations in the regulation of the BM state.

Inflammation and the regulation of lymphopoiesis

The BM as a source of inflammatory factors. The presence of systemic

inflammatory factors, such as IL-6, TNFα, and IL-1, is a hallmark of aging and

obesity (Baylis et al., 2013, Jung and Choi, 2014, Vasto et al., 2007). For

example, a study examining obese mice identified visceral adipose tissue as a

source of IL-1 and proposed that IL-1 can travel systemically to promote

myelopoiesis in the BM (Nagareddy et al., 2014). In addition to the increase in

systemic inflammatory factors, our study leads us to propose that the BM

microenvironment could become a local source of IL-6, TNFα, and IL-1, which

inhibit B lymphopoiesis (Figure 3.10, Dorshkind, 1988a, Hirayama et al., 1994,

Maeda et al., 2005, Maeda et al., 2009, Ratliff et al., 2013, Ueda et al., 2004).

This idea is supported by studies that suggest inflammatory cells accumulate in

the BM during aging and obesity. In fact, adipocytes accumulate in the BM during

aging and obesity (Adler et al., 2014, Krings et al., 2012), and can act as a

source of IL-6 (Figure 3.15 and Fried et al., 1998, Van Snick, 1990). TNF

producing ABCs also increase in the BM during aging (Ratliff et al., 2013), as do

MDSCs (Enioutina et al., 2011), which we suggest could be a source of IL-1 in

fatty BM. Overall, we propose that the presence of these cells make the BM

microenvironment a local source of inflammatory factors that alter hematopoiesis,

as well as a source of factors that potentially contribute to the increased systemic

inflammation seen in aging and obesity.

The effect of inflammasome activation on lymphopoiesis. A major

pathway shown to contribute to the inflammation observed in aging and obesity is

through inflammasome activation (Vandanmagsar et al., 2011, Youm et al., 2013). The NLRP3 inflammasome, for example, is critical for integrating danger signals that accumulate during aging and obesity, and for triggering active IL-1 production by myeloid lineage cells. Since adipocyte-derived molecules induce IL-1 producing myeloid cells (Figure 3.9), it is logical that inflammasome activation is involved in the adipocyte-mediated induction of MDSCs and the loss of B lymphopoiesis. In fact, blocking the NLRP3 inflammasome with glybenclamide prevented MDSC accumulation, and effectively boosted B lymphopoiesis (Figure 3.16). These data suggest that targeting the inflammasome pathway, instead of individual molecules produced by adipocytes, could be an effective means to enhance the production of naïve B cells.

One limitation of the above finding is that this experiment was only performed *in vitro*. Additional studies will be needed to determine the effectiveness of blocking the NLRP3 inflammasome *in vivo*. However, a study of T lymphopoiesis and aging found that blocking the NLRP3 inflammasome *in vivo* prevented thymic atrophy and the decline of T lymphopoiesis (Youm et al., 2012). Similar to our findings, the authors suggested that adipocyte-derived inflammasome activators initiated the decline of T lymphopoiesis, and could be prevented through blockade to the NLRP3 inflammasome. These data, in combination with our study, implicate NLRP3 inflammasome activation in the negative regulation of both B and T lymphopoiesis.

S100A9 and the amplification of inflammation

Characterization of BM from >2 month old rabbits allowed us to identify a

previously unrecognized negative regulator of B lymphopoiesis. We found that

S100A9+ myeloid cells increase in the BM of >2 month old rabbits (Figure 3.22),

which is consistent with studies that found increased expression of *S100A8* and

S100A9 during aging and obesity (Sekimoto et al., 2012, Swindell et al., 2013).

While the expression of these inflammatory proteins increase in many tissues

with age (Sekimoto et al., 2012), it was unknown whether *S100A8* and *S100A9*

also increase in the BM. Interestingly, we found that *S100A9* increases in the BM

of >2 month old rabbits, correlating with increased BM fat and the loss of B cell

development.

We identified that *S100A9* expression increases in the BM of >2 month old

rabbits by analyzing the SVF BM pellet containing BM cells separated from the

adipocyte layer. As *S100A8* and *S100A9* are known to be expressed by adipose

tissue, one possibility why we did not find increased expression of *S100A8* could

be because we did not assay mature adipocytes. Consistent with this idea, a

recent study found while *S100A8* and *S100A9* are expressed in adipose tissue,

S100A8 expression was primarily from adipocytes and *S100A9* expression came

from the SVF pellet (containing myeloid cells) (Sekimoto et al., 2012). In fact, we

suggest S100A8 does contribute to the adipocyte-induced inhibition of B

lymphopoiesis, as *S100A8* is expressed by 3T3.L1 adipocytes (Hiuge-Shimizu et

al., 2011, Sekimoto et al., 2012), which we used as a source of ACM. Further,

S100A8 has been implicated in NLRP3 inflammasome activation (Simard et al.,

2013) and MDSC induction (Sinha et al., 2008). Future studies will be needed to confirm the contribution of S100A8 to adipocyte-mediated inhibition of B lymphopoiesis.

The ability of S100A9 to inhibit B lymphopoiesis (Figure 3.23) further supports the idea that inflammatory factors negatively regulate B cell development. It was very interesting to us that S100A9 did not act on HSCs or MPPs like IL-1 (Figure 3.24), but instead induced the expression of *IL-6*, *TNF*, and *IL-1β* in BM myeloid progenitors (Figure 3.25). One interpretation of these data is that S100A9 acts to amplify inflammation, a known role of S100A9 in inflammatory processes (Cesaro et al., 2012). In fact one study found that while S100A9 monomers are relatively unstable, stimulation of cells with the inflammatory factors IL-1β and TNFα resulted in the formation of S100A9 homodimers that were incredibly stable and resistant to proteolytic digestion (Riva et al., 2013). In combination with our findings, these data suggest S100A9 inhibits B lymphopoiesis indirectly acting through BM myeloid cells to amplify the production of inflammatory factors that in turn inhibit B cell development.

Therapeutic strategies to boost B lymphopoiesis during aging and obesity

Our study has uncovered multiple targets for intervention to enhance B lymphopoiesis in scenarios with fatty BM. The mechanism we identified can be targeted at the level of the adipocyte, the MDSC, or at the downstream effector molecules. Below are potential strategies that can be used individually, or in combination, to target each step in the mechanism of adipocyte-mediated inhibition of B cell development.

Targeting the adipocyte

Calorie restriction. Adipocytes induce the mechanism of inhibition described in this dissertation, and we suggest targeting adipocytes has the potential to prevent all downstream events leading to inhibition. One mechanism to target the adipocyte is through diet and exercise.

Calorie restriction was shown to be beneficial in the prevention of thymic atrophy and the decline of T cell development. Calorie restriction delayed thymic decline, while obesity accelerated this decline (Yang et al., 2009a, Yang et al., 2009b). Additionally, one study found that high fat diet resulted in increased adipocytes in the BM and reduced B cell development (Adler et al., 2014), implying that calorie restriction might be beneficial to boost B lymphopoiesis.

Alternatively, calorie restriction appears to regulate the thymus and BM marrow differently. Several studies in humans and mice found that calorie restriction actually increased BM fat (Bredella et al., 2009, Cawthorn et al., 2014, Devlin et al., 2010). Further, Cawthorn et al. found that during calorie restriction BM adipose tissue expands and becomes the primary systemic source of adiponectin (Cawthorn et al., 2014), a factor known to inhibit B cell development (Yokota et al., 2003). Although adiponectin is an anti-inflammatory molecule which might offset the negative regulation of B lymphopoiesis through inflammatory factors, it is unknown if calorie restriction will restore B lymphopoiesis in the presence of adiponectin.

Exercise. While calorie restriction increases BM fat, exercise was found to reduce BM adipose tissue volume. In fact, Styner et al. found this to be true in

healthy as well as obese mice (Styner et al., 2014). The BM adipose tissue

reducing effects of exercise are probably due to mechanical stimulus. For

example in rabbits, Bilwani and Knight found that older rabbit MSCs were more

prone to differentiate into adipocytes (inhibit B cell development) instead of

osteoblasts (support B cell development) (Bilwani and Knight, 2012). Exercise

could reverse this effect as a study in mice found that *in vivo* mechanical

stimulation of MSCs promoted MSC differentiation into osteoblasts instead of

adipocytes (Rubin et al., 2007). Consistent with this observation mechanical

stimulation of MSCs was found to decrease PPAR-γ signaling, which is critical for

adipocyte differentiation (Case et al., 2013). Mechanical stimulus also negatively

regulates adipocyte differentiation by increasing β-catenin levels (Case et al.,

2010, Sen et al., 2008). Because β-catenin levels are induced downstream of

WNT receptors and MSCs from older rabbits have decreased expression of

frizzled 4 (WNT receptor), exercise could be beneficial to prevent/reduce

adipocyte accumulation in rabbit BM.

Diet and exercise. Diet and exercise in combination could be very

beneficial to enhance B cell development. The studies above suggest that anti-

inflammatory conditioning of BM adipose tissue through calorie restriction will

reduce the inflammatory state of the BM, in effect removing inhibitory molecules.

In addition, exercise appears to be beneficial in regulating the volume of marrow

adipose tissue and promoting osteoblast generation. This would provide more

space for hematopoiesis and potentially keep adiponectin levels at a manageable

level for B lymphopoiesis to occur.

Targeting myeloid-derived suppressor cells

Since MDSCs have been identified as a therapeutic target in many cancers, there are a plethora of strategies available to target these cells. Current strategies act to deplete MDSCs, block MDSC development, inactivate MDSC effector mechanisms, and induce differentiation of MDSCs into mature myeloid cells that lack suppressive activity (Wesolowski et al., 2013).

Deplete MDSCs. Gemcitabine is one cytotoxic agent used to deplete MDSCs in lung and mammary cancers (Suzuki et al., 2005). This drug has also been used in combination with an anti-IL-6R neutralizing antibody to block the accumulation of MDSCs (Sumida et al., 2012). Other drugs that are toxic to MDSCs include 5-fluorouracil and Docetaxel (Kodumudi et al., 2010, Talmadge and Gabrilovich, 2013, Vincent et al., 2010, Wesolowski et al., 2013). One study using 5-fluorouracil to deplete MDSCs in mice with thymoma found that 5-fluorouracil did not affect most other immune cell lineages. However 5-fluorouracil treatment did result in an increased number of B cells (Vincent et al., 2010), which the authors suggest is to compensate for the loss of MDSCs. Overall, these drugs are an effective means to deplete MDSCs, although it will be important to verify their safety at doses used to target cells in the BM.

Block MDSC development. Several strategies to block MDSC development target signaling pathways responsible for MDSC formation. For example MDSCs require STAT3 activation for development and survival, therefore inhibiting this pathway will result in fewer MDSCs (Sansone and Bromberg, 2012, Wesolowski et al., 2013). In fact, a small molecule has been

developed to target STAT3 phosphorylation (Lin et al., 2010) and could be used to target MDSCs.

Inactivate MDSC effector mechanisms. We found that MDSCs inhibit B lymphopoiesis and suppress T cell responses via different mechanisms. While there are many strategies to block MDSC-mediated suppression of T cell responses, such as blocking arginase and iNos, the treatments developed to target these mechanisms cannot be used to block the inhibition of B lymphopoiesis. Targeting MDSC effector mechanisms in the context of B lymphopoiesis will be discussed in the next section.

Promote MDSC differentiation into non-suppressive myeloid cells. MDSCs are a population of immature myeloid cells, and it is now understood that inducing differentiation into mature myeloid cells is an effective strategy to target MDSCs in the context of cancer. One such agent capable of doing this is the vitamin A metabolite all-trans-retinoic acid (ATRA). ATRA treatment of mice was found to induce MDSC maturation into macrophages, granulocytes, and dendritic cells (Kusmartsev et al., 2003). Overall, ATRA treatment reduced the number of MDSCs in several tumor models (through inducing maturation), increased anti-tumor immune responses, and improved the effect of 2 different anti-tumor vaccines. Another study found ATRA treatment of MDSCs isolated from cancer patients induced differentiation in these cells (Kusmartsev et al., 2008), implicating this as an effective strategy to use in humans. In addition to ATRA, other agents that can be used to induce differentiation include vitamin A, vitamin D, and CpG (TLR9 agonist) (Wesolowski et al., 2013). These are effective

treatments to reduce MDSC numbers in cancers, however the molecule used to enhance B lymphopoiesis must be chosen carefully. For example, while CpG can induce the maturation of MDSCs to less suppressive myeloid cells, CpG recognition by TLR9 was also found to induce CLPs to differentiate into dendritic cells (Welner et al., 2008). Therefore adaptation of this and other strategies for the modulation of hematopoiesis will require additional studies to identify any undesirable outcomes.

Targeting adipocyte and/or MDSC effector molecules

IL-1. Adipocyte-induced MDSCs inhibit B lymphopoiesis through the production of IL-1. One strategy to boost B lymphopoiesis is to target IL-1. This is possible with the drug anakinra, which is an IL-1R antagonist commonly used to treat rheumatoid arthritis (Kavanaugh, 2006). Anakinra may prove useful in preventing the effects of IL-1, but perhaps is not the best strategy because this is the most downstream target identified for adipocyte-mediated inhibition of B lymphopoiesis. One of the most promising therapeutic strategies we identified will be targeting adipocyte products.

Preventing inflammasome activation. Our results suggest that preventing NLRP3 inflammasome activation to promote B lymphopoiesis could be very effective. Glybenclamide treatment (NLRP3 inflammasome inhibitor) of BM cultures containing adipocyte factors prevented MDSC accumulation and enhanced B lymphopoiesis. Blocking the inflammasome acts at the level of adipocyte products preventing downstream events to improve B cell development (removing MDSCs and IL-1 from the equation). Preventing NLRP3 activation in

aged mice was shown to block the atrophy of the thymus, improving T

lymphopoiesis (Youm et al., 2012). If targeting the inflammasome is also found to

be beneficial for B lymphopoiesis *in vivo*, modulating NLRP3 inflammasome

activation could be a powerful treatment to improve general lymphopoiesis. In

fact, aged NLRP3$^{-/-}$ mice have a significant increase in MPPs compared to aged

WT mice (Youm et al., 2012), which might provide a larger progenitor pool for

lymphocyte development. Additional studies will be needed to address this

question.

Statins, inflammasome activation, and B lymphopoiesis

Prior to the current study, our laboratory attempted to prevent/restore

rabbit B lymphopoiesis using lovastatin. Statins are widely prescribed for their

LDL-cholesterol lowering effects, which are mediated through inhibiting the

enzyme hydroxymethylglutaryl-CoA reductase. The logic for using lovastatin to

enhance B lymphopoiesis in rabbits came from studies that showed statins also

inhibit adipocyte differentiation (Nakata et al., 2006, Nicholson et al., 2007), and

therefore was used as a strategy to target the accumulation of adipocytes in

rabbit BM. While preliminary results using lovastatin treatment were promising,

the overall ability to enhance B lymphopoiesis was limited. This dissertation

provides new insight into why the effect on B lymphopoiesis was limited and a

potential strategy to improve B cell development using lovastatin.

In addition to the functions of statins described above, statins were found

to activate the NLRP3 inflammasome in macrophages leading to IL-1β

production (Henriksbo et al., 2014, Mandey et al., 2006). This mechanism

appears to contribute to an increased incidence of type II diabetes seen in patients using statins (Culver et al., 2012, Henriksbo et al., 2014, Ridker et al., 2012). In fact, inflammasome activated myeloid cells are found in patients with type II diabetes (Lee et al., 2013). Similar to using NLRP3$^{-/-}$ mice, Henricksbo et al. found that statin induced insulin resistance and induction of IL-1β secretion by macrophages could be blocked by preventing NLRP3 inflammasome activation with glybenclamide treatment (inflammasome inhibitor) (Henriksbo et al., 2014). These authors suggested that glybenclamide treatment (and other NLRP3 inflammasome inhibitors) in combination with statins will prevent insulin resistance due to statin use. In light of our findings, a combination therapy using statins to prevent adipocyte accumulation and NLRP3 inflammasome inhibitors to prevent statin-induced IL-1β production could be a promising strategy to enhance B lymphopoiesis.

The rabbit as a model system and remaining questions

We envision that the insights gained from this dissertation will be relevant to many situations resulting in fatty BM, such as aging and obesity. As discussed previously, characterization of rabbit hematopoiesis in our study and by others suggests the rabbit is an accelerated model of aging hematopoiesis. The phenotypes observed correlate with an increase in BM adipose tissue occurring by two to four months of age, which is consistent with the accumulation seen in elderly humans (Li et al., 2013). While the rabbit model is valuable to gain an understanding of the processes that occur in aged BM, can the rabbit also be used to model other BM situations?

The rabbit as a model of bone marrow failure

Could rabbits be used as a model for other BM pathologies, such as BM failure? Additional studies will be needed to assess the plausibility of this notion, but available evidence suggests there are some similarities between the BM of >2 month old rabbits and the BM of patients undergoing BM failure. Aplastic anemia and myelodysplastic syndrome (MDS) are both forms of BM failure leading to cytopenias in one or more hematopoietic lineages; including defective production of B lineage cells (Ogata et al., 2006, Ribeiro et al., 2006, Sandes et al., 2012). While ultimately different, the similarities between these diseases often make aplastic anemia and MDS hard to distinguish in some patients. In aplastic anemia, the BM fills with fat (Takaku et al., 2010). Originally it was thought that fat fills the empty BM space, but it has now been posed that adipocytes could cause or contribute to the disease (Islam, 1988, Young, 2013). Certainly this is similar to the process that occurs naturally by two to four months in rabbits. Rabbits shut down the production of new B lymphocytes at this time, but it is unknown if the underlying mechanism could be considered a form of preprogramed BM failure.

MDS also results in altered hematopoiesis that can progress into leukemia, and sometimes exhibits infiltration of BM fat similar to that seen in aplastic anemia (Rovo et al., 2013). Defective hematopoiesis in MDS is often characterized by a large number of hematopoietic progenitors undergoing apoptosis (Gersuk et al., 1998, Raza et al., 1995). The BM in these patients is influenced by inflammatory factors such as IL-1β, TNFα, and Fas-ligand (Gersuk

et al., 1998, Shetty et al., 1996), which can contribute to apoptosis seen in the

BM (Hirai, 2003). This is consistent with increased *IL-1β* expression in BM from

>2 month old rabbits and the increases of inflammatory factors seen during

aging.

How changes in BM MSCs influence the development of MDS is not fully

understood. Several studies suggest MSCs from patients with MDS have

chromosomal abnormalities (Blau et al., 2007, Flores-Figueroa et al., 2005).

Additionally, Borojevic et al. found that plating cord blood hematopoietic

progenitors on BM stromal cells from MDS patients resulted in altered

hematopoiesis, and suggest the BM stroma in MDS might promote the disease

(Borojevic et al., 2004). Further, Raajimakers et al. found that genetic deletion of

Dicer1 or Sbds (mutated in Schwachman-Bodian-Diamond syndrome) in mouse

osteoprogenitors resulted in a BM phenotype resembling MDS. Additional studies

will be needed to assess whether the changes that occur to MSCs in MDS are

similar to the changes occurring in MSCs by two months of age in rabbit BM.

While there are similarities between BM failure and the changes occurring

in rabbit BM, it may be too early to tell if the rabbit is a good model to study BM

failure. Aplastic anemia and MDS both contain a known autoimmune component

that contributes to BM failure (Barrett and Sloand, 2009), which is not known to

occur in rabbit BM. In terms of hematopoietic progenitors, there is a wealth of

information suggesting that genetic defects in hematopoietic progenitors

contribute to MDS (Hirai, 2003). Our data in rabbits (Figure 3.20 and Kalis et al.,

2007), suggest the loss of B lymphopoiesis is mediated by changes in the

microenvironment as opposed to hematopoietic progenitor defects. Additional

differences between available information on rabbit hematopoiesis and BM

failure include; 1. MDS can progress into leukemia, but we rarely find leukemia

develop in our rabbit colony; 2. Rabbits continue to live for years after the arrest

of B lymphopoiesis that occurs at two months of age, while BM failure usually

results in death if untreated; and 3. Although B cell development is lost in rabbits,

decreases in peripheral white blood cell numbers are not known to occur.

Overall, while the rabbit might serve as a good model to assess how changes in

the BM microenvironment impact hematopoiesis, rabbit BM does not exhibit

other common features of BM failure. Most notably, even in the absence of B

lymphopoiesis the rabbit immune system remains intact.

Maintaining adaptive immunity in the absence of B lymphopoiesis

 B lymphopoiesis shuts down early in the life of rabbits, generating a very

perplexing question. How do rabbits remain immune competent? In fact, the

arrest of B lymphopoiesis does not prevent rabbits from responding to new

antigens. Adult rabbits are commonly used to generate polyclonal antibodies to

various antigens. Additionally, rabbit monoclonal antibody technology, a powerful

tool developed by Knight and colleagues uses adult rabbits to generate highly

specific monoclonal antibodies (Spieker-Polet et al., 1995). This suggests the

rabbit must have a mechanism to maintain the ability to respond to a diverse

array of antigens in the absence of B lymphopoiesis. One possible explanation is

that rabbit GALT maintains antibody diversity throughout the life of rabbits. As

described in Figure 1.4, B cells made in the BM traffic to rabbit GALT and

undergo further maturation. It is possible that unknown mechanisms in GALT

support the maintenance of B lineage cells. Alternatively, perhaps rabbit B

lineage cells can self-renew. One study found that rabbit B cells express the

molecule CD5 (Raman and Knight, 1992), a molecule used to identify B1a cells

in mice. Although it is unknown if rabbit B cells can self-renew, mouse B1a cells

are maintained throughout life by self-renewal (Forster and Rajewsky, 1987,

Hayakawa et al., 1985, Hayakawa et al., 1986, Herzenberg et al., 1986,

Herzenberg and Kantor, 1993). Future studies will be needed to understand how

B cells are maintained, as this may alleviate the demand for B lymphopoiesis in

the BM and contribute to the arrest of new B lymphocyte production.

Understanding changes in MSCs before and after two months of age

Bilwani and Knight found that there are fewer MSCs present in the BM of

>2 month old rabbits (the time when B lymphopoiesis arrests), and that the MSCs

are more prone to differentiate into adipocytes compared to MSCs from younger

rabbits (Bilwani and Knight, 2012). Because adipocytes initiate the mechanism of

inhibition described in this dissertation, preventing the accumulation of

adipocytes in the BM could result in enhanced B lymphopoiesis. It will be

important for future studies to identify what triggers changes in MSCs at two

months of age in rabbits.

Similar to hematopoietic progenitors, intrinsic changes to MSCs or

extrinsic changes in the systemic environment might contribute to increased

potential to become adipocytes over osteoblasts. While this altered potential is

not fully understood, one study found that serum from adult rabbits induced

adipocyte differentiation in several human, mouse, and rat

osteoblast/osteosarcoma cell lines (Diascro et al., 1998). These data suggest a

systemic factor in rabbits could influence this differentiation decision. Further

analysis of adult rabbit serum identified the fatty acids palmitic, oleic, and linoleic

acids were responsible for inducing adipocyte differentiation (Diascro et al.,

1998). What causes these fatty acids to be present in adult rabbit serum, and the

genetic and epigenetic effects they have on MSCs is yet to be determined.

An alternative mechanism that may modulate MSC adipogenic vs

ostobalastic potential could come from altered WNT signaling in older rabbits.

RDA analysis comparing BM MSCs from a young and a 2 year old rabbit found

frizzled 4 (WNT ligand receptor) expression was decreased in MSCs isolated

from the 2 year old rabbit (Siewe et al., 2011). This is important because

decreased WNT signaling results in low β-catenin levels, and having low

amounts of active β-catenin in MSCs promotes adipocyte differentiation (Sen et

al., 2008). More studies will be needed to truly understand the mechanisms

initiating the accumulation of adipocytes in BM. This may be due to changes in

diet and exercise leading to changes in serum fatty acids, and/or currently

undefined genetic/epigenetic mechanisms.

Additional studies are also needed to better understand the features of

regulated BM adipose tissue (supports hematopoiesis) vs constitutive BM

adipose tissue (does not support hematopoiesis), and how these types of fat

contribute to the state of the BM microenvironment.

Conclusion

This study has improved our understanding of how adipocytes influence the BM microenvironment and affect hematopoiesis. We propose a model occurring in fatty BM that is initiated by adipocytes and results in altered hematopoiesis (Figure 4.2). Adipocytes produce inflammasome activating molecules that promote the accumulation of MDSCs/inflammatory myeloid cells which, in turn produce IL-1. IL-1 acts on hematopoietic progenitors and stromal cells to produce an inflammatory state that drives MPPs to differentiate to the myeloid lineage, instead of the B lineage. The inflammatory state negatively regulating B lymphopoiesis and promoting myelopoiesis can be amplified by S100A9 production from myeloid cells (Figure 4.3). S100A9 acts on BM myeloid cells to induce *IL-1*, *IL-6*, and *TNFα* expression, known inhibitors of B lymphopoiesis (Dorshkind, 1988a, Hirayama et al., 1994, Maeda et al., 2005, Maeda et al., 2009, Ratliff et al., 2013, Ueda et al., 2004).
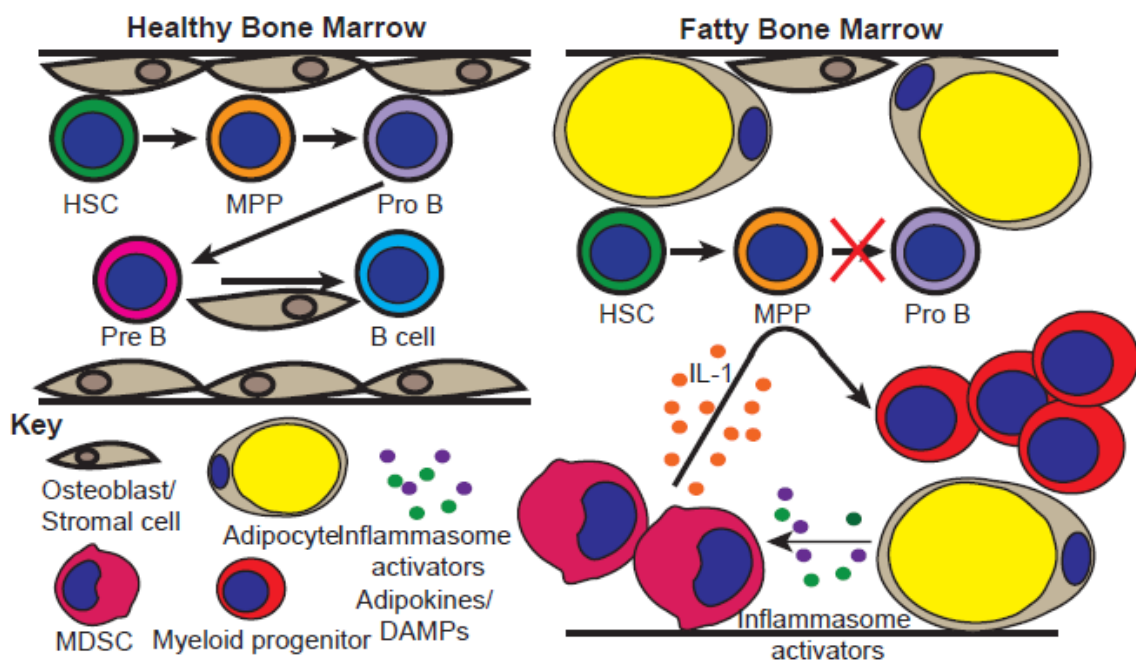
Figure 4.2 Negative regulation of B lymphopoiesis in adipocyte-rich bone marrow. Healthy BM is characterized by the presence of osteoblasts and stromal cells that support B lymphopoiesis. In fatty BM, adipocytes accumulate while supportive stromal cells are decreased in number. Adipocytes actively influence hematopoiesis by producing inflammasome activating molecules, as well as inflammatory cytokines. Adipocyte-rich BM exhibits an increased myeloid compartment, which contains a population of cells that inhibit B lineage development. Overall, inflammatory factors derived from adipocytes and inflammatory MDSCs create an environment that promotes myelpoiesis and negatively affects B cell development. Adapted from Kennedy et al. 2016.
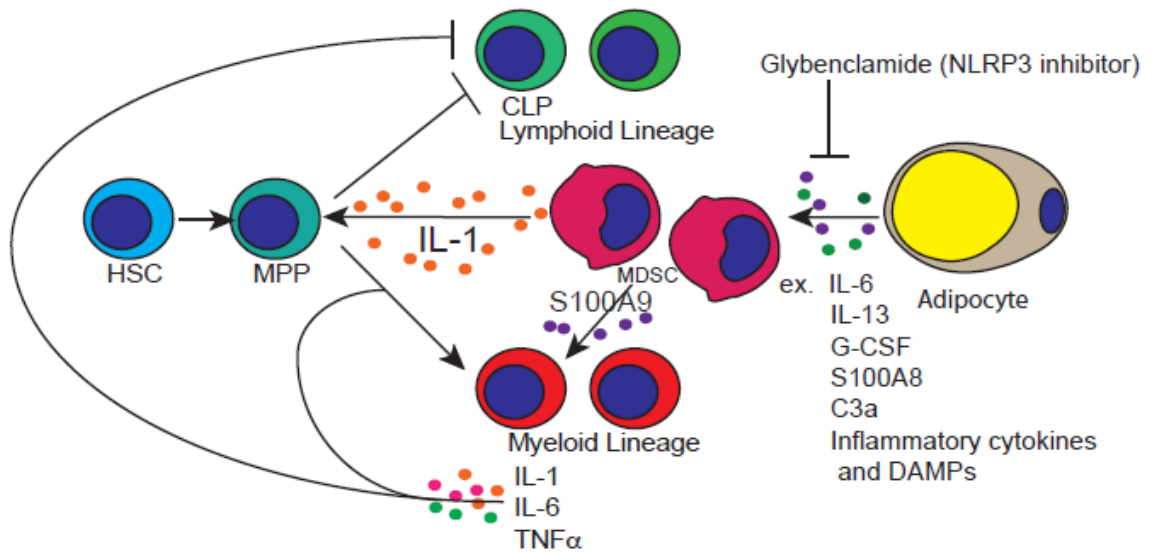
Figure 4.3 Effect of S100A9 on hematopoiesis. Inhibition of B lymphopoiesis is initiated by inflammasome activating adipocyte factors (can be blocked with glybenclamide) and mediated through IL-1 producing MDSCs (as described in Figure 4.1). In addition to this mechanism, the presence of S100A9 further amplifies inflammation in the BM. S100A9 derived from myeloid cells in fatty BM does not act at the MPP stage like IL-1. Instead, S100A9 induces *IL-1β* expression in BM myeloid progenitors cells which can feed into the inhibition of B lymphopoiesis already described for IL-1. Further, S100A9 also induces the production of *IL-6*, and *TNFα* in BM myeloid cells which can directly inhibit B lymphopoiesis. Adapted from Kennedy and Knight, 2015.

B cells and antibodies are critical for generating productive immune responses. Situations resulting in decreased output of new B cells into the

periphery put the host at risk for infection. Our study highlights the importance of the BM microenvironment (consisting of stromal and hematopoietic cells) in the regulation of B lymphopoiesis, and how changes to this environment negatively regulate B lineage development. We provide mechanistic insight into how adipocytes and MDSCs inhibit B lymphopoiesis, and identified several therapeutic targets for intervention. Accumulation of BM fat occurs in multiple pathologies (eg. obesity, BM failure, aging), we expect the knowledge gained in this dissertation will be used to develop treatments aimed at increasing B lymphopoiesis in a broad context of pathologies resulting in altered white blood cell development due to increased BM fat.

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VITA

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