DIETARY MANIPULATION OF NATURAL KILLER CELL BIOLOGY THROUGH REFEEDING OF PREVIOUSLY CALORICALLY RESTRICTED MICE

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ABSTRACT

DIETARY MANIPULATION OF NATURAL KILLER CELL BIOLOGY THROUGH REFEEDING OF PREVIOUSLY CALORICALLY RESTRICTED MICE

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The dynamic role of natural killer (NK) cells in immunology has been demonstrated in numerous fashions, proving they are much more than "natural killers". However, NK cells are perhaps still the least well understood lymphocyte, due to their relatively low abundance and the limited transgenic models available for study. An innate immune cell that plays a critical role in providing early immunity against viral infections and cancers, NK cells are becoming increasingly recognized for shaping and directing immune responses. Accounting for approximately 5-25% of peripheral blood mononuclear cells in humans and 5-10% of lymphocytes in murine circulation, NK cells have very similar functional attributes in both species, making mice an ideal model system for the study of NK cell biology. Studies in mice have revealed the critical importance of NK cells in providing tumor surveillance as well as early protection from viral infections. Preclinical studies have underscored the utility of using NK cells as an immunotherapeutic technique to combat various cancers, while NK cell function is often associated with positive or negative outcomes in various disease states. Furthermore, various lifestyle factors have been found to positively or negatively influence NK cell function. Among these lifestyle factors, diet has gained notoriety as being capable of influencing the homeostasis and function of NK cells. However, the mechanisms by which diet influences NK cells are not fully understood, highlighting the need for a better understanding of the molecular and cellular mechanisms by which diet influences NK cell development. Thus, our laboratory has extensively studied the effects of the restriction of energy intake, or caloric restriction (CR)

on NK cell function and biology at the organismal, cellular, and molecular level. Here we describe a series of experiments investigating the role of energy intake on immunity to influenza virus, with a focus on NK cells. We describe a series of studies that identify the specific changes to NK cells induced by CR, both beneficial and potentially damaging. In these experiments we show that CR results in fewer NK cells with a mature phenotype, and that expression of transcription factors critical for NK cell maturation are reduced by CR. We also demonstrate that thymic derived NK cells are present in normal numbers in CR mice and have enhanced function. In a series of experiments demonstrating the intricate relationship between immunity and metabolism we show how refeeding of CR mice restores NK cell homeostasis and function, both before, and during influenza infection. Using *in vitro* techniques combined with *ex vivo* analysis of metabolic signaling pathways, we provide potential mechanisms by which CR impairs NK cell maturation. These studies serve to highlight the critical role of optimal nutrition in maintaining NK cell homeostasis and function. To our knowledge, this dissertation is the first data presented that clearly details the effects of CR on NK cell development and homeostasis, as well as the molecular and biochemical pathways mediating this effect.

Dedication

This work is dedicated to my parents, John and Susan who have always supported and encouraged me, and to my brother Alex, whose free spirit I have always admired

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My education and training at Michigan State University has been an enlightening experience, and I have matured both academically and emotionally as a result. In truth, this work would not have been possible without the guidance and trust of my advisor, Dr. Elizabeth Gardner. During my research I have also been thankful to receive the support of my labmates, Dr. David Duriancik, Dr. Eleni Beli, and Brooke Roman. Without their help many of the experiments described in this dissertation would have been impossible. I had the pleasure of joining the laboratory only shortly after Eleni joined, and it was under her guidance that I learned many of the techniques that I used to pursue my research ideas. These techniques were further refined with the help of David and I am very thankful to him for sharing his experience with me. Furthermore, Eleni and David were always brimming with new ideas and it was very inspirational to work with both of them. Fellow graduate students in the Linz, Pestka, and Fenton lab (especially Eric) have always been available for thoughtful discussion as well as leisurely conversation, and for this I am thankful. I also would like to thank my guidance committee for all their help, including: Dr. Jenifer Fenton, Dr. Peter Mancuso, Dr. John Gerlach, and Dr. Norman Hord. I owe special thanks to Dr. Fenton, through collaborative projects with her laboratory I have had to opportunity to learn many new skills and ideas.

I would also like to thank the Department of Food Science and Human Nutrition for their support of my education. The amount of resources they invest in their graduate students makes me proud to be a member of this department, and I will be forever in their debt.

Of course, though out the last several years I have always been able to rely on my family, especially my parents, John and Susan for their thoughtful input and emotional guidance, and it is to them that I dedicate this dissertation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

7-AAD	7 aminoactinomycin D
ADP	Adenosine diphosphoate
AIN	American institute of nutrition
AL	Ad libitum
AMPK	AMP activated kinase
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATP	Adenosine triphosphoate
B220	Cluster designated molecule 45R
BM	Bone marrow
BrdU	Bromodeoxyuridine
CALERIE	Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy
CD	Cluster designated
CR	Caloric restriction
CTL	Cytotoxic T lymphocyte
CXCR	CXC chemokine receptor
d	Day
Db/db	Leptin receptor deficient
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
DX5	Cluster designated molecule 49b

E4bp4	Nuclear factor interleukin 3 regulated
E:T	Effector:Target
ELISA	Enzyme linked immunosorbant assay
Eomes	Eomesodermin
FACS	Flourescent activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Flt-3L	Fms-related tyrosine kinase 3 ligand
GATA-3	Trans-acting T-cell specific transcription factor GATA-3
GC	Glucocorticoid
GM-CSF	Granulocyte macrophage colony stimulatory factor
GR-1	Ly6C and Ly6G
HA	Hemmagluttinin
HAU	Hemmagluttination units
iDC	Immature classical dendritic cell
IF	Intermittent fasting
IFN	Interferon
IFNR	Interferon receptor
Ig	Immunoglobulin
IL	Interleukin
iNK	Immature natural killer
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus activated kinase
KIR	Killer cell immunoglobulin-like receptor

KLRG1	Killer cell lectin-like receptor subfamily G, member 1
IRF	Interferon response factor
LKB1	Liver kinase B1
LN	Lymph node
LPS	Lipopolysaccharide
Ly49	Killer cell lectin-like receptor subfamily A
Ly	Lymphocyte antigen complex
mAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
mDC	Mature classical dendritic cell
MFI	Median fluorescent intensity
mg	Milligram
MHC	Major histocompatability complex
mL	Milliliter
mM	Millimolar
mo	Months old
mNK	Mature natural killer
mTOR	Mammalian target of rapamycin
NIA	National institute on aging
NIH	National institute of health
NK	Natural killer
NKC	Natural killer complex
NKG2A	Cluster designated molecule 159a
NKG2C	Cluster designated molecule 159c
NKp46	Cluster designated molecule 335

NKT	Natural killer T
ng	Nanogram
Ob/ob	Leptin deficient
PBS	Phosphate buffered saline
PE	R-phycoethyrin
PEM	Protein energy malnutrition
PerCP	Peridinin chlorophyll protein complex
p.i.	Post infection
PI3K	Phosphoinositide 3 kinase
PMA	Phorbol-12-myristate-13-acetate
PR8	Puerto Rico 8 1934
pS6	Phosphorylated S6
RBC	Red blood cell
RF	Refed
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
S1P5	Sphingosine 1-phosphate 5
SDF-1α	Stromal derived factor 1 alpha
SEM	Standard error of the mean
STAT	Signal transducers and activators of transcription
T-bet	T-box transcription factor TBX21
TCA	Tricaboxylic acid cycle
TCR	T cell receptor
TF	Transcription factor
Th	T helper

TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T regulatory cell
TRAIL	TNF related apoptosis inducing ligand
U	Units
-/-	Homozygous knockout
β2m	Beta-2 microglobulin
µg	Microgram

INTRODUCTION:

BACKGROUND:

Throughout history the search for eternal life has been a constant pressure for humanity; from the quest for the Holy Grail, to the search for the fountain of youth, humans have relentlessly pursued longevity. Now in the 21st century, we turn not to religion or mythology for answers, but to science. It is predicted that by the year 2050 there will be over 370 million persons over 80 years of age [1], highlighting the social pressures for understanding the mechanisms behind the aging process. As early as 1936 scientific evidence provided by McCay and colleagues suggested that restriction of calorie intake without causing malnutrition in laboratory animals could extend lifespan [2, 3]. Since, numerous studies have utilized chronic calorie restriction (CR) in order to promote longevity and healthy aging in laboratory settings. Among the multitude of findings concerning the pro-longevity effects of CR, the ability of CR to delay the decline in immune function associated with aging (immunosenescence) has received a great deal of attention.

It is the CR paradigm that has been the focus of my research and the research presented in this dissertation. Because of the interest in understanding the mechanisms by which CR inhibits immunosenescence, this work has focused on the development and function of an immune cell which our laboratory has previously shown to be influenced by CR [4]. Indeed, as early as the 1980s it was recognized that CR influenced the function of NK cells in aged mice [5], however it was not until 25 years later that the repercussions of this observation were documented. Gardner reported that aged CR mice were more susceptible to influenza infection, and that this was accompanied by reduced natural killer (NK) cell cytotoxicity in the lungs during influenza infection [4]. In 2008, Ritz et al. reported a similar finding, but that young CR

mice were also more susceptible to influenza infection, suggesting the impairment in NK cell cytotoxicity observed previously exists independent of aging.

Despite the widespread use of CR as a dietary intervention in the laboratory to suppress autoimmunity, reduce cancer incidence, inhibit immuonsenesence, prevent metabolic diseases, and limit age-associated disease incidence [6], the degree to which CR influences the immune system is still poorly characterized [7]. A major goal of this dissertation was to expand on the current literature regarding the influence of CR on immune homeostasis and the phenotype of the immune system. This was performed in young 6 month old (mo) mice in order to study the influence of CR on immune homeostasis independent of aging. Furthermore, utilizing recent studies examining the influence of metabolic signaling on T cell function, we suggest a role for intracellular signaling through the mammalian target of rapamycin (mTOR) kinase in regulating NK cell development.

OVERALL HYPOTHESIS:

Ritz et al. and Gardner both reported that NK cell cytotoxicity is reduced in the lungs of CR mice during influenza infection. Cytotoxicity is a functional attribute only associated with mature NK (mNK) cells, except in unique circumstances [8]. Furthermore, mNK cells are among the only NK cells to seed the lungs of mice [9, 10], and it has been reported CR mice have fewer NK cells in the lungs during influenza infection [11]. Finally, CR mice exhibit increased susceptibility to influenza during the first week of infection, suggesting the immunological defect responsible for this observation is likely related to the function of innate immunity. The combination of these observations lead to the formulation of the guiding hypothesis for this work: The early susceptibility to influenza virus observed in CR mice is due

to impaired NK cell development or function. We remained cognizant of the fact that there they may be other immunological changes to in CR mice which also may contribute to increased susceptibility to influenza virus. This dissertation tests this hypothesis through two specific aims (not including sub-aims) 1) identify key changes to the innate immune system resulting from CR and 2) evaluate the efficacy of a nutritional intervention to restore immune homeostasis. Importantly, we expanded upon the original hypothesis from the Gardner lab and investigated whether phenotypic and functional changes to NK cells resulting from a CR diet can be attributed to altered signaling through mTOR, a pathway known to be influenced by CR in numerous different cell types. Thus, this work provides novel data on the influence of CR on the phenotype of immune cells, specifically NK cells, as well as molecular mechanisms potentially responsible for these observations.

CHAPTER SUMMARIES:

Chapter 1 is a literature review that lays the foundation for the research problems that are addressed in this work. It will begin by giving a brief overview of the immune system, with a brief outline of how the immune system recognizes and responds to pathogens. Because the metabolism of immune cells is a relatively new field there is significant attention focused on the concept of "immunometabolism" at the end of this chapter, which highlights and reviews our current knowledge on this subject.

Chapter 2 is a continuation of the literature review with a specific focus on NK cells, which are the primary cell type that was the focus of this work. First, previously published data regarding the developmental stages of NK cells will be discussed. The markers that are used to identify various stages of NK cell development will be explained, and the potential significance

of these cell surface makers will be identified. Furthermore, research regarding the critical role of various cytokines responsible for controlling various stages of NK cell development shall be discussed. This will be elaborated upon by providing an explanation of our current understanding of how transcription factors (TF) regulate NK cell development in the bone marrow (BM) and periphery. This work will explain how studies using transgenic mice that are homozygous knockouts (-/-) for various TFs or other signaling molecules have been used to elucidate the influence of various molecules on NK cell development and homeostasis.

Recent research has identified various roles for NK cells in a variety of immune responses. Indeed, these cells have a multifaceted role in immunity and new aspects of NK cell function are frequently described [12]. Chapter 2 will address the different mechanisms by which NK cells participate in immune responses, including cell mediated cytotoxicity, production of cytokines, and immunological editing. Because NK cells are capable of responding to target cells without prior antigen sensitization, their function must be tightly regulated. An entire sub-section of Chapter 2 is devoted to explaining our current understanding of how self-tolerance is achieved by NK cells and identifying the various areas in which we lack understanding.

Following the background information on how NK cells develop, function, and are regulated, Chapter 2 discusses identified roles for NK cells in viral infections, including influenza infection. Furthermore, the mechanisms by which NK cells become activated in order to provide immune defense against viral infection and cancer are explained. Following the explanation of activation mechanisms of NK cells, the second to last section of Chapter 2 explains the current clinical applications of NK cells, with a specific focus on NK cell therapies to fight cancers. Chapter 2 concludes with a section highly relevant to the rest of this

dissertation, in which research regarding the influence of diet on NK cell development, function, and homeostasis is reviewed.

Chapter 3 is a continuation of the literature review, however the focus has shifted to the dietary paradigm known as CR. Here the history of CR is outlined as well as the proper dietary protocol for what is considered the "gold-standard" of CR research. Discrepancies between dietary approaches are outlined, as well as the various dietary formulas that have been used over the years in order to conduct CR research. This is an especially important section, as many CR protocols result in malnutrition, a confounding factor in many CR studies.

Chapter 3 continues with the known immunological effects of CR. In order to understand the mechanisms by which CR reduces immunosenescence is first explained. Chapter 3 then summarizes the ability of CR to reduce immunosenescence and also discusses the influence of CR on other aspects of immunity. CR has also been shown to reduce the incidence of spontaneous tumors, and thus has been cited as a potential preventative treatment for cancers. This is discussed briefly in Chapter 3 as well as how the immunological changes induced by CR potentially can explain the anti-cancer effect of CR. Also discussed here are the molecular pathways by which CR seems to exert many of its anti-cancer effects.

Finally, Chapter 3 focuses on the influence of CR on immune function in the face of intact pathogens. There have been several studies highlighting the fact that CR animals challenged with infectious diseases seem to have increased susceptibility. While the precise immunological reasons for these observations are still unknown and under investigation, Chapter 3 reviews the current literature available of this subject.

Chapter 4 is the beginning of primary research presented in this dissertation regarding preliminary studies in which CR mice were refed (RF) by being given unlimited access to food.

In order to determine whether influenza-induced NK cell function could be restored in CR mice, young adult (6 mo) male C57BL/6 mice were refed I control diet *ad-libitum* for two weeks before infection with Puerto Rico 8 1934 (PR8) influenza A. Refeeding improved survival and attenuated the decline in NK cell function during infection as is observed in CR mice. This is evidenced by increased numbers, percentages of NK cells, and an increase in CD69 expression on NK cells on d 3 post-infection (p.i.) in RF mice. These observations suggest that refeeding for a defined period before, and perhaps throughout, influenza season may provide the energy needed to counter the deleterious effects of CR on NK cell function, especially during exposure to newly emerging strains of influenza. Data in this chapter have been published in *The Journal of Nutrition* and can be found at: http://www.ncbi.nlm.nih.gov/pubmed/20534876

Chapter 5 contains experiments in which our current understanding of NK cell biology is applied to the CR paradigm in order to understand the full effect of CR on NK cell development. This chapter describes the influence of CR on NK cell phenotype and function in the absence of infection. Mature BM derived NK cells are shown to be reduced while thymic derived NK cells are shown to develop in a normal fashion. Because these cells have unique functional attributes, this results in a skewing of the function of the total NK cell pool. NK cells from CR mice were more capable of producing pro-inflammatory cytokines, likely related to the thymic phenotype displayed by these NK cells. On the other hand interferon (IFN)-γ production was impaired in NK cells from CR mice, as this functional attribute is generally associated with classical BM derived NK cells. CR is to be a potent dietary intervention, yet the mechanisms by which the CR increases lifespan have yet to be fully understood. These data presented in this chapter are the first in-depth analysis of the effects of caloric intake on NK cell phenotype and function. Furthermore the observations found in Chapter 6 represent important implications regarding

potential ways in which CR alters NK cell function prior to infection or cancer. Data in this chapter have been published in *The Journal of Immunology* at: http://www.ncbi.nlm.nih.gov/pubmed/23241894

Chapter 6 contains experiments investigating the extent to which refeeding of CR mice is capable of restoring the changes to NK cells that are described in Chapter 5. Indeed, this chapter shows that refeeding of previously CR mice for 3 weeks is required to restore NK cell homeostasis in the lung and spleen. NK cell frequency, subset distribution and the proliferation and apoptosis of NK cells is investigated at various time points during the refeeding period. Furthermore, the chapter ends by examining the effect of refeeding on NK cell function at baseline as well as during influenza infection.

Chapter 7 details a series of experiments using BD Phosflow^{TM} signaling technology in order to study alterations to metabolic signaling pathways that are associated with both CR and immune homeostasis. The experiments in Chapter 7 were designed to investigate potential mechanisms that would explain the changes to NK cells that are described in Chapter 6. This chapter begins by examining the metabolic phenotype of NK cells and NK cell subsets in comparison to other lymphocytes. A major observation in this chapter is that NK cells from CR mice appear to have reduced signaling through the mTOR pathway. Therefore, we utilize a brief treatment with rapamycin in order to induce pharmacological blockade of mTOR signaling in NK cells and study the consequence of this blockade on several aspects of NK cell biology. Cytokine cultures are used to activate mTOR signaling and mature NK cells *in vitro* and the ability of cytokines to mature NK cells after brief treatment with rapamycin is examined. The phenotype of NK cells is also determined following suppression of mTOR signaling with regards to metabolic transporters and NK cell receptor expression. Finally, because IFN-γ production is

a paramount aspect of NK cell function, we examined whether brief rapamycin treatment would influence the responsiveness of NK cells to cytokines or ligation of activating receptors with regards to IFN-γ production.

Chapter 8 concludes this dissertation with a brief summary of the previous chapters as well as suggestions for the future direction of this research. A summary figure is provided that describes our proposed model of how energy intake influences metabolic signaling pathways and ultimately influences the terminal maturation of NK cells.

Finally, the Appendix of this dissertation contains experiments in which we describe alterations to the distribution of leukocytes within the BM of young adult C57BL/6 mice resulting from CR. Using multicolor flow cytometric analysis the Appendix describes B cell development in CR BM which remains unaffected up to the prepro stage, after which pro and pre-B cells were found at a significantly reduced frequency and exhibited increased apoptosis in CR mice. Other changes to BM leukocytes are described here, including a decrease in lymphocytes that was accompanied by an increase in the proportion of monocytes, granulocytes, and mixed progenitor lineages in the BM. Within the granulocyte lineage, there was a significant increase in the percent of granulocytes that were fully differentiated mature neutrophilic granulocytes. Finally, using enzyme linked immunosorbent assays (ELISAs) to quantify growth factors present in the BM microenvironment, it is shown that CR alters levels of leptin and corticosterone, two hormones known to play a significant role in the development and homeostasis of lymphoid and myeloid lineages.

CHAPTER 1:

BACKGROUND

1.1 IMMUNE SYSTEM OVERVIEW:

The immune system defends the host from pathogenic bacteria and viruses through a variety of mechanisms, ranging from providing simple barriers against pathogens [13], to cellular recognition of pathogens at the molecular levels [14]. The protection provided by the immune system is generally classified as either innate or adaptive immunity. A large variety of cell types make up the immune system and based on the mechanisms by which invading microorganisms and malignancies are recognized, immune cells are categorized as innate or adaptive. Through coordinated interactions, innate and adaptive immunity protect against bacterial, fungal, parasitic, and viral infections as well as cancers and malignant cells [15]. At the most basic level, the immune system recognizes molecular patterns that are associated with infections or cancers and responds appropriately (14). The characteristics of an immune response are dictated by the types of immune cells involved and which specific intracellular signaling cascades are activated. Characterization of immune responses has resulted in a wide variety of specific immune responses [16]; however the principals of immune function remain the same. If a pathogen or malignancy is not cleared during the early innate immune response then adaptive immune cells are recruited to help fight the pathogen or cancer.

The best defining characteristic used to separate the innate immune system from the adaptive is the specificity that the different arms of the immune system use to recognize targets. The innate immune system recognizes intact structures that are commonly conserved molecular patterns often found on pathogens, also known as pathogen associated molecular patterns, or

molecules released from damaged or stressed cells, known as damage associated molecular patterns [14, 17]. These patterns range from ligands expressed by self-cells that share sequence homology with major histocompatability complex (MHC) proteins, to components of bacterial cell walls such as lipopolysaccharide (LPS) [14]. Utilizing a variety of both extracellular and intracellular receptors, innate immune cells recognize and respond to these "danger" signals in the appropriate fashion, dictated by the type of structure detected. Cells characterized as belonging to the innate immune system include phagocytes such as macrophages and neutrophils [18], lytic cells such as NK cells [19], and newly described innate cells capable of producing pro-inflammatory cytokines such as interleukin (IL)-17 [20]. Phagocytes take up pathogens or apoptotic and damaged cells within phagosomes, and destroy and digest these targets [21]. Digestion of pathogens occurs within phagolysosomes which are the product of fusion between the phagosome containing a microbe and a lysosome [22]. Phagolysosomes contain digestive enzymes as well as harbor a low pH, allowing for multiple modes of pathogen destruction [22]. Phagocytes can also destroy phagocytosed pathogens via the production of toxic reactive oxygen species (ROS) such as superoxide and hydrogen peroxide molecules, or through production of anti-microbial peptides [23]. Lytic cells on the other hand, induce death of targets through directed and targeted release of proteins such as perforin and granzyme that activate cell death pathways [19, 24]. Furthermore, lytic cells often express receptors such as tumor-necrosis-factor related apoptosis inducing ligand (TRAIL) and Fas-ligand (FasL) that can directly induce cell death on a target cell upon ligation of the cognate receptor on a cell targeted for destruction [19].

Nonetheless, recognition of a target by phagocytes or lytic cells also results in the induction of an inflammatory response through the production of cytokines and chemokines by both local tissue specific cells and innate immune cells [13]. These inflammatory signals recruit

and activate immune cells as well as promote non-cellular microenvironmental changes which also protect the organism from pathogens [13]. Also, under basal conditions non-cellular factors such as compliment and protease enzymes limit pathogen replication and entry to the organism, while under inflammatory conditions these non-cellular factors are upregulated to protect the organism by directly killing the invading pathogen [13].

As mentioned previously, innate immunity relies on the detection of conserved molecular patterns in order to provide a directed immune response. Adaptive immunity, on the other hand, is much more specific, recognizing short polypeptide sequences unique to a particular antigen or microbe [25]. In order to mount an adaptive response, T cells recognize short polypeptide sequences presented on MHC molecules by antigen presented cells (APCs), such as dendritic cells (DCs) and B cells [26]. While both DCs and B cells are classified as APCs, B cells are unique in that they are also capable of antibody production [26], an element critical to host defense [27]. Antibodies are produced by a specialized type of B cell, known as a plasma cell, which forms after a B cell encounters an antigen that is recognized by the B cell receptor (BCR) (27). Antibodies act through several mechanisms to protect the host from pathogens. These mechanisms include: neutralization of the harmful molecule, as is observed with toxins; coating of a pathogen to activate the compliment cascade, the activation of proteins that ultimately kill the target microorganism; promotion of opsonization, in which antibody coated microorganisms such as bacteria are more easily phagocytosed by phagocytes due to recognition of the Fc portion of an antibody by Fc receptors on phagocytes; and the induction of antibody dependent cell mediated cytotoxicity, in which Fc receptors on lytic cells recognize the Fc portion of an antibody that is bound to a target cell, resulting in degranulation by lytic cells and target cell death [27, 28].

Upon recognition and uptake of an antigen, endosomes within the cytoplasm of DCs and B cells fuse with lysosomes forming endolysosomes and resulting in antigen degradation [29]. Through a series of protein interactions within the endolysosome, degraded antigen in the form of polypeptides are eventually loaded onto either MHC-I or MHC-II molecules which are expressed on the cell surface [29, 30] and presented to naïve T cells [30]. A naïve T cell refers to a T cell that has not previously encountered antigen for which its T cell receptor (TCR) recognizes. The TCR is a structure composed of an α-chain and β-chain, and is found in a complex with the cluster designated (CD) 3 molecule. The TCR repertoire displayed by T cells is generated through genetic recombination that occurs during T cell development, and is regulated so that autoreactive T cells, or those with a TCR specific for self-peptides are eliminated during development [31]. Naïve T cells are produced in the thymus, after differentiation into T cell precursors in the BM, and recirculate through lymphoid and non-lymphoid tissues where they interact with APCs [32].

Upon presentation of antigen to a T cell with the cognate TCR specific for the polypeptide sequence on the corresponding MHC molecule, APCs provide secondary and tertiary signals which program T cells to have a specific response to the pathogen from which the antigen originated [33]. T cell responses have been studied extensively, and T cell subsets can be identified based on the cytokines that the cell is capable of producing as well as the T cell co-receptor expressed by that specific cell. T helper (Th) cells express the CD4 molecule, and are capable of producing a variety of cytokines which orchestrate the immune response [34]. The general paradigm is that depending on whether the immune response requires cell mediated immunity or humoral immunity Th cells will produce either Th1 or Th2 type cytokines, respectively. Th1 cells express the TF T-box transcription factor TBX21 (T-bet) and produce

cytokines including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-2 [34]. The other major Th cell subset is Th2 cells, these cells express trans-acting T-cell specific transcription factor GATA-3 (GATA-3) and produce IL-4, IL-5, IL-6, IL-10, and IL-13 [34]. Cytolytic T lymphocytes (CTLs) express the CD8 molecule, produce IFN-γ, and are capable of lysing target cells, such as infected cells or tumor cells [35]. Recent studies have identified novel T cell populations that are identified by their unique TF expression profile. For example, Foxp3 is expressed by anti-inflammatory T regulatory cells (Tregs), while retinoic orphan receptor-γ is expressed by pro-inflammatory Th17 cells [36, 37]. These cells not only have unique expression of TFs, but also have unique functional attributes, which are only now coming to the forefront in immunology.

1.2 IMMUNOMETABOLISM:

With the rising incidence of metabolic diseases such as type II diabetes and diet induced obesity, our need for understanding how alterations to metabolic signaling influences overall health and well being has dramatically increased. Among the physiological systems found to be impaired in those with metabolic abnormalities, there is significant evidence that immune function is compromised [38]. Innate and adaptive immune cells represent a dynamic population of cells that undergo homeostatic proliferation, have relatively short (7 days) or long (years) half-lives, and undergo rapid proliferation and migration upon activation [39]. This sort of plasticity has lead researchers to investigate the metabolic pathways that are involved in the dynamic responsiveness exhibited by these cell populations. Thus, immune cell metabolism or "immunometabolism" is an emerging field which is only recently becoming understood. Clearly, these cells require adenosine triphosphate (ATP) for survival and homeostatic

maintenance, but the biochemical pathways responsible for ATP generation in lymphoid and myeloid cells have generated significant interest [40].

In general, the immune system exists in a resting state, in which unactivated cells serve as sentinels, awaiting pathogenic insult. Upon activation, a variety of changes occur within these immune cell populations, directly related to changes in gene expression and metabolism [39, 41]. Rapid proliferation resulting in clonal expansion, as is exhibited by T cells after encountering their cognate antigen, requires upregulation of numerous genes related to changes in functional capacity as well as glucose and nutrient transport [39]. Rapid proliferation and the corresponding protein synthesis associated with proliferation requires a large amount of ATP, highlighting the essential role of metabolism during immune cell activation [39]. Thus, metabolic signals during immune activation largely influence the outcome of an immune response. In immune cells ATP generation occurs through several pathways. The primary mode of metabolism exhibited by unactivated or quiescent immune cells is glycolysis, during which glucose is broken down into pyruvate [39]. During this process ATP is created through donation of phosphate groups to ADP during the conversion of glucose to pyruvate. In a secondary metabolic step, pyruvate is shunted into the tricarboxyillic acid (TCA) cycle as aceytl-CoA and is used to produce ATP through oxidative phosphorylation. Interestingly, numerous studies have demonstrated that certain immune cells forgo oxidative phosphorylation and instead rely on aerobic glycolysis [39, 42], in which pyruvate in converted to lactic acid in the cytosol rather than being shunted to the TCA cycle in the mitochondria. Immune cells are also capable of metabolizing amino acids or fatty acids through aminolysis or β oxidation, respectively [39]. Perhaps unsurprisingly, different metabolic pathways are utilized by different immune cells in

different states of activation, and this can be largely influenced on the availability of oxygen and substrate [39, 40].

Neutrophils and macrophages are among the first cell types to respond to infection and inflammation, with large numbers of neutrophils being recruited to sites of infection. Migration of neutrophils has been found to be largely dependent on aerobic glycolysis or "Warburg metabolism" [43, 44], in which despite abundant oxygen, glucose is broken down into pyruvate in the cytoplasm and metabolic intermediates are used for the generation of ATP. During aerobic glycolysis, pyruvate is diverted into lactate production rather than acetyl Co-A for the TCA cycle, a metabolic process primarily observed in cancerous cells. It has been proposed that the reliance of neutrophils on this mode of metabolism reflects the need for effective neutrophil migration and function in tissues that may have become hypoxic due to inflammation [45]. The use of aerobic glycolysis to generate ATP has also been demonstrated in activated macrophages and activated DCs [46, 47]. As would be expected with the major metabolic pathway utilized by these cell types, neutrophils and activated DCs consume little oxygen and have few mitochondria [39, 45], thus limiting the use of the TCA cycle during periods of high metabolic demand.

Critical for the control of infections and cancers, T cells exist in numerous subsets with unique functional attributes. These different subsets exhibit reliance on different metabolic pathways, and to some degree, manipulation of T cell metabolism can influence the function and fate of T cells [48]. As mentioned previously, clonal expansion as is exhibited by activated T cells, results in metabolic changes in T cells, and the metabolism demonstrated by these rapidly proliferating cells is similar to what is observed in rapidly proliferating cancer cells (Warburg metabolism) [49, 50]. Upon activation and clonal expansion, $CD8^+$ effector cells, known as CTLs upregulate the glucose transporter GLUT1, as well as enzymes involved in the breakdown

of glucose for energy such as hexokinase 1, hexokinase 2, and phosphofructokinase [51]. Furthermore, the high amino acid demand placed on effector cells due to high biosynthetic demands, appears to be met by the upregulation of nutrient transporters such as the large neutral amino acid transporter CD98 and the transferrin receptor CD71 [52-54]. Following the resolution of an immune response, some T cells become long-lived memory cells, which can exist in the periphery for years and only require ATP to prevent autophagy and ensure survival [54]. In contrast to activated effector T cells which rely on Warburg metabolism, it has been proposed that memory T cells utilize β oxidation to metabolize fatty acids in the mitochondria via the TCA cycle for ATP production [55, 56]. Indeed, utilizing pharmacological inhibition of fatty acid oxidation inhibits the development of memory T cells, suggesting that the switch from metabolizing glucose to fatty acids is an essential part of memory T cell development [55, 56]. Thus, the switch from anabolic growth as is observed in effector T cells to catabolic processes relying on oxidative phosphorylation appears to be a critical step in the transition from an effector cell to a memory cell. In a similar fashion, anti-inflammatory Tregs have been shown to heavily rely on fatty acid oxidation for production of ATP [57], while Th17 cells, an inflammatory cell with an opposite immunological role to that of Tregs, utilize glycolysis [58]. Thus, while metabolic manipulation of immune cell function and differentiation is not common, manipulation of certain metabolic pathways may result in the resolution or enhancement of immune responses and have application in the clinic.

In order to develop strategies to manipulate immune cell metabolism, further research is required to elucidate the mechanisms by which metabolic pathways are regulated in immune cells. In general, immune cells utilize metabolic pathways observed in other cell types, however there exists some unique aspects of immune cell metabolism that are worth noting. Molecular

and biochemical studies have revealed the serine/theronine kinases Akt1-3, AMP activated kinase (AMPK), liver kinase B1 (LKB1), and mTOR not only serve as nutrient sensors in immune cells, but also regulate immunological fate and development [48, 59]. Cell signaling regulated by Akt, AMPK, and LKB1 have all been cited as playing a role in regulating the fate decision of $CD8^+$ T cells during the transition from effector cells into memory cells [52, 55]. For example, by regulating the expression of chemokine receptors and integrins, Akt signaling controls trafficking of $CD8^+$ T cells which ultimately influences the fate of these cells following activation [54, 60].

Long recognized as a critical regulator of cell proliferation and growth, the role of the serine/threonine kinase mTOR is now becoming increasingly appreciated in immune function. Pharmacological blockade of mTOR signaling through the use of rapamycin results in substantial changes to innate and adaptive immune cell function and development [59]. While studies support the notion that both DCs and B cells require mTOR signaling for normal function; the role of mTOR in regulating T cell development, homeostasis and fate is the best characterized of the immune cells. Generally associated with integrating extracellular cues and intracellular signals, mTOR gauges energy availability and in turn regulates the appropriate cellular response [61], however these signals are delivered through unique means in lymphocytes as evidenced by studies regarding mTOR signaling in T cells.

Classical environmental cues influence mTOR signaling in T cells such as intracellular signals related to energy status delivered through the AMPK-family of proteins including AMPK and LKB1 [48, 54]. Growth factors are known to activate mTOR in numerous cell types, thus it is unsurprising that growth factors have also been shown to mediate mTOR signaling in lymphocytes [59], however the specific growth factors responsible for mTOR activation in T

cells differ from classical mTOR agonists. For example, IL-2 is a major T cell stimulatory cytokine, and IL-2 signaling stimulates the mTOR pathway, promoting anabolic growth in effector T cells [62]. Similarly, IL-7, a cytokine critical to T cell survival also activates metabolic pathways, including mTOR in T cells [63, 64]. The mTOR pathway also negatively regulates the transition from anabolic metabolism to catabolic metabolism that is observed during the transition from effector $CD8^+$ T cells to memory $CD8^+$ T cells [54]. Constitutive mTOR activation inhibits the formation of memory T cells and inhibits β oxidation in T cells, while inactiving mTOR with rapamycin generates memory cells, despite anabolic growth factor signaling [55, 56, 62]. Interestingly, leptin, is an mTOR agonist in a variety of cell types [65], activates mTOR signaling in Tregs and in turn influence Treg function [66]. Neutralization of leptin signaling or deletion of the leptin receptor in Tregs reverses the anergic state typically displayed by Tregs, resulting in robust proliferation following TCR stimulation [67]. Furthermore, $CD4^+$ T cells, especially Tregs are capable of producing leptin, resulting in autocrine regulation during immune responses [66]. Other non-classical pathways of mTOR activation have also recently been highlighted in T cells. Stimulatory signals delivered from DCs are essential for T cell activation, and DC mediated activation of T cells during the initiation of an immune response requires mTOR signaling [68]. Signals received from DCs that activate mTOR include stimulatory signals delivered through TCR-MHC interactions [68], costimulatory signals delivered through ligation of CD28 [68], and signaling resulting from proinflammatory cytokines produced by DCs such as IL-4 and IL-12 [59, 69].

CHAPTER 2:

NATURAL KILLER CELLS

2.1 NATURAL KILLER CELL DEVELOPMENT AND MATURATION:

In 1975 scientists described a leukocyte in the blood, spleen, and BM of mice that was capable of eliciting background or 'natural' cytotoxicity against tumor cells [70-72]. This observation lead to the investigation of the ability of these cells to lyse target cells without prior antigen sensitization, and thus these cells were termed "natural killer" cells [71, 72]. The aforementioned studies set the foundation for the study of NK cells and the developmental and functional processes that control NK cell cytotoxicity. Mice lacking functional NK cells are highly susceptible to developing spontaneous tumors as well as display increased susceptibility to viral infections [73], thus NK cells play an important role in protecting the host from viral infections and cancers. Initially, NK cells were classified as innate immune cells due to their ability to become activated without the help of APCs and the fact that target recognition occurs on the cellular level [19, 24]. However, recent studies have identified novel functions of NK cells, resulting in confusion whether NK cells should be classified as innate or adaptive cells.

NK cells are lymphocytes, derived from a common lymphoid precursor during hematopoiesis in the BM. In mice, NK cells are classified by expression of the cell surface marker CD161c (NK1.1) or CD335 (NKp46) and the lack of expression of T and B cell antigen receptors, TCR and surface immunoglobulin (BCR), respectively [74]. In certain strains of mice such as BALB/c and 129Svj, NK cells do not express NK1.1 [75], thus NKp46 has been suggested to be a better marker for NK cells across different murine strains [75]. However, certain newly identified innate immune cells also express NKp46 and lack expression of CD3 or

the TCR [76]; classifying NK cells based on surface marker expression is difficult. Most studies focusing on NK cell biology utilize C57Bl/6 mice, and NK cells in this strain express NK1.1 and do not express CD3, TCRs, or BCRs [74], thus we have also utilized C56Bl/6 mice in our research.

In mice, NK cells are found throughout lymphoid and non-lymphoid tissues in varying frequency. Mature NK cells seed peripheral tissues such as the blood, lung, uterus and small intestine after development [9, 77, 78]. Immature NK cells (iNK) can be found in the spleen, liver, thymus, LN and BM; the BM contains a large reservoir of iNK cells as well as the earliest known NK cell precursors [8, 10], supporting the notion that the BM is the major site of NK cell development. This concept is further supported by the fact that destruction of the BM microenvironment with estradiol or BM seeking isotopes (89S) results in depletion or impairment of NK cells [79, 80].

Interactions with BM stromal cells as well as cytokines and growth factors within the BM shape the microenvironment that is critical for the development of fully functional NK cells [81]. In the earliest stage of development, NK cell precursors differentiate from common lymphoid precursors in a lymphotoxin-α dependent manner, such that lymphotoxin-α deficient mice lack NK and NK T cells [82]. Transgenic mice and i*n vitro* studies have given further insight into the required cytokines for NK cell development, elucidating a role for stem cell factor, IL-7, fms-related tyrosine kinase 3 ligand (Flt-3L), IL-15, leptin, and stromal derived factor 1α (SDF-1α) in NK cell development and homeostasis [83-85]. Culture of NK cell precursors with a cocktail of cytokines generates cells that functionally resemble NK cells, but lack certain receptors critical for NK cell function and recognition of self [74]. In mice, these self-receptors belong to the killer cell lectin-like receptor subfamily A (Ly49) family and are encoded by a family of genes in

the natural killer complex (NKC) on chromosome 6 in mice and humans [86]. In order for NK cells to acquire these critical receptors, contact with self-stromal cells within the BM microenvironment *in vivo* or the BM stromal cell line OP-9 *in vitro* is required [87]. Combining knowledge generated from transgenic mice and *in vitro* studies; it is now accepted that a combination of cytokine signals and cell:cell interactions are required for fully functional NK cells to develop.

The developmental process of NK cells has been conceptualized into 3 steps, with distinct stages existing within each individual step [88]. The first step is the differentiation of common lymphoid precursors into a restricted NK/NKT cell precursor in the BM (**Figure 1**). NK/NKT cell precursors express of the IL-2/IL-15Rβ (CD122), are responsive to IL-2 and IL-15, and can be found in either the BM or thymus of adult mice [85]. Depending on the environmental signals, upregulation of TFs specific for NK cell or NK-T cell lineage commitment takes place [89]. While the entire biochemical and molecular pathway for NK cell commitment and differentiation remains to be fully described, studies have shown that the TFs Ikaros, PU.1 and Id-2 are required for the development of NK cell precursors in the BM [89]. Following commitment to IL-15 responsive NK cell precursors, NK cells enter step 2, a stage described as immature NK (iNK) cell development. During early iNK cell development, NK cells in B6 mice acquire the prototypical NK cell marker NK1.1 [8]. Further phenotypic changes occur as iNK cell develop, including expression of Ly49s, CD314 (NKG2D), CD244 (2B4), CD16, and CD94, all encoded by genes within the NKC gene complex [90]. Cell surface receptors found on other lymphocytes such as CD117, CD127 (IL-7Rα), and CD360 (IL-21R) are also expressed at this stage of NK cell development [8].

After acquisition of markers expressed on iNK cells, NK cells remain in the BM to complete what is described as the classical NK cell maturation process [85], or iNK can egress from the BM and seed peripheral tissues to complete a somewhat less well understood non-classical maturation program in the liver, spleen, LNs, or thymus [85, 91]. The TFs interferon response factor (IRF) 2, Eomesodermin (Eomes), and GATA-3, control the upregulation of the maturation markers CD49b (DX5), CD11b, and CD43, and the downregulation of markers of iNK cells such as CD127, TRAIL, and CD51 during maturation in the BM (Figure 1)[89, 91]. The acquisition of DX5 marks the transition of an iNK to an early mNK cell [8], where at least some functional competence is also acquired [8, 92]. NK cell functional competence is defined as the capacity to produce IFN-γ in response to activating stimulus [93], although this is commonly assessed using IL-2 + IL-12 to elicit IFN-γ production [8]. There are a variety of NK cell functions that will be covered later in this manuscript, suffice to say, the various functional responses are largely influenced by the type of tissue that an NK cell completes its developmental processes within, as well as the cytokine environment in which NK cell activation takes place [85, 94].

In the BM, NK cells that enter the early mNK cell stage undergo rapid proliferation. Bromodeoxyuridine (BrdU), a nucleotide analog, can be detected after short term pulsation only within the subset of NK cells expressing DX5 and lacking markers of later maturation [8]. The stimulus for proliferation has yet to be fully described, however, interactions with stromal cells through the Ly49 family of receptors is thought to play an important role, as every $BrdU^{+}$ NK cell expresses at least one self-MHC specific Ly49 receptor [92], indicating these cells have recently proliferated. Furthermore, studies in BM chimeric mice have identified that extracellular interactions between Ly49s and self-MHC are required for NK cell proliferation in

the BM [81]. Finally, after a round of proliferation, NK cells begin step three of BM maturation and upregulate CD11b and CD43, the final markers typically acquired in the BM [8, 74]. NK cells then egress from the BM through BM sinusoids and into the blood where chemokines and cytokines guide NK cells to lymphoid and non-lymphoid tissues [84]. Egress is regulated by CXC chemokine receptors (CXCR) CXCR3 and CXCR4, and the sphingosine 1-phosphate receptor, S1P5 [95-97]; NK cells migrate down a chemokine gradient to their respective niches.

In the BM almost all NK cells express CD27, however in the periphery NK cells can be classified into functional subsets by defining NK cells based on expression of CD27 in conjunction with CD11b (**Figure 2**) [10]. Individual subsets have unique function, partially related to the mRNA expression patterns discovered through genomic analysis of fluorescent activated cell sorting (FACS) sorted cells [98]. For example, $CD27^{-}$ $CD11b^{+}$ cells are capable of the highest degree of cytotoxicity, largely because they express higher levels of perforin and granzyme mRNA [98]; thus these cells are more prepared to respond with immediate synthesis of the lytic molecules through which NK cells kill target cells. Another example of differential function related to respective NK cell subsets is that NK cells expressing both CD27 and CD11b have been demonstrated to respond more robustly to cytokines such as IL-12 and IL-18, and demonstrate the greatest degree of cytotoxicity against infected DCs [9, 10], perhaps due to reduced expression of inhibitory receptors [98]. Through a stepwise progression, NK cells in the per iphery mature from $CD27^{+}$ $CD11b^{-}$ to $CD27^{+}$ $CD11b^{+}$ and finally into $CD27^{-}$ $CD11b^{+}$ [10, 98]], with the latter pool containing the most mature, terminally differentiated NK cells that express the killer-immunoglobulin like receptor 1 (KLRG1) [99, 100]. Due to differences in chemokine receptor expression, NK cell subsets seed lymphoid and non-lymphoid tissues at different frequencies [10]. For example, the majority of NK cells found in the lungs and blood

are $CD27^{+}$ $CD11b^{+}$ or $CD27^{-}$ $CD11b^{+}$ NK cells [9, 10]. The spleen and liver on the other hand, contain a large portion of iNK and early mNK cells that express CD27 [9, 10]. The LNs also contain mostly $CD27^{+}$ NK cells which may or may not express CD127 (IL-7Rα) [10, 101].

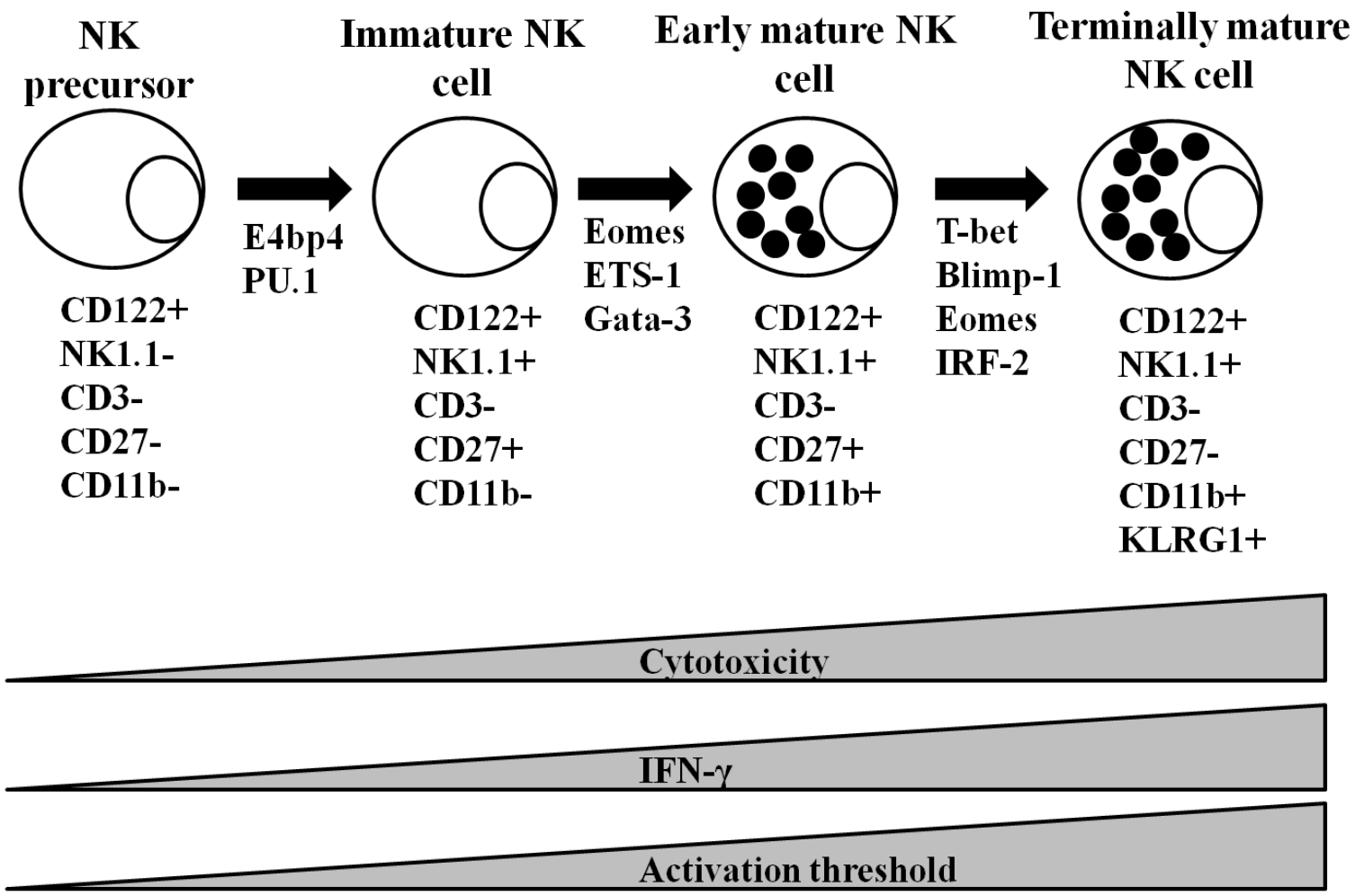

Figure 1: Flow cytometry can be used to characterize NK cells into maturational stages based on surface phenotype. The process by which NK cell maturation takes place is defined by the upregulation of cell surface markers and transcription factors [8].

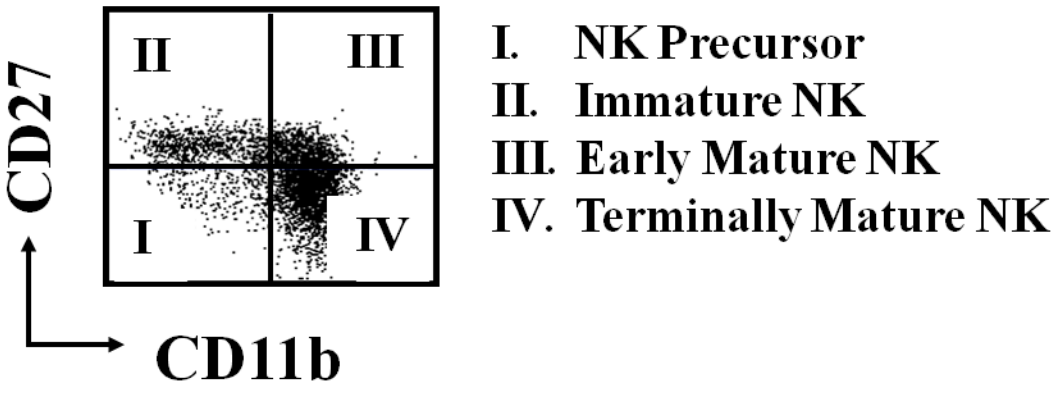

Figure 2: NK cells can be divided into functional subsets. Splenic NK cells can be divided into functional subsets using flow cytometry based on expression of CD27 and CD11b [9].

2.2 TRANSCRIPTIONAL REGULATION OF DEVELOPMENT:

Compared to T and B cells, the transcriptional regulation of NK cell development is poorly understood. Studies in transgenic mice have lead to the identification of numerous TFs that play a role in NK cell development; however the gene targets of these TFs and the time course of their expression remains to be established. Among the TFs known to be important to NK cell development, function and homeostasis are Ets-1, Pu.1, MEF, T-bet, IRF-2, GATA-3, nuclear factor interleukin 3 regulated (E4bp4), Id-2, Bcl11b, Blimp-1, Eomes, and Tox [87, 89]. The majority of transgenic models still contain mNK cells, although often at a reduced frequency, that may or may not exhibit impaired function depending on the stimulus used to interrogate NK cells. This observation highlights the multifactorial and overlapping role of TFs in NK cell development. Also, it should be noted that impaired NK cell function is highly specific to the type of NK cell function that is of interest or being studied. For example, T-bet is thought to regulate granzyme B and perforin production, but has little to do with IFN-γ production [102], thus T-bet deficient NK cells only exhibit one aspect of impaired function [103]. Furthermore, it is not always clear whether the defects in NK cell development are due to intrinsic defects in NK cells or due to extrinsic defects altering the microenvironment in which NK cells develop or become activated.

As most NK cells are thought to develop in the medullary cavity of the BM from BM precursor cells, studies have sought to identify the biochemical signature of developing NK cells in this compartment. Transcriptional regulation of the earliest stages of NK cell development is thought to be under the control of PU.1, E4bp4, and Id-2 (Figure 1) [89, 104]. SP1-/- mice lack the PU.1 protein and die before birth. However, studies harvesting hematopoietic cells from fetal liver of SP1-/- mice reveal that transplant of these cells into a wild-type host results in

significantly fewer NK cells generated from SP1-/- hematopoietic stem cells, suggesting a role for PU.1 in the development of NK cells [77, 105]. Interestingly, T and B cells cannot be generated from SP1-/- hematopoietic stem cells, thus leading to the conclusion that PU.1 is a protein required for the differentiation of common lymphoid precursors [77]. Similarly, MEF-/- mice display significantly fewer NK cells in peripheral tissues and these NK cells do not proliferate as efficiently, suggesting a role for MEF in the early proliferation of NK cell precursors [106], a step required to generate sufficient iNK cells. E4bp4 is a TF that is expressed at significantly higher levels in NK cells than in other lymphocytes [107, 108], leading to the hypothesis that E4bp4 is critical for NK cell development. Knockout of E4bp4 results in mice that have very few detectable NK cells in the periphery, and importantly in the BM [108]. NK cell precursors are found to be present at a normal frequency in the BM of E4bp4-/- mice, however mNK cells are almost entirely absent, while iNK cells are found at a significantly reduced frequency [108]. Furthermore, E4bp4 has been found to be downstream of IL-15 signaling, and because of the critical role of IL-15 in NK cell homeostasis, E4bp4 has been identified as a TF required for classical NK cell development and maturation [107]. Non-classical NK cells can also be found in the periphery; these NK cells express high levels of CD127, have low expression of CD11b, have limited cytotoxicity, but are robust producers of cytokines such as IFN-γ, granulocyte macrophage colony stimulating factor (GM-CSF), and TNF-α [101, 109]. The most commonly studied non-classical NK cells are also known as thymic NK cells, due to the fact that athymic (Foxn1-/-) mice have very few $CD127^{+}$ NK cells in the periphery [101]. Thymic NK cells express high levels of GATA-3 and GATA-3-/- mice have no detectable thymic NK cells, identifying a critical role for GATA-3 in the development of thymic NK cells [101].

Beyond commitment of CLPs into NK cell precursors, TFs have also been identified that play a role in the transcriptional regulation of NK cell maturation. Id-2 -/- mice have ten-fold less NK cells than their wildtype littermates, although iNK cell frequency is not influenced in the BM [110], suggesting Id-2 is a TF important for the early maturation of NK cells. T-bet has also been identified as being important for later stages of NK cell maturation that take place in the periphery as T-bet deficient (Tbx21-/-) mice lack expression of the TF T-bet and also have fewer mature NK cells in the periphery [103]. Soderquest et al. showed adoptive transfer of Tbx21-/- NK cells into a Tbx21 sufficient host results in peripheral maturation of NK cells so that they become terminally differentiated $CD27^{-}$ $CD11b^{+}$ NK cells (Figure 1-2) [111]. This observation was attributed to T-bet dependent monocyte *trans*-presentation of IL-15 on the IL-15Rα to NK cells [111]. However, in disagreement with this observation are data published by Gordon et al. in which experiments utilizing BM chimeras suggests cell intrinsic, rather than extrinsic, expression of T-bet is absolutely required for peripheral NK cell maturation [112]. Regardless, among the TFs known to be important for NK cell development, T-bet plays a critical role in the maturation of NK cells outside of the BM. Furthermore, T-bet has also been identified to regulate expression of S1P5, which is absolutely required for NK cells to egress from the BM [97, 113]. Thus, some TFs regulate multiple aspects of NK cell biology through regulation of maturation, trafficking or function. Blimp-1 is a TF that is found to be upregulated in senescent T-cells [114], and plays a role in the terminal maturation of NK cells in the spleen as NK cells from Blimp-1 deficient animals are arrested in stages of early maturation, possibly because Blimp-1 acts downstream of T-bet [114]. Similarly, IRF-2-/- or GATA-3-/- mice display normal NK cell numbers in the spleen and liver, but the maturation status of NK cells in these animals is reduced [115, 116].

2.3 NATURAL KILLER CELL FUNCTION:

Perhaps the most astounding aspect of NK cell biology is the rapid ability of NK cells to kill target cells without prior antigen sensitization, yet they display a high degree of self-tolerance [74, 91]. While T cell tolerance is achieved through selective processes during development, as well as tolerance induced by regulatory cells [31, 117], NK cell tolerance has only recently become understood. NK cells express a variety of cell surface receptors that result in activating or inhibitory signals and upon encountering a target cell NK cells receive both positive and negative signals through these receptors, ultimately resulting in intracellular signals that encourage or inhibit target lysis [92, 93]. However, NK cells are more than killer cells, they are also capable of robust cytokine and chemokine production, leading to the acceleration or resolution of an immune response [118].

Because NK cells can be found in virtually every tissue, their role in an immune response is quite complicated and highly tissue specific. For example, mucosal NK cells are the only NK cells capable of producing IL-22, an anti-inflammatory, pro-constructive cytokine [119], shown to promote healing of epithelial cells and tissue regrowth [120]. NK cells found in LNs are generally immature or thymic derived NK cells, capable of robust production of IFN-γ, TNF-α, and GM-CSF, cytokines associated with pro-inflammatory responses [101]. Despite their robust capacity to produce cytokines, NK cells in LNs express low levels of the antibody dependent cell mediated cytotoxicity receptor FcRγIII (CD16) and exhibit a low degree of cytotoxicity [101], thus their major role in immune responses is likely to shape the cytokine milieu within the LNs or other tissues into which they home. Interestingly, despite the recent advances in understanding the biology this subset of NK cells, little is known about the specific pathogens to which they respond, and their immunological relevance is only speculative. NK cells found in

the blood are generally mNK cells and display the highest degree of cytotoxicity but limited capacity to produce TNF-α and GM-CSF [9]. Not only do these mNK cells display the capacity to directly lyse target cells, but they are also capable of antibody dependent cell mediated cytotoxicity and redirected cell lysis, in which antibodies specific for NK cell activating receptors are bound to the Fc receptor of a target cell and activate NK cells through stimulation of NK cell activating receptors [121]. NK cells also have been shown to interact with DCs in inflamed tissues and in LNs [122], and these cells reciprocally activate each other, further highlighting the situational and multifaceted role of NK cells in immune surveillance and immune responses [122, 123]. The unique function associated with tissue specific NK cells supports the notion that NK cells are not a homogenous population of cells and that their function is highly dependent on the environment which NK cells become activated within.

As discussed earlier, NK cell function is related to the maturation status of NK cells, likely due to the transcription of mRNA in various mNK cell subsets as well as expression of specific TFs known to play a role in production of cytokines and cytolytic molecules [87]. However, *natural killing,* the process by which NK cells become activated to produce cytokines and granules of cytolytic molecules are exocytosed (degranulation), is regulated by opposing signals received through activating and inhibitory receptors [124]. In mice, self tolerance is achieved by NK cells through the critical signals received by an NK cell resulting from the interaction between inhibitory Ly49 receptors and self-MHC-I [124]. This acts as a steady state inhibitory signal that activating signals must overcome before NK cell function can be elicited against a target cell (123). Lack of MHC-I on a target cell results in limited inhibitory signals in NK cells and NK cells generally react by degranulating and producing cytokines [124, 125]; the ability of NK cells to recognize and rapidly kill self-cells lacking MHC-I has been termed as the

"missing self" hypothesis [92]. Indeed, some viruses and tumor cells downregulate MHC-I in order to escape $CD8^+$ T cell mediated immunity [126], however NK cells are believed to have evolved in parallel with this escape mechanisms in order to provide the host with protection [127]. It is also by this mechanism that NK cells recognize the T cell lymphoma, YAC-1 cells, a commonly used target cell [92].

In general, two major structural classes of activating and inhibitory receptors are expressed on the NK cell surface; immunoglobulin (Ig)-like and lectin-like receptors [128]. In mice, a large portion of activating and inhibitory receptors are lectin-like receptors and belong to the Ly49 family of receptors [129]. Ig-like receptors are integral membrane bound proteins, most commonly found on human NK cells and belong to the killer Ig-like receptor (KIR) family [125]. Despite little structural homology between the Ly49 family receptors and KIRs, there exists a great deal of similarity in the function of these receptors. The cytoplasmic tail of inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMS) that recruit SRC homology 2–domain-containing protein tyrosine phosphatases such as SRC homology-domain containing phosphates 1 and 2, while the cytoplasmic tail of activating Ly49s or KIRs associate with DAP12 or DAP10 kinases through immunoreceptor tyrosine-based activating motifs (ITAMS) [128]. Upon ligation of activating or inhibitory receptors, signaling takes place resulting in the recruitment and phosphorylation or dephosphorylation of ITIMs and ITAMS, thus regulating phosphorylation of downstream signaling molecules and ultimately resulting in activation or inhibition of NK cells [128]. Thus, through a balance achieved by phosphatase and kinase activity on ITIMs and ITAMs, NK cell activation or inhibition prevails based on the phosphorylation state of downstream signaling molecules.

The ITIM containing inhibitory receptors Ly49C and Ly49I have been demonstrated to directly recognize self-MHC-I in C57Bl/6 mice [92, 124], an observation resulting in the proposal that self tolerance may be achieved in NK cells through the delivery of a strong inhibitory signal [92, 124]. However, a major limitation to our understanding of the self-tolerance demonstrated by NK cells is the fact that mice or humans deficient in β2 microglobulin (β2m) or transporter associated protein which lack functional MHC-I, do not display signs of autoimmunity [128]. In fact, NK cells from β2m-/- mice are hyporesponsive when stimulated through major activating receptors such as NK1.1 or Ly49D [92]. This has lead to the proposal that NK cells are "licensed" or "educated" during development in the BM through interactions between inhibitory receptors and self-MHC-I [124]. In C57Bl/6 mice stimulation of NK cells with anti-NK1.1 results in IFN-γ production [92]; however almost all of the NK cells capable of producing IFN-γ after NK1.1 stimulation express Ly49C/I, thus Ly49C/I^{+} NK cells represent the "licensed" NK cell pool in C57Bl/6 mice [92]. Adoptive transfer studies, in which hyporesponsive BM derived NK cells from β2m-/- mice were transferred into a wild type host demonstrated that normal NK cell function is restored upon introduction of NK cells into a MHC-I sufficient environment [130]. Thus, a hypothesis has emerged proposing self-MHC-I interacts with inhibitory receptors on developing NK cells during maturation, resulting in the production of functionally mNK cells. Furthermore, NK cells that express receptors specific for self-MHC molecules preferentially proliferate during development in the BM [131], suggesting signaling through an inhibitory receptor promotes proliferation of iNK cells, a step critical for NK cell maturation [8]. However it should be noted, a small subset of NK cells develop in mice that do not express any inhibitory receptors specific for self MHC-I molecules yet display normal function [132], highlighting the limited understand we currently have of this process. There

have since been numerous hypotheses as to how NK cell function is governed during the development and attempts to relate this to maturational status in the BM, however no single theory has prevailed and more work in this field remains to be done. Nonetheless, achieving self tolerance is essential as NK cells must be able to elicit maximal effector function upon encountering a target cell, yet display tightly controlled cytotoxicity due to the fact that they do not need prior antigen sensitization before killing.

Despite the lack of structural homology between human and murine activating and inhibitory receptors, the shared function of these receptors emphasizes the critical role of these receptors as this is likely an evolutionarily conserved mechanism by which NK cell function is regulated [127]. There have been at least 20 activating and inhibitory receptors described in both mice and humans, yet a single NK cell will randomly express between three and five of these receptors [128], resulting in a pool of NK cells with a high degree of heterogeneity. The functional implications of this heterogeneity is perhaps best demonstrated by the fact that "licensed" NK cells represent the more responsive NK cells *in vitro* [92], yet "unlicensed" NK cells (NK cells lacking inhibitory receptors specific for self MHC) are activated and provide protection during the immune response to murine cytomegalovirus (MCMV) [133, 134]. This phenomenon was at least partially attributed to the fact that "licensed" NK cells receive a high degree of inhibitory signals during *in vivo* immune responses, while "unlicensed" NK cells that receive pro-inflammatory cytokine signals become rapidly activated and elicit effector responses [133].

2.4 ANTI-VIRAL PROPERTIES OF NATURAL KILLER CELLS:

While NK cell "licensing" is attributed to interactions between self-MHC molecules and inhibitory receptors [128], NK cell reactivity during *in vivo* immune responses is influenced by a combination of cytokine induced activation and ligation of activating and inhibitory receptors [130]. As mentioned earlier, NK cells play a major role in the immune response to MCMV, and the degree of detail unto which the NK cell response to MCMV has been characterized is quite extensive and serves as an excellent example of the multifaceted mechanisms by which NK cells respond during *in vivo* responses (**Figure 3**). The requirement for NK cells in protection from MCMV has been demonstrated by numerous laboratories and is highlighted by the fact that NK cells express Ly49H [135], an activating receptor specific for the MCMV encoded protein m157 which is displayed on MHC molecules on the surface of MCMV infected cells [135]. During the NK cell response to MCMV, $Ly49H^+$ NK cells rapidly proliferate, increasing in number by 5-10 fold over the course of 5-7 days as is demonstrated by BrdU incorporation [136]. Specific and non-specific activation of NK cells occurs during this time period, DCs and macrophages produce TNF-α, IL-12 and IL-18, cytokines which stimulate and activate both $Ly49H^+$ and $Ly49H^-$ NK cells and promotes NK cell maturation [133, 137]. NK cells activated during the immune response to MCMV display a mature phenotype, express CD69, produce IFN-γ, and undergo terminal maturation resulting in upregulation of KLRG1 and downregulation of CD27 [138, 139]. NK cells responding to MCMV can be "licensed" NK cells that also express Ly-49H or "unlicensed" NK cells that express Ly-49H and receive strong proinflammatory stimulus [133]. Thus, through specific and non-specific activation of "licensed" and "unlicensed" NK cells during the NK cell response to MCMV, a heterogeneous pool of NK cells are activated to

provide protection at various time points during infection [133]. Upon the resolution of the immune response to MCMV, the pool of NK cells expressing Ly-49H contracts due to apoptosis of activated NK cells, similar to the contraction of antigen specific $CD8^+$ T cells [140]. Interestingly, upon rechallenge with MCMV there appears to be a more rapid and robust reactivation and proliferation of Ly-49H expressing NK cells, suggesting NK cells may have memory like characteristics [141]. Further exploration of this observation revealed memory NK cells are mature, long lived, self renewing cells, capable of secondary and tertiary expansion upon rechallenge with MCMV that likely reside in the BM [141, 142]. Memory NK cells express high levels of lymphocyte antigen complex (Ly) 6C, KLRG1, CD94, CD69, CD159a (NKG2A), and NKp46, although the phenotype of these cells appears to depend on the pathway by which NK cell memory has been induced and differs between humans and mice [141, 143]. These cells have been shown to develop in a DC and macrophage derived, IL-12 dependent fashion during the primary immune response [138]. In humans infected with HCMV, a similar population of NK cells have been described that express high levels of CD159c, an activating receptor found on some human NK cells. The generation of memory NK cells has also been described in mice following immunization with chemical haptens [144]. These NK cells reside in the liver and express the chemokine receptor CXCR6, and importantly, provide memory-like immunity against influenza virus and have also been shown to mediate contact hypersensitivity [145]. Our understanding of memory NK cells is still in its infancy, however observations of this nature emphasize the heterogeneous nature of NK cell responses as well as the difficult task of classifying NK cells as innate or adaptive immune cells.

There are few identified viral ligands of NK cell activating receptors. However, NKp46, a major activating receptor expressed on all murine NK cells and a large portion of human NK

cells, directly recognizes viral HA from influenza A virus [146], and some suggest NKp44 may also recognize influenza HA of some subtypes [147]. The recognition of viral HA has been narrowed down to 3 branched O-glycan sequences on the NKp46 glycosylation site, although this may vary based on the influenza A subtype being studied [146, 148, 149]. The role of NK cells during influenza virus infection is somewhat controversial. However, recent evidence suggests an important role for NK cells in providing resistance against influenza virus. Mice or humans with reduced NK cell numbers at baseline have been shown to be more susceptible to influenza virus [4, 150]. For example, aged or CR mice have significantly fewer lung NK cells and both have been shown to exhibit increased weight loss and mortality following influenza virus infection [11, 151]. Similarly, depletion of NK cells prior to influenza infection with antibodies results in increased weight loss, increased viral titers and greater mortality [152]. Replacement of NCR1, the gene that encodes for NKp46, with a gene encoding for a green fluorescent protein results in a non-functional NKp46 receptor and results in increased susceptibility to influenza virus [149]. Mice with genetic mutations resulting in NK cells incapable of producing cytolytic molecules such as perforin and granzyme are also more susceptible to influenza [153], while mice with genetic mutations resulting in hyper-reactive NK cells are more resistant to infection with influenza virus [154]. These studies demonstrate the requirement of NK cells in providing protection from influenza virus, through cytolytic function NK cells kill infected cells, limiting virus replication (Figure 3). Recent studies also suggest a role for NK cells in providing resolution from the inflammatory response seen during influenza. IL-22 is a newly described cytokine shown to promote the regeneration of tracheal and bronchial epithelial cells post influenza infection [119] (Figure 3), and its major source towards during the resolution phase of the immune response to influenza infection are mNK cells. Mice deficient in

IL-22 production fail to recover from influenza and continue to exhibit weight loss 10 days p.i. [119]. Thus, NK cells provide protection from influenza virus early in the immune response by limiting viral titers and provide pro-recovery signals to host epithelial cells through production of cytokines.

NK cells are one of the largest lymphocyte populations in the lung [155], an observation that supports the hypothesis that they are important cells in providing innate protection from respiratory infections, especially viral infection [12, 155, 156]. However, because NK cells represent a large portion of leukocytes and thus the immune defense in the lung, it has been proposed that influenza virus has evolved mechanisms to specifically evade NK cells [157]. As replication of influenza virus begins within hours after infection, one theory is that NK cell numbers in the lung are too low to completely control influenza virus early in infection [157]. This is supported by the observation that there is not dramatic trafficking of NK cells to the lung until 3-5 days p.i. [151, 158]; thus NK cell pool in the lungs may not expand at a fast enough rate to control the rapidly replicating influenza virus. Similarly, lung NK cells do not proliferate well in response to influenza infection evidenced by limited BrdU incorporation [158], a dramatic difference between influenza infection and MCMV infection. Further studies have demonstrated that influenza virus has evolved a host of mechanisms by which it limits or evades NK cell mediated killing of infected cells. Mutation of HA and neuraminidase on a seasonal basis is a strategy used by influenza in order to avoid antibody neutralization, forcing yearly vaccination strategies. However, it was not until recently that it was accepted that mutations to HA also affect NK cell recognition of HA through NKp46 [159]. Overexposure of NK cells to HA has also been reported to inhibit NK cell function, high levels of replicating influenza may overwhelm NK cells, resulting in feedback inhibition or upregulation of suppressor of cytokine

signaling proteins [160]. Several studies have illustrated that both murine and human NK cells can be infected by influenza virus *in vitro* and *in vivo*, possibly because they express salicylic acid receptors [161, 162], resulting in diminished NK cell cytotoxicity against targets lacking self-MHC molecules [157]. This impairment in NK cell cytotoxicity was achieved without directly influencing the NK cell receptor repertoire, suggesting influenza inhibits NK cell cytotoxicity by altering signaling cascades within NK cells [157]. Indeed, it was later shown that infection of NK cells with influenza virus results in the downregulation of the CD3ζ chain, a signaling molecule required for NKp46 to properly activate NK cells through phosphorylation of Syk and ERK [162]. Furthermore, production of IFN-γ by NK cells that are infected with influenza is impaired when induced through ligation of major activating receptors such as NKp46, NKG2D, NK1.1 and Ly49D [163]. Interestingly, this occurred despite the fact that there was not a change in the expression of these receptors, supporting the hypothesis that infection of NK cells by influenza virus alters signaling intracellular cascades [157]. NK cell expression of inhibitory receptors is not reported to be changed during influenza infection, however, influenza reorganizes the plasma membrane of host cells resulting in changes to cell surface lipid microdomains [164]. Overall, this has been proposed to result in the enhancement of interactions between self-MHC molecules and inhibitory receptors on NK cells, suppressing NK cell reactivity to influenza infected cells [157]. Thus, evidence supports the concept that NK cells play a role in providing immunity to influenza virus infection; the virus has also evolved compensatory mechanisms by which NK cell mediated killing of virally infected cells is diminished or thwarted.

2.5 ACTIVATION OF NATURAL KILLER CELLS:

Activation of NK cells occurs through multiple mechanisms during influenza infection. *In vitro* stimulation of splenocytes with influenza virus results in production of IFN-γ and granzyme B within 8-12 hours by NK cells [165]. In a similar fashion, human NK cells recognize influenza infected DCs in a NKG2D and NKp46 dependent fashion and become activated by both type I IFNs (IFN-α/β) and IL-12 produced by DCs during *in vitro* co-culture [156]. *In vivo,* NK cell cytoxicity is increased during influenza infection and peaks between 2 and 3 days p.i. [11, 153]. Influenza induced NK cell cytotoxicity is attributed to cytokines produced by DCs and macrophages as well as epithelial cells [157]. IFN-α/β is probably the best studied of the cytokines known to promote influenza induced NK cell cytotoxicity. Importantly, knockout of IFN-α/β receptor results in no detectable IFN-γ or granzyme B production by NK cells [165]. Type I IFNs act through direct activation of NK cells by type I IFNs as adoptive transfer of NK cells that lack the receptor required for IFN-α/β signaling (IFNR-/-) into a wild-type influenza infected host results in limited activation of IFNR-/- NK cells [165]. Furthermore, direct activation of NK cells during influenza infection is thought to require signaling through janus activated kinase (JAK) / signal transducers and activators of transcription (STAT) pathways as IFN-α/β results in phosphorylation of both STAT1 and STAT4 in NK cells [165]. IL-12 and IL-18 have been shown to play a critical role in dictating the NK cell response to other viral infections [138, 153], yet mice lacking expression of IL-12 or IL-18 receptors displayed enhanced NK cell activation following influenza infection and unimpaired function [165], thus there appears to be some conflicting data regarding the role of IL-12 and IL-18 in activating NK cells during influenza infection.

DCs precursors are one of the first immune cells recruited to sites of inflammation, after which DC precursors mature into immature classical DCs (iDCs) which are highly skilled at antigen uptake and are stimulated to become mature classical DCs (mDCs) [32]. DC maturation results in significant changes to DC morphology and cell surface marker expression, allowing for phenotypic characterization of iDCs and mDCs by FACS [166]. Furthermore, these changes also significantly influence the function of DCs as mDCs have reduced capacity for antigen uptake, but express high levels of costimulatory molecules and are more efficient cytokine and chemokine producers [32]. Typical convention is that as classical DCs mature, they upregulate C-C chemokine receptor 7, a chemokine receptor that allows them to traffic to LNs where they can activate naïve T cells in order to initiate an adaptive immune response (32). However, it has also been observed that NK cells and classical DCs interact in LNs and inflamed tissues (93), which has potential implications for an immune response.

Prior to the initiation of an adaptive immune response, crosstalk between NK cells and DCs has been demonstrated to take place in both humans and mice [167, 168]. Plasmacytoid DCs and classical DCs recruit NK cells to sites of inflammation through production of type I IFN and chemokines following activation of TLRs [169]. Encountering activated immature DCs (iDCs) in inflamed tissues results in NK cell mediated lysis of these iDCs (Figure 3) [170, 171]. During the period of time that NK cells are recruited to sites of inflammation, iDCs mature into mDCs, which also results in the upregulation of MHC-I molecules which results in limited killing of mDCs by NK cells due to increased inhibitory signals delivered to NK cells [122]. Furthermore, during this time peroid NK cells and mDCs reciprocally activate each other through production of IFN-γ + TNF-α and IL-12 + IL-18 (Figure 3), respectively [169]. DCs can also activate NK cells in a contact dependent manner by *trans*-presentation of IL-15 [172]. By

"editing" the DC pool to remove infected iDCs, NK cells select for mDCs to coordinate the immune response, and further potentiate the activity of mDCs with IFN-γ and TNF-α [173, 174].

NK cell mediated cytolysis of iDCs in inflamed tissues results in the selection of mDCs to traffic to lymphoid tissues to stimulate T cells (93, 172). However, NK cells can also rendezvous with activated mDCs in lymphoid tissues and orchestrate T cell responses [175, 176]. Because T cells are polarized towards Th1 responses primarily by IFN-γ and IL-12 [177, 178], crosstalk between activated DCs and NK cells that result in enhanced production of IFN-γ by NK cells is thought to directly stimulate Th1 immunity (Figure 3) [122, 179]. Interestingly, the NK cells that reside in LNs display a unique dependence on the thymic microenvironment for development, are capable of robust cytokine production, and display limited cytotolytic capacity [101]. Because of this observation, it is thought little DC editing takes place in LNs and that most NK:DC crosstalk that takes place in these tissues is occurs via cytokines [19, 180]. NK cell derived IFN-γ stimulates DCs to produce IL-12, while NK cell derived TNF-α increases maturation and thus expression of costimulatory molecules on DCs [122, 181, 182]. The increased expression of costimulatory molecules and increased secretion of IL-12 by DCs which, combined with NK cell derived IFN-γ, heavily favors the development of Th1 type immunity. Thus, during infections that require CTLs, macrophage activation, and control of intracellular pathogens, NK cells participate by producing IFN-γ and TNF-α to assist with adaptive immunity, and also kill infected cells directly through release of granzyme and perforin.

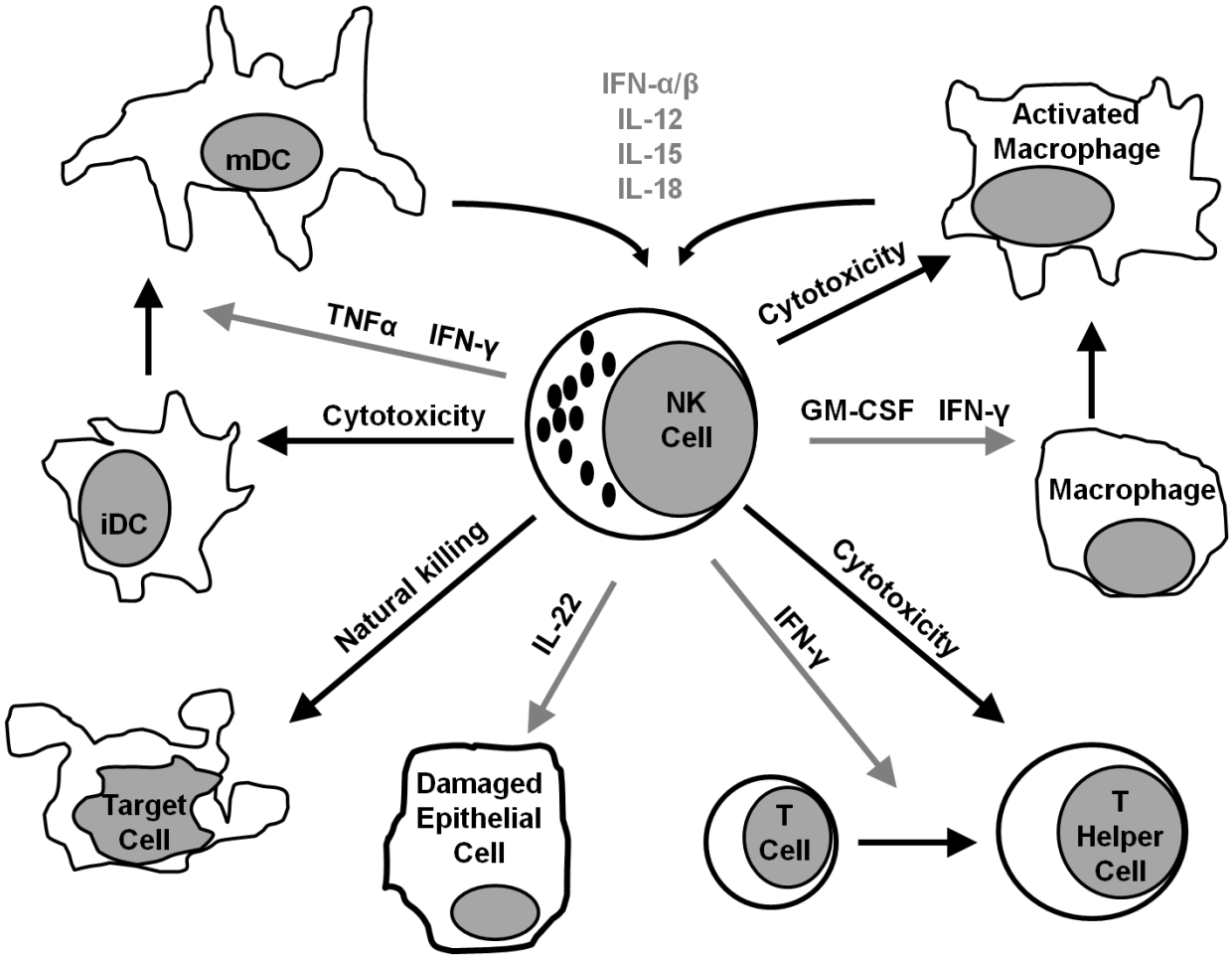

Figure 3: Multifaceted role of NK cells in an immune response. NK cells exhibit a multitude of functional responses that help shape the proper immune response. Upon activation NK cells can directly kill target cells (natural killing) or can produce cytokines to activate other cell types such as T cells, iDCs and macrophages. If necessary NK cells can exhibit cytotoxicity against self cells such as iDCs, T helper cells, and activated macrophages in order to properly regulate immune responses. Cytokines produced by mDCs and activated macrophages stimulate NK cells enhancing NK cell cytotoxicity and cytokine production. NK cells can also produce the pro-regenerative cytokine, IL-22 to help promote healing of damaged epithelial cells. Adapted from [122].

2.6 CLINICAL SIGNIFICANCE OF NATURAL KILLER CELLS:

The *natural killing* exhibited by NK cells has lead to a great deal of interest in harnessing NK cells for pre-clinical and clinic applications for populations at high risk of infection or developing cancer. In humans, NK cell activity has been identified as an important predictor of mortality in aged populations; among numerous factors examined, only low NK cell activity was identified as a predictor of risk of infection and short survival time during infection [183]. Our laboratory has demonstrated reduced NK cell maturation and numbers in aged mice, suggesting aging impairs NK cell function [151]. However, data suggests healthy aging occurs in individuals who maintain high NK cell cytotoxicity despite increasing age [184], as centenarians often have high NK cell activity and numbers [185]. Thus, methods to increase NK cell function and numbers may provide life-extending and anti-aging effects in humans.

NK cells make up approximately 30% of leukocytes in circulation in humans, and also make up a large portion of the lymphocytes found in lymphoid and non-lymphoid tissues [186]. Because NK cells also posses the ability to respond to antibodies through Fc receptor mediated activation [187, 188], there exists the potential to utilize NK cell mediated killing by antibody dependent cell mediated cytotoxicity in the clinic. Furthermore, NK cell immunotherapies have very few reported adverse side effects [189, 190], therefore therapies utilizing NK cells are highly desirable if found to be effective. Because the natural target of NK cells are malignant or transformed self-cells, most pre-clinical and clinical studies have focused on utilizing this aspect of NK cell biology to benefit cancer patients [190, 191]. A wide range of cancers such as carcinomas, melanomas, and leukemias have all been treated using NK cells from analogous and autologous sources [190]. Perhaps some of the most promising results are demonstrated by clinical trials in which patients with acute myeloid leukemia who have received NK cell

immunotherapy [192-194]. In one clinical study, pediatric patients with acute myeloid leukemia who received an NK cell transplant in combination with chemotherapeutics had a 2 year improvement in their survival rate [195]. It bears mentioning that in treatment of leukemia's donor derived NK cells are often purposefully KIR mismatched, that is to say, the KIR expressed by the donor NK cells do not recognize host MHC-I molecules. While this has the potential to increase the severity and incidence graft versus host disease [196], it also allows for enhanced reactivity of NK cells against tumors, as host MHC-I molecules do not provide inhibitory signals to donor derived NK cells [197]. Despite the fact that in some studies there are no demonstrative changes in tumor size or metastasis [198], there is a consistent increase in NK cell activity in patients receiving NK cell immunotherapy, which may have other immunological benefits for the patient.

2.7 DIET AND NATURAL KILLER CELLS:

The significance of maintaining a diet sufficient in vitamins, minerals, and protein has been appreciated as being essential for maintaining optimal immune function. Studies detailing the immunosuppressive effects of starvation, micronutrient deficiency, and excess energy intake indicate metabolism is intricately linked to immunity [199]. Leptin, a hormone that shares significant homology with cytokines, is produced by adipocytes in response to energy availability and has been suggested to play a major role in coordinating the allocation of physiological resources with energy availability [200, 201]. In this fashion leptin acts on immune cells by signaling through the long-form of the leptin receptor often referred to as LepR or ObR in the literature (199). Mice that are genetically deficient in leptin (*ob/ob*), or its receptor (*db/db*), are obese and present with significant alterations to multiple physiological systems,

including the immune system [202], are more susceptible to a variety of pathogens [203], and have a shortened lifespan [204]. Interestingly, while diet-induced obesity results in hyperleptinemia, there appears to be limited feedback inhibition regarding the influence of leptin on appetite [205], leading to the hypothesis that leptin insensitivity develops during diet-induced obesity, which may result in similar immune defects as is observed in *ob/ob* mice. Thus, dietary changes resulting in sub-optimal macronutrient intake has the ability to significantly alter the production of systemic hormones that directly influence immune cell function or development. Indeed, Lord et al. were among the first to correlate the immunosuppressive effects of starvation with the hypoleptinemia that accompanies restricted caloric intake [206]. T cells, B cells, and macrophages have all been described as expressing the leptin receptor and capable of responding to leptin stimulation [66, 207-210]. Similarly, NK cells express the leptin receptor and leptin alters NK cell homeostasis and function [211, 212]. Thus, states of excessive or restricted energy intake may to influence NK cell homeostasis or function.

Among the various immunological impairments found in *ob/ob* or *db/db* mice, NK cells are found at a reduced frequency in peripheral tissue such as liver, blood and spleen of *db/db* mice [211, 213]. Furthermore, NK cells from these mice display impaired cytotoxicity and although the exact mechanism has not been established, culture of a human NK cell line with leptin increases NK cell cytotoxicity in a dose dependent fashion [212]. In 2009, Lo et al. demonstrated that leptin plays a critical role in protecting NK cells from apoptosis during development in the BM, resulting in fewer mature NK cells being produced by the BM of *db/db* mice (212). Further analysis revealed NK cells from the BM of *db/db* mice expressed lower levels of the anti-apoptotic protein Bcl-2 and higher levels of the pro-apoptotic protein Bax [213]. As circulating leptin levels are directly related to the amount of body fat found on an

animal, we hypothesized CR mice would have a similar impairment in NK cell maturation as is observed in *db/db* mice. Indeed, data presented later in this dissertation confirms this hypothesis. Furthermore, obesity, a state of excess energy intake which results in impaired leptin signaling [201, 204], has been documented as resulting in altered NK cell function and homeostasis [214]. These data indicate leptin and energy intake play an essential role in supporting normal NK cell development and function. Interestingly, studies have shown that leptin is capable of enacting its immunoregulatory function directly through ligand-receptor interactions on the cell surface [210] and indirectly by regulating hormone production through the central nervous system [215]. While *in vitro* data suggest leptin has a direct role in regulating NK cell function and survival, it remains unclear whether other impairments found in leptin deficient animals are regulated by direct or indirect leptin action.

Numerous other studies have reported other dietary factors shown to influence NK cell behavior. NK cell frequency can be increased in mesenteric LNs by supplementation of SMAD3-/- mice with galacto-oligosaccharides [216], while in humans *Lactobacillus casei Shirota* supplementation enhanced NK cell activity [217, 218]. Similarly, *Lactococcus lactis subsp. cremoris* supplementation enhances IL-12 and IL-18 production by macrophages and DCs, which in turn increases IFN-γ production by NK cells [219]. In 2012 You et al. demonstrated that several strains of *bifidobacterium* also significantly increase IFN-γ production by human NK cells, although this observation depended on the age of the participants [220]. The immunosuppressive effect of ethanol consumption is well documented and NK cells are known to be negatively influence by chronic ethanol consumption in both humans and mice (220). In mice, chronic ethanol consumption depletes NK cells in the periphery and BM, while thymic NK cell development is relatively unimpaired [221]. Furthermore, chronic ethanol consumption

impairs NK cell function in the face of MCMV, likely due to poor lytic capacity of hepatic NK cells [222]. Elegant studies have recently demonstrated a role for vitamin D in the development and maturation of NK T cells in mice [223], and similar studies have shown that knockout of a gene that encodes for vitamin D3 upregulated protein results in significantly reduced NK cells [224], thus vitamin D appears to play a role in NK cell homeostasis. It should be noted that both these studies used transgenic mice lacking specific response elements that are acted upon by vitamin D and do not necessarily recapitulate *in vivo* vitamin and mineral deficiencies as they actually occur. Interestingly, there is at least one report that excessive vitamin D exerts a suppressive effect on NK cells. *In vitro* treatment of NK cells with vitamin D3 suppressed NK cell cytotoxicity and inhibited NK cell proliferation in response to mitogens in a dose-dependent fashion [225]. On the other hand, high dose supplementation with vitamin E increases NK cell cytotoxicity in patients with pre-existing conditions such as cancer [226]. Furthermore, maintaining adequate micronutrient status positively correlates with maintaining NK cell cytotoxicity into advanced aged [227]. Numerous other dietary factors such as black and brown rice bran, breast vs. formula feeding, fucocidan administration and active hexose correlated compound supplementation positively impact NK cell function, however detailed mechanistic explanations for these observations are lacking [228-231]. Certain other systemic and hormonal factors such as prostaglandins, and hormones such as epinephrine, norepinephrine, corticosterone, insulin, and growth hormone have also been shown to influence both lymphocyte and myelocyte function [210, 232-235], although whether NK cells homeostasis is influenced by these factors remains less well known [236], although corticosterone does appear to suppress NK cell function, there is little data regarding the mechanism by which this is achieved [237].

CHAPTER 3:

CALORIC RESTRICTION

3.1 BACKGROUND:

Among dietary interventions that have been shown to impact immune cell development and function, CR is among the best characterized. Initially identified because as a pro-longevity dietary intervention; CR increases the lifespan of multiple species of laboratory animals [238, 239] and studies have been funded by the National Institute on Aging (NIA), designed to address whether CR is feasible in humans [240]. Referred to as the "frugal phenotype," physiological changes that occur as a result of CR are the result of an organism attempting to allocate the available resources to essential physiological processes [241]. This results in numerous changes to multiple physiological systems, including: hormonal changes, changes to core body temperature, changes to the resting metabolic rate of the organism, and other musculoskeletal and metabolic adaptations [239, 242]. Experimental models of neurodegenerative diseases and autoimmune diseases indicate disease parameters are improved by CR irrespective of aging [243, 244], while impairments in renal function, cardiac function, immune function that occur during the aging process have all been shown to be improved by CR [245, 246]. Aged rodents on a CR diet have reduced incidence of spontaneous tumors [247], and CR slows the proliferation of tumor cells [248], suggesting CR may be used as an anti-cancer treatment.

Because of the numerous applications of CR for the treatment of various pathological conditions along with its ability to slow the aging process, CR is used in many different laboratory settings to examine disease outcomes. One of the hallmarks of a properly implemented CR protocol is the occurrence of food restriction without resulting in micronutrient deficiencies [249]. However, a recent meta-analysis revealed that approximately 60% of CR

protocols do not specify the use of vitamin and mineral supplementation [249]. Furthermore, the degree of restriction varies widely between studies (8-60%), with 40% restriction being the most common, as does the age of CR onset [249]. As early as the 1920's CR protocols were designed to incorporate micronutrient supplementation. Indeed, McCay and colleagues, cited as the first to report the pro-longevity effects of CR, were aware that micronutrient deficiencies could accompany food restriction studies and therefore supplemented rats in their studies with cod liver oil and yeast [3]. Years later in the 1980s, Weindruch and Walford developed diets supplemented with vitamins and minerals in order to study the influence of CR on mitochondrial function and immune function independent of micronutrient deficiencies [250-252]. Thus, an integral part of the CR regime is the prevention of malnutrition despite reduced caloric intake. In the 1990s the National Institute of Health (NIH) moved to limit discrepancies between studies due to different dietary protocol and eventually designed the American Institute of Nutrition (AIN)-93 maintenance diet, and the AIN-93 growth diet, which are designed for adult or growing and/or lactating rodents, respectively [249]. The AIN-maintenance diet, while containing vitamins and minerals, provides numerous micronutrients in quantities that are significantly below what is recommended by the Nutrition Research Council (NRC) for rats or mice [249]. For example, vitamin B12 and vitamin K levels are below the NRC recommendations for *ad libitum* (AL) rats and mice [249]. Other micronutrients, such as vitamin B6, calcium, manganese, iron, molybdenum, and selenium, are at the lower limit of what is recommended by the NRC [249]. Thus, when these diets are used in CR protocols and chow intake is severely restricted, there is an increased risk of vitamin or mineral malnutrition. Because of the need for supplementation of CR diets as well as the fact that AIN diets do not contain the recommended amounts of micronutrients, the NIH has since released a specific formula to be used in CR

protocols, known as NIH-31, which is supplemented to contain enhanced levels of the micronutrients mentioned previously and is endorsed by the NIA. Although data regarding the influence of consuming AIN-93 diets on longevity is lacking, there is at least one study published in 2002 which found rats consuming a restricted AIN-93 diet did not live as long as rats consuming a restricted NIH-31 diet [253]. The inadequacy of the AIN-93 diet is also evidenced by studies during which Swiss mice consuming AIN-93 at a 40% restricted level were found to have a lower body mass than mice consuming an isocaloric CR diet supplemented with vitamins and minerals [249]. Furthermore, upon supplementation of vitamins and minerals to the diets of mice that were previously consuming AIN-93, weight loss was attenuated, despite no change in caloric intake [249]. Similar studies in rats have shown that rats consuming a diet supplemented with vitamins and minerals during restriction compared to rats consuming unsupplemented diets results in a significantly greater body weight in supplemented rats, despite the fact both groups consumed isocaloric diets [249], highlighting the difference between supplemented and unsupplemented diets for use during CR protocols.

Several of the advantageous biological adaptations that occur as a result of CR are similarly influenced by every other day feeding, also known as intermittent fasting (IF) [254, 255]. IF is a restriction protocol in which the subjects are fed AL every other day, and then either fasted, or fed very few calories the day after AL feeding. However, thus far IF has mostly been used in short-term restriction studies that are not designed to determine whether IF can influence maximal and median lifespan [249]. Of the two studies designed to determine whether long-term IF could influence lifespan, there are controversial results. In 1990, Goodrich et al. found IF to result in a similarly metabolic phenotype to CR mice and to increase maximal lifespan of congenic mice. However, in 2008 it was reported by a different group that IF had no

influence on any aspect of lifespan, despite resulting in activation of numerous metabolic pathways observed during CR [256]. Also, to the best of our knowledge, no IF study published to date has supplemented the subjects with vitamins and minerals; AL chow is offered to the animals on the feeding day [249]. It should also be noted that in studies assessing the influence of IF on food intake and body weight, rats that were on a IF protocol consumed a comparable quantity of food as AL animals, although they presented with a lower body weight, suggesting a decrease in metabolic efficiency [249, 254, 257]. Despite this fact, IF animals still weighed more than CR animals, emphasizing the difference between IF and CR [249]. Furthermore, IF does not seem to result in the changes in body composition that are observed with CR [254]. IF is a relatively new dietary intervention, and while there have been promising results regarding the metabolic effects of IF, yet there are still many unanswered questions regarding whether IF protocols are comparable to CR protocols. Specifically it remains to be determined whether IF has the same beneficial effects as CR on different disease states as well as aging and the prevention of the age associated decline in immune function. However, it should be noted that the implementation of IF in a human population may be more practical than CR.

Currently there are two independent reports regarding whether CR extends lifespan in non-human primates [258, 259]. However, despite this disagreement, studies examining whether CR is efficacious in the human population have already begun at under the guidance of the NIA [240]. This project, known as the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) is a large multicenter trial being conducted in the United States [240]. The goal of this project is to achieve 25% restriction in a non-obese population, and to determine if similar metabolic and cardiovascular benefits that have been observed in laboratory animals on a CR diet can be replicated in free living human beings [240]. Adherence is

monitored using a double labeled water technique and the primary outcomes being measured are core temperature and resting metabolic rate [240]. The secondary outcomes measured include: immune function, cardiovascular risk markers, insulin sensitivity and secretion, neuroendocrine function, quality of life, cognitive function and oxidative damage [240]. There are several theories as to the efficacy of CR in humans. Preliminary results suggest that successfully reaching the degree of restriction that is outlined in the study design is very difficult, as CR participants appear to be reaching between 12-18% restriction after 6 months [260]. Because of the wide breadth of organisms that respond favorably to the CR paradigm, some suggest that there is a good chance that humans will also respond with favorable adaptations that will translate into improved longevity [261]. Furthermore, several small independent trials are reporting positive findings regarding the efficacy of CR in humans [262, 263], supporting the notion that more CR research is required in humans. With the rapidly rising rate of obesity in Western countries [264], it has also been suggested that if modest CR can be achieved in the CALERIE study than it will simply limit the incidence of obesity-related disease and mortality.

3.2 CALORIC RESTRICTION AND IMMUNOSENESCENCE:

The decline in immune function that occurs with aging, also known as immunosenescence, has been characterized as influencing numerous immune cell populations. This results in a decline in the ability of the elderly to fight infections such as influenza and pneumonia [265]. Indeed, over 80% of influenza related mortalities are in people age 65 or greater [266]. Rates of autoimmunity are also higher in the elderly, suggesting the ability of the immune system to separate self and non-self becomes dysregulated during the aging process [267]. Age related changes have been reported in NK cell and neutrophil number, and some

studies have found changes to DC function, although this remains somewhat controversial [268]. Perhaps the most significant changes to the immune system that occur during the aging process are alterations to adaptive lymphocytes [269]. The thymus is the major site of T-cell development, and a significant body of literature has shown that thymic involution accompanies aging. This results in diminished production of naïve T cells, and poor selection against autoreactive T cell clones [267, 270]. The other major site of adaptive immune cell development, the BM, exhibits a loss in hematopoietic volume during the aging process, which results in reduced production of B cells in some studies [271].

In addition to the impaired ability of aged mice or humans to produce T cells and B cells at rates comparable to younger mice or humans, aging has also been shown to influence the function of adaptive lymphocytes. For example, antibodies produced by B cells from elderly humans have reduced affinity for antigens, likely due to the inability of B cells in aged individuals to class switch from immunoglobulin (Ig) M producing B cells into IgG producing B cells [271]. Furthermore, B cells from aged individuals express fewer costimulatory molecules, resulting in poor stimulation by follicular DCs, and impairing humoral immune responses [271, 272].

A major contributor to impaired T cell function is aged humans is the accumulation of senescent memory cells and the loss of naïve lymphocytes that occurs with aging [273]. Production of naïve cells decreases due to thymic involution [274], and the remaining naïve cells display abnormal proliferative responses that accelerate their differentiation into memory cells [275]. The loss of a diverse TCR repertoire has numerous implications for the ability of $CD4^+$ and $CD8^+$ T cells to respond to new pathogens and this is exacerbated by other functional impairments also arise in T cells during the aging process [275]. There are numerous studies

citing the fact that at the cellular level, signal transduction is impaired in T cells from aged rodents [276]. For example, stimulatory and costimulatory signaling though the TCR and CD28 is less robust in T cells from aged mice, suggesting a direct impairment in the intracellular signaling cascade [276]. Naïve T cells from aged rodents also exhibit impaired proliferative responses when compared to naïve T cells from young rodents [277] as well as impaired functional responses. Studies performed as early as 1973 showed that CR improves T cell proliferation in aged mice [278], this concept is well documented in the literature though it is often attributed to the fact that CR results in less senescent T cells in the periphery [279, 280].

The combination of thymic involution and the reduction in hematopoietic volume in the BM, results in fewer antigen inexperienced or naïve T and B cells [7]. The overall implication of fewer naïve lymphocytes in an aged organism results in a limited antigen repertoire available to respond to infections not recognized by memory cells. As memory cells accumulate they outcompete naïve T and B cells for immunological space and growth factors, further limiting the antigen repertoire [281, 282]. In this respect, the consumption of a CR diet during the aging process has beneficial effects; thymic involution is inhibited by CR and the T cell antigen repertoire increased [274]. It remains unknown whether the decline in B cell immunosenescence is capable of being altered by a CR regime. However, CR results in fewer peripheral B cells [283], and is beneficial in ameliorating autoimmune diseases in which B cell autoaggression is part of the etiology [2, 284]. These observations suggest CR may also regulate some aspects of B cell biology, yet further studies are required to confirm or refute this hypothesis.

3.3 BIOLOGICAL MECHANISMS OF CALORIC RESTRICTION:

There are numerous hypotheses regarding the mechanisms by which CR delays aging and extends lifespan in rodents. CR increases secondary metabolism in the liver; upregulation of genes encoding for cytochrome p450 enzymes likely results in more rapid detoxification of carcinogens that may be used to induce cancer in experimental settings [285]. Others studies have found CR reduces the mutation rate of rapidly proliferating somatic cells [286], a mechanism by which the progression of some cancers are suggested to be limited by a CR diet [239]. Furthermore, production of pro-inflammatory cytokines and molecules, such as reactive oxygen species (ROS), leptin, IL-6 and TNF-α are reduced by a CR diet [239, 280, 287], which may limit the total inflammatory damage inflicted upon on organism over time. In fact, the reduced mutation rates resulting from CR that is observed in somatic cells has been indirectly related to reduced oxidative damage to DNA in several studies [286, 288]. Obesity is also associated with increased cancer risk [289], and CR has been clearly demonstrated to reduce the incidence of obesity as well as result in a reduced body fat percentage [288, 290]. Adiponectin, an adipokine which is thought to play a role in inhibiting tumorigenesis [291, 292], is increased in some studies of CR, suggesting another possible mechanism by which cancer risk is reduced by CR [239]. Furthermore, as leptin has been suggested to be pro-carcinogenic [293], reduced circulating leptin levels resulting to altered body composition mediated by CR has lead to theory that reduced circulating leptin levels also contribute to the reduced incidence of cancer in CR studies [294]. Another mechanism by which CR has been hypothesized to reduce cancer incidence is that CR mice have reduced endogenous glucose production [295]. Because cancer cell proliferation is known to require large amounts of glucose [296], reducing exogenous glucose intake and endogenous glucose production via CR may also result in fewer blood

glucose spikes with lower amplitude, lowering the total glucose available to tumor cells on a daily basis [239, 295]. Insulin and insulin-like growth factor 1 are highly anabolic hormones that are produced in response to raised blood glucose [297], as is often observed in the postprandial state. In turn insulin and insulin-like growth factor 1 act as a growth factors for normal cells as well as cancer cells [297, 298]. Thus, tightly regulated blood glucose levels as is observed in mice on a CR regime, has the potential to reduce cancer cell growth and proliferation [239, 296]. Because adiponectin is a major determinant of insulin sensitivity and glucose utilization [299], it has been conjectured that adiponectin is indirectly involved the anti-cancer effect of a CR diet [239]. Many of the potential mechanisms by which CR reduces cancer incidence are interlinked; likely it is a combination of these mechanisms that results in an overall multifactorial risk reduction.

Examination of the CR phenotype and microenvironment has yielded many hypotheses on the mechanism(s) by which CR increases lifespan and reduces cancer risk. Many of these hypotheses converge on intracellular signaling pathways directly related to integrating energy availability signals at the cellular and molecular level. The AMPK pathway is a pathway regulated by the intracellular ratio of AMP:ATP, acting as a direct energy sensor, AMPK controls many processes that are related to tumor development [300]. Processes required for rapidly proliferating cells such as the progression through the cell cycle, protein synthesis, and pro-survival signals are all partially dependent on AMPK activity [300]. Furthermore, AMPK signaling, growth factor signaling, and cytokine and adipokine signaling converge on a common signaling pathway which regulates cell survival and growth in relation to intracellular and extracellular signals [239]. Downstream of this signaling pathway is mTOR and p53, proteins that ultimately control cell fate [301, 302]. The TF p53 is a tumor suppressor, intimately

involved in the aging process as well as cell-cycle control, DNA repair, apoptosis and cellular stress responses [302]. Inhibition of p53 activity is thought to be directly result in tumor progression, and thus this TF serves as a target for cancer therapy [302]. Activation of p53 has been observed in CR mice, however, mice with homozygous knock-out of genes encoding for p53 still show a benefit of being on a CR diet with respect to tumor burden [303], suggesting CR induced activation of p53 alone cannot explain the anti-cancer effects of CR [239]. The serine threonine kinase mTOR serves as a master coordinator of nutrient availability signals at a cellular level [301]. Activation of mTOR results in robust proliferation and protein synthesis, while inhibition of mTOR results in cellular quiescence [301]; mTOR gauges extracellular energy levels and coordinates the appropriate cellular response. Rapamycin, a CR mimetic, directly inhibits the activity of mTOR and reduces tumor burden in rodents and also extend lifespan [304, 305]. Studies using rapamycin to inhibit the mTOR pathway have linked mTOR inhibition with reduced cancer incidence in rodents [305]. CR results in chronically low levels of growth factors, adipokines, and limited energy reserves, and suppressed signaling by through pathways results in decreased phosphorylation of activating motifs on mTOR as well as its downstream targets such as ribosomal S6 kinase [306, 307]. Under normal homeostatic conditions mTOR activation is an essential step in cellular growth, however in cancer cells overly active mTOR signaling has been attributed to enhancing tumor proliferation and growth [307]. Thus, by limiting mTOR activation through changes in energy availability, CR has been proposed to suppress mTOR related tumor growth.

While tumor immunology is still in its infancy, it is well accepted that immune cells constantly survey and kill malignant cells and neoplasms [308]. Two of the main cells required for this function are CTLs and NK cells [308]. Cancer incidence increases and immune function

decreases as an organism ages [265], leading to the proposal that therapies that limit immunosenescence have the potential to prevent or slow cancer progression in aged organisms [7]. CR has been clearly demonstrated to increase the frequency of CTL precursors known as $CD8^+$ T lymphocytes [279, 283]. Furthermore, CR has also been proposed to have differential effects on NK cell biology [5, 309]. Although a subject a controversy, NK cell function likely decreases with age [310], leading to the investigation of whether CR is capable of limiting the decline in NK cell function during the aging process. However, results are mixed, and generally point to limited efficacy of the ability of CR to improve NK cell function. Weindruch et al. found that aged CR mice had reduced basal NK cell cytotoxicity compared to aged AL matched controls, although this defect could be overcome by stimulation with polyinosinic:polycytidylic acid [5]. We have recently reported that young adult mice on a CR diet have reduced NK cell frequency in peripheral tissues and that NK cells found in these peripheral tissues have a less mature phenotype than those found in tissues of their AL littermates [309]. These less mature NK cells had reduced capacity to produce IFN-γ and granzyme B compared to NK cells from AL controls [309]. However, NK cells from CR mice exhibited increased reactivity to tumor cells during *ex vivo* co-culture studies, suggesting that there may be both negative and beneficial effects of CR on NK cell function. Overall, CR may have beneficial effects on tumor immunity, however further studies are warranted to discern whether the anti-cancer effects of CR are due to the direct action of CR on tumors, whether this is mediated through the immune system, or both.

3.4 CALORIC RESTRICTION AND SUSCEPTIBILITY TO PATHOGENS:

The interrelationship between diet and immune function has been a long time focus of both nutritional scientist and immunologists. Insufficient intake of protein has detrimental

immunological consequences. Inadequate or excess intake of micronutrients has also been recognized as having negative consequences on the immune system [311, 312]. However, as discussed previously, CR exists independent from malnutrition and therefore any negative or positive effects of CR should be directly or indirectly related to changes induced by reduced caloric intake (248). This brings up an interesting point in regards to the influence of CR on immune function; studies have clearly demonstrated that CR enhances the function of T cells in aged mice by inhibiting the age-related decline in T cell proliferative capacity and function as well as providing enhanced APC function in aged rodents [278, 313]. Based on these observations one would assume aged CR animals would be more resistant to infectious diseases when compared to aged AL mice. Indeed, studies by Effros et al. support this conclusion; following vaccination of aged AL and aged CR mice it was discovered that T cells from aged CR mice demonstrated increased antigen specific T cell proliferation upon restimulation of with virus *in vitro* [313]. Influenza specific antibody titers were also higher in aged CR animals than in aged AL mice [313], although it was not delineated if this was due to increased Th cell activity or increased B cell function. This study concluded that CR increased immunity to influenza virus in aged mice; however a major pitfall of the experiments used to come to this conclusion was that primary intranasal infection of aged CR mice was never tested. This major pitfall was addressed by studies from our laboratory in which Gardner clearly demonstrated that aged CR mice were more susceptible to primary, intranasal infection with influenza than aged AL mice [4].

Further study of the immunological consequences of CR on the immune response to influenza virus revealed aged CR mice succumbed to influenza infection at time points before an adaptive immune response could be mounted [4]. Furthermore, it was found that there was

reduced pulmonary NK cell function in aged CR mice following influenza infection when compared with influenza infected aged matched controls, suggesting CR results in impaired innate immunity. In 2008 Ritz et al. showed that the CR induced defect in innate immunity exists independent of age, as young CR mice were also more susceptible to influenza virus, demonstrated reduced pulmonary NK cell cytotoxicity during influenza infection, and had increased lung viral titers early during infection [11]. Although this challenged the paradigm that CR enhances immune function, several other studies have since reported increased susceptibility of CR mice to primary infections. It has since been shown mice on a CR diet are more susceptible to primary infection by several types of bacteria, parasitic worms, and viruses [314].

Unfortunately, the mechanisms by which CR mice are more susceptible to primary infections are still poorly understood. Perhaps the best characterized immunological defects reported in CR mice are our laboratory's studies which indicate NK cell dysfunction or deregulation plays a major role in the increased susceptibility to primary influenza infection. However, due to the nature of the immune response required to combat other infections which CR mice are more susceptible, it is unlikely NK cell dysfunction can completely explain the reasons by which CR mice are immunocompromised. For example, CR mice appear more susceptible to parasitic worm infection [315], which requires a Th2 response, largely dominated by $CD4^+$ T cells that produce IL-4, IL-5, IL-10 and IL-13 [34]. This in turn activates and recruits eosinophils and also induces B cells to produce antibodies [27, 34]. Very little is understood about the effect of CR on Th2 responses, or the influence of CR on the development of eosinophils and B cells, although CR does appear to have suppressive effects on B cell autoantibody production [6]. It was shown by Harrison et al. that CR initiated at weaning

maintained naïve $CD4^+$ and $CD8^+$ T cell numbers during the aging process [316], while others have shown beneficial effects of CR on $CD4^+$ and $CD8^+$ T cell proliferation and/or function [276, 278, 313, 317]. Thus, it is unlikely impaired $CD4^+$ T cell activity is at fault for increased susceptibility of CR mice to pathogens. Data presented in this dissertation further confirms that CR has detrimental effects on B cell development; however the relationship between impaired B cell lymphopoiesis and increased susceptibility to infection has not been explored in CR mice.

One of the major differences between studies indicating CR has beneficial effects on immune function and contradictory studies indicating CR results in increased susceptibility of organisms to pathogens lies within the methods used to assess immune function [199, 314]. For example, a study indicating CR has a beneficial effect on immunity to influenza virus relied on non-physiological measures of immune function; utilizing *in vivo* sensitization of cells to influenza by intraperotoneal injection with antigen, followed by isolation of splenocytes or cells from LNs, and restimulation with antigen [313]. Clearly, intraperotoneal sensitization with antigen is unlikely scenario outside of a laboratory setting; however, these data indicate CR may have beneficial effects on vaccination efficacy, a major problem in the elderly [7]. In the absence of *in vivo* sensitization, several studies have examined the influence of CR on *in vitro* measures of immune function, such as mitogen induced proliferation, or cytokine production following stimulation of T cells with anti-CD3/CD28 antibodies [6]. Furthermore, while CR is accepted to limit accumulation of senescent memory T cells and preserve the naïve $CD4^+$ and $CD8^+$ T cell pool [316], very little is known about how these cells respond to intact, unadultered pathogens, *in vivo*.

Currently, there are at least four independent reports of increased susceptibility of young adult CR mice to infection with intact pathogens [11, 315, 318, 319]. Using a model of polymicrobial peritonitis induced by cecal puncture ligation, Sun et al. showed CR mice were more susceptible to bacterial infection and the resulting sepsis [318]. This susceptibility existed despite *in vitro* and *ex vivo* data suggesting the opposite. For example, macrophages from young CR mice appeared less inflammatory, had reduced responsiveness to LPS and impaired ability to produce Th1 type cytokines *ex vivo* [318]. Expression of CD14, the major receptor for LPS, did not differ between AL and CR macrophages, yet macrophages from CR mice did not mature in response to LPS stimulation as robustly as macrophages from AL mice [318]. LPS is a major contributor to septic shock (intraperitoneal injection of mice with LPS can be used to model sepsis), it was thought that CR would protect mice from septic shock. However, *in vivo* CR mice suffering sepsis due to cecal puncture ligation were more likely to succumb to septic shock than young AL fed mice, and suffered significantly increased mortality [318]. The authors of this study discovered that despite suppressed macrophage function *in vitro,* cytokines commonly attributed to being produced by macrophages during sepsis were increased in CR mice [318]. Furthermore, when the capacity of macrophages to produce cytokines was assessed following the induction polymicrobial sepsis, it was found that Th1 cytokine production was significantly increased by macrophages from CR mice compared to macrophages from AL fed mice [318]. Clearly, there are dissimilarities in the results described in studies using intact organisms versus studies using molecular structures from these pathogens. Susceptibility to lethal bacterial infection was reported in other models of CR in which bacterial infection promotes disease. Utilizing *helicobacter hepaticus* infection to induce colitis, McCaskey et al. found mice on a CR diet had increased mortality as early as 1 week p.i. with *helicobacter hepaticus*, well before the

initiation of colitis [319]. While the immunological causes for this observation were not examined in detail, there are discrepancies between the influence of CR on immunological outcomes *in vitro* and *in vivo*.

CR mice also display increased susceptibility to infection with parasitic worms [315]. It was found CR mice displayed no difference in the number of eosinophils present to combat infection, and had higher levels of IgG1 antibodies [315]. Again, one would expect these immunological observations to support the notion that CR has positive effects on the immune response to infection, yet higher levels of IgG1 production were attributed to an increase in baseline IgG1 levels, and not thought to be a result of the immune response to infection. Nonetheless, the small intestines of CR mice contained a greater numbers of parasitic worms up to 30 days p.i., and parasitic worms displayed increased capacity to produce eggs, a major indicator of parasite health [315]. While the authors were unable to explain the reasons for increased susceptibility, they hypothesized reduced ROS production, a hallmark of CR [239], may limit the ability of immune cells in CR mice to fight pathogens. Indeed, parasitic worms produce antioxidants as part of their own defense mechanism [321], suggesting ROS production is a major component of host defense against parasitic worms.

It is also possible that CR mice simply lack the energy reserves to combat primary infections [320, 321]. Influenza induces an acute state of anorexia, in which severity of infection is directly related to the percent of body weight lost [322]. CR mice did not differ from AL mice in the percent of weight lost [11], however, it is entirely possible that they cannot afford to lose over 20% of their body weight in 3-4 day period, as is often observed in AL fed mice (11). Studies examining the relationship between body weight and influenza severity are limited. However, in our laboratory we refed (RF) young previously CR mice with an AL diet for 14

days until body weight and body composition were returned to levels comparable to AL fed mice. Following the refeeding period, RF mice were infected intranasally with 100 hemmagluttination units (HAU) of influenza virus and were found to have improved survival compared with their CR littermates [323]. RF mice also appeared to have an improved pulmonary immune response to infection (329), which will be discussed in more detail later in this dissertation.

CHAPTER 4:

SHORT-TERM REFEEDING OF PREVIOUSLY CALORIE RESTRICTED C57BL/6 MALE MICE RESTORES BODY WEIGHT AND BODY FAT AND ATTENUATES THE DECLINE IN NATURAL KILLER CELL FUNCTION AFTER PRIMARY INFLUENZA INFECTION

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4.1 ABSTRACT:

A hallmark of CR is a decrease in total body fat, which is thought to increase lifespan and maintain immune function. However, we have shown that during primary influenza infection, CR induces rapid weight loss, impairs natural killer (NK) cell function, and increases mortality in young and aged mice. To determine whether influenza-induced NK cell function could be restored in CR mice, young adult (6 mo) male C57Bl/6 mice were fed an CR diet or refed (RF) control diet ad-libitum for two weeks before infection with PR8 influenza A. An initial hyperphagic response was observed in RF mice, characterized by increased food intake, rapid weight gain, and restoration of body fat and fat depots by 5-7 days of refeeding to levels comparable to control ad-libitum (AL) mice. Refeeding improved survival and attenuated the decline in NK cell function during infection, evidenced by increased numbers, percentages, and

CD69 expression by d 3 post-infection in RF mice. Interestingly, an altered metabolic phenotype was observed during infection of RF mice, with plasma leptin concentrations increased compared with CR mice, but lower than AL mice. In contrast, adiponectin concentrations of RF mice were lower than those of both CR and AL mice. These data suggest that refeeding for a defined period before, and perhaps throughout, influenza season may provide the energy needed to counter the deleterious effects of CR on NK cell function, especially during exposure to newly emerging strains of influenza, to which vaccines are limited or unavailable.

4.2 INTRODUCTION:

There is a critical need to evaluate the primary response to influenza infection, especially to newly emerging strains of influenza to which we have had no prior exposure. The recent emergence of the novel swine 2009-H1N1 influenza virus poses a real threat to a large population under the age of 65. This influenza strain has the potential to infect 30-50% of the population, leading to as many as 1.8 million hospitalizations, and causing between 30,000-90,000 deaths. This is further complicated because the 2009-H1N1 vaccine supply has been either limited or unavailable, and for most of the population, vaccination is likely to occur after the peak of influenza infection. It takes several weeks to develop protective immunity in vaccinated individuals. This delay in adaptive immunity is likely to diminish the usefulness of the vaccine in protecting from influenza infection [324] and puts a greater reliance on having a functional primary response to combat this virus. There is additional reason for concern since both seasonal and H1N1 influenza strains are circulating simultaneously in the current 2009-10 influenza season. While ~90% of influenza-related deaths occur from seasonal influenza in people 65 years and older, mortality from H1N1 during the early 2009 outbreak was highest among people 25 to 49 years of age (39%), followed by people 50 to 64 year of age (25%) and people 5 to 24 year of age (16%) [324]. Therefore, a large percent of the population may be at risk for potentially contracting influenza from one or both strains.

Caloric restriction is a nutritional paradigm that has been widely used to study life extension in rodents. Several studies have shown that diets ranging from 30-70% CR increase median and maximal lifespan of rodents by up to approximately 65% and 50%, respectively, over those fed AL diets [3, 325]. Similar results have been obtained in multiple rodent species and strains, dogs, and non-mammalian species, such as fish and flies [239]. Longitudinal studies

of rhesus and squirrel monkeys fed a 30% CR diet suggest a comparable decrease in morbidity and mortality rates [258, 325], but there are very limited data regarding the effects of CR on maximal lifespan in non-human primates.

Energy restriction has been repeatedly shown to have positive effects on both non-specific and adaptive immunity in rodents [6]. It is now accepted that in addition to life extension, one of the hallmarks of CR is to delay the onset of age-related changes in immune function. Many reports have indicated that CR reduces the incidence of spontaneous tumors and cancers in aged rodents [247], maintains T cell proliferation, cytokine production, and cytotoxic T lymphocyte activity [6]. CR also enhances splenic lymphocyte proliferation and improve the antibody response after vaccination of mice with influenza [313]. However, our recent studies clearly indicate that young and aged mice fed an CR diet demonstrated decreased survival, delayed lung virus clearance, and markedly impaired NK cell function during an acute primary immune response to influenza infection [4, 11]. Therefore, the current study was designed to determine whether short-term refeeding of young adult CR mice with AL diet was beneficial in response to an acute infection with influenza virus. Such studies are clinically relevant to identify possible nutritional paradigms to increase caloric intake prior to and during influenza season, which occurs during the same defined time period each year. Further, increased caloric intake may be essential to support the primary response of susceptible populations to newly emerging strains of influenza, to which vaccines are limited or unavailable.

4.3 MATERIALS AND METHODS:

Animals and diets

Specific pathogen-free young adult (6 mo) AL and young adult (6 mo) CR male C57BL/6 mice were purchased from the NIA colony maintained by Charles River Laboratories (Wilmington, MA). The animal use protocol for this study was approved by Michigan State University Institutional Animal Care and Use Committee. Upon arrival, mice were housed individually in micro-isolator cages in the AAALAC-accredited containment facility at MSU and were acclimated at least 10-14 days prior to the initiation of each experiment. Both CR (NIH-31/NIA-fortified) and AL (NIH-31) diets were purchased from the NIA, the compositions of which have been reported in detail previously [11]. The composition of the CR diet is sufficient in micronutrients and minerals, but results in restriction of total energy intake by approximately 40%. The CR regimen is designed to gradually achieve 40% restriction in mice by 3 mo of age, such that they were weight stable upon arrival at 6 mo of age.

Feeding protocol

Briefly, on d -14, CR mice were randomly assigned to one of the following dietary groups: 1) CR group, which continued to consume CR diet or 2) re-fed group, in which CR mice were transferred onto NIH-31 diet, which they consumed freely. The third diet group (AL) was comprised of mice consuming NIH-31 diet upon arrival and throughout the duration of the study. The feeding protocol continued until from d -14 until d 0 upon which mice were anesthetized and infected with influenza virus.

Food intake, body weight and body composition

Food intake and body weight were monitored daily during the two week feeding protocol. All mice were weighed daily between 08:00-09:00, after which they were fed. Body composition was measured on d -14, -13,-11,-9,-7,-5, and 0. The protocol to assess body composition using the EchoMRI-500 (Echo Medical Systems, Houston, TX) has been validated and described in detail previously [326]. Briefly, after calibration using a rapeseed oil standard, individual mice are then placed in a holding tube to restrict movement. Body composition is determined within 30-45 s using standard programs validated for utilization in mice. The advantages of this system are that it is rapid, allows for repeated measurements, and does not require anesthesia, enabling mice to recover immediately after MRI.

Virus and infection

The method to isolate mouse-adapted influenza A/Puerto Rico/8/34 (H1N1, PR8) from specific pathogen-free eggs (B & E Eggs) has been described in detail [151]. For infection, mice were first anesthetized by intraperitoneal (i.p.) injection with Avertin (2,2,2-tribromoethanol, Sigma) and then infected i.n. with 10^4 x the 50% tissue culture infectious dose ($TCID_{50}$), calculated as 100 HAU [152], of PR8 in saline. Previous studies in our laboratory have routinely found that this dose of PR8 induces a measurable innate immune response with little to no mortality during d 3-4 p.i. Both nutritional and immune parameters were assessed d 0-3 p.i.

Lymphocyte isolation

The isolation of mononuclear cells from spleens and lungs has been described in detail [11]. Cell suspensions from spleens and collagenase-digested lungs were layered on Histopaque-1083 (Sigma) for density gradient centrifugation and were resuspended at appropriate concentrations to quantify NK and $CD8^+$ T cells by flow cytometry.

Flow cytometry

Cells from lung or spleen were resuspended in FACS buffer (0.1% sodium azide, 1% FBS, in PBS) containing various combinations of the following fluorochrome-conjugated antibodies (eBioscience or BD Bioscience) at concentrations ranging from 1:100 to 1:300: CD3 (Peridinin chlorophyll protein complex (PerCP)-Cy5.5), CD19 (PerCP-Cy5.5), CD8 (PerCP-Cy5.5), NK1.1 (R-phycoethyrin (PE)-Cy7 or Allophycocyanin), B220 (Allophycocyanin), Nkp46 (Alexa Flour647 or Fluorescein isothiocyanate (FITC)), and CD69 (FITC). Cells were incubated in staining cocktails on ice in the dark for 30 min. Samples were then acquired on a LSR II flow cytometer (Becton Dickinson) or a C6 flow cytometer (Accuri) and analyzed using FlowJo software (Tree Star).

Leptin and Adiponectin concentrations

Plasma concentrations of leptin and adiponectin were quantified by ELISA according to the manufacturer's instructions (R&D Systems; Minneapolis, MN). Plates were read at 450 nm

wavelength using a Synergy® HT plate reader (Bio-Tek; Winooski, VT) and concentrations were determined using a standard curve for each protein (11).

Fat pad weight

Fat pads were excised after 14 days refeeding and before challenge with influenza. Animals were anesthetized, followed by cervical dislocation. Inguinal and gonadal fat pads were immediately removed and were weighed immediately.

Quantifying adipocyte accumulation in BM

Femur and tibia from CR, RF and AL mice were harvested, fixed in 10% formalin, decalcified in 14% Ethylenediaminetetraacetic acid, soaked in a 1:1 solution of equal volumes of 2% aqueous osmium tetroxide (OsO_4) and 5% potassium dichromate to stain marrow-born adipocytes. The intact bones then were imaged at 6 μm resolution using micro-focus conebeam X-ray computed tomography (μCT40, Scanco Medical AG, Brüttisellen, Switzerland) at 55 kV and 145 μA, collecting 2000 projections per rotation at 300 msec integration time. Three-dimensional images were reconstructed using standard convolution and back-projection algorithms with Shepp and Logan filtering, and rendered within a 12.3 mm field of view at a discrete density of 4,629,630 voxels/mm^3 (isometric 6 μm voxels). The resulting images revealed high contrast 3D image arrays of adipocytes distributed throughout the fixed BM. Discrete adipocytes were segmented from background using a constrained Gaussian filter, summing total marrow fat volume for each sample.

Statistics

Statistical analyses were performed using Sigma Stat (Systat). Values in the text are means ± SEM. Survival data were analyzed using the Kaplan-Meier estimates with censoring. Log rank tests were used to determine statistical significance of survival curves among diet groups. Body composition, food intake and weight were analyzed using repeated measures ANOVA. Flow cytometric data and plasma adipokine concentrations were analyzed by 2-way ANOVA, with diet and time as main effects. Following 2-way ANOVA, differences in cell populations and adipokine concentration were determined using Student's t test for post-hoc analysis of differences between diet groups, and Tukey's test for post-hoc analysis of differences over time. Differences in adipocytes in BM were analyzed assessed using one-way ANOVA followed by unpaired two-tailed t-tests, corrected for multiple comparisons. Nonparametric data were analyzed using Kruskal Wallis test. Statistical significance was accepted at $P < 0.05$.

4.4 RESULTS:

Short-term feeding increases food intake and restores body weight and percentage of body fat in RF mice

Food intake and body weight of CR, RF and AL mice were monitored daily for two weeks prior to influenza infection. During d 1 of refeeding, RF mice initially demonstrated a hyperphagic response, during which food intake rapidly and markedly increased over that consumed at baseline (**Figure 4A**). By d 5 of refeeding, food intake steadily declined and was comparable to intakes of AL mice. Increased food intake was accompanied by a concomitant restoration of body weight (**Figure 4B**) and percentage of body fat (**Figure 4C**) in RF mice that were

comparable to those seen in AL mice by d 6 of refeeding. During the latter half of the refeeding protocol, food intake, body weight and body composition of RF mice were stable.

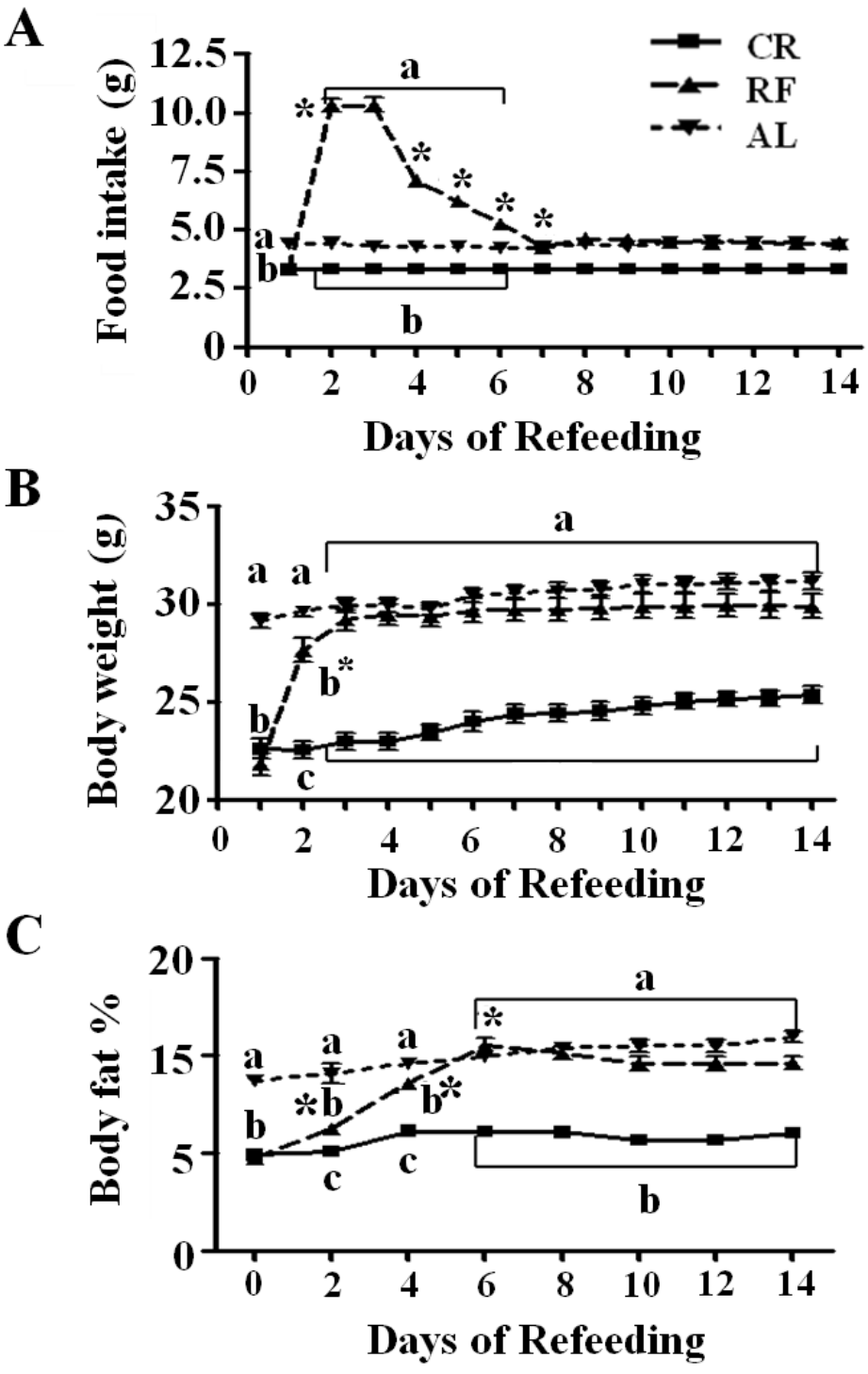

Figure 4: Refeeding of CR mice is characterized by a hyperphagic response. Food intake (A), body weight (B), and body fat percentages (C) of CR, RF and AL mice through 14 d of feeding.

(Figure 4 cont'd) Values are means ± SEM, n=12-15 mice per group. Means at a time without a common letter differ, P<0.001. * Different from the preceding time point, P<0.001.

Short-term refeeding increases inguinal and gonadal fat pad depots, but does not restore adipocyte accumulation in BM of RF mice

On d 0 of infection, we readily observed inguinal and gonadal fat pads in RF and AL, but not in CR mice. Both inguinal and gonadal fat pad weights were quantified indicating that fat weight was equivalent for RF and AL mice, but could not be detected in CR mice (**Table 1**). The restoration of fat depots of RF mice prompted us to assess BM-derived adipocyte volumes in femora and tibiae of CR, RF and AL mice on d 0 of infection (Table 1). Adipocyte volumes in both femurs and tibiae from AL mice were reduced compared with both CR and RF mice (Table 1). This indicates that RF had a phenotype more similar to CR rather than AL mice. These data suggest that refeeding favors restoration of body fat depots to meet increased energy storage demands prior to infection, but does not reduce the observed increase in BM adiposity of CR mice [327].

TABLE 1 Weights of inguinal and gonadal fat and adipocyte volume in bone marrow from femurs and tibias on d 0 of infection [1].

	Diet groups		
	CR	**RF**	**AL**
Fat pad weight, g			
Inguinal	**ND[b]**	**0.39 ± 0.07[a]**	**0.26 ± 0.04[a]**
Gonadal	**ND[b]**	**0.43 ± 0.03[a]**	**0.42 ± 0.01[a]**
Adipocyte volume, mm^3			
Femur	**1.13 ± 0.07[a]**	**1.10 ± 0.81[a]**	**0.001 ± 0.007[b]**
Tibia	**5.06 ± 1.37[a]**	**4.51 ± 1.09[a]**	**1.61 ± 0.006[b]**

[1] Values are means ± SEM, n=6 per group. Means without a common letter differ, $P < 0.01$. ND = Not detected

Short-term feeding improves survival of CR mice during influenza infection

After the two week feeding protocol, CR, RF and AL mice were infected with PR8 and survival was monitored through d 7 p.i. (**Figure 5)**. Sixty percent of CR mice did not survive infection through d 7, having a median survival of 5 days, which was significantly reduced compared with AL mice, all of which survived through d 7 p.i. In contrast, only 20% of RF mice succumbed to influenza infection by d 7 p.i. Importantly, the percent survival estimates of RF mice did not differ from AL mice through d 7 p.i.

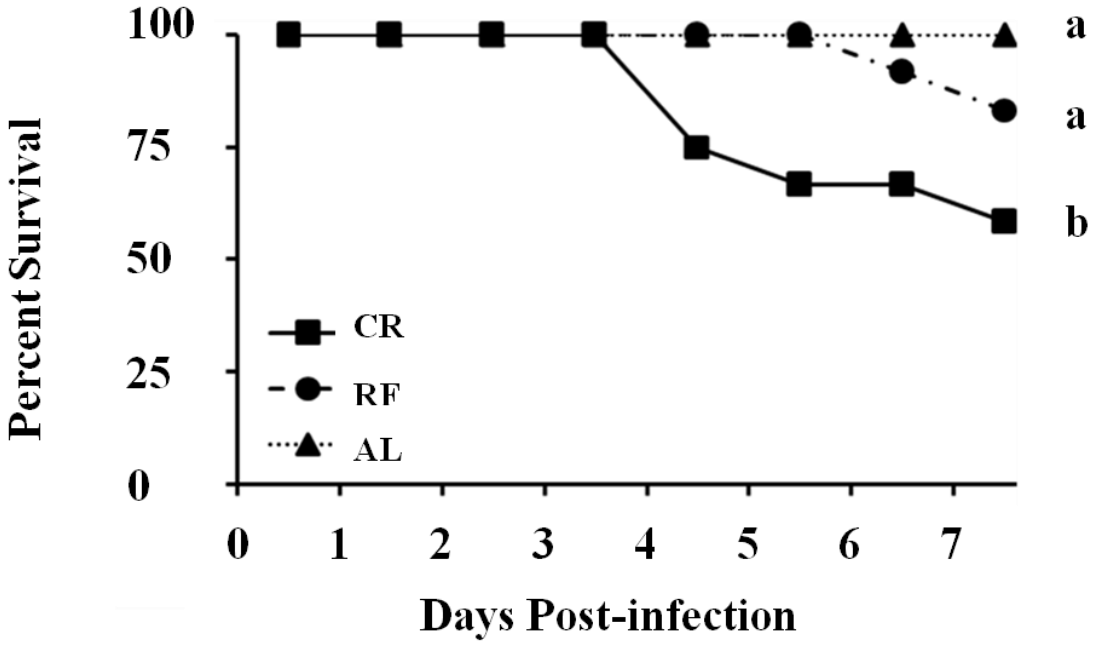

Figure 5: Survival of CR, RF and AL mice during infection with 100 HAU influenza. Percent survival during influenza infection of CR, RF and AL mice. Values are means ± SEM, n=12-15 mice per diet group. a, Survival of RF and AL mice was significantly different from CR, $P < 0.05$.

Short-term refeeding attenuates the decline in percentages and numbers of NK cells during influenza infection of previously CR mice

NK cell percentages (**Figure 6A**) and numbers (**Figure 6B**) were also quantitated in lungs of CR, RF and AL mice through d 3 of infection. At baseline, neither the percentages nor numbers of NK cells were significantly different among the three diet groups. However, the percentages of NK cells in lungs of RF mice were significantly higher than CR mice, but did not differ from, AL mice on d 3 p.i. While NK cell numbers were significantly lower in CR mice compared with AL and RF mice on d 2 and 3 of infection, these values did not differ between RF and CR mice.

Caloric restriction alters the kinetics, percentages and numbers of NK cells expressing the activation marker, CD69

The surface marker, CD69 was used to assess early NK cell activation in lungs of CR, RF and AL during influenza infection. There was a rapid and significant increase in percentages (**Figure 7A**), but not numbers (**Figure 7B**), of $CD69^+$ NK cells of CR mice on d 1 p.i., relative to those seen in RF and AL mice. However, CR mice could not maintain a large pool of activated NK cells during the first three days of infection, evidenced by significantly decreased numbers of $CD69^+$ NK cells in lungs compared to both RF and AL mice on d 2 and 3 p.i.

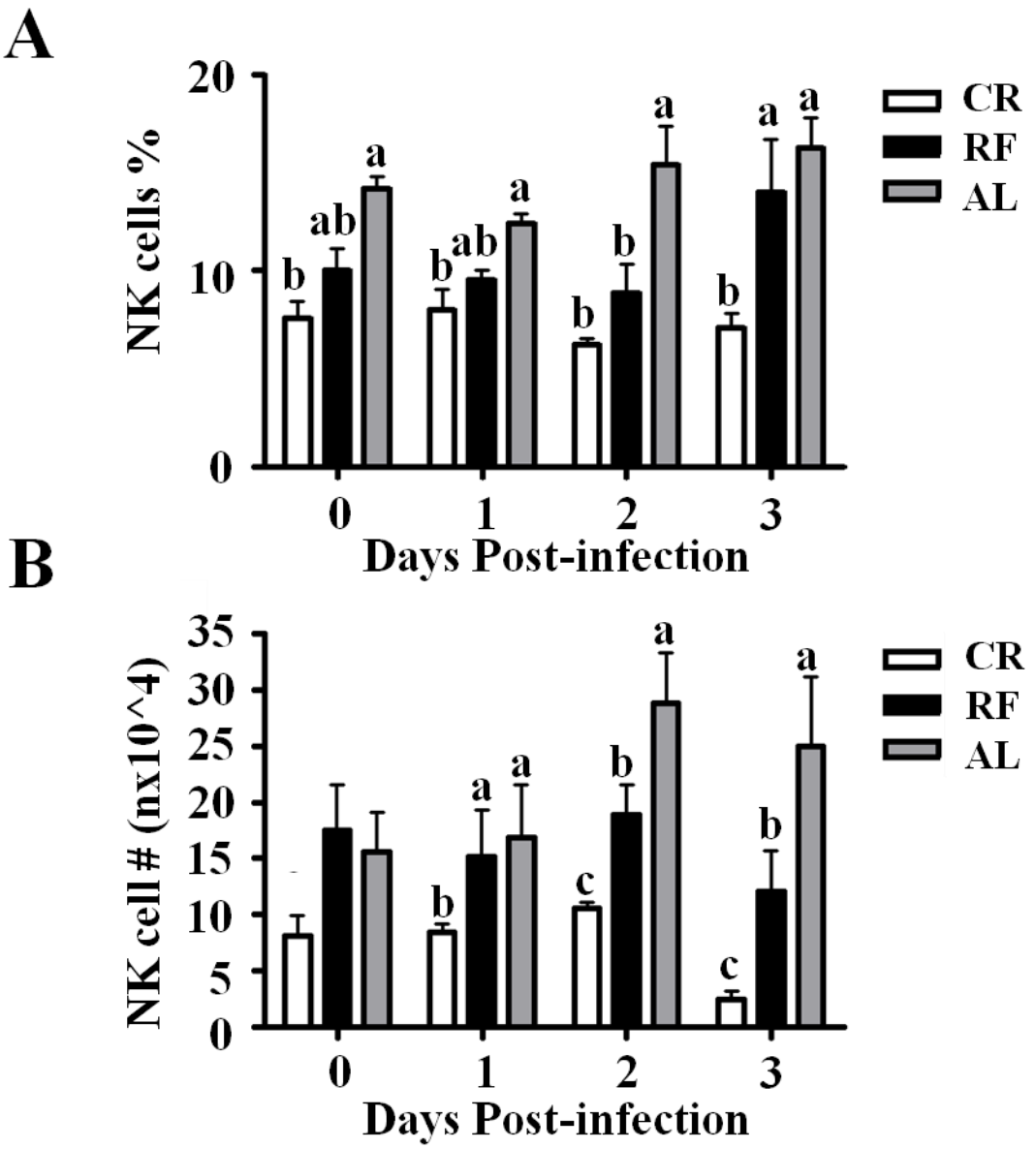

Figure 6: NK cell percentages and numbers in lungs of CR, RF and AL mice before and influenza infection. The percent (A) and number (B) of NK cells ($NK1.1^{+}$ $CD3^{-}$) in the lungs of CR, RF, and AL mice was quantified using flow cytometry. Values are means ± SEM, n=6 mice per group. Means at a time without a common letter differ, $P<0.01$. X,Y,Z Different from the preceding time point, $P<0.05$.

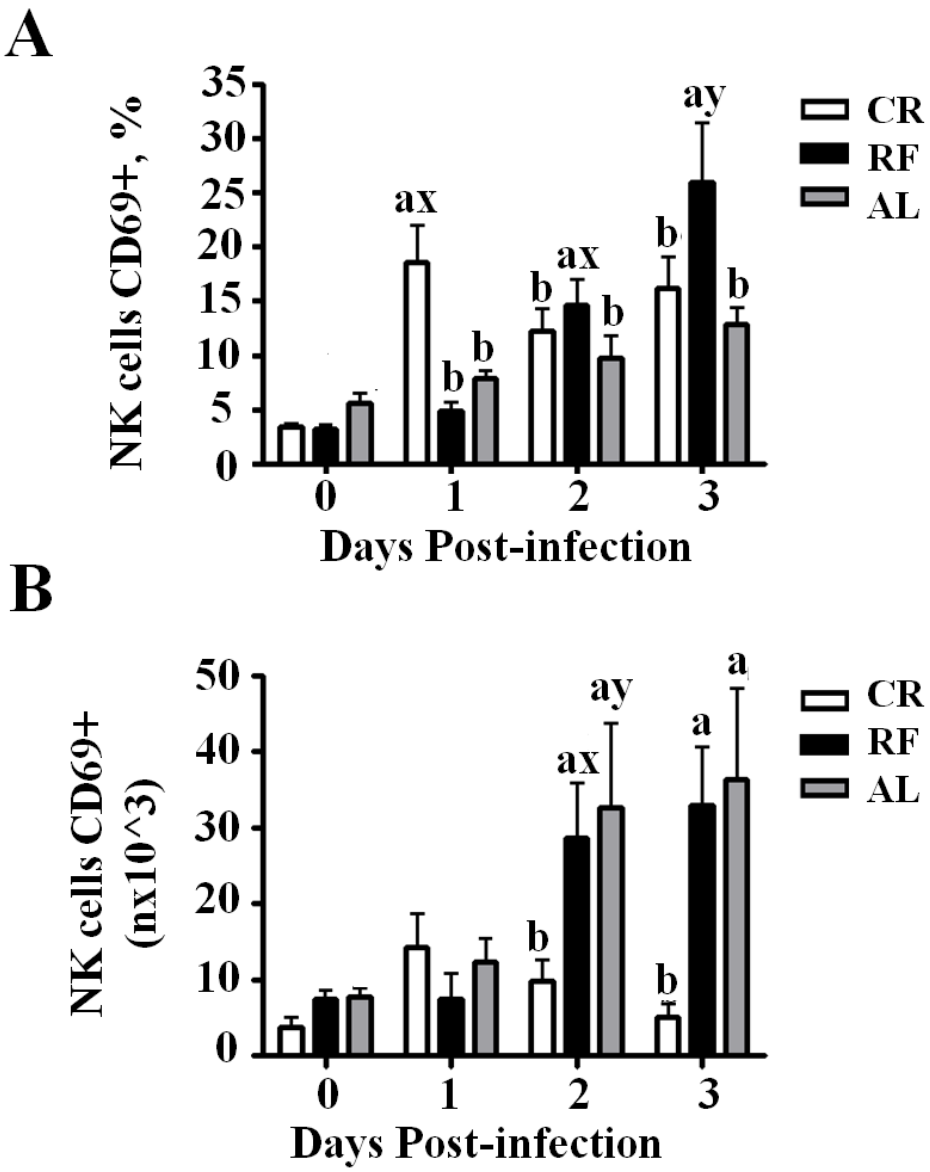

Figure 7: Percentage and numbers of $CD69^{+}$ NK cells in the lungs of CR, RF and AL mice before and during influenza infection. The percent (A) and number (B) of NK cells ($NK1.1^{+}$ $CD3^{-}$) expressing CD69 in the lungs of CR, RF, and AL mice was quantified using flow cytometry. Values are means ± SEM, n=6 mice per group. Means at a time without a common letter differ P<0.01. X, Y, Z Different from the preceding time point, P<0.01.

Short-term feeding differentially affects leptin and adiponectin concentrations in plasma during influenza infection

Adipokines both function to regulate energy metabolism and to influence innate immunity. The adipokines, leptin and adiponectin, have both been shown to alter immunity, including NK cell function [211, 328]. The adipokine leptin is a hormone secreted mainly by adipocytes and regulates food intake and weight control; recently it was demonstrated that leptin supports innate immunity, including NK cell function [211, 329]. However, adiponectin an anti-inflammatory adipokine, has also been shown to suppress inducible NK cell cytotoxicty and impair function [328]. The concentrations of leptin and adiponectin were measured during the course of infection of CR, RF and AL mice. The AL group exhibited a transient rise in plasma leptin concentrations on d.1 p.i. most likely due to the observed increased food intake upon recovery from anesthesia. CR mice had lower concentrations of leptin, compared with both RF and AL mice on d 0, 1, and 3 p.i. (**Table 2**). However, plasma leptin concentrations were comparable in RF and AL mice on 2 d p.i. when NK cell numbers were also greater than CR mice (2 d p.i.). In contrast, adiponectin concentrations in RF mice were lower on d 1 p.i., compared with either CR or AL mice. By d 3 p.i., adiponectin concentrations were comparable between CR and RF mice, but had steadily declined in AL mice such that they were lower than those of CR and RF mice.

TABLE 2 Plasma concentrations of leptin and adiponectin before and after influenza infection [1].

	Diet groups		
	CR	RF	AL
Leptin, µg/L			
Day 0	1.46 ± 0.28^{b}	3.37 ± 0.34^{b}	7.84 ± 1.06^{a}
Day 1	1.24 ± 0.15^{b}	3.22 ± 0.15^{b}	11.3 ± 02.28^{ax}
Day 2	1.55 ± 0.27^{a}	3.56 ± 0.88^{a}	2.66 ± 0.61^{ay}
Day 3	1.02 ± 0.16^{b}	2.71 ± 0.54^{a}	4.97 ± 1.15^{a}
Adoponectin, µg/L			
Day 0	12.14 ± 0.52^{a}	9.72 ± 3.10^{a}	12.94 ± 2.44^{a}
Day 1	16.06 ± 0.39^{a}	10.49 ± 0.68^{b}	16.34 ± 1.94^{a}
Day 2	18.38 ± 1.52^{a}	13.69 ± 2.19^{a}	13.76 ± 0.66^{a}
Day 3	17.72 ± 1.48^{a}	17.22 ± 0.26^{ax}	10.28 ± 02.11^{b}

[1] Values are means ± SEM, n=5 per group. Means without a common letter differ, $P < 0.01$. X,Y,Z indicate different from the preceding time point within the same group.

4.5 DISCUSSION:

The present study was designed to elucidate the effects of short-term refeeding of CR mice on metabolic parameters known to impact innate immune function during primary influenza infection. Our study indicates that refeeding previously CR mice with AL diet induced a hyperphagic response as early as d 1 of refeeding. This resulted in complete restoration of body weight and percent body fat comparable to AL mice by day 5 of the 14 d feeding protocol. In addition, quantifiable inguinal and gonadal fat depots were observed by d 0 of infection. The hyperphagic response after short- or long-term CR, characterized by a rapid increase in body

weight, has been reported previously in humans and rodent models [330, 331]. However, to our knowledge, our study is the first to include comprehensive analysis of body composition to determine the kinetics of body fat restoration and relate these changes to outcome from acute virus infection.

In accord with previous observations [4, 11], CR mice exhibited increased mortality, marked weight loss, and impaired NK cell function during the early innate response to primary influenza infection. Interestingly, RF mice exhibited anorexic effects of acute influenza infection, as evidenced by reduced food intake and lethargy (data not shown), that were more similar to that of CR, compared with AL mice. However, survival, and the percentages, numbers and activation of NK cell in lungs, were markedly improved in RF compared with CR mice. In addition, the percentages of activated NK cells in lungs of RF mice were increased relative to CR mice at d 3 p.i. This suggests that short-term refeeding may not completely restore NK cell function, but does maintain the activated NK cell pool throughout the early phase of infection. Refeeding also resulted in increased numbers and percentages of $CD8^+$ T cells in the lungs by d 3 p.i. compared to CR mice (data not shown), which may contribute to improved survival of RF mice compared with CR mice through d 7 of infection.

While it could be argued in the current study that refeeding did not restore all parameters of NK cell function to levels comparable to that of AL mice, the more relevant comparison is between CR and RF mice. While the diet of AL mice was consistent through the entire study, CR and RF mice were fed the same diet up until the two week feeding protocol. Therefore, it would not be expected for the responses of RF mice to be comparable to AL mice given the short duration of refeeding. However, this refeeding protocol clearly attenuated the decline in NK cell

function observed in CR mice, resulting in overall improved outcome from acute influenza infection.

A critical observation in the current study indicated that the restoration of body weight, body fat percentage, and fat depots may be to provide the energy necessary to support NK cell function during the early innate response to influenza infection. We hypothesized that this restoration of body fat may positively alter plasma concentrations of leptin and adiponectin, both of which have been shown to modulate energy metabolism and directly impact NK cell cytotoxicity and cytokine production [210, 211].

Leptin is produced mainly by adipocytes and acts on the hypothalamus to regulate appetite and energy expenditure and to suppress energy intake via a feedback mechanism [332]. Thus, leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice exhibit an obese phenotype [204]. Leptin also plays an important role in immunity since it is released during an infection, and the leptin receptor (Ob-Rb) is expressed by immune cells, including hematopoietic cells, T cells, B cells, NK cells, and macrophages [202, 210]. Further, leptin regulates NK cell cytotoxicity and apoptosis in *db/db* mice, which have impaired NK cell cytotoxicity and a greater percentage of NK cells undergoing apoptosis in BM [213]. In addition, inhibition of leptin signaling increases apoptosis of NK cells from WT animals [213].

Although body fat was restored in RF mice prior to infection, leptin concentrations were not statistically equivalent to AL until d 2 p.i, when concentration of plasma leptin in AL mice were comparable to levels in both CR and RF. Similarly, despite restoration of body weight, body fat percentage, and fat pad depots, high numbers of marrow adipocytes remained in RF mice. This phenomenon has been observed during CR and in ob/ob mice, suggesting that leptin has a critical role in regulating BM adipocity [327, 333]. It is also reasonable to suggest that

either leptin production and/or leptin receptor function on NK cells is not fully restored in RF mice, rendering them incapable of mounting an innate immune response equivalent to that of AL mice after influenza infection. This may account for the ability of RF mice to increase NK cell numbers and percentages relative to CR mice, but not to comparable levels seen in AL mice through d 3 p.i. These data are supported by previous studies [203, 334] indicating that exogenous leptin administration improves survival and reduces bacterial load in *ob/ob* mice

Lower leptin secretion in response to decreased body fat is designed to improve survival under hostile conditions [335], such as starvation, by shifting energy utilization towards vital metabolic processes, such as cardiac and respiratory functions. In contrast, AL mice have large lipid filled adipocytes that are programmed to rapidly release fatty acids for acute energy needs, such as during a primary infection. Thus, AL mice are able to respond to this acute stress via systemic catabolism of fat for energy by an increase in gluconeogeneis, lypolysis and glycolysis to maintain an adequate immune response [321]. Based upon these observations, it is likely that the combination of insufficient body fat and decreased circulating leptin concentrations impede the ability of CR mice to meet metabolic energy demands necessary to support NK cell function in the face of an acute infection.

Adiponectin is secreted from white adipose tissue into circulation and is inversely correlated with body fat percentage in adults [336]. Therefore, we predicted that adiponectin levels in RF mice would be comparable to AL mice throughout infection. However, although our body composition data indicated that body fat was rapidly restored in RF mice, adiponectin levels were lower in RF compared with AL mice before infection. Interestingly, plasma concentrations of adiponectin steadily increased during infection of RF mice, but sharply declined in AL mice, despite both groups losing a significant percentage of body weight during

infection (data not shown). Thus, despite restoration of body fat, it is plausible that adiponectin may not be as responsive to short-term refeeding.

Adipocyte type, function, gene expression, secretion levels, and deposition are altered following caloric restriction [337], which could explain the increased marrow adipocyte volume in CR mice in our study. Further, since NK cells develop and mature in BM, we further speculate that CR also alters the microenvironment necessary for NK cell maturation. Indeed, adipocyte accumulation in BM has negative impacts on hematopoiesis, resulting in quiescent stem cells and negatively regulating hematopoietic activity. This may lead to limited expansion of short term progenitors [338]. Importantly, adiponectin has been reported to inhibit progenitor differentiation, resulting in a larger pool of undifferentiated hematopoietic stem cells [339]. Thus, increased adiponectin concentrations during infection of CR and RF mice may further inhibit the development of NK cells during lymphopoiesis in BM. Previous reports support this hypothesis indicating adiponectin negatively regulates inducible NK cell cytotoxicity, decreasing IFN-γ production and reducing IL-2 induced Fas-L expression [328]. Furthermore, adiponectin also primes macrophages to become anti-inflammatory M2 macrophages, which promote tissue repair and remodeling [340]. Adiponectin concentrations were increased in CR and RF mice at d 3 p.i. compared to AL mice; while we did not investigate whether macrophage priming was affected in CR and RF mice, this could have significant implications to the innate immune response to influenza. For example, increased M2 macrophages would exert anti-inflammatory effects, if viral titers remain high at this time point, immunosuppressive activity by M2 macrophages could suppress innate immune function. Future studies should examine the relationship between CR and macrophage function and polarization.

Future research will examine the role of longer term feeding and the effect on adipose tissue distribution after refeeding. We will also address specific changes in NK cell development based upon the observation that the cellular components of the BM appear altered. In addition, characterization of the adipose tissue changes in expression, function, and cell morphology are key to understanding how energy balance impacts innate immune response to influenza infection. Taken together, these studies indicate that it may be beneficial to increase energy intake for a defined time period to prepare for yearly influenza season. This dietary intervention may improve the primary response of susceptible individuals to newly emerging strains of influenza, to which vaccination is limited or unavailable.

CHAPTER 5:

NATURAL KILLER CELL MATURATION AND FUNCTION ARE ALTERED BY CALORIC RESTRICTION IN C57BL/6 MICE

Data in this chapter have been published in: Clinthorne JF, Beli E, Duriancik DM, Gardner EM. *Natural Killer Cell Maturation and Function are Altered by Caloric Restriction in C57Bl/6 Mice* 2013. *J Immunology*. Jan 15;190(2):712-22.

5.1 ABSTRACT:

Natural killer (NK) cells are a heterogenous population of innate lymphocytes with diverse functional attributes critical for early protection from viral infections. We have previously reported a decrease in influenza-induced NK cell cytotoxicity in 6 mo old C57Bl/6 calorically restricted (CR) mice. In the present study, we extend our findings on the influence of CR on NK cell phenotype and function in the absence of infection. We demonstrate that reduced mature NK cell subsets results in increased frequencies of $CD127^+$ NK cells in CR mice, skewing the function of the total NK cell pool. NK cells from CR mice produced TNF-α and GM-CSF at a higher level, while IFN-γ production was impaired following IL-2 + IL-12 or anti-NK1.1 stimulation. NK cells from CR mice were highly responsive to stimulation with YAC-1 cells such that $CD27^-$ $CD11b^+$ NK cells from CR mice produced granzyme B and degranulated at a higher frequency than $CD27^-$ $CD11b^+$ NK cells from ad-libitum (AL) fed mice. CR is a potent dietary intervention, yet the mechanisms by which the CR increases lifespan have yet to be fully understood. These findings are the first in-depth analysis of the effects of caloric intake on NK

cell phenotype and function and provide important implications regarding potential ways in which CR alters NK cell function prior to infection or cancer.

5.2 INTRODUCTION:

Caloric restriction is a dietary intervention that extends the lifespan of laboratory animals [341]. While excess energy intake has been associated with increased incidence of disease, CR has been found to decrease the severity of autoimmune disease, and decrease the incidence of cardiac, kidney, or nervous system dysfunction [325, 341-343]. Other benefits of CR include decreased triglycerides and blood pressure, lower central adiposity, improved insulin sensitivity, and delayed age-related immunosenescence [7, 344]. It has been established that in laboratory conditions, CR reduces the incidence of spontaneous tumors and cancers in aged rodents, and slows the age-related decline in T cell proliferation, cytokine production, and cytotoxic T lymphocyte activity that is often observed during aging [6, 320]. Lifelong CR of mice preserves thymopoiesis in the face of aging, and enhances influenza specific antibodies and splenic lymphocyte proliferation after vaccination of mice with influenza [6, 313]. These beneficial changes to the adaptive immune system have been well characterized; however, it has also been found that CR influences innate immune function [318, 345]. Several decades ago, Weindruch et al. reported that CR resulted in decreased in splenic natural killer (NK) cell cytotoxicity compared to aged matched controls, although this could be ameliorated by polyinosinic:polycytidylic acid [5]. More recently, we have shown CR results in increased susceptibility to primary influenza infection and decreased influenza induced NK cell cytotoxicity in young and aged mice [4, 11]. This was accompanied by the observation that NK cell numbers and frequency are decreased in the spleen of young CR mice [11]. Overall, these findings have raised concerns about on the effects of CR on innate immunity, and may predispose CR individuals to suffer more severe primary infections [315, 318]. However, at this

time few studies have focused on understanding the effects of CR on innate immune cell development and function.

Natural killer cells are responsible for recognizing virally infected cells, as well as transformed cells including neoplasms and tumor cells [155, 346, 347]. Development of NK cells takes place mainly in the BM and signals from stromal cells and cytokines, result in the microenvironment required for NK cell generation [8, 81]. NK cell commitment takes place through upregulation of the shared IL-2/IL15Rβ chain (CD122), followed by acquisition of the NK cell marker NK1.1 in B6 mice [8, 88]. Interactions with stromal cells within the BM regulate gene expression leading to programmed expression of surface molecules including integrins, cytokine receptors, and a family of NK cell receptors [98, 108, 348, 349]. NK cell maturation is classified by using both surface phenotype and functional capacity. Phenotypic maturation takes place in stepwise fashion; expression of the integrin CD49b (DX5) is used to define early mature NK cells [74]. Following acquisition of DX5, NK cells in the BM upregulate CD11b and CD43, which correlates strongly with the capability of an NK cell to produce large amounts of IFN-γ [8]. Once mature, NK cells seed various lymphoid and nonlymphoid peripheral tissues, with the majority of NK cells expressing high levels of DX5, CD11b, and CD43, [8, 77].

After emigrating from the BM via the blood and seeding peripheral tissues, $DX5^+$ NK cells continue to adapt to their environment; the down regulation of CD27 and TNF-related apoptosis inducing ligand (TRAIL), and upregulation of KLRG1 are associated with peripheral NK cell maturation [10, 99]. The application of the marker CD27 has allowed the $DX5^+$ NK cell pool to be further divided into subsets in mice in which there is a linear progression from $CD27^+$

$CD11b^-$ early mature NK cells to $CD27^+$ $CD11b^+$ (double positive; DP) NK cells followed by development into $CD27^-$ $CD11b^+$ NK cells. These phenotypic changes further reflect changes to NK cell function, as DP NK cells exhibit a greater responsiveness to *in vitro* culture with DCs, while $CD27^-$ $CD11b^+$ $KLRG1^+$ NK cells are terminally differentiated NK cells that are tightly regulated with reduced capacity to proliferate or elicit effector function during viral infection, such as MCMV [99, 100].

Recent studies have begun to identify the molecular mechanisms that regulate NK cell development, egress, and differentiation. These studies have identified TFs required for NK cell commitment and differentiation into mature NK cells. Among these TFs, PU.1, Id2 and E4bp4 are thought to regulate the earliest developmental stages, while other studies have established a role for the TFs T-bet, Eomesodermin (Eomes), Blimp-1, IRF-2, and GATA-3 in the generation of terminally differentiated $CD43^+$ $KLRG1^+$ NK cells [101, 115, 350, 351]. Similar to their role in T cell function, several of these TFs regulate aspects of NK cell function. For example, T-bet is involved in the expression granzyme B in NK cells, while NK cells lacking Eomes are capable of producing more TNF-α than their $Eomes^+$ counterparts [350].

Based on the above studies, we sought to understand how CR impacts NK cell development and function independent of aging or infection in 6 mo old mice. Here we show that NK cells are reduced in frequency in peripheral tissues and exhibit an altered phenotype in the spleens of CR mice. We characterize these changes by analyzing the expression of a variety of cell surface markers associated with the maturation process such as CD94, CD127, DX5, CD11b, CD43 and KLRG1. Most studies of murine NK cells focus on splenocytes, which normally express low levels of CD127. However we discovered that CR results in an increased

fraction of NK cells that express CD127 in the spleen and LNs, accompanied with an increase in the frequency of NK cells in the thymus, of which the majority are $CD127^{+}$. These cells are thought to have low cytotoxic potential, produce a variety of cytokines and are normally found at the highest frequency in the thymus and LNs. Comparison of the distribution of NK cell subsets between AL and CR mice revealed CR results in significant reductions to the $CD27^{-}$ $CD11b^{+}$ NK cell pool, suggesting caloric intake plays a role in regulating peripheral NK cell maturation or homeostasis. We used various stimuli to test the functional competence of NK cells from CR mice, and found that alterations to NK cell function in CR mice are specific to the stimulus used. Our results suggest that CR significantly alters NK cell subset distribution, resulting in a heterogeneous pool of NK cells displaying unique functional characteristics.

5.3 MATERIALS AND METHODS:

Mice and diets

Specific pathogen-free young adult (6 mo) AL and young adult (6 mo) CR male C57Bl/6 mice were purchased from the NIA colony maintained by Charles River Laboratories (Wilmington, MA). The animal use protocol for this study was approved by Michigan State University Institutional Animal Care and Use Committee. Upon arrival, mice were housed individually in micro-isolator cages in the AAALAC-accredited containment facility at Michigan State University and were acclimated at least 10-14 days prior to the initiation of each experiment. Both CR (NIH-31/NIA-fortified) and AL (NIH-31) diets were purchased from the NIA, the compositions of which have been reported in detail previously [11]. The composition of the CR diet is sufficient in micronutrients and minerals, but results in restriction of total energy intake. The CR regimen initiated by the NIA is designed to gradually achieve 40% restriction in mice by

4 mo of age, such that they are weight stable upon arrival at 6 mo of age. All experiments were repeated at least twice using 4-5 mice per diet treatment per experiment, unless otherwise noted.

Body composition, food intake and metabolic profile

Body composition (fat, lean, water) was determined by daily magnetic resonance imaging (MRI) during the feeding protocol. Mice were individually housed allowing food intake to be recorded each day. All mice were weighed daily between 0800 and 0900, after which they were fed. The protocol to assess body composition using the EchoMRI-500 (Echo Medical Systems) has been validated and described in detail previously [323]. Briefly, after calibration using a rapeseed oil standard, individual mice are placed in an MRI holding tube. The advantages of this system are that it is rapid, allows for repeated measurements, and does not require anesthesia, enabling mice to recover immediately after MRI. Serum concentrations of corticosterone, albumin, and leptin were quantified by commercially available ELISA kits according to the manufacturer's instructions (Assaypro, Life Diagnostics, R&D, respectively). Serum concentrations of glucose, cholesterol and triglycerides were determined using colorimetric assays as per the manufacturer's instructions (Cayman Chemical). Plates were read at 450 nm wavelength using a Synergy HT plate reader (Bio-Tek) and concentrations were determined using a standard curve for each respective assay.

Lymphocyte isolation

Following euthanasia, blood was collected by cardiac puncture into heparinized syringes. Following cardiac puncture, spleens, lungs, LNs (inguinal, auxiliary, and brachial), and thymus, were excised and weighed. Isolation of mononuclear cells from spleens and lungs has been

previously described [323]. Briefly, single cell suspensions were obtained from spleens using homogenization. BM cells were isolated from the femur and tibia by flushing with a 25 5/8 needle and syringe containing roswell park memorial institute medium **(**RPMI) 1640. The resulting cell suspensions were lysed of red blood cells (RBCs) using an ammonium chloride buffer. Lungs were excised, weighed and minced using a Miltenyi GentleMACs system. Cells were then incubated for 30 minutes at 37^{o} C in RPMI 1640 containing 5% FBS, 1mg/mL collaganase D (Roche, Indianapolis, IN, USA) and 80 Kuntz Units DNAse (Roche). Cell suspensions from digested lungs or blood were diluted with PBS and layered onto 1083-histopaque (Sigma-Aldrich) for isolation of mononuclear cells by density gradient centrifugation. Isolation of cells from LNs and thymus was performed by pressing the LNs and thymus through 40μm cell strainers (BD Falcon). All cell suspensions were washed in PBS and resuspended for counting using Trypan Blue viability dye.

Flow cytometry

Cells from various tissues were resuspended in FACS buffer (0.1% sodium azide, 1% FBS, in PBS) at a concentration of $2x10^{7}$ cells/mL. $1\text{-}4x10^{6}$ cells were incubated on ice for 10 minutes with anti–CD32/CD16 antibody (2.4G2) (BD Bioscience) in order to block FcγII/III receptor–mediated non-specific binding. Samples were then incubated with a cocktail containing various combinations of the following fluorochrome-conjugated antibodies (eBioscience, BD Bioscience, or Biolegend) at optimal concentrations determined in our laboratory: NK1.1 (Allophycocyanin or PE-Cy7), CD3 (Alexa Fluor700 [500A2]), CD94/NKG2 (PE [HP-3D9]), CD27 (PE or PerCP-eFluor710 [LG.7F9]), CD127 (PE or PerCP-Cy5.5 [A7R.34]), CD51 (Biotin [RMV-7]), CD49b (Allophycocyanin or PE-Cy7 [DX5]) CD11b (PE-Cy7 or V500

[M1/70]), GITR (FITC [DTA-1]), B220 (Allophycocyanin [RA3-6B2]), CD43 (Allophycocyanin-Cy7 [1B11]), Ly49C/I/F/H (FITC or PE [14B11]), Ly49-G2 (Allophycocyanin [4D11]), Ly49D (FITC [4E5]), Ly49H (Biotin [3D10]), KLRG1 (Allophycocyanin [2F1]). Biotinylated antibodies were detected using Streptavidin conjugated PerCP-Cy5.5 or Allophycocyanin-Cy7. Cells were incubated in staining cocktails on ice in the dark for 30 min. To detect TF expression, cells were fixed and permeabilized using eBioscience Foxp3 staining kit according to the manufacturer's instructions, then incubated with antibodies against T-bet (PE-Cy7 [4B10]) and Eomes (Alexa Fluor488 [Dan11mag])(eBioscience). Viable lymphocytes were gated based on light scattering properties, after which NK cells were characterized as $NK1.1^{+}$ $CD3^{-}$ unless otherwise noted. Samples were analyzed using an LSR II flow cytometer (BD Bioscience) or a FACS Canto II flow cytometer (BD Bioscience) with FlowJo software (Tree Star).

Cytokine, granzyme B production and degranulation

NK cell capacity to produce IFN-γ and degranulate were measured using flow cytometry according to previously published methods [151]. Briefly, high affinity 96 well plates (Thermo-Fisher) were coated with a monoclonal antibody against NK1.1 (25 µg/mL [PK136]) or NKp46 (15 µg/mL [29A1.4]) for 18 hours at 4^{o} C. Plates were then washed with PBS 3 times and freshly prepared splenocytes ($1\text{-}4x10^{6}$) in complete media were added. Alternatively, splenocytes in complete media were added to uncoated 96 well plates and IL-2 (1000U) + IL-12 (10ng/mL) or YAC-1 cells (10:1 E:T ratio) were added. Anti-CD107a (FITC or PE-Cy7 [1D4B]), a marker of degranulation, was also added to NK1.1 and YAC-1 stimulated splenocyte

cultures [352]. Plates were incubated for 4-8 hours during which brefeldin A and monensin added after the first 30 minutes. In order to elicit GM-CSF and TNF-α production, NK cells were incubated with IL-2 (1000U) and IL-12 (10 ng/mL) for 18 hours, followed by phorbol-12-myristate-13-acetate (PMA) (50 ng/mL), Ionomycin (1 μg/mL), with brefeldin A being added during the last 4 hours to block cytokine secretion and raise intracellular cytokine stores as reported by Vosshenrich et al (34). Following incubation, cells were stained with lineage specific antibodies then fixed and permeabilized using BD cytofix/cytoperm kits according to manufactures' protocol. Intracellular cytokines and granzyme B were detected using monoclonal antibodies against IFN-γ (FITC or PE-Cy7 [XMG1.2]), granzyme B (PE [GB11]), TNF-α (PE [MP6-XT22]), and GM-CSF (FITC [MP1-22E9]) (BD Biosciences).

Statistics

Statistics were performed using GraphPad Prism 4 software (La Jolla, CA). Values in text are means ± SEM. Body composition, food intake, weight, serum metabolic profile, immune cell populations and NK cell function were analyzed using student's T-test to determine significant differences between diet groups. Statistical significance was set at $p<0.05$.

5.4 RESULTS:

Physiological parameters influenced by CR

CR is initiated at 14 weeks of age (10% restriction), and at 15 weeks of age the restriction is increased to 25%. Finally, at 16 weeks mice are fed a 40% restricted diet that is maintained throughout the life of the animal. For this study, our CR protocol supplied mice with 3 grams of food daily in the form of a vitamin and mineral supplemented cookie supplied by the NIA, while

AL mice consumed approximately 4.53 grams of food daily (**Figure 8A**). Our data indicate feeding CR mice with a 3 gram cookie resulted in a 34% restriction rather than 40%; however we still observed physiological changes indicating our CR protocol induced CR characteristics, which is supported by the notion that even mild CR (10-25%) is effective in increasing lifespan [151, 341]. Restriction resulted in mice with reduced body weight (Figure 8A) achieved by a reduction in both lean and fat mass, as well as reduced body fat percentages (**Figure 8B**). True CR exists independently from PEM, a deficiency resulting in nutritional stress shown to negatively impact the immune system and increase circulating GCs [249]. To determine if this CR protocol was inducing PEM we assessed circulating levels of corticosterone, the major endogenous GC (**Figure 8C**) and serum albumin levels, a marker of protein status (Figure 8C) [249]. Consistent with the reports of others, CR resulted in increased circulating corticosterone, however we found no difference in serum albumin levels between CR and AL mice, indicating NIH-31/NIA-fortified diet contains sufficient protein (18%). These findings support the notion that CR is a nutritional stress resulting in increased circulating GCs, but any immunological observations are independent of PEM [287]. Other physiological parameters influenced by CR included reduced levels of serum cholesterol, triglycerides and leptin, but normal blood glucose (Figure 8C-8D).

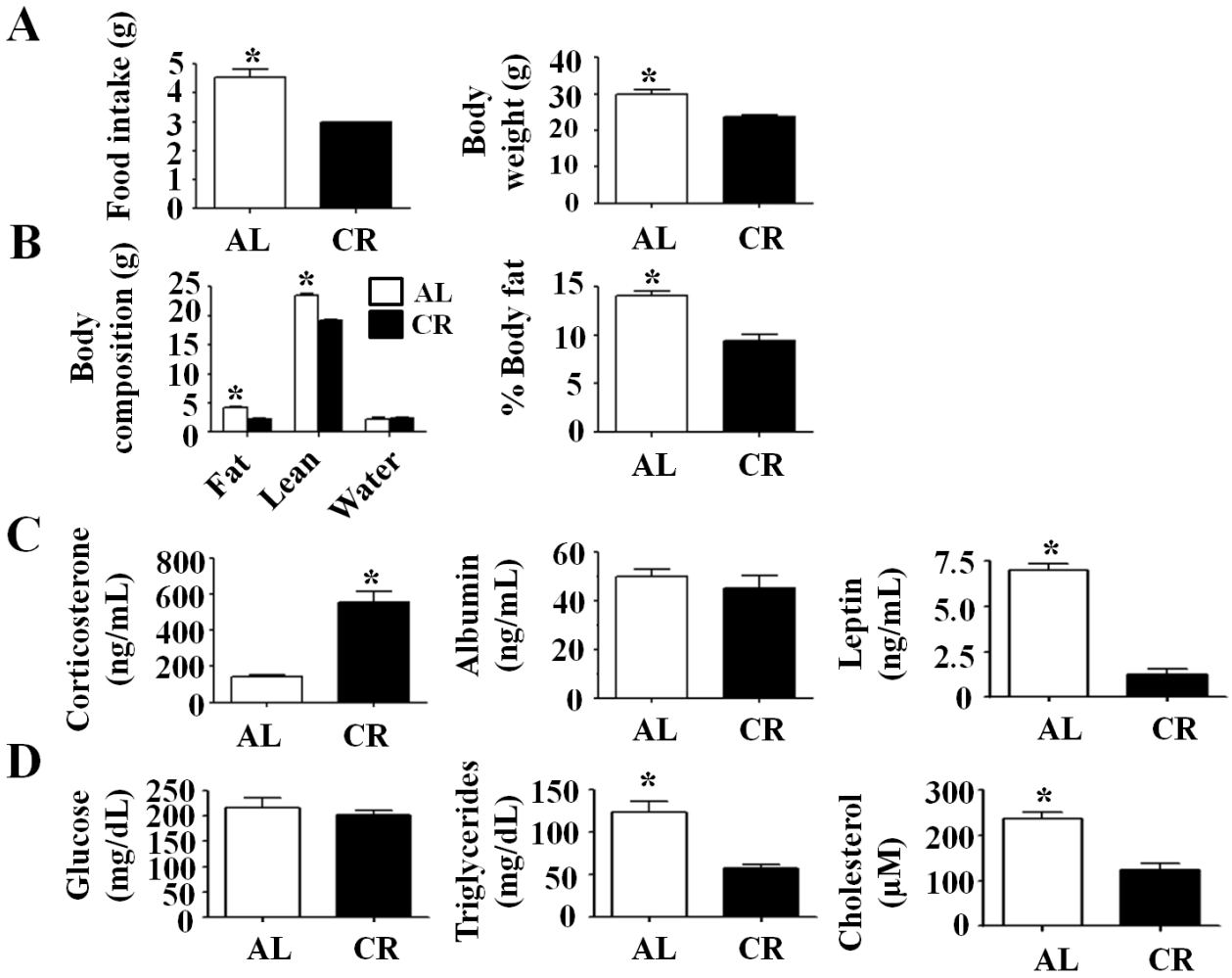

Figure 8: Food intake and physiological parameters altered by CR. A, Food intake and body weight was recorded daily for 7 days and averages for AL and CR mice are shown. B, Body composition was assessed by MRI on the day animals were sacrificed and

(Figure 8 cont'd) body fat percentage was calculated as the portion of fat mass relative to total mass. C, Circulating levels of corticosterone, albumin, and leptin were determined in serum from AL and CR mice by ELISA on the day of sacrifice. D, Serum glucose, triglycerides, and cholesterol from AL and CR mice were measured on day of sacrifice by colorimetric assays. Data are means ± SEM. * indicates significance, $p<0.05$ (n=8-10 mice/group).

CR results in altered NK cell in distribution in peripheral tissues

We have previously shown that the percentage of $NK1.1^+$ lymphocytes in the spleen, but not the lungs, are reduced in CR mice [11]. However, it is possible that in this prior analysis NKT cells were included. Therefore we assessed NK cell percentages ($NK1.1^+$ $CD3^-$) in the spleen, blood, BM, LNs, and lungs of 6 mo old AL and CR mice and found that CR results in a significant reduction in the frequency of cells that are NK cells in the spleen, lungs and blood (**Figure 9A**). In contrast, NK cells were found to be present at normal frequencies in LNs and at an increased frequency in the BM (Figure 9A). Because of differences in body weight and spleen mass, we normalized the absolute number of NK cells in various tissues to the weight of each tissue harvested (**Figure 9C-E**). As NK cell frequency was reduced in the lungs and spleen, we expected to find a reduced number of NK cells after normalizing cell numbers to the weight of the respective tissues. Indeed, there were fewer NK cells in the lungs and spleens of CR than AL mice on "a per mg of tissue" basis (Figure 9D-E). There was no detectable difference in NK cell numbers in the BM of CR mice compared to AL mice (Figure 9C). Thus, the reduced frequency of NK cells in CR is not due to an increase in another cell population, but rather reflects a direct change to NK cells resulting from CR as both frequency and numbers of NK cells are reduced.

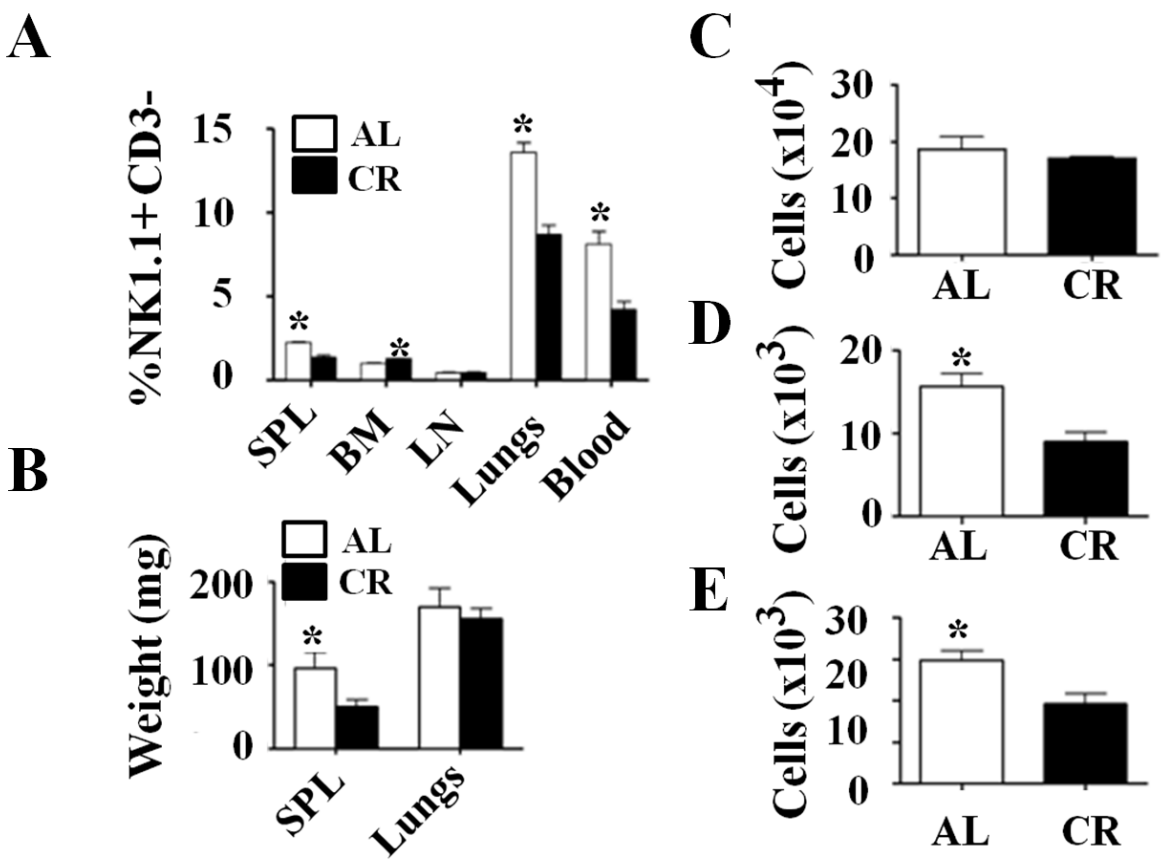

Figure 9: Tissue weight and distribution of NK cells in CR mice. A, The percentage of $NK1.1^{+}$ $CD3^{-}$ cells out of total lymphocytes was determined in various tissues known to contain NK cells in AL and CR mice. B, Wet tissue weights from AL and CR mice were taken immediately

(Figure 9 cont'd) following sacrifice of AL and CR mice. NK cell numbers from BM (C), spleen (D), and lungs (E) of CR and AL mice shown as the number of NK cells per femur or per mg of tissue. The absolute number of NK cells was calculated based on the frequency of NK cells out of total cells analyzed by flow relative to wet tissue weight. Data are means ± SEM. * indicates significance $p<0.05$ (n=5 mice/group/experiment). Experiments were repeated twice.

CR alters expression of NK cell maturation markers

Natural killer cells are a heterogeneous population of cells, undergoing a developmental process within the BM before seeding peripheral tissues [8, 99]. Based on our observation that CR results in changes to NK cell frequency in the spleen, we investigated whether this was due to a reduction in total NK cells or reflected that a specific stage of maturation was reduced in CR. We found CR results in decreased percentages of splenic NK cells expressing the maturation markers CD11b, CD43 and KLRG1, but not DX5 (**Figure 10A**). Similarly, fewer NK cells expressed Ly49C/I/F/H, indicating that NK cells in CR mice exhibit an immature phenotype (**Figure 10B**). We also found NK cells from CR mice displayed increased expression of CD51, CD127 and CD94 (Figure 10A), markers normally not expressed at high levels on mature NK cells [8]. Further phenotypic marker examination showed that NK cells from CR mice exhibited higher expression of several activation markers such as CD69, B220, and GITR (Figure 10B). Overall, CR results in NK cells with an activated and immature phenotype, leading us to hypothesize that a lack of mature NK cells are the cause for reduced NK cell frequency and number in peripheral tissues of CR mice.

Activating and inhibitory receptor expression is altered by CR

Our data indicates that the frequency of mNK cells is altered by CR, therefore we investigated whether expression of receptors involved in NK cell function were also influenced by CR. We found no difference in the median fluorescent intensity (MFI) of the activating receptors NKp46 and NKG2D, but did observe a decreased frequency of NK cells stained with a mAb that recognizes Ly49C/I/F/H [128]. Furthermore, we observed a decrease in the frequency of NK cells expressing Ly49D, Ly49H and Ly49G2 (data not shown). Because Ly49s are acquired during the maturation process, we investigated whether CR influenced the Ly49 receptor repertoire on mature ($CD11b^{+}$) and immature ($CD11b^{-}$) NK cells from CR and AL mice. $CD11b^{+}$ NK cells from CR mice expressed slightly, but significantly reduced levels of Ly49H, Ly49G2, and Ly49C/I/F/H than $CD11b^{+}$ NK cells from AL mice (**Figure 10C**). Similarly, $CD11b^{-}$ NK cells from CR mice expressed significantly lower levels of Ly49H, Ly49G2, and Ly49C/I/F/H although neither group showed significantly different expression of Ly49D (Figure 10C).

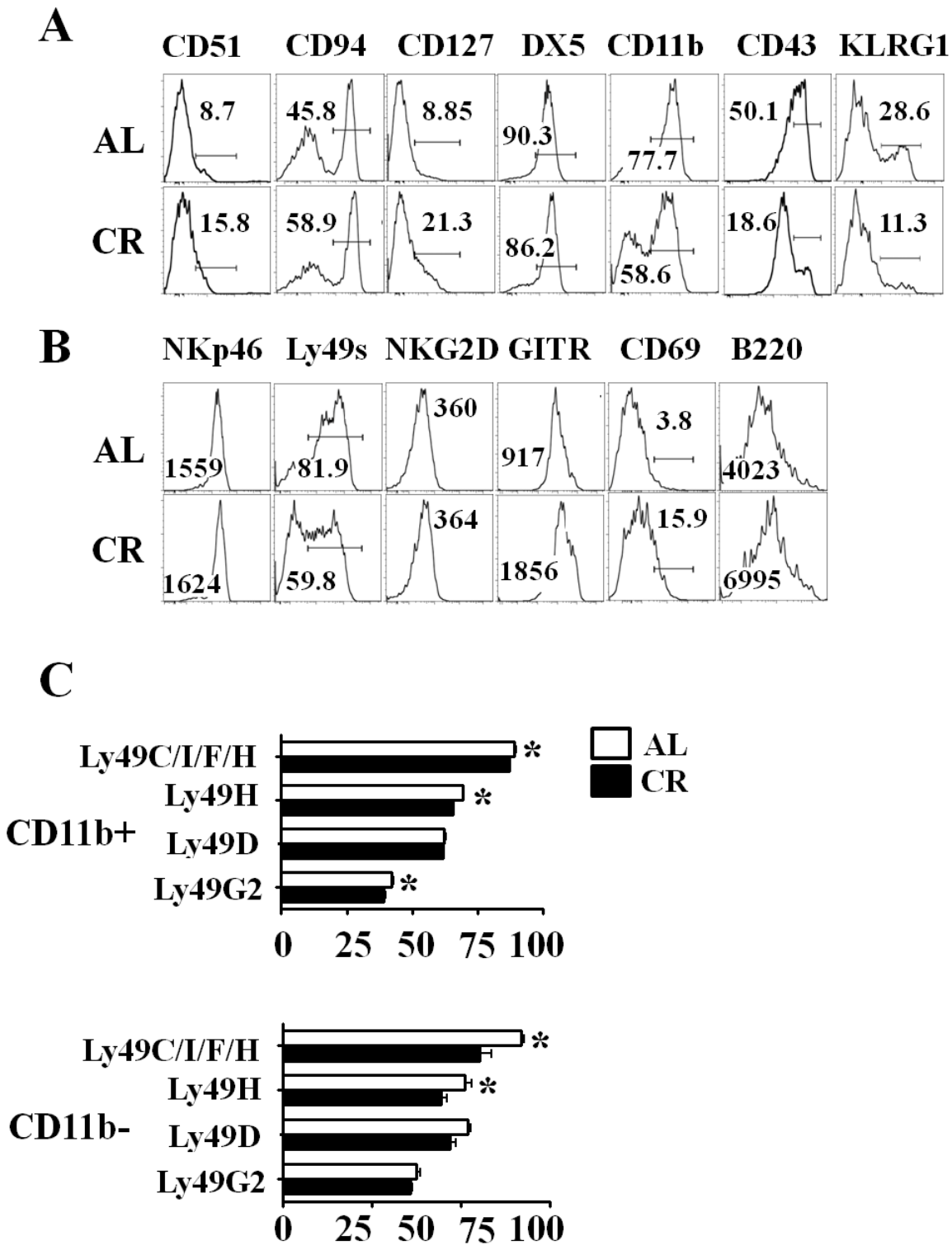

Figure 10: Characterization of the surface phenotype of splenic NK cells in CR mice. Histograms are representative and contain either percentage of NK cells within the positive gate

(Figure 10 cont'd) for the indicated cell surface antigen or the MFI of the indicated marker (when no gate is shown). A, Expression of surface markers associated with NK cell maturation on splenic NK cells gated NK1.1^{+} CD3^{-} from 6 mo old AL and CR mice. B, Expression of NK cell receptors and activation markers on splenic NK cells from CR and AL mice. C, Ly49 repertoire on both CD11b^{+} (top) and CD11b^{-} (bottom) NK cells. Data are mean ± SEM. * indicates significance $p<0.05$ (n=5 mice/group/experiment). Experiments were repeated twice.

CD127^{+} NK cells are increased in frequency but not number in the BM, spleen and LNs of CR mice

NK cells expressing the IL-7Rα (CD127) are normally found at a high frequency in the thymus and LNs; however the origin of CD127^{+} NK cells remains to be fully resolved [101, 353]. Athymic (*foxn1-/-*) mice demonstrate a significant reduction in the frequency of CD127^{+} NK cells in the spleen and LNs, supporting the notion that the thymus is a major source of CD127^{+} NK cells in peripheral tissues [101]. However, it has also been postulated the thymic NK cell developmental pathway is an extension of a pathway normally occurring in the BM [88]. Analysis of CD127 expression on NK cells from the spleen, LNs, and BM of CR mice revealed a significantly greater proportion of NK cells in CR mice expressed CD127 in all three tissues (**Figure 11A**). Because we observed a significant increase in CD127 expression on NK cells from CR mice (Figure 11A), but changes in the frequency of total NK cells (Figure 10A), we compared the absolute number of CD127^{+} NK cells between AL and CR mice (**Figure 11B**). While CD127^{+} NK cells represented a larger percentage of the total cell pool (data not shown), when we compared the absolute number of CD127^{+} NK cells present in AL and CR mice we

found no difference in $CD127^+$ cell numbers in the spleen, LNs, or BM (Figure 11B) suggesting that $CD127^+$ NK cell numbers are maintained in CR mice, while other NK cell subsets are reduced. The surface phenotype of $CD127^+$ NK cells in CR mice also differed slightly from $CD127^+$ NK cells from AL mice: in the spleen, DX5 and CD11b were both expressed at higher levels on $CD127^+$ NK cells from CR mice than $CD127^+$ NK cells from AL mice (**Figure 11C**), while these cells had low expression of Ly49s compared to $CD127^-$ NK cells from either diet group. Similar to the spleen, DX5 expression was found to be higher on BM $CD127^+$ NK cells from CR mice compared to $CD127^+$ BM NK cells from AL mice (Figure 11C). Because the thymus is known to be important for the generation of $CD127^+$ NK cells, we assessed $CD127^+$ NK cell frequencies in the thymi of CR and AL mice by first gating $NK1.1^+$ $CD3^-$ cells (**Figure 11D)** and comparing the frequency of NK cells expressing CD127, approximately 80% of NK cells in both AL and CR (Figure 11D). Compared to AL mice, NK cells were increased in frequency (**Figure 11E**) in the thymi of CR mice, but not in number after correcting for differences in thymic size (Figure 11E), while total cells within the thymus were significantly reduced with CR (**Figure 11F**). Taken together, the fact that $CD127^+$ NK cells are present in comparable numbers between AL and CR mice, combined with similar numbers of $CD127^+$ NK cells "per mg thymus", suggest thymic output of NK cells is normal in CR mice and the increased frequency represents changes in frequencies of other NK cell populations in CR.

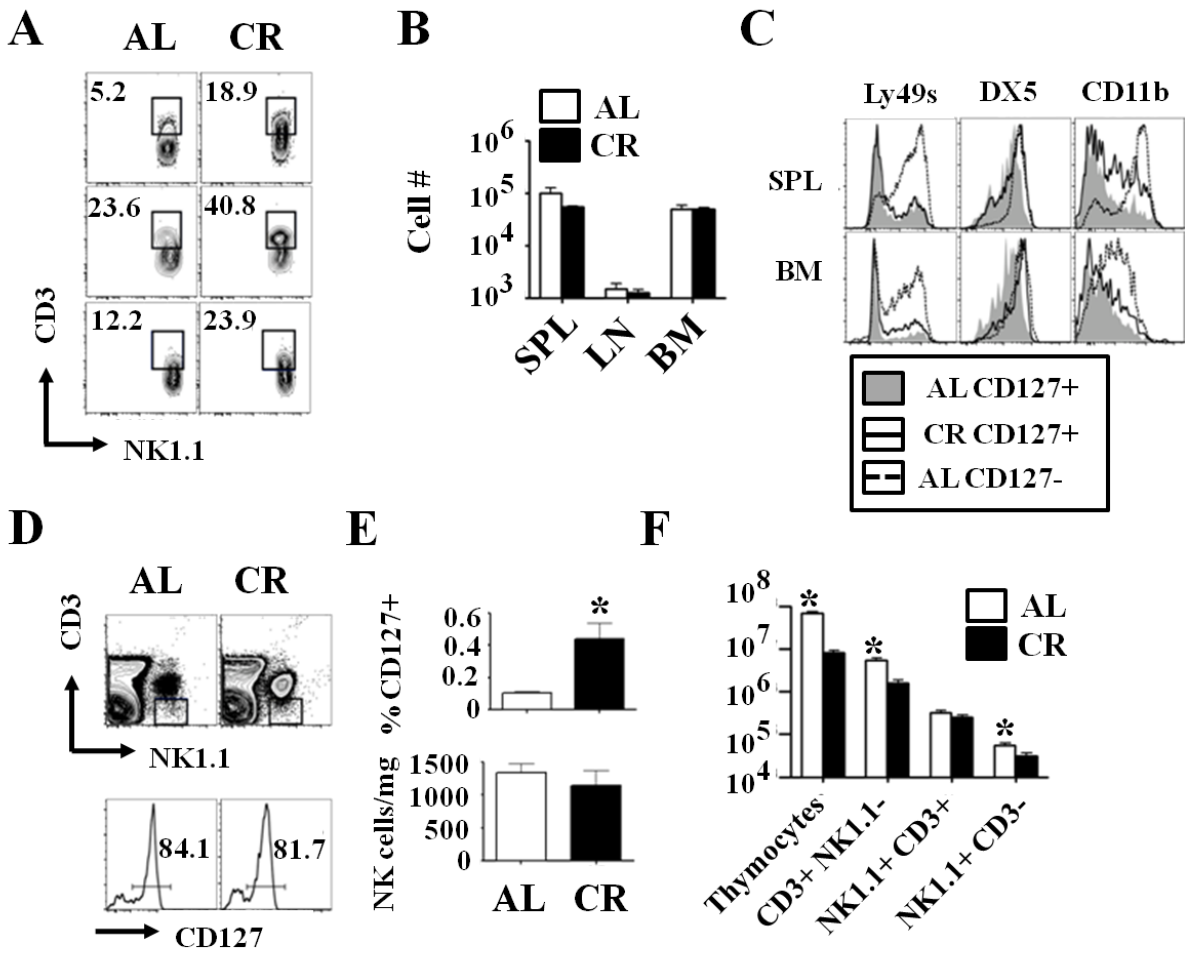

Figure 11: A greater fraction of NK cells from CR mice express CD127. A) CD127 expression on spleen (SPL), LN, and BM NK cells from AL and CR mice. B) The absolute number of $CD127^+$ NK cells ($NK1.1^+$ $CD3^-$) in the spleen, LN, and BM of AL and CR mice. C) Surface phenotype of splenic and BM $CD127^+$ NK cells from gates indicated in (A). Filled gray histogram represents $CD127^+$ NK cells from AL, solid line represents $CD127^+$ NK cells from CR, and dotted line represents splenic $CD127^-$ NK cells from AL mice. D) Gating strategy for identification of thymic NK cells which are identified as $NK1.1^+$ $CD3^-$ (top) and $CD127^+$ (bottom). E) Frequency of NK cells in the thymus represented both as frequency of thymocytes (top) and number of NK cells per mg of thymus collected (bottom). F, Absolute counts of various cell populations identified in the thymus of AL and CR are shown. Data are mean ± SEM, * indicates significance, $p<0.05$ (n=5 mice/group/experiment). Flow cytometry plots are

(Figure 11 cont'd) representative and contain the percentage of NK cells positive for the indicated gates. Experiments were repeated twice.

$CD127^+$ NK cell cytokine production but not cytotoxicity is significantly altered by CR

NK cells can be classified into distinct functional subsets based on the cell surface phenotype and degree of cytokine production and cytotoxicity exhibited. In humans, $CD16^{bright}$ $CD56^{dim}$ NK cells are enriched in LNs and produce high levels of cytokines but have limited cytotoxicity, and it has been reported murine $CD127^+$ NK cells have similar functional attributes [101]. Because we observed an increased frequency of $CD127^+$ NK cells in the spleen and LNs of CR mice, we measured the capacity of LN and splenic NK cells to produce TNF-α and GM-CSF. Following stimulation, significantly more NK cells from CR mice stained positive for TNF-α and GM-CSF (**Figure 12A-B),** correlating with the increased frequency of $CD127^+$ NK cells (Figure 12A). When gating on $CD127^+$ NK cells from either the spleen and LNs of CR mice we found these cells produced TNF-α and GM-CSF at a higher frequency than $CD127^+$ NK cells from AL mice (**Figure 12C**), indicating a direct change in the activity of these cells on a per cell basis. Next, because we observed an increase in cytokine production by $CD127^+$ NK cells from CR mice we investigated whether these cells exhibited higher cytotoxic potential than $CD127^+$ NK cells from AL mice when stimulated with YAC-1 cells. In general, $CD127^+$ NK cells produced less granzyme B and degranulated at a lower frequency than $CD127^-$ NK cells in both CR and AL mice (data not shown), however we observed no difference in the frequency of

$CD127^{+}$ NK cells staining positive for granzyme B or CD107a between CR and AL mice (Figure 12C).

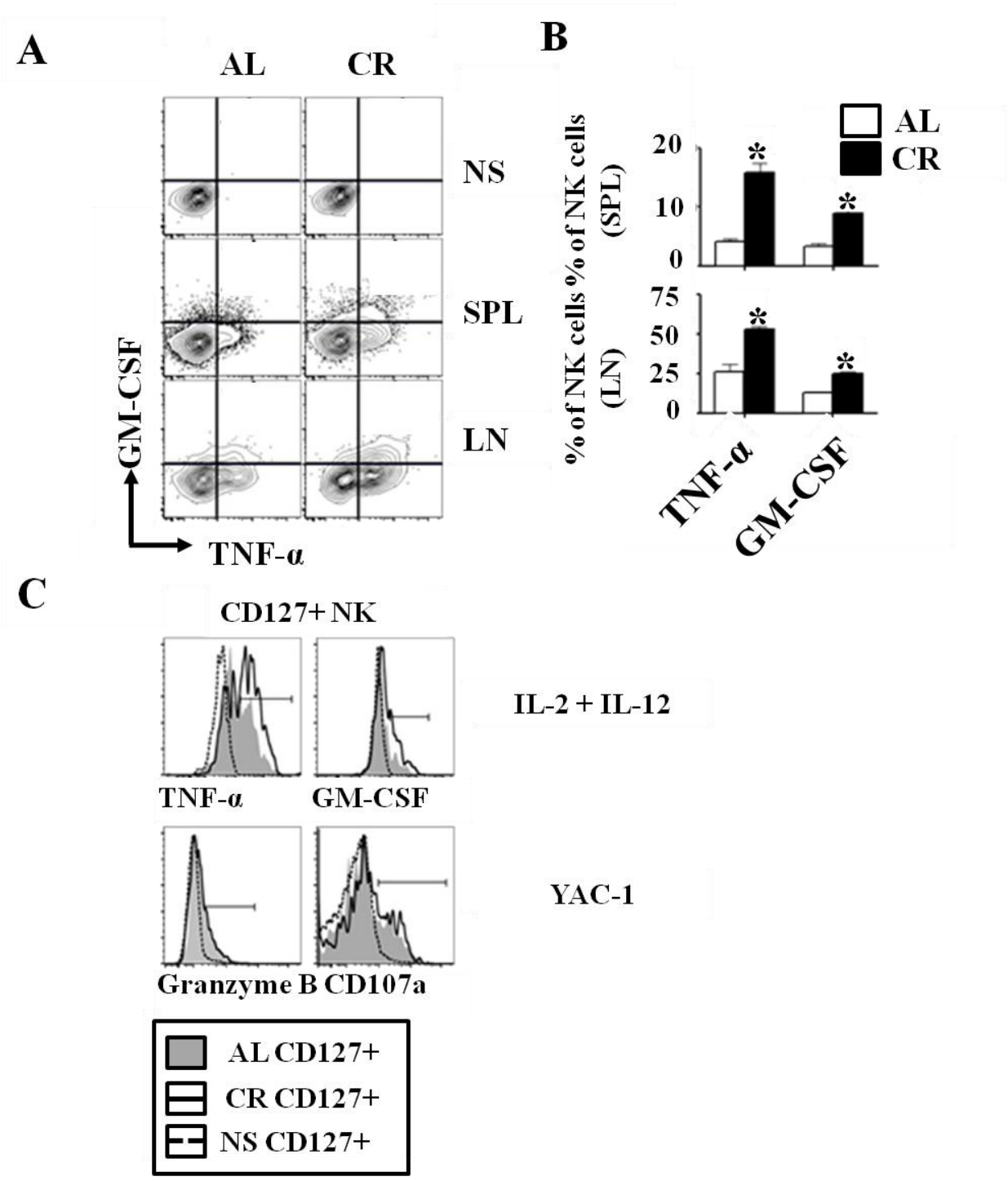

Figure 12: Functional characterization of $CD127^{+}$ NK cells from CR mice. A) Analysis of cytokine production by NK cells ($NK1.1^{+}$ $CD3^{-}$) from AL and CR mice in nonstimulated (NS) controls (top), cells isolated from spleen (middle) and LNs (bottom) stimulated with IL-2

(Figure 12 cont'd) (1000U/ml), IL-12 (10ng/ml) and PMA (50ng/ml) + Ionomycin (1µg/ml). B) Summary of the frequency of cytokine producing NK cells in the spleen (top) and LNs (bottom) of AL and CR mice. C) Splenic lymphocytes were gated NK1.1^{+} CD3^{-} CD127^{+} and analyzed for cytokine production and cytotoxicity. Histograms of TNF-α and GM-CSF production following IL-2 + IL-12 stimulation (top) and granzyme B and CD107a (bottom) staining following stimulation with YAC-1 cells (10:1 E:T ratio) in CD127^{+} NK cells from CR and AL mice. Filled grey histogram represents AL, solid line represents CR, and dotted line represents cells with no stimulation (NS) from CR mice. Data are mean ± SEM, * indicates significance, $p<0.05$ (n=5 mice/group/experiment). Flow plots and histograms are representative and contain the percentage of NK cells positive for the indicated gates. Experiments were repeated twice.

NK cell subset distribution is sensitive to energy intake

In mice, upregulation of CD11b on NK cells is associated with functional maturity; however Hayakawa et al. have proposed that CD11b^{+} NK cells can be divided into functional subsets based on expression of CD27. We found that terminally differentiated NK cells (CD27^{-} CD11b^{+}) made up a significantly smaller portion of NK cells in both the spleen and BM of CR mice (**Figure 13A**), although terminally differentiated NK cells are found at a relatively low frequency in the BM (Figure 13A). Immature NK cells (CD11b^{-}) and NK cells coexpressing CD27 and CD11b (double positive or DP) represented a larger portion of the total NK cell pool in CR mice compared to AL mice (Figure 13A). To determine whether immature and DP NK cells were actually increased or whether this was due to a decrease in mature NK cells, we compared the frequency of NK cell subsets out of total splenocytes we found a two-fold reduction in the frequency of DP NK cells and a four-fold reduction in CD27^{-} CD11b^{+} NK cells in the spleen of CR mice (**Figure 13B**). Comparison of the frequency of CD27^{-} CD11b^{-} (double

negative or DN) or $CD27^{+}$ $CD11b^{-}$ NK cells among total splenocytes revealed no differences between CR and AL. Upon assessing the frequency of NK cell subsets in the BM relative to total cells harvested, we found $CD27^{+}$ $CD11b^{-}$ and DP NK cells were increased in CR mice. This finding can be extrapolated to the observed increased frequency of NK cells in the BM of CR mice (Figure 13A) which is due to an increase in $CD27^{+}$ $CD11b^{-}$ and DP NK cells. These data suggest that while modest changes to NK cells exist within the BM of CR mice, the majority of differences present in CR mice are found in peripheral NK cell tissues such as the spleen.

CR results in differential expression of T-bet and Eomes in NK cells

While the precise molecular mechanisms that regulate NK cell maturation remain to be elucidated, it has been suggested that terminal maturation of NK cells is at least partially dependent on the TFs Eomes, and T-bet [350]. In order to understand whether changes in NK cell maturation were related to altered TF expression, we analyzed the expression pattern of T-bet and Eomes within splenic NK cell subsets from CR and AL mice (**Figure 13C).** With respect to Eomes, we found fewer DN, $CD27^{+}$ $CD11b^{-}$, and DP NK cells from CR mice expressed Eomes when compared to the corresponding NK cell subsets from AL mice (Figure 13C). There was no difference in the expression of T-bet when comparing $CD27^{+}$ $CD11b^{-}$ or DP NK cells from CR or AL mice. However, we detected DN and $CD27^{-}$ $CD11b^{+}$ NK cells from CR mice expressed significantly lower levels of T-bet than $CD27^{-}$ $CD11b^{+}$ NK cells from AL mice (Figure 13C). Finally, after finding $CD27^{-}$ $CD11b^{+}$ NK cells expressed altered levels of T-bet, we compared the frequency of $CD27^{-}$ $CD11b^{+}$ NK cells from AL and CR mice

expressing terminal maturation markers KLRG1 and CD43 (**Figure 13D)**, as the upregulation of these markers is thought to be at least partially dependent on T-bet expression. Consistent with reduced T-bet expression, both CD43 and KLRG1 were decreased on $CD27^{-}$ $CD11b^{+}$ NK cells from CR mice suggesting terminal differentiation of NK cells in CR mice is incomplete.

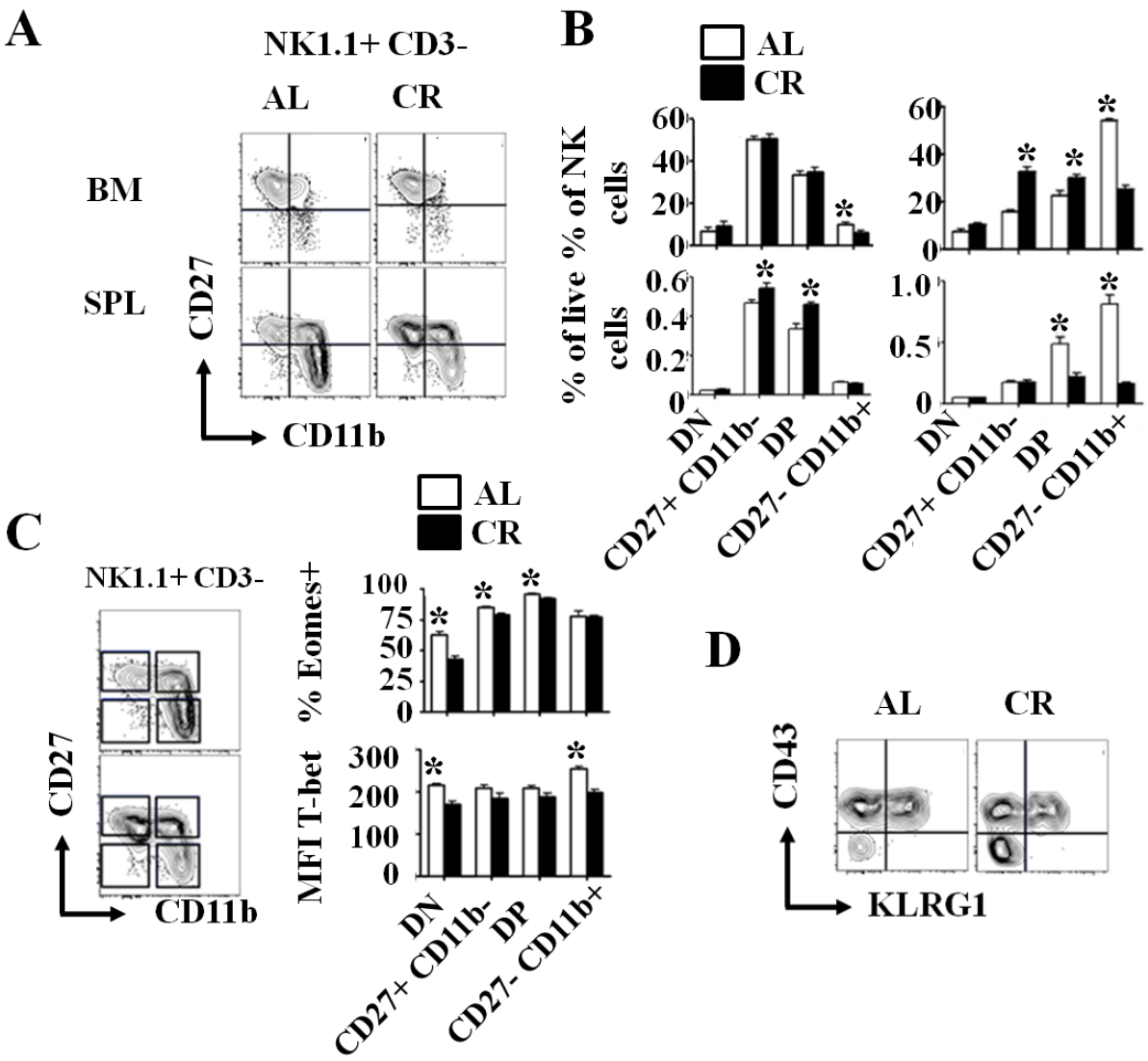

Figure 13: Altered distribution of NK cell subsets in the BM and spleen of CR mice. A) Distribution of NK cell ($NK1.1^{+}$ $CD3^{-}$) subsets based on expression of CD27 and CD11b in the BM (top) and spleens (bottom) of AL and CR mice. B) Summary of the frequency of NK cells in each subset both as a percentage of NK cells (top) and as a percentage of total cells

(Figure 13 cont'd) recovered (bottom). NK cell subsets were defined as $CD27^-$ $CD11b^-$ (DN), $CD27^+$ $CD11b^-$, $CD27^+$ $CD11b^+$ (DP), and $CD27^-$ $CD11b^+$. C) Gating strategy for TF analysis (left) and summary of TF expression in splenic NK cells from AL and CR mice (right). D) Expression of KLRG1 and CD43 on splenic $CD27^-$ $CD11b^+$ NK cells from AL and CR mice. Data are mean ± SEM, * indicates significance $p<0.05$ (n=4-5 mice/group/experiment). Flow plots are representative and contain the percentage of NK cells positive for the indicated gates. Experiments were repeated twice.

NK cells from CR mice have altered functional responses

We found NK cells from CR mice to be phenotypically immature and have altered distribution of functional subsets, and therefore began a series of experiments to determine if these cells were also functionally immature. We found that BM NK cells from CR and AL mice were equally capable of IFN-γ production, although this trended to be lower in CR mice (**Figure 14A**), possibly due to the decrease in $CD27^-$ $CD11b^+$ NK cells in the CR BM (Figure 14A). On the other hand, stimulation of splenic NK cells with IL-2 + IL-12 or anti-NK1.1 resulted in significantly fewer NK cells producing IFN-γ from CR mice (**Figure 14A-B**). In order to determine whether the observed changes in NK cell IFN-γ production were due to a functional impairment or simply because of altered distribution of NK cell subsets, we compared IFN-γ production by $CD27^+$ $CD11b^-$, DP, and $CD27^-$ $CD11b^+$ NK cells from CR and AL mice following stimulation known to elicit IFN-γ production by mature NK cells (IL-2 + IL-12) (Figure 14A) [354]. We observed no difference in IFN-γ production between NK cell subsets from AL and CR mice, suggesting the observed changes in IFN-γ production are due to

alterations in NK cell subset distribution. NK cells from CR mice stimulated with plate bound anti-NK1.1 or anti-NKp46 degranulated at a lower frequency than NK cells from AL mice, as defined by reduced surface CD107a (**Figure 14B-C**). Similarly, granzyme B production was significantly diminished in NK cells from CR mice following activation receptor ligation by antibodies (Figure 14B-C). Interestingly, we found stimulation with YAC-1 cells resulted in increased surface CD107a and IFN-γ by NK cells from CR mice (**Figure 14D**) as well as enhanced CD69 expression (data not shown) compared to NK cells from AL mice. While the use of anti-NK1.1 or anti-NKp46 to activate NK cells resulted in a decreased frequency of NK cells from CR mice producing granzyme B, we found no difference in granzyme B production between CR and AL NK cells following YAC-1 stimulation (**Figure 14D**). Taken together, our data suggests CR influences the distribution of NK cell subsets and results in NK cells that are less functional following stimulation with cytokines or plate bound antibodies, but retain high levels of responsiveness when faced with target cells.

CR results in functional changes to CD27$^-$ CD11b$^+$ NK cells

While IFN-γ production by NK cell subsets was comparable following cytokine stimulation, we observed NK cells from CR mice responded robustly to YAC-1 cells *in vitro* (Figure 14D) and asked whether our any of our previous observations, such as decreased expression of markers of terminal maturation on CD27$^-$ CD11b$^+$ NK cells from CR mice (Figure 13F) was related to increased responsiveness. We stimulated NK cells from AL and CR mice with YAC-1 cells and measured the functionality of individual NK cell subsets (**Figure 14E**). The function of CD27$^+$ CD11b$^-$ and DP NK cells was comparable between AL and CR mice; however CD27$^-$ CD11b$^+$

NK cells from CR mice produced granzyme B and degranulated at a higher frequency than $CD27^{-}$ $CD11b^{+}$ NK cells from AL controls (Figure 14E). IFN-γ production was not different between any of the NK cell subsets analyzed following YAC-1 stimulation (data not shown), suggesting the increase in IFN-γ^{+} NK cells from CR mice was related to changes in the distribution of NK cell subsets defined using expression of CD27 and CD11b.

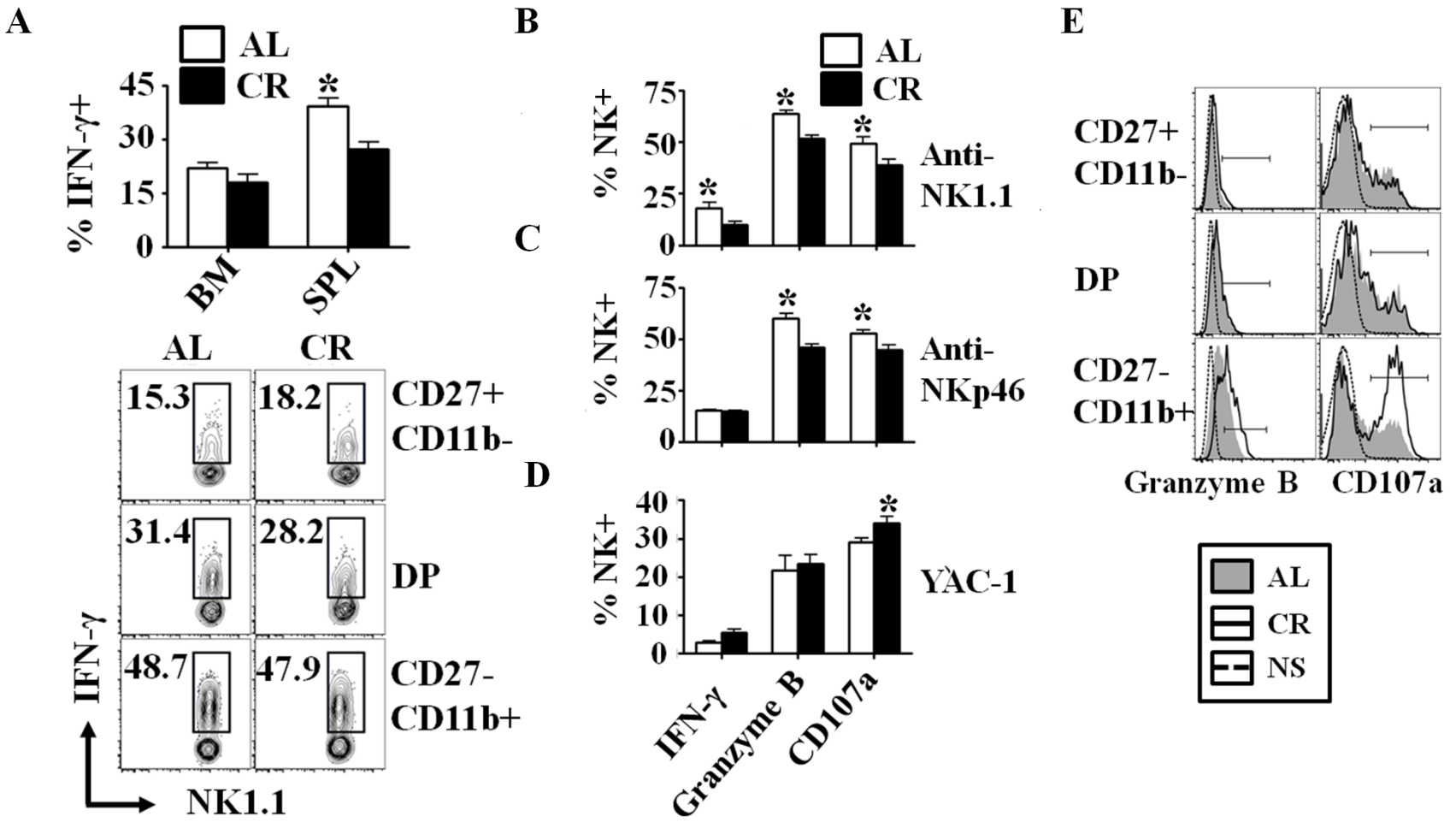

Figure 14: Function of NK cells from CR mice is altered after interrogation with various stimuli. A) IFN-γ production by BM and splenic NK cells ($NK1.1^{+}$ $CD3^{-}$) stimulated with IL-2 (1000U/ml) + IL-12 (50ng/ml) (top) and the frequency of splenic NK cell subsets producing IFN-γ from AL and CR mice (bottom). B-D) Splenic NK cells from AL and CR mice were stimulated with (B)

(Figure 14 cont'd) anti-NK1.1 (25µg/ml), (C) anti-NKp46 (15µg/ml), and (D) YAC-1 cells (10:1 E:T ratio) and $DX5^+$ $CD3^-$ cells were analyzed for production of IFN-γ, granzyme B, and surface CD107a. E) Histograms representing granzyme B and CD107a staining in NK cell subsets following stimulation of splenic NK cells with YAC-1 cells. NK cells from AL and CR mice were gated $DX5^+$ $CD3^-$ and the indicated NK cell subset was analyzed for granzyme B or CD107a expression. Filled grey histogram represents AL, solid line represents CR, and dotted line represents cells from CR mice that received no stimulation (NS). Data are mean ± SEM, * indicates significance, $p<0.05$. (n=5 mice/group/experiment). Flow plots and histograms are representative and contain the percentage of NK cells positive for the indicated gates. Experiments were repeated twice.

5.5 DISCUSSION:

It has been put forth that NK cells are sensitive to dietary manipulation; excessive and restricted energy intake, alcohol consumption, vitamins and minerals as well as bioactive food components have all been suggested to influence NK cell cytotoxicity or NK cell development [224, 355-362]. However, detailed analysis of how dietary manipulation influences NK cell function and homeostasis is limited. Although CR has repeatedly been demonstrated to have beneficial effects on T cell senescence, the implications of CR on innate immunity and NK cell homeostasis have been understudied. Prompted by our observation that NK cells are reduced in frequency and numbers in most peripheral tissues of CR mice, we further investigated functional and developmental changes to the NK cell pool in adult CR mice. In addition to reduced frequency of total NK cells, our results suggest that generation of NK cells in the BM is relatively unimpaired in CR, while the generation and/or maintenance of NK cells in peripheral tissues such as the spleen appears most affected. Furthermore, using CD27 and CD11b to classify NK cell subsets, our data indicate CR mainly influences the homeostasis of mature NK

cell subsets in mice, as DN and $CD27^+$ $CD11b^-$ NK cells represented a comparable fraction of total splenocytes, but $CD11b^+$ NK cells were significantly reduced.

In contrast to reduced mature NK cells, we observed an increased frequency $CD127^+$ NK cells not only in LNs of CR mice, but also in the spleen and BM of CR mice, suggesting that $CD127^+$ NK cells compose a larger portion of the NK cell pool in CR mice. Because we observed comparable numbers of $CD127^+$ NK cells between AL and CR mice, we hypothesize that $CD127^+$ NK cell output is not impaired in CR while classical mature NK cell development is impaired, resulting in a greater frequency of NK cells being $CD127^+$. $CD127^+$ NK cells are normally recognized as having poor cytolytic potential but high pro-inflammatory cytokine production [101], thus we were not surprised to find NK cells from the LNs and spleens of CR mice produced TNF-α and GM-CSF at a higher frequency than NK cells from AL mice. However, when we compared the capacity of $CD127^+$ NK cells from AL mice and CR mice to produce TNF-α and GM-CSF we consistently observed higher production of these cytokines by $CD127^+$ NK cells from CR mice. The unique functional characteristics of $CD127^+$ NK cells have been attributed to their limited Ly49 receptor repertoire [101]; however we did not detect any differences in the frequency of splenic $CD127^+$ NK cells expressing Ly49C/I/F/H. On the other hand, we show DX5 and CD11b are expressed at higher levels on $CD127^+$ NK cells from CR mice; tempting us to speculate that perhaps increased cytokine production is related to a more mature phenotype of these cells. Alternatively, BM $CD127^+$ NK cells from CR mice

expressed higher levels of Ly49 receptors, possibly acquiring increased functional competence early in development.

In this study, we show NK cells from CR mice are impaired in their ability to respond to stimulation through cytokine and activation receptors; however we also demonstrate that NK cells from CR mice respond more robustly to YAC-1 cells than NK cells from AL mice. The observation that NK cells may harbor an immature phenotype yet retain cytotoxicity against YAC-1 cells has been reported previously [103, 115, 116], suggesting that cytotoxicity is acquired at an early stages of NK cell development [116]. Furthermore, the hyperresponsiveness of CR NK cells to YAC-1 cells is at least partially related to the increased frequency of DP NK cells in CR mice, as these cells are known to respond more robustly to YAC-1 cells, have a lower activation threshold, and exhibit cytotoxicity against YAC-1 cells though both NKG2D-dependent and independent mechanisms [10]. We also observed enhanced responsiveness to YAC-1 cells in the $CD27^{-}$ $CD11b^{+}$ NK cell subset from CR mice compared to AL mice. This phenomenon appears limited to NK cell activation mediated through cell-cell interactions, as we did not observe differences between the function of $CD27^{-}$ $CD11b^{+}$ NK cells from AL and CR mice after stimulation with cytokines or antibodies against major activating receptors. Investigation of potential causes for this observation revealed lower KLRG1 expression on $CD27^{-}$ $CD11b^{+}$ NK cells from CR mice, which is often associated with hyporesponsive NK cells [100, 363]. Thus, it is likely the increased responsiveness of CR NK cells to YAC-1 cells is due to both an increased frequency of DP NK cells as well as increased cell-cell responsiveness of $CD27^{-}$ $CD11b^{+}$ NK cells.

Little is known about the molecular events required for acquisition of KLRG1 and down regulation of CD27 on $CD11b^+$ NK cells in the periphery; however this process is thought to be mediated through coordinated expression of several TFs [364, 365]. NK cell development and homeostasis relies on numerous transcription factors such as Id2, IRF-2, Eomes, T-Bet, GATA-3, Blimp1, and E4bp4 [350, 364]. It has been suggested that terminal maturation of NK cells takes place in the spleen in a T-bet dependent manner, with upregulation of KLRG1 and CD43 being severely impaired in T-bet deficient (*tbx21-/-)* mice, while Eomes appears to play an opposite role by promoting downregulation of markers associated with immature NK cells [350, 365]. In this study we investigated whether T-bet or Eomes deficiencies in NK cells from CR mice could explain the observed reduction in terminally mature NK cells. We found T-bet to be expressed at lower levels in DN, and importantly $CD27^-$ $CD11b^+$ NK cells. Based on this finding, we further analyzed the surface phenotype of $CD27^-$ $CD11b^+$ NK cells in CR mice and found that significantly fewer $CD27^-$ $CD11b^+$ NK cells expressed CD43 and KLRG1. Our data suggest dietary regimes, such as CR, can result in altered expression of TFs important for NK cell maturation such as Eomes and T-bet possibly resulting in changes to NK cell phenotype and function.

Recent studies have highlighted the central role of the energy-sensitive serine/threonine kinase, mTOR in regulating the expression of TFs such as Eomes and T-bet in T cells. Inhibition of mTOR by treatment of $CD8^+$ T cells with a CR mimetic, rapamycin, results in the inhibition of IL-12 induced T-bet expression, suggesting a direct relationship between energy status and development of lymphocytes into effector subtypes [366]. However, while becoming increasingly established in T cells, the relationship between metabolism and NK cell maturation

and function is somewhat less well understood. Leptin, a cytokine involved in the upstream activation of phosphoinositide 3 kinase (PI3K) and Akt, was shown here to be reduced in CR mice [65, 367]. Importantly, NK cells express the leptin receptor (CD295/ObR), and leptin plays an important role in maintaining NK cell numbers, making our observations pertaining to decreased leptin in CR mice a potential candidate through which some of the immunomodulatory effects of CR are mediated [211, 213]. In NK cells, inhibition of PI3K signaling, a kinase with a central role in the integration of metabolic signals upstream of mTOR, results in phenotypic and functional changes to NK cells, some of which are similar to the results reported here in our model of CR such as high expression of CD127 and reduced terminal maturation [368, 369].

In the BM and periphery IL-15 has been firmly established to play a critical role in the generation and maintenance of NK cells [370]. Mice deficient in IL-15 have few detectable NK cells and IL-15 signaling regulates NK cell development in the BM and NK cell homeostasis and terminal maturation in the periphery [371]. Because we observed a significant reduction in NK cells in CR mice, one hypothesis is that CR may result in reduced levels of IL-15 *trans*-presentation. This is supported by the observation that CR results in apoptosis of senescent memory T cells in aged mice, which are thought to be dependent on IL-7 and IL-15 [371-373]. However, we observed no difference IL-15Rα levels on splenic monocytes from CR mice (Gardner, unpublished observation) and did not observe a significant reduction in NK cells in the BM, resulting in inconclusive findings about the implications of CR on IL-15 production or *trans*-presentation. Furthermore, CR reduces chronic inflammation that arises during aging or in models of chronic inflammation through reducing production of inflammatory cytokines such as C reactive protein, IL-6, TNF-α, and leptin, as well as increasing circulating GCs [374]. Overall these changes result in a significantly altered cytokine milieu *in vivo*, leading us to hypothesize

that the changes to NK cells observed are due to a multitude of adaptations to the CR homeostatic environment, confounding the isolation of a single specific cause.

It should be noted that the tissues we observed decreases in NK cell frequency in CR mice generally house NK cells that have made significant maturational progress. For example, in AL mice**,** tissues such as the lungs and blood house mostly terminally differentiated $CD27^{-}$ $CD11b^{+}$ NK cells [10], a subset of NK cells which we show to be significantly reduced in CR mice. This suggests the lack of mature NK cells in CR mice contributes greatly to the decreased frequency of NK cells observed throughout the body of CR mice. The inability of NK cells to robustly populate the lungs and blood of CR mice could also be related to decreased expression of chemokine receptors, as CR has been previously shown to influence chemokine receptor expression on T cells [375]. Among these chemokine receptors, S1P5 regulates emigration of NK cells from BM sinusoids in a T-bet dependent manner; suggesting reduced T-bet expression may be the cause for increased BM NK cells in CR mice. However, we do not believe this to be the case, as we only observed decreased T-bet expression in DN and $CD27^{-}$ $CD11b^{+}$ NK cells, rather than $CD27^{+}$ $CD11b^{-}$ or DP NK cells, the likely candidates for BM egress. Furthermore, we do not believe impaired emigration from the BM of CR mice can completely explain the decreased frequency of NK cells in peripheral tissues of CR mice, as the increased frequency of NK cells in the BM did not result in differences between the total number of NK cells recovered from the femurs of CR and AL mice.

Caloric restriction without malnutrition is a dietary intervention used in both gerontological and oncological research, yet CR protocols are varied, despite attempts at unifying and standardizing the dietary intervention [249]. However, we employed the best documented and

studied CR protocols, established by the NIA, which has been repeatedly shown to increase the lifespan of laboratory animals when initiated early in life [341]. Furthermore, our studies in young adult CR mice allow us to study the effect of CR on NK cells independent of aging. We and others have found adult mice subjected to CR early in life suffer increased susceptibility to pathogens, thus raising the question of whether CR is only useful in a laboratory setting [4, 246, 283, 287, 315, 318, 376]. Infection via these pathogens results in substantial weight loss, which could be detrimental to CR mice because of limited energy reserves [320]. However, it is also plausible that CR initiated before adulthood results in immunological changes such as those presented here, that increase susceptibility to specific pathogens, limiting the usefulness of this intervention in humans. However, future studies are required to determine if this is specific for respiratory viruses as we have shown**,** or whether other viral infections such as herpes simplex virus-1, mousepox, and MCMV, pose a greater threat to CR mice as well [346, 377]. Our study utilizes dietary manipulation in a mouse model lead us to wonder if CR has a similar effect on NK cells in humans. Indeed, the NIA has begun a series of human trials in order to determine the efficacy of CR in humans; this study, known as CALERIE, should allow for further study of the influence of CR on immune function in humans [378]. Furthermore, because CR is designed as a dietary intervention to delay aging, it will be interesting to determine whether any age related changes in NK cell phenotype that occur in mice are ameliorated or exacerbated by CR in humans and mice [151, 377].

CHAPTER 6:

THREE WEEK REFEEDING OF PREVIOUSLY CALORICALLY RESTRICTED MICE RESTORES PERIPHERAL NK CELL HOMEOSTASIS

Clinthorne JF, Duriancik DM, Roman BE, Gardner EM

6.1 ABSTRACT:

Natural killer cells are a heterogeneous population of innate lymphocytes with the ability to produce cytokines or lyse target cells without prior antigen sensitization. We have previously reported NK cells from 6 mo C57Bl/6 CR mice display a less mature phenotype and are less capable of producing IFN-γ upon stimulation with cytokines. In the present study, we examine whether refeeding of CR mice restores NK cell phenotype and function to levels observed in ad-libitum (AL) fed mice. We demonstrate that while CD11b expression is restored within 10 days of refeeding, restoration of terminally mNK cell subsets requires three weeks of refeeding. Furthermore, we show NK cells from refed (RF) are more resistant to apoptosis and are proliferating at a higher frequency than NK cells from CR mice. NK cell function is restored in 3 week RF mice, such that IFN-γ production is comparable to NK cells from AL mice following NK cell activation with cytokines. Because we previously reported increased susceptibility of CR mice to influenza virus, we also assessed whether NK cells from RF mice exhibited enhanced functional responses to influenza infection than NK cells from CR mice. Following influenza infection, NK cells from RF mice exhibit increased IL-22 production and decreased TNF-α production when compared with NK cells from CR mice.

6.2 INTRODUCTION:

Caloric restriction is a dietary intervention that extends the lifespan of laboratory animals [341]. One of the mechanisms by which CR extends lifespan is through altering immune function; T and B cell development and function and the function of innate immune cells can be influenced by CR [6]. Furthermore, there is a decrease in the incidence of cardiac, kidney, or nervous system dysfunction in aged laboratory animals on a CR diet [325, 341-343]. When examined independent of aging, CR has been found to decrease the severity of autoimmune disease [2, 244], suggesting that CR has the potential to suppress immune responses. In agreement with this hypothesis, we have shown CR of young mice results in increased susceptibility to primary influenza infection and decreased influenza induced NK cell cytotoxicity in the lungs [4, 11]. Others have reported similar findings, CR mice appear more susceptible to various bacterial and parasitic infections [314], although the specific immunological causes are difficult to identify due to the numerous physiological systems influenced by CR. However, in our studies, increased susceptibility to influenza infection was accompanied by decreased NK cell numbers and frequency in the lungs of CR mice both before and during influenza infection [11]. Overall, these findings have raised concerns about on the effects of CR on innate immunity, and may predispose CR individuals to suffer more severe primary infections [315, 318].

Natural killer cells are responsible for recognizing altered self-cells such as malignant cells or virally infected cells [346]. Through a well defined process NK cells develop in the BM and signals from stromal cells and cytokines result in the differentiation of common lymphoid progenitors into NK cell precursors [8, 81]. The earliest stages of NK cell commitment are identified by the upregulation of the shared IL-2/IL15Rβ chain (CD122), followed by acquisition

of the NK cell marker NK1.1 in B6 mice and lack of the CD3 molecule [8, 88]. Programmed expression of surface molecules including integrins, cytokine receptors, and a family of NK cell receptors takes place during NK cell maturation, resulting in the development of early mNK cells expressing CD49b (DX5) in the BM [98, 108, 348, 349]. Following acquisition of DX5, NK cells undergo rapid proliferation in the BM and upregulate CD11b and CD43, which correlates with the capability of an NK cell to produce IFN-γ and elicit other functional responses [379]. Mature NK cells then egress from the BM and seed various lymphoid and nonlymphoid peripheral tissues, with the majority of peripheral NK cells expressing high levels of the maturation markers CD11b and CD43 [8, 77].

Expression of CD27 can been used to separate the peripheral $DX5^+$ NK cell pool into subsets in which there is a step-wise progression from $CD27^+$ $CD11b^-$ early mNK cells to $CD27^+$ $CD11b^+$ NK cells followed by development into terminally mature $CD27^-$ $CD11b^+$ NK cells [10, 98]. Terminally mNK cells then upregulate KLRG1 [99], currently the best known marker of terminal NK cell maturation. Among the TFs known to influence NK cell development, a role for T-bet, Eomes, Blimp-1, IRF-2, and GATA-3 has been identified to be important in the generation of terminally differentiated, $CD43^+$ $KLRG1^+$ NK cells [101, 115, 350, 351].

Because of the known role of NK cells in protecting the host from viral infections [155], including influenza [149], our previous studies examined whether NK cells from CR mice exhibited an altered phenotype or altered function. During the course of these studies we demonstrated that NK cells from CR mice were less mature, and terminally differentiated $CD27^-$ $CD11b^+$ NK cells were reduced in frequency and number in peripheral tissues. The immature

phenotype of NK cells from CR mice was reflected in the reduced ability of the NK cell pool to produce IFN-γ following cytokine stimulation, leading us to conclude that classical mature BM derived NK cells were reduced in the periphery by a CR diet. Based on the aforementioned studies, we sought to determine whether NK cell development and function could be restored by allowing CR mice unlimited access to NIH-31 diet. In agreement with our previous studies, mice rapidly regained body weight and body composition was normalized shortly thereafter. Interestingly, NK cell frequency in the spleen increased to levels over that found in AL mice after 10 days of refeeding, however, the maturation status of the splenic NK cell pool was not restored until after 20 days of refeeding. The ability of NK cells to traffic to the lung following refeeding was also examined and we observed that NK cell frequency had normalized in the lungs of RF mice after 20 days refeeding, but not after 10 days.

6.3 MATERIALS AND METHODS:

Mice and diets

Specific pathogen-free young adult (6 mo) AL and young adult (6 mo) CR male C57Bl/6 mice were purchased from the NIA colony maintained by Charles River Laboratories (Wilmington, MA). The animal use protocol for this study was approved by Michigan State University Institutional Animal Care and Use Committee. Upon arrival, mice were housed individually in micro-isolator cages in the AAALAC-accredited containment facility at Michigan State University and were acclimated at least 7 days prior to the initiation of each experiment. Both CR (NIH-31/NIA-fortified) and AL (NIH-31) diets were purchased from the NIA, the compositions of which have been reported in detail previously [11]. The composition of the CR diet is sufficient in micronutrients, but results in restriction of total energy intake. The CR

regimen initiated by the NIA is designed to gradually achieve 40% restriction in mice by 4 mo, such that they are weight stable upon arrival at 6 mo.

Body composition, food intake and adipokine concentrations

Body composition (body fat percentage) was determined by daily magnetic resonance imaging (MRI) during the feeding protocol. Mice were individually housed allowing food intake to be recorded each day. All mice were weighed daily between 08:00-09:00, after which they were fed. The protocol to assess body composition using the EchoMRI-500 (Echo Medical Systems) has been validated and described in detail previously [323]. Prior to collection of data, a daily calibration is performed using a rapeseed oil standard, after which individual mice are placed in an MRI holding tube and the MRI performed. The advantages of this system are that it is rapid, allows for repeated measurements, and does not require anesthesia, enabling mice to recover immediately after MRI. Serum concentrations of corticosterone and leptin were quantified by commercially available ELISA kits according to the manufacturer's instructions (Assaypro, R&D, respectively). Plates were read at 450 nm wavelength using a Synergy HT plate reader (Bio-Tek) and concentrations were determined using a standard curve for each respective assay.

Lymphocyte isolation

Following euthanasia, blood was collected by cardiac puncture into heparinized syringes. Following cardiac puncture, spleens, and lungs and femurs were excised. Isolation of mononuclear cells from spleens and lungs has been previously described [323]. Briefly, single cell suspensions were obtained from spleens using homogenization. BM cells were isolated from the femur and tibia by flushing with a 25 5/8 needle and syringe containing RPMI 1640. Lungs

were excised, weighed and minced using a Miltenyi GentleMACs system. Cells were then incubated for 30 minutes at 37^{o} C in RPMI 1640 containing 5% FBS, 1mg/mL collaganase D (Roche, Indianapolis, IN, USA) and 80 Kuntz Units DNAse (Roche). The resulting cell suspensions were lysed of RBCs using an ammonium chloride buffer. All cell suspensions were washed in complete media (RPMI 1640 + 10% FBS) and resuspended for counting using Trypan Blue viability dye.

Flow cytometry

Cells from various tissues were resuspended in FACS buffer (0.1% sodium azide, 1% FBS, in PBS) at a concentration of $2x10^{7}$ cells/mL. $1\text{-}4x10^{6}$ cells were incubated on ice for 10 minutes with anti–CD32/CD16 antibody (2.4G2) (BD Bioscience) in order to block FcγII/III receptor–mediated non-specific binding. Samples were then incubated with a cocktail containing various combinations of the following fluorochrome-conjugated antibodies (eBioscience or BD Bioscience) at optimal concentrations determined in our laboratory: NK1.1 (Allophycocyanin or PE-Cy7), CD3 (Alexa Fluor700 [500A2]), CD27 (PE or PerCP-eFluor710 [LG.7F9]), CD49b (Allophycocyanin or PE-Cy7 [DX5]), and CD11b (PE-Cy7 or V500 [M1/70]). Cells were incubated in staining cocktails on ice in the dark for 30 min. For measurement of *in vivo* proliferation, lineage stained cells were fixed with eBioscience Foxp3 staining kit, permeabilized and incubated with anti-Ki-67 (Alexa Fluor488 [B56]) for 30 minutes. Viable lymphocytes were gated based on light scattering properties, after which NK cells were characterized as $NK1.1^{+}$ $CD3^{-}$ unless otherwise noted. Samples were analyzed using an LSR II flow cytometer (BD

Bioscience) or a FACS Canto II flow cytometer (BD Bioscience) with FlowJo software (Tree Star).

Ex vivo measurement of apoptosis

To induce apoptosis, cells from the indicated tissues were resuspended in single cell suspensions at $1x10^7$ cells/mL in RPMI 1640 for serum starvation and incubated at 37^o C for 6-10 hours [213]. After incubation cells were centrifuged and resuspended in FACS buffer for surface staining. Cells were stained on ice with lineage specific antibodies after which they were washed with Annexin V binding buffer and stained with Annexin V and 7-AAD according to manufacturer's protocol (BD Bioscience). Samples were acquired on a FACS Canto II.

Cytokine production

NK cell capacity to produce IFN-γ was measured using flow cytometry according to previously published methods [151]. Briefly, splenocytes in complete media were added to uncoated 96 well plates and IL-12 (20ng/mL) + IL-18 (40ng/mL) or IL-2 (1000U/mL) + IL-12 (20ng/mL). Cultures were incubated for 8 hours during which brefeldin A was added after the first 60 minutes. Following incubation, cells were stained with lineage specific antibodies then fixed and permeabilized using BD cytofix/cytoperm kits according to manufactures' protocol. Intracellular cytokines were detected using monoclonal antibodies against IFN-γ (FITC or PE-Cy7 [XMG1.2]) or granzyme B (PE [GB11]) (BD Biosciences). Production of IL-22 and TNF-α was measured over the course of influenza infection by incubating lung cells *ex vivo* with PMA 50 ng/mL), Ionomycin (1 µg/mL) for 8 hours during which brefeldin A was added after the first 60 minutes. Cells were then stained with lineage specific antibodies then fixed and permeabilized

using BD cytofix/cytoperm kits according to manufactures' protocol. Intracellular cytokines were detected using monoclonal antibodies against IL-22 (PE [140301]) (R&D Systems) or TNF-α (eFluor450 [MP6-XT22]) (eBiosciences).

Statistics

Statistics were performed using GraphPad Prism 4 software (La Jolla, CA). Values in text are means ± SEM. Body composition, serum adipokines, NK cell frequency, and basal NK cell function were analyzed using a one-way ANOVA to determine statistical significance between diet groups. Cytokine production in response to influenza infection was measured across time using two-way ANOVA to determine statistical significance between diet groups over time. Statistical significance was set at $p<0.05$.

6.4 RESULTS:

Influence of 20 days refeeding on body weight

We have previously shown body weight and body composition were recovered within 14 days of refeeding. Here we show that this restoration remained stable throughout the refeeding period implemented in our studies resulting in mice of weight comparable to AL (**Figure 15A**). Because CR is a nutritional stress, resulting in increased glucocorticoids, we investigated if 20d refeeding would alleviate that stress and return corticosterone levels to normal. Indeed, serum corticosterone levels were significantly higher in CR mice (**Figure 15B**), than AL or 20d RF mice, and no difference was detected between AL and 20d RF. As leptin is a major immunomodulatory adipokine known to influence NK cells, we also measured leptin levels in AL, CR, and 20d RF mice. Interestingly, despite restoration of body weight and normalized

corticosterone levels, we found that leptin levels were greater than CR, but not as high as AL in 20d RF mice (**Figure 15C**).

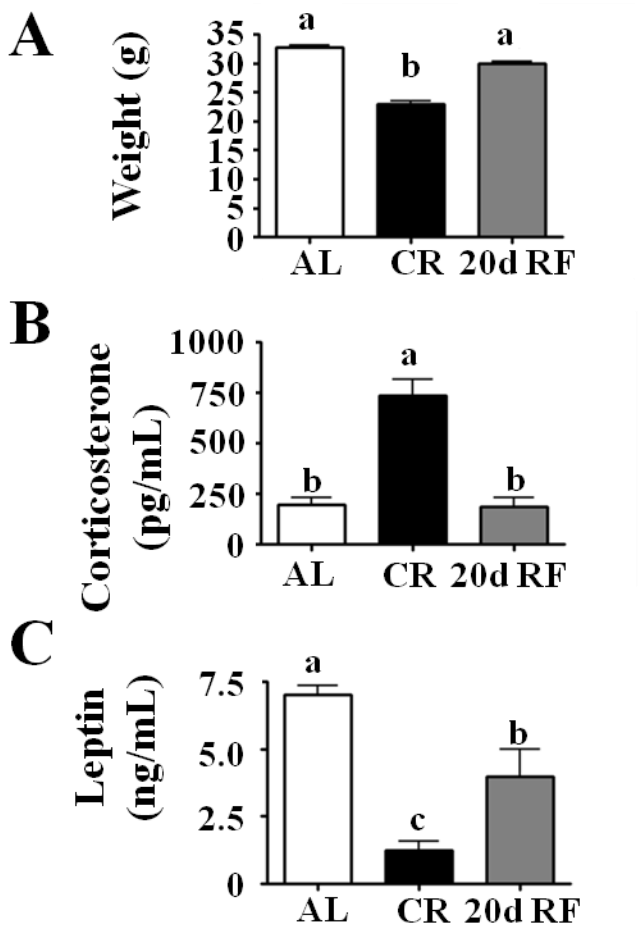

Figure 15: Influence of 20 day refeeding on body weight, corticosterone, and leptin. A) Body weight was measured after 20 days of refeeding and weights were averaged for each group. Serum samples were taken from AL, CR, and 20d RF mice and levels of corticosterone (B) and leptin (C) were determined using ELISA. Data are mean ± SEM. Means without a common letter differ, $p < 0.05$ (n=4-8 mice/group).

Peripheral NK cell frequency is restored by 20 days refeeding of CR mice

Because CR results in fewer NK cells in the spleen, a major source of NK cells in mice, we were interested in whether refeeding would restore NK cell frequency. 10 days of refeeding resulted in spleens that contained more NK cells than was observed in AL or CR mice (**Figure 16A**). However, NK cell percentage in the lungs remained unchanged despite 10 days of refeeding (**Figure 16B**). Therefore, we continued our refeeding paradigm until 20 days and reexamined the NK cell frequencies in both the spleen and lungs and observed NK cells to be restored to levels comparable to AL mice in both tissues.

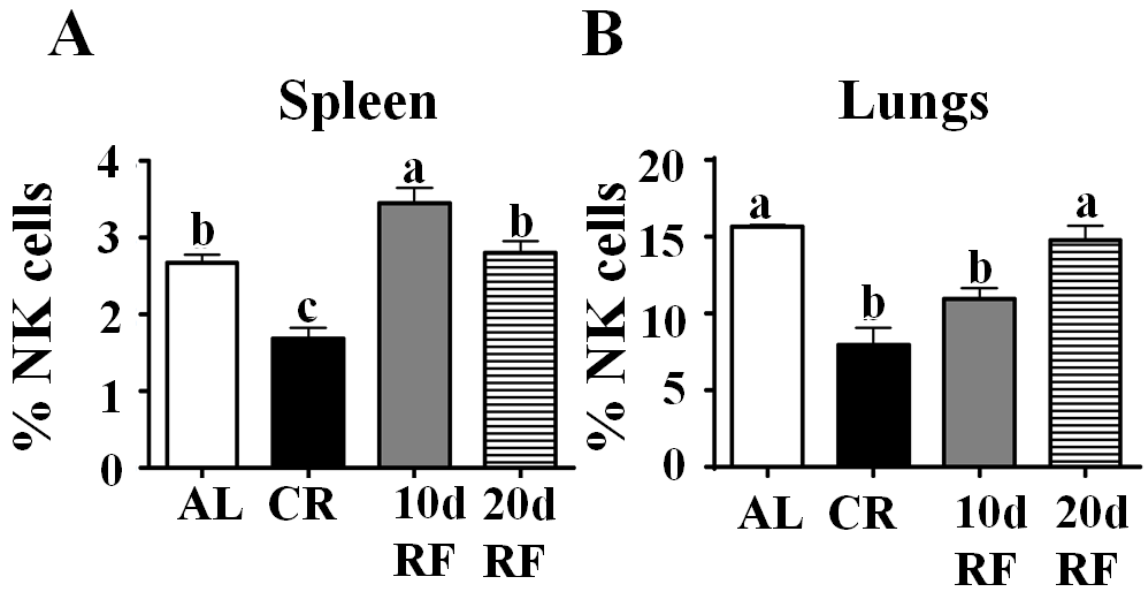

Figure 16: Effect of refeeding on NK cell frequency. NK cell frequency was measured in the spleen (A) and lungs (B) of AL mice, CR mice, or CR mice that were refed (RF) for 10 and 20 days. NK cells were gated $NK1.1^{+}$ $CD3^{-}$. Data are mean ± SEM. Means without a common letter differ, $p < 0.05$ (n=5-8 mice/group).

Refeeding stimulates proliferation of NK cells while reducing CR induced NK cell apoptosis

During the refeeding protocol we examined the rate at which NK cells were proliferating and undergoing apoptosis in AL, CR, and 10d RF mice. Refeeding resulted in a high frequency of NK cells that expressed the Ki-67 antigen in the BM (**Figure 17A**) and fewer NK cells undergoing apoptosis in the periphery (**Figure 17B**). Furthermore, NK cells from CR mice exhibited greater levels of apoptosis than NK cells from AL mice (Figure 17B), a possible explanation for reduced NK cells in peripheral tissues of CR mice.

Terminally differentiated NK cells are restored in the spleen and lungs after 20 days refeeding

Because we previously observed a significant decrease in the frequency of NK cells that were mNK cells in the spleens of CR mice, we investigated the influence of refeeding on NK cell subset distribution in both the spleen and lungs of mice. Consistent with our previous report, the terminally differentiated NK cell pool was significantly reduced in the spleens of CR mice (**Figure 18A**), and for the first time we show that terminally differentiated NK cells are also reduced in the lungs of CR mice. 10 days of refeeding resulted in an increase in $CD27^{+}$ $CD11b^{+}$ NK cells, however the terminally differentiated NK cell pool in the spleen and lungs of 10d RF mice still remained reduced compared to AL mice. As we had determined 20 days of refeeding recovered NK cell frequencies to comparable levels as is observed in AL mice, we investigated whether 20d RF mice also had similar NK cell subset distribution. Indeed, 20 days refeeding restored splenic NK cell subset division to a relatively normal distribution, and importantly, restored the frequency of terminally differentiated NK cells in the lungs of 20d RF mice (**Figure 18B**).

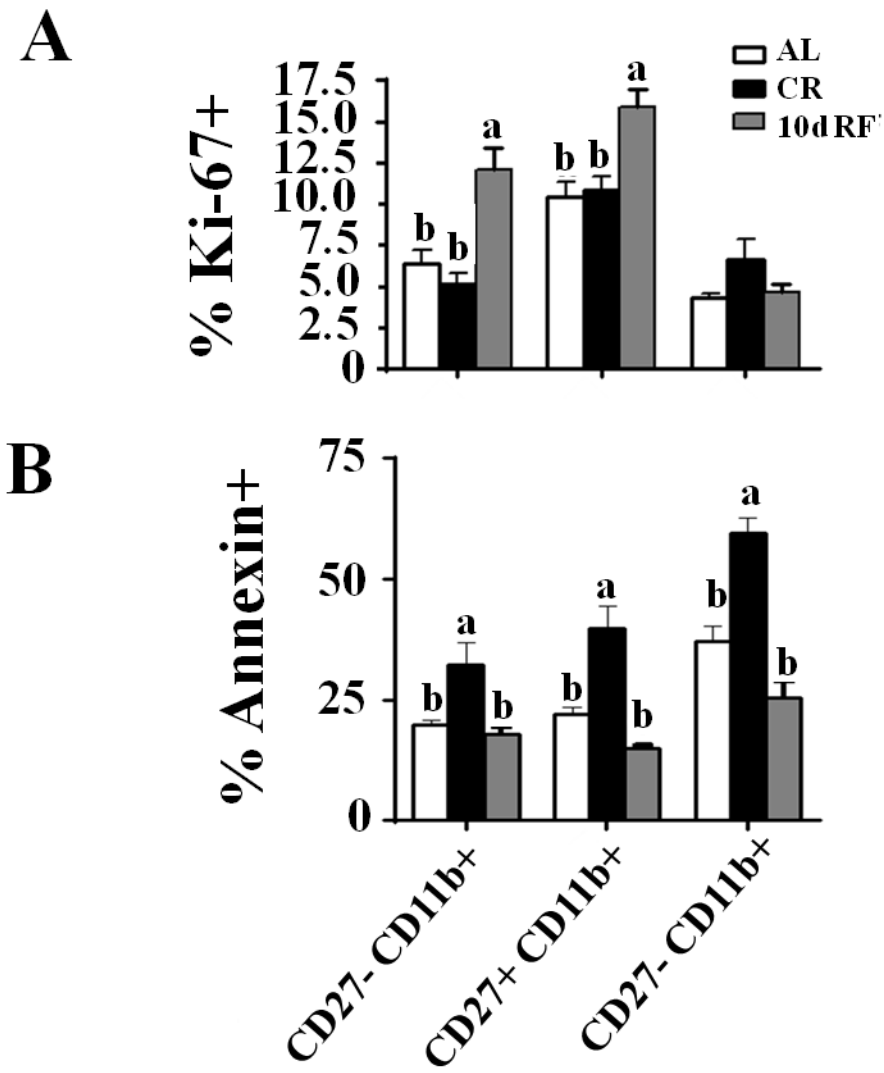

Figure 17: Proliferation and apoptosis of NK cells from AL, CR, and 10d RF mice. The proliferation (A) of BM NK cells ($NK1.1^{+}$ $CD3^{-}$) and apoptosis (B) of splenic NK cells ($NK1.1^{+}$ $CD3^{-}$) was measured in AL, CR, and 10d RF mice. Proliferation was quantified using intracellular detection of Ki-67 while apoptosis was quantified in NK cell subsets by staining cells with Annexin V. Data is mean ± SEM. Means without a common letter differ, $p < 0.05$ (n=4-6 mice/group).

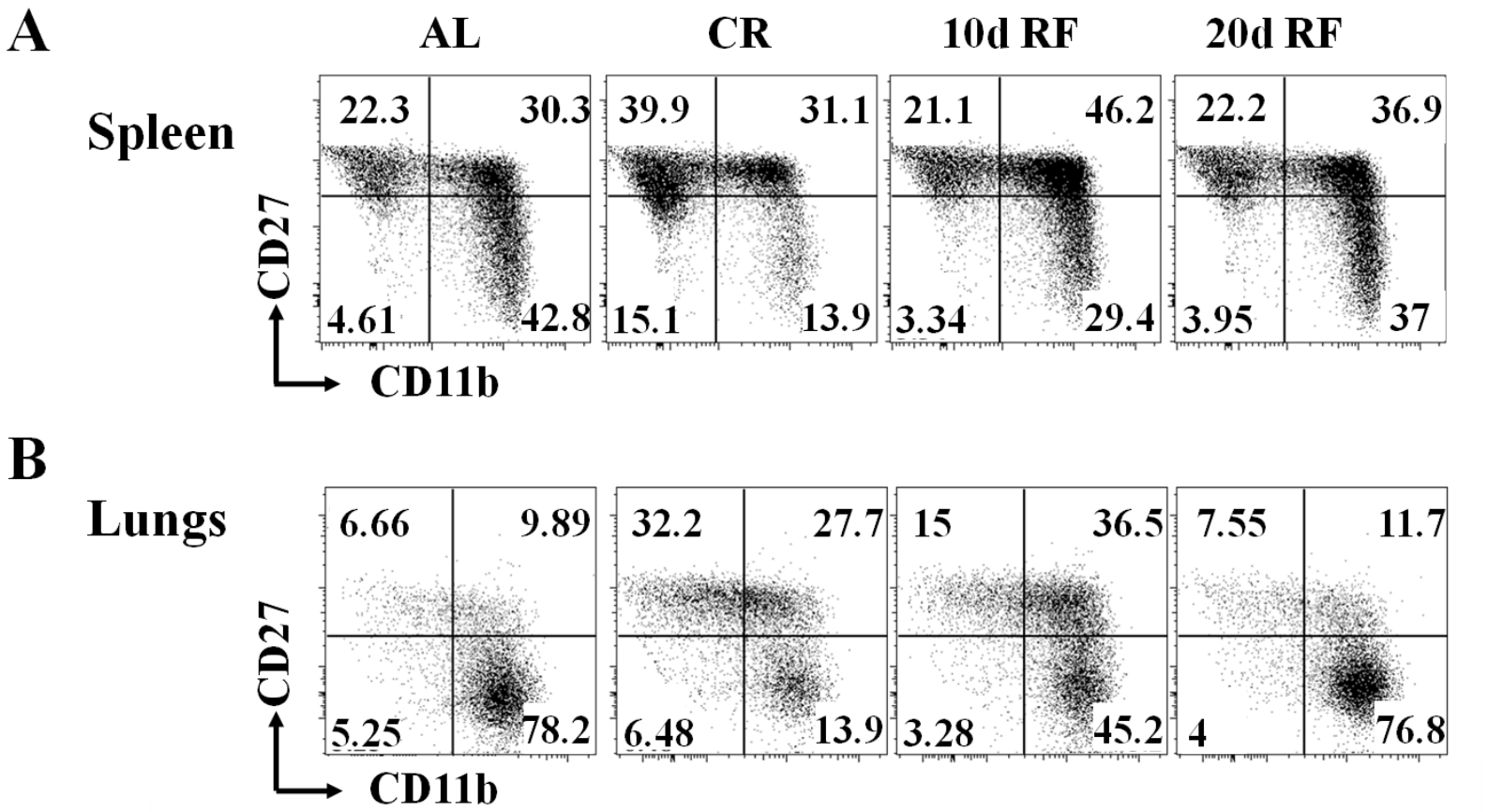

Figure 18: Influence of refeeding on NK cell subset distribution in peripheral tissues. NK cells from AL, CR, or CR mice that were refed for 10 and 20 days were divided into the depicted subsets based on expression of CD27 and CD11b. A) NK cell subsets in

(Figure 18 cont'd) the spleen. B) NK cell subsets in the lungs. NK cells were gated $NK1.1^+$ $CD3^-$. Flow plots are representative. Data are representative of experiments containing 4-8 mice per group.

NK cell function is restored by 20d refeeding

We had previously shown that impaired NK cell function as is observed in young adult CR mice is directly related to the fact that there are fewer mNK cells in the CR NK cell pool [309]. Upon discovering that 20d refeeding restored NK cell subset homeostasis in the spleens of mice, were interested in whether these NK cells were also functionally mature. Therefore, we stimulated NK cells with a variety of stimulants to determine if NK cells from 20d RF mice were functionally mature. Importantly, we discovered that there was a comparable frequency of NK cells that stained positive for IFN-γ between AL and 20d RF mice, a significantly higher frequency than was observed in CR, after stimulation with IL-12 + IL-18 or IL-2 + IL-12 (**Figure 19A-B**). In order to study the cytotoxic capability of NK cells after refeeding, we also investigated whether NK cells from 20d RF mice could produce granzyme B in response to cytokine stimulation. Indeed, refeeding not only resulted in comparable IFN-γ production, but granzyme B production was also restored in NK cells from 20d RF mice (Figure 19A-B)

Terminally mNK cells are cited as the NK cell primarily responsible for IL-22 production in the lungs during influenza infection [119]. Because CR mice had fewer terminally differentiated NK cells in their lungs, we hypothesized they would have impaired IL-22 production during influenza infection. On the other hand, TNF-α is produced primarily by immature and thymic derived NK cells [101], of which CR mice have an increased frequency, leading us to investigate the dichotomy between anti-inflammatory cytokine production and pro-

inflammatory cytokine production by NK cells from AL and CR mice (**Figure 20A-B**). Furthermore, because we found terminally differentiated NK cell frequency was restored in 20d RF mice in both the spleen and lungs, we examined whether influenza induced cytokine production was also normalized by this refeeding. Indeed, NK cells from RF mice produced more IL-22 than NK cells from CR mice during influenza infection and we no longer observed a high frequency of NK cells producing TNF-α during late stages of infection, as was observed in NK cells from CR mice. Thus, it appears that 20d RF mice not only had normalized NK cell phenotype, but also a functional NK cell response to influenza infection that was comparable to NK cells from AL mice.

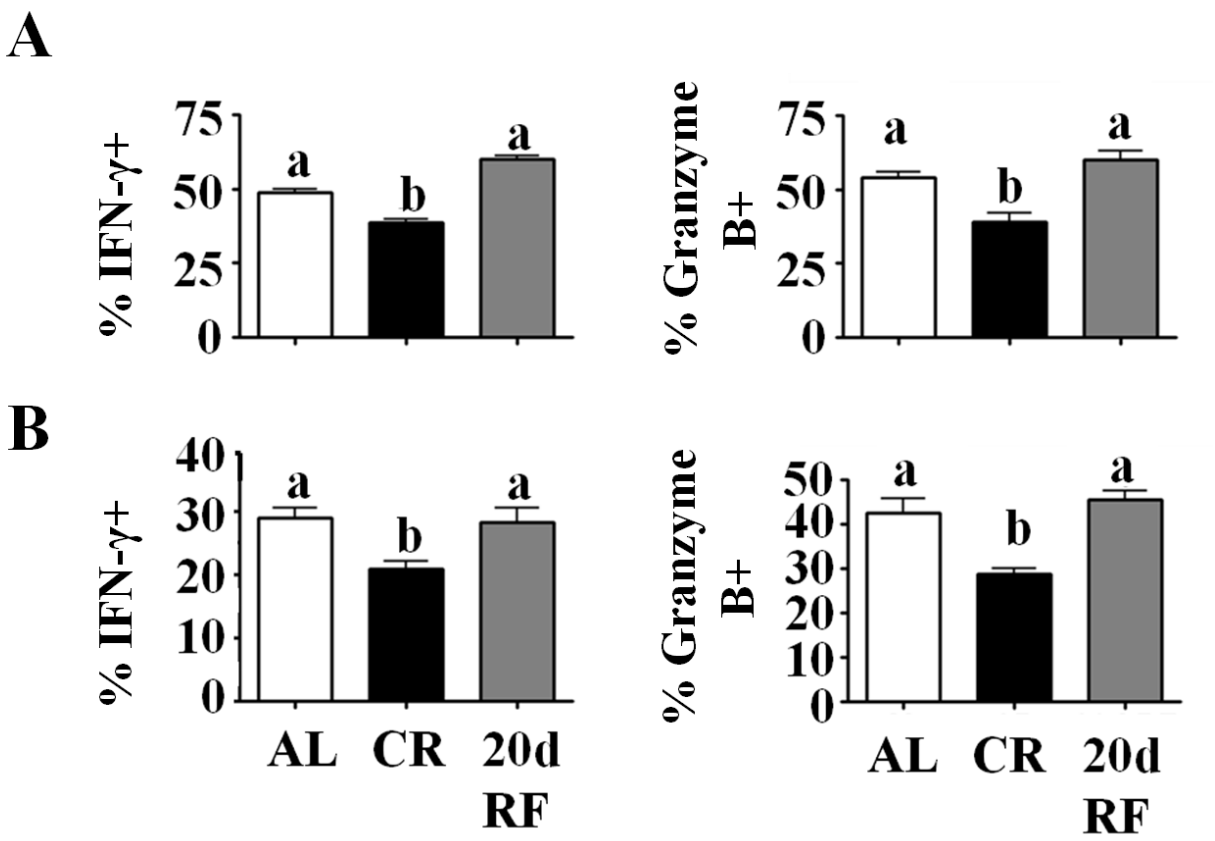

Figure 19: 20d refeeding restores NK cell response to cytokine stimulation. Splenocytes were cultured for 8 hours in IL-12+ IL-18 (A) or IL-2 + IL-12 (B). The frequency of NK cells staining positive for either IFN-γ (left) or granzyme B (right) from AL, CR, and 20d RF mice was determined using flow cytometry. NK cells were gated $NK1.1^{+}$ $CD3^{-}$. Data are mean ± SEM. Means without a common letter differ, $p < 0.05$ (n=4-5 mice/group).

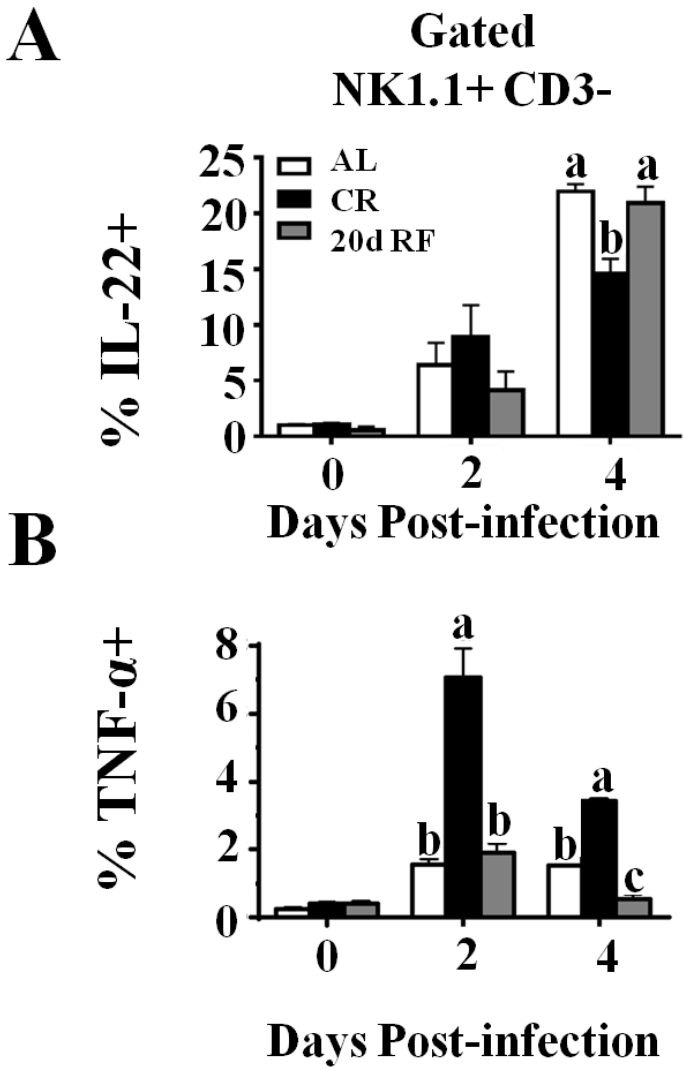

Figure 20: Cytokine production by NK cells from AL, CR, and 20d RF mice during influenza infection. Mice were infected with 100 HAU of influenza virus and the NK cell response was measured in the lungs at 0, 2, and 4 days post infection. Lung cells were incubated with protein transport inhibitors, after which cells were stained with lineage and cytokine specific antibodies. NK cells were gated $NK1.1^{+}$ $CD3^{-}$. Data are mean ± SEM. Means without a common letter differ, $p < 0.05$ (n=4-5 mice/group).

6.5 DISCUSSION:

Natural killer cells provide innate immune surveillance and act as early responders in the face primary influenza infection [157]. NK cells that are $CD27^{-}$ $CD11b^{+}$ are the largest subset of NK cells found in the lungs prior to influenza infection [10, 380], thus it stands to reason that these cells provide immediate immune protection while other innate and adaptive lymphocytes are recruited to fight a viral infection in the lungs. We found CR results in significantly fewer $CD27^{-}$ $CD11b^{+}$ NK cells throughout the periphery, especially the lungs, suggesting to us that this deficiency might contribute to the increased susceptibility of CR mice to influenza infection. Furthermore, IL-22 a critical pro-regenerative cytokine involved in the recovery process from influenza [120], is produced almost exclusively by mNK cells in the lungs of mice in response to influenza infection [119]. While there is evidence that NK cells are sensitive to dietary changes [213, 216, 309, 362], data presented in this manuscript is among the first evidence suggesting NK cell homeostasis and function can be directly manipulated by changes in energy intake over a relatively short period of time.

We found refeeding to result in a hyperphagic response; despite the restoration of body weight and body composition as early as 10 days post-refeeding, NK cell homeostasis was not fully restored. The lifespan of an NK cell in mice has been measured to be between 14-17 days [381], leading us to hypothesize that refeeding needs to be carried out for at least this amount of time to generate a new mature pool of NK cells. Interestingly, this also suggests that refeeding did not immediately drive early mNK cells to become terminally mNK cells. It is not known whether there is a requirement for sufficient nutrient related signals to be present during the development of NK cells. However, one possibility is that an NK cell must receive strong nutritional signals throughout its development in order for it to be programmed to become a

mNK cell. This would suggest potential epigenetic imprinting, in which the decision to remain an iNK cell was made during the CR phase, and cannot be reversed despite dietary changes. Thus, in this study we extended our feeding protocol to encompass the time period necessary to restore NK cell homeostasis. After extending our RF protocol and observing that NK cell subsets were restored, we tested whether NK cells were proliferating at a comparable rate between AL and 20d RF mice and if the ability of NK cells to survive serum starvation was also comparable. NK cells from CR mice were more susceptible to apoptosis following short term cultures in serum free media, suggesting a potential mechanism by which NK cell numbers are reduced in CR mice. CR resulted in normal NK cell proliferation, defined by expression of the cell cycle protein Ki-67 [382], an unexpected observation as CR mice have very few NK cells. However, it is possible that NK cells proliferate in a normal fashion in CR mice in response to the lymphopenia and the resulting vacant immunological "space" created by apoptosis of mNK cells [381, 383].

The relationship between immune cell development and homeostasis has only recently become appreciated. Signaling molecules classically associated with metabolic pathways such as AMPK, LKB1, and mTOR, have all been recently identified to play a role in the development of T cells, DCs, and B cells, yet their role in the development and function of NK cells remains a relative mystery [40, 384]. In T cells, constitutive mTOR blockade during activation results in development of the Treg phenotype [67], thus it can be inferred that constitutive signaling through mTOR can regulate T cell fate [67], it is possible a similar situation takes place during NK cell development. Furthermore, this process is known to be particularly sensitive to metabolic signaling delivered by leptin [66]. Here we show that despite a restoration of body weight, 20d RF mice did not have the same levels of circulating leptin as is observed in AL mice.

Whether this means that there is insufficient leptin to stimulate immunity is unclear, but it remains a possible explanation for the delay in NK cell development. Importantly, CR results in mTOR inhibition in a variety of cell types [307, 385], leading us to hypothesize that CR alters NK cell development through manipulation of metabolic pathways, specifically mTOR. Future studies should assess whether there is a direct connection between energy intake, mTOR signaling, and NK cell development, which would provide insight into potential mechanisms by which CR and RF alter and restore NK cell homeostasis, respectively.

CHAPTER 7:

ROLE OF MAMMALIAN TARGET OF RAPAMYCIN IN THE TERMINAL MATURATION OF NK CELLS

Clinthorne JF, Durianick DM, Gardner EM. In preparation for submission to *J Immunology.*

7.1 ABSTRACT:

Natural killer cells are potent anti-viral and anti-cancer innate lymphocytes capable of lysing target cells without the need for prior antigen sensitization. We have recently established that CR mice have impaired NK cell maturation and reduced numbers of NK cells in peripheral tissues, leading us to hypothesize that there is a link between metabolism and NK cell maturation. In this article, we examine the metabolic signature of NK cells in relation to T cells and B cells and investigate the metabolic pathways altered by a CR diet in NK cells. We demonstrate CR results in significantly reduced phosphorylation of ribosomal protein S6 in NK cells, suggesting that CR suppresses mTOR signaling. We investigate the consequences of mTOR inhibition on NK cell maturation and function through brief exposure of NK cells to an inhibitor of mTOR prior to cytokine-induced NK cell maturation. The transition of NK cells into a terminally mature phenotype occurs in the periphery as a result of interaction with accessory cells or signals derived from cytokines and upregulation of TFs associated with NK cell maturation, a transition which we show to be suppressed by mTOR inhibition. These findings define mTOR as a target for pharmacological manipulation of NK cell phenotype and function and have potential implications designing therapeutic interventions to combat various diseases in which metabolic dysregulation is at the heart of the disease etiology.

7.2 INTRODUCTION:

With the rising incidence of metabolic diseases such as type II diabetes and diet induced obesity, our need for understanding how alterations to metabolic signaling influences overall health and well being has dramatically increased. Among the physiological systems found to be impaired in those with metabolic abnormalities, there is significant evidence that immune function is compromised [38]. In order to develop strategies to manipulate immune cell metabolism, further research is required to elucidate the mechanisms by which metabolic pathways are regulated in immune cells. Molecular and biochemical studies have revealed the serine/theronine kinases Akt1-3, AMP activated kinase (AMPK), liver kinase B1 (LKB1), and mTOR not only serve as nutrient sensors in immune cells, but also regulate immunological fate and development [48, 59].

Long recognized as a critical regulator of cell proliferation and growth, the role of the serine/threonine kinase mTOR is now becoming increasingly appreciated in immune function. Pharmacological blockade of mTOR signaling through the use of rapamycin results in substantial changes to innate and adaptive immune cell function and development [59]. While studies support the notion that T cells, DCs and B cells require mTOR signaling for normal function, however the role of mTOR in the development and function of NK cells remains relatively unknown.

NK cells are innate lymphocytes with the unique ability to respond to and lyse target cells without prior antigen sensitization [346]. Effector function is elicited by NK cells through various mechanisms, including production and secretion of a variety of cytokines and chemokines as well as lytic molecules such as perforin and granzymes [346]. Upon encountering a target cell, NK cells recognize the presence or absence of specific ligands on the cognate cell, ultimately resulting in signals that encourage or inhibit NK cell mediated lysis of

targets [128]. Activation of NK cells with cytokines results in similar effector function [346], highlighting the multifaceted role of NK cells in an immune response to infections and cancers.

Through a distinct maturation process that has been divided into specific developmental stages NK cells become terminally mature cells expressing high levels of CD11b, CD43, and KLRG1 [8, 99]. NK cells in different developmental stages differ not only in expression of surface markers, but also express different TFs and have uniquely different functional responses [85, 112]. While the molecular mechanisms by which NK cell development are becoming increasingly understood, the precise biochemical signature of developing NK cells remains to be fully elucidated. Gordon et al. revealed a novel checkpoint for NK cell maturation in which the TFs Eomes and T-bet are critical for the generation of mature NK cells [112], while other TFs such as E4bp4, Id2, IRF-2, GATA-3, and Blimp-1 are all thought to play a role in the maturation process [89].

Cytokines play a critical role in the activation and differentiation of NK cells [83], with IL-15 perhaps being the most critical as NK cells are nearly undetectable in IL-15-/- mice [370]. *In vivo,* IL-12 is required for the generation of memory NK cells during MCMV infection [138], while NK cells from mice lacking normal IL-18 signaling have impaired NK cell responses [386]. Furthermore, *in vitro* studies show NK cells to have altered expression of maturation markers or TFs during stimulation with different γ-chain cytokines [83].

We have previously reported CR results in fewer mNK cells, and NK cells from CR mice express lower levels of the TFs Eomes and T-bet [309]. Here, we use a mouse model of CR to explore the role that extracellular energy signals play in the development and function of NK cells. Biochemical analysis of NK cells from CR mice showed significantly decreased levels of pS6, suggesting the low energy state of CR resulted directly influences NK cell metabolism.

Further analysis of the consequences of mTOR inhibition in NK cells revealed that the upregulation of specific markers of maturation such as CD43 and KLRG1 was inhibited if mTOR inhibition occurred prior to culture in the cytokines IL-12 and IL-18. Acute mTOR inhibition in NK cells resulted in cells that failed to upregulate metabolic transporters in response to cytokine stimulation, using flow cytometry we also demonstrate that mTOR inhibition also suppressed upregulation of TFs required for peripheral NK cell maturation, drawing a parallel to our previously reported observations regarding the influence of CR on the expression of TFs by NK cells.

7.3 MATERIALS AND METHODS:

Mice and diets

Specific pathogen-free young adult (6 mo) AL and young adult (6 mo) CR male C57Bl/6 mice were purchased from the NIA colony maintained by Charles River Laboratories (Wilmington, MA). The animal use protocol for this study was approved by Michigan State University Institutional Animal Care and Use Committee. Upon arrival, mice were housed individually in micro-isolator cages in the AAALAC-accredited containment facility at Michigan State University and were acclimated at least 10-14 days prior to the initiation of each experiment. Both CR (NIH-31/NIA-fortified) and AL (NIH-31) diets were purchased from the NIA, the compositions of which have been reported in detail previously [11]. The composition of the CR diet is sufficient in micronutrients and minerals, but results in restriction of total energy intake. The CR regimen initiated by the NIA is designed to gradually achieve 40% restriction in mice by 4 mo of age, such that they are weight stable upon arrival at 6 mo of age. Refeeding was performed by allowing CR mice unlimited access to NIH-31 diet for the indicated time points.

All experiments were repeated at least twice using 4-5 mice per diet treatment per experiment, unless otherwise noted.

Lymphocyte isolation

For restimulation assays, spleens were excised from AL fed mice and isolation of mononuclear cells was performed as previously described. Briefly, single cell suspensions were obtained from spleens using homogenization. The resulting cell suspensions were lysed of RBCs using an ammonium chloride buffer. All cell suspensions were washed in PBS and resuspended for counting using Trypan Blue viability dye.

Acute mTOR inhibition

After the preparation of single cell suspensions, splenocytes were incubated for 90 minutes at 37^{o}C in complete media containing 100mM rapamycin [66]. Following acute mTOR inhibition, cells were washed extensively in serum-free media (Lonza), before being resuspended at the appropriate concentrations for downstream applications.

Flow cytometry

Following incubations splenocytes were resuspended in FACS buffer (0.1% sodium azide, 1% FBS, in PBS) at a concentration of $2x10^{7}$ cells/mL. $1\text{-}4x10^{6}$ cells were incubated on ice for 10 minutes with anti–CD32/CD16 antibody (2.4G2) (BD Bioscience) in order to block FcγII/III receptor–mediated non-specific binding. Samples were then incubated with a cocktail containing various combinations of the following fluorochrome-conjugated antibodies (eBioscience, BD Bioscience, or Biolegend) at optimal concentrations determined in our laboratory: NKp46 (PE

[29A1.4]) NK1.1 (Allophycocyanin or PE-Cy7 [PK136]), CD3 (Alexa Fluor700 [500A2]), CD94/NKG2 (PE [HP-3D9]), CD27 (PE or PerCP-eFluor710 [LG.7F9]), CD127 (PE or PerCP-Cy5.5 [A7R.34]), CD51 (Biotin [RMV-7]), CD49b (Allophycocyanin or PE-Cy7 [DX5]) CD11b (PE-Cy7 or V500 [M1/70]), GITR (FITC [DTA-1]), B220 (Allophycocyanin [RA3-6B2]), CD43 (Allophycocyanin-Cy7 [1B11]), Ly49C/I/F/H (FITC or PE [14B11]), and KLRG1 (Allophycocyanin [2F1]). Cells were incubated in staining cocktails on ice in the dark for 30 min. To detect TF expression, cells were fixed and permeabilized using eBioscience Foxp3 staining kit according to the manufacturer's instructions, then incubated with antibodies against T-bet (PE-Cy7 [4B10]), Eomes (PerCP-Cy5.5 [Dan11mag])(eBioscience) and Blimp-1 (Alexa Fluor488 [646702]) (R&D Systems). Viable lymphocytes were gated based on light scattering properties, after which NK cells were characterized as $NK1.1^{+}$ $CD3^{-}$ unless otherwise noted. Samples were analyzed using an LSR II flow cytometer (BD Bioscience) or a FACS Canto II flow cytometer (BD Bioscience) with FlowJo software (Tree Star).

Detection of phosphorylated proteins by flow cytometry

For *in vivo* detection of phosphorylated proteins, spleens from AL or CR mice were immediately excised following euthanasia and placed into BD fix/lyse. Homogenization of spleens was performed in BD fix/lyse buffer and single cell suspensions were counted using trypan blue viability dye. Following counting, cells were resuspended at the appropriate concentration and permeabilized using BD Perm Buffer III according to manufacturer's instructions. Fixed and permeabilized cells were resuspended at the appropriate concentration for flow cytometric analysis as described. Alternatively, for time course phosphoprotein analysis, single cell suspensions were prepared as previously described, and at each requisite time point, cells were

immediately fixed with BD cytofix and permeabilized with ice cold BD Perm Buffer IIl. Cells were simultaneously stained using lineage specific monoclonal antibodies and antibodies specific for phosphoproteins of interest at room temperature for 1 hour. Antibodies against pS6 (FITC [2F9 (pS235/236)]) and pAkt (Allophycocyanin [C31E5E (pT308)]) were acquired from Cell Signaling Technologies, anti-pSTAT3 (Pacific Blue [4/P-STAT3 (pY705)]) and anti-pSTAT5 (PE-Cy7 [47/Stat5(pY694)]) were acquired from BD Biosciences. Following staining, cells were washed twice in FACs Buffer, and samples were acquired on an LSRII (BD) and analyzed using FlowJo software (Treestar).

NK cell stimulation

The influence of stimulants to induce NK cell maturation was determined using previously published methods. Briefly, high affinity 96 well plates (Thermo-Fisher) were coated with a monoclonal antibody against NK1.1 (25ug/mL [PK136]) or NKp46 (15ug/mL [29A1.4]) for 18 hours at 4^{o} C. Plates were then washed with PBS 3 times and freshly prepared splenocytes (1-$4x10^{6}$) in complete media were added. Alternatively, splenocytes in complete media were added to uncoated 96 well plates and IL-12 (20ng/mL) and IL-18 (40ng/mL) or YAC-1 cells (10:1 E:T ratio) were added. Plates were incubated for 10 hours after which cells were harvested from plates and stained with monoclonal antibodies for determination of NK cell phenotype. In order to detect IL-12 + IL-18 induced S6 phosphorylation, NK cells were incubated with plates IL-12 (20ng/mL) and IL-18 (40ng/mL) for the indicated time points after which cells were immediately fixed with BD cytofix. Following fixation, flow cytometry was performed as reported.

Statistics

Statistics were performed using GraphPad Prism 4 software. Values in text are means ± SEM. Influence of diet on MFI of phosphorylated protein and changes to NK cell phenotype were analyzed using student's T-test to determine significant differences between treatments. Changes to body weight and food intake were measured using repeated measures ANOVA. Influence of stimulation on NK cell maturation and function was determined using ANOVA. Statistical significance was set at $p<0.05$.

7.4 Results and Discussion:

NK cells from CR mice have a unique biochemical signature

Recent studies have identified the critical role of various metabolic signaling pathways in the development and homeostasis of adaptive lymphocytes [39]. Furthermore, investigation into metabolic pathways utilized to produce ATP by leukocytes has revealed significant differences between innate and adaptive immune cells. However, to date, none have examined or compared the activity of metabolic pathways in regulating NK cell homeostasis. Comparison of the expression of metabolic transporters CD71 and CD98 between NK cells, T cells, and B cells, revealed NK cells expressed CD71 and CD98 at levels comparable to T cells, yet different than B cells (**Figure 21A**). Cells that rely on aerobic glycolysis to produce ATP such as neutrophils [39], have been found to have a fewer mitochondria than is observed in quiescent lymphocytes that utilize oxidative phosphorylation [45]. Mitochondrial density in NK cells was found to be reduced compared to other lymphocytes tested (Figure 21A), which may be reflective of resting NK cells utilizing metabolic pathways more commonly associated with innate immunity rather than quiescent adaptive lymphocytes [47]. Because mTOR activity is thought to play a

significant role in the differentiation and homeostasis of lymphocytes [48, 69], we compared the level of S6 phosphorylation between lymphocytes as an indicator of mTOR activity [306]. NK cells displayed comparable mTOR activity to T cells and the mTOR activity in both these lymphocytes was higher than what was observed in B cells (Figure 21A). There have been previously described similarities between $CD8^+$ T cells and NK cells [140], and here we show resting mTOR activity also appears to be comparable between T cells and NK cells, based on S6 phosphorylation, although levels of pS6 in specific T cell subsets were not investigated. Because the NK cell pool can be divided into various subsets representing functional and maturational status [10], we investigated markers of metabolic status in NK cell subsets based on expression of CD27 and CD11b (Figure 19B). Early mature NK cells that coexpress CD27 and CD11b (Double positive, DP) displayed greater mitochondrial density than immature ($CD27^+$ $CD11b^-$) or terminally mature ($CD27^-$ $CD11b^+$) and higher levels of intracellular pS6 than terminally mature NK cells (Figure 21C). When comparing mature ($CD11b^+$) NK cell subsets, NK cells that are DP have been found to be the most responsive to DC coculture or IL-12 + IL-18 stimulation [10], which may be partially explained by increased metabolic activity as shown here.

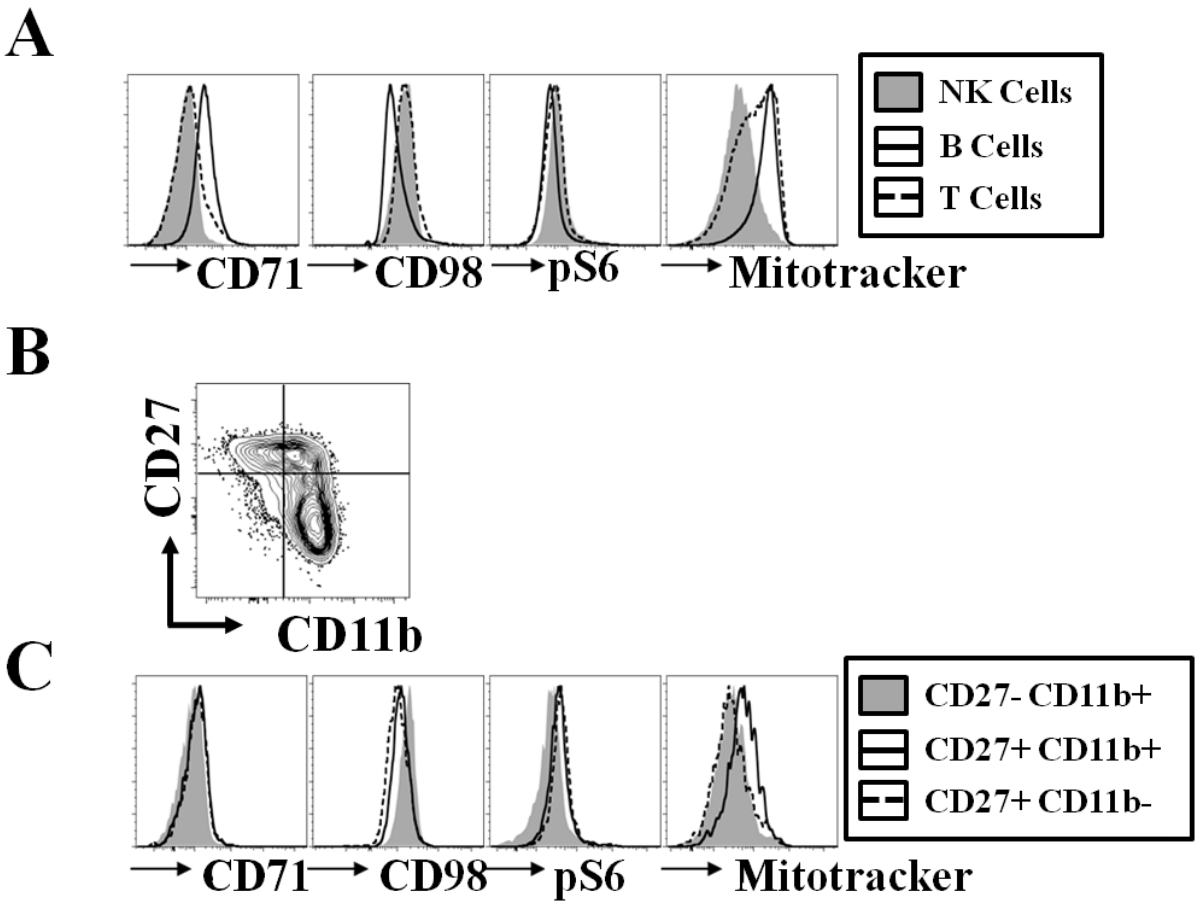

Figure 21: The metabolic phenotype of NK cells. A**)** NK cells (NKp46^{+} CD3^{-} MHC-II^{-}), T cells (CD3^{+} B220^{-} MHC-II^{-}), and B cells (B220^{+} MHC-II^{+} CD3^{-}) were stained with lineage specific or phosphospecific antibodies or stained with mitotracker and the intensity of staining was compared between lymphocyte subsets. B) NK cells were divided into the depicted NK cell subsets corresponding to NK cell maturation status and (C) the metabolic activity was compared between these subsets. Histograms are representative. Data are representative of individual experiments containing 3-4 mice per experiment.

NK cells are sensitive to metabolic homeostasis in vivo

We have previously reported mice on a CR diet have fewer mature NK cells [309], leading us to hypothesize this is at least partially due to metabolic changes induced by reduced energy intake. Levels of phosphorylated proteins were compared between AL and CR mice by immediately placing spleens into fixative following harvest. Using antibodies tested in our laboratory to react with fixed epitopes, NK cells were defined as $NKp46^{+}$ $CD3^{-}$ $MHC\text{-}II^{-}$ and levels of phosphorylated proteins that are downstream of potential metabolic pathways influenced by CR were compared between AL and CR mice (**Figure 22**). Among the various signaling pathways screened, we discovered CR resulted in significant inhibition of phosphorylation of ribosomal protein S6 (S6) (Data not shown), a protein directly downstream of the mTOR signaling pathway [306], which is in line with the concept that one mechanism by which CR increases lifespan is through global mTOR inhibition [239]. A signaling pathway involved in the regulation mTOR activity in lymphocytes occurs through PI3K-Akt signaling [54]; however we did not observe a difference in the phosphorylation of Akt in NK cells from AL and CR mice, suggesting alternative pathways may be responsible for diminished mTOR activity in NK cells from CR mice (**Figure 22A-B**). CR influences multiple metabolic pathways [239], thus it is conceivable that reduced mTOR activity in NK cells from CR mice reported was a result of metabolic signals delivered through alternative pathways. A negative regulator of mTOR activity, AMPK becomes activated under conditions of bioenergetic stress such as CR to reduce anabolic processes that consume ATP [239]. Importantly AMPK activation has been linked to increased resistance to stress induced apoptosis in lymphocytes [59, 387], a hallmark of CR. While not assessed in this study, future studies are warranted to determine the potential relationship

between AMPK and its upstream regulators on NK cell biology regarding conditions of bioenergetic stress.

There are metabolic differences between different stages of NK cell development (Figure 21C); because we have previously reported significant differences in the maturation status of splenic NK cells between AL and CR mice, NK cells were divided into immature ($CD27^+$ $CD11b^-$), early mature ($CD27^+$ $CD11b^+$) and terminally differentiated ($CD27^-$ $CD11b^+$) subsets to determine whether the observed decreased S6 phosphorylation was due to altered subset distribution in CR mice or due to global mTOR inhibition in NK cells (Figure 22A-B). S6 phosphorylation was consistently reduced in NK cells from CR mice, independent of maturational changes to NK cells existing in CR mice. Both NK and T cell homeostasis and function are influenced by leptin and in T cells, leptin exerts at least some of its immunomodulatory effects through influencing mTOR signaling [65, 67]. Because we have previously observed CR mice have decreased circulating leptin, we examined whether NK cells from CR mice exhibited impaired Stat3 phosphorylation. Interestingly, we observed a slight, but significant increase in Stat3 phosphorylation in NK cells from CR mice, which may be related to the increased leptin sensitivity conferred by CR (Figure 22A-B). NK cell sensitivity to metabolic changes is further evidenced by studies in which we allowed CR mice unlimited food intake for 48 hours, in which they consumed approximately 18 grams of food (**Figure 22C**). There was a rapid increase ribosomal S6 phosphorylation rapidly increased in response to refeeding, suggesting a direct relationship between caloric intake and mTOR activity in NK cells (**Figure 22D**).

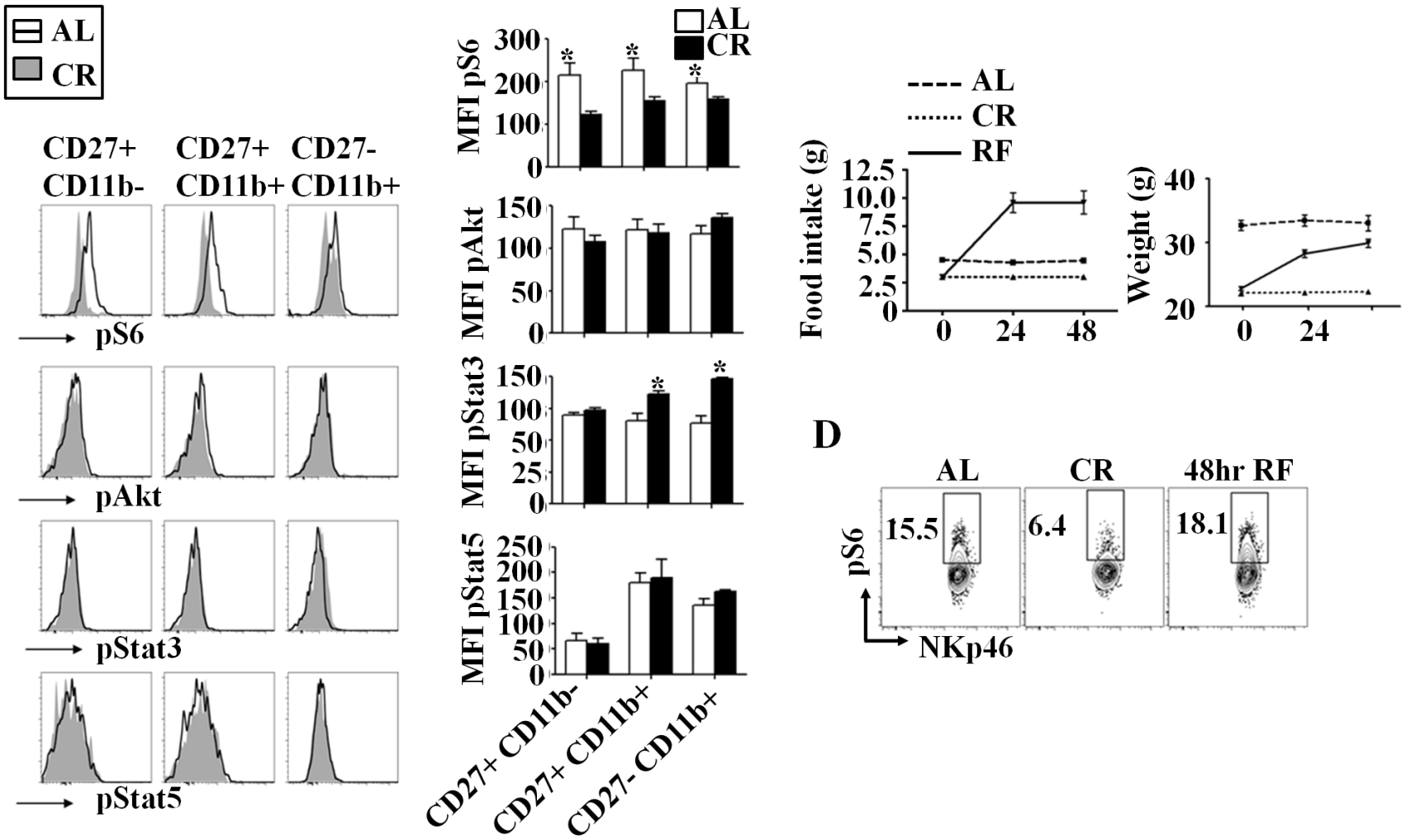

Figure 22: The metabolic homeostasis of NK cells is sensitive to changes in energy intake. Spleens from AL and CR mice were immediately placed into fixative and permeablized as described in the methods section. The phosphorylation status of

(Figure 22 cont'd) proteins associated with metabolic signaling pathways were compared between NK cell ($NKp46^{+}$ $CD3^{-}$ $MHC\text{-}II^{-}$) subsets from AL and CR mice. A) NK cells from AL and CR mice were divided into the described NK cell subsets and protein phosophorylation was compared between AL and CR NK cell subsets. B) Graphical summary of the MFI's of flow data described in (A). C) 48 hour refeeding of CR mice results in significant increases in food intake (left) and body weight (right). D) Increased energy intake is accompanied by increased phosphorylation of S6 in NK cells. Flow plots and histograms are representative. * indicates significance, $p<0.05$. Data are representative of individual experiments containing 3-4 mice per diet group. Experiments were repeated at least twice.

IL-12 + IL-18 stimulation activates mTOR in NK cells

We have previously reported CR results in significant changes to NK cell phenotype, resulting in NK cells that are less mature [309]. In order to determine whether metabolic activation plays a role in NK cell maturation, we tested multiple NK cell stimulants for their ability to induce NK cell maturation (**Figure 23A-B**) and found IL-12 + IL-18 stimulation to be the most effective at inducing a mature phenotype in splenic NK cells [83]. In order to understand the consequence of mTOR inhibition on NK cell maturation and compare this observation to the reduced maturation status of CR mice, we stimulated NK cells that were pretreated with rapamycin or vehicle (Dimethyl sulfoxide [DMSO]) with IL-12 + IL-18 for 16-18 hours. Stimulation of NK cells with IL-12 + IL-18 resulted in significant phosphorylation of S6 that peaked between 8-12 hours and was still detectable at 18 hours post-stimulation (**Figure 23A-B**). S6 phosphorylation was the direct result of mTOR activation as acute mTOR inhibition via treatment of cells with rapamycin inhibited IL-12 + IL-18 induced phosphorylation for up to 18 hours compared to NK cells were treated with DMSO prior to IL-12 + IL-18 stimulation. While perhaps unsurprising, activation of metabolic pathways resulting from cytokine stimulation, specifically through the mTOR

pathway, has not been previously reported in NK cells. These data lead us to question whether mTOR inhibition in NK cells results in substantial changes to the NK cell response to IL-12 + IL-18.

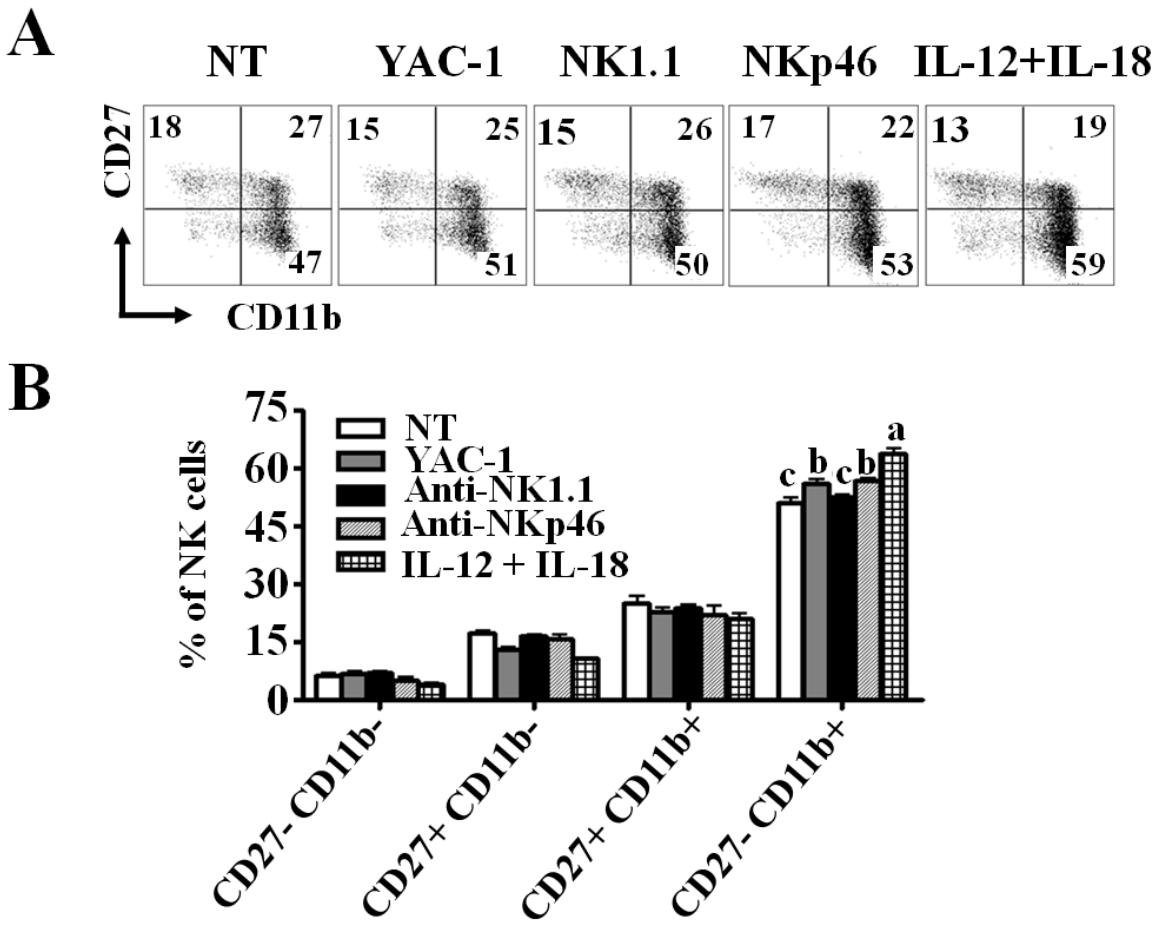

Figure 23: Culture of NK cells in cytokines elicits NK cell maturation. Splenocytes were incubated with various stimulants for 10 hours and NK cell maturation was analyzed. A) Representative flow plots depicting expression of CD27 and CD11b on unstimulated NK cells (NT) or NK cells stimulated with YAC-1 cells, anti-NK1.1, anti-NKp46, or IL-12 + IL-18. B) Graphical summary of the frequency of NK cells that were $CD27^{-}$ $CD11b^{-}$ $CD27^{+}$ $CD11b^{-}$, $CD27^{+}$ $CD11b^{+}$, or $CD27^{-}$ $CD11b^{+}$. Data are mean ± SEM. Means without a common letter are significantly different, $p<0.05$ (n = 9-10).

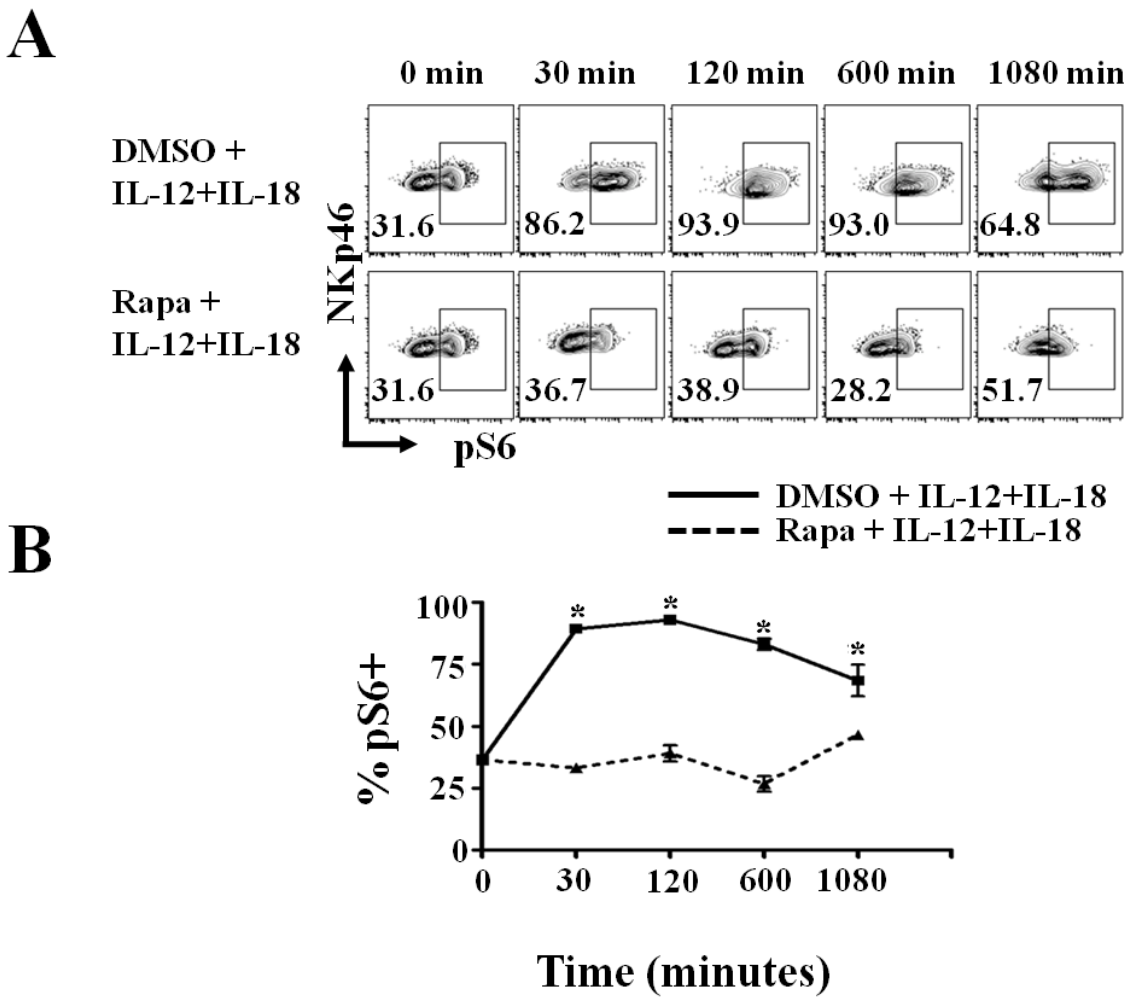

Figure 24: IL-12 + IL-18 induced S6 phosphorylation requires mTOR. Splenocytes were cultured in the presence rapamycin (100mM) or vehicle (DMSO), washed extensively and then stimulated with IL-12 (20ng/mL) and IL-18 (40ng/mL) the indicated time points after which they were fixed and stained for flow cytometric analysis. A) NK cells ($NKp46^{+}$ $CD3^{-}$ $MHC\text{-}II^{-}$) were electronically gated and the level of phosphorylated S6 was determined in IL-12 + IL-18 stimulated and cells pretreated with rapamycin and then stimulated with IL-12 + IL-18. B) Graphical summary of the percent of NK cells staining positive for pS6 over the indicated time course. Flow plots and histograms are representative. * indicates significance between different treatments, $p<0.05$. Data are representative of individual experiments containing 3 mice per group.

T-bet driven NK cell maturation is dependent on mTOR signaling

IL-12 and IL-18 are known to play critical role in the activation of NK cells during the immune response to MCMV [175, 388], in which NK cells rapidly proliferate and develop a mature $KLRG1^{+}$ phenotype [99]. Here we show that compared to other NK cell stimulants, short term cultures containing IL-12 + IL-18 increased the frequency of mature NK cells within the NK cell pool without affecting NK cell proliferation or apoptosis (**Figure 25A-B**). It has previously been reported that long term culture with IL-12 and IL-18 results in upregulation of KLRG1 and CD43 [83], resulting in NK cells expressing increased levels of maturation markers, similar to what is observed during MCMV infection. Indeed, IL-12 and IL-18 are major cytokines produced by DCs in order to activate NK cells in response to viral infection [123, 181]. Treatment of NK cells with IL-12 + IL-18 for 10 hours resulted in significant changes to NK cell phenotype and subset distribution, which was impaired if mTOR was acutely inhibition prior to the initiation of cytokine stimulation (**Figure 26A-B**). One possibility was that mTOR inhibition simply resulted in decreased protein synthesis, thus NK cells did not upregulate maturation markers in response to IL-12 + IL-18 stimulation. However, we believe that this is unlikely, as acute mTOR inhibition resulted in decreased downregulation of CD27 in response to IL-12 + IL-18 stimulation as well as the fact that NK cells upregulated CD51, Ly49s, B220, and GITR in response to IL-12 + IL18 in a normal fashion (Figure 26A).

Our data indicated rapamycin treatment blocked phenotypic changes in NK cells that were related to the maturational status of these NK cells. For example, early mature NK cells are $CD27^{+}$ $CD11b^{+}$, while terminally differentiated NK cells downregulate CD27 while maintaining CD11b expression and upregulating KLRG1 [99, 111]. Based on this observation we assessed whether mTOR inhibition altered cytokine driven terminal maturation of NK cells by

determining the frequency of NK cells that displayed a terminally mature phenotype ($CD27^-$ $KLRG1^+$ or $CD27^-$ $CD11b^+$) following culture (Figure 26B). Acute mTOR inhibition prior to culture in IL-12 + IL-18 resulted in fewer NK cells displaying a terminally mature phenotype when compared to NK cells that were pretreated with vehicle prior to culture, suggesting terminal maturation of NK cells is in some part dependent on mTOR activity (Figure 26A-B). Interestingly, studies examining the influence of immunosuppressive drugs commonly prescribed prior to hematopoietic stem cell transplant on NK cell function have reported that rapamycin in particular suppresses NK cell function [389, 390]. These studies were conducted using cultured NK cells, and it was demonstrated that the inclusion of rapamycin to cultures resulted in a phenotypically altered NK cell pool when compared to untreated cultured NK cells [391]. While it is difficult to compare murine NK cells to human NK cells due to a lack of shared markers, NK cells from both species have been divided into distinct maturational stages [93]. Cultures of human NK cells rely on cytokines to maintain cell viability resulting in a shift of the NK cell receptor repertoire such that NK cells express higher levels of markers normally associated with more mature NK cells such as NKp44, 2B4, and CD56 [391]. Eissens et al. demonstrated that during culture of NK cells in cytokines upregulation of these same markers and this was found to be inhibited by rapamycin [391], supporting our hypothesis that mTOR plays a role in the regulating the mature phenotype of NK cells.

There exist parallels in NK cell and $CD8^+$ T cell functional responses [140], leading us to speculate signaling cascades regulated by mTOR in $CD8^+$ T cells might also be sensitive to mTOR signaling in NK cells. Metabolic activation resulting from IL-12 signaling influences T-bet and Eomes expression and in turn influences memory $CD8^+$ T cell development [69].

Similarly, memory NK cells require IL-12 for development during MCMV infection [138]; however whether mTOR signaling is involved in responsiveness to IL-12 in NK cells is not known. Terminal maturation of peripheral splenic NK cells is dependent on coordinated expression of several TFs. Peripheral NK cell maturation is dependent upon T-bet, Eomes, and Blimp-1 expression [89, 114]. In T cells, expression of Eomes and T-bet are intimately linked to mTOR activity [67, 69], leading us test whether expression of these TFs may also be regulated by mTOR in NK cells. Eomes upregulation was unimpeded in NK cells pretreated with rapamycin before culture in IL-12 + IL-18. However, IL-12 + IL-18 induced T-bet and Blimp-1 upregulation was significantly reduced by acute mTOR inhibition prior to the initiation of culture (**Figure 26C**). Terminal NK cell maturation is dependent on T-bet; T-bet is required for NK cells to upregulate CD43 and KLRG1, and the transition from $CD27^{+}$ $CD11b^{+}$ to $CD27^{-}$ $CD11b^{+}$ NK cells *in vivo* is dependent on T-bet expression [103, 111]. Our data are in agreement with this finding as mTOR inhibition resulted in reduced upregulation of TFs associated with T-bet expression and inhibited the upregulation of T-bet in response to cytokine induced maturation (Figure 24C). While it is unknown what the direct effect of altering the dichotomy of the expression of T-bet and Eomes in NK cells, balanced TF expression is critical for regulating aspects of T cell differentiation, thus it is possible carefully balanced T-bet and Eomes expression are required for NK cell differentiation as well.

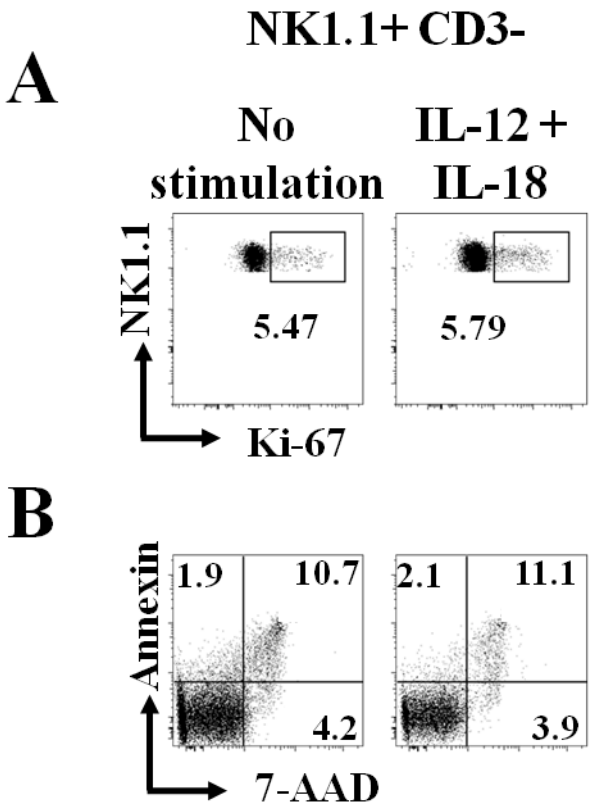

Figure 25: Short-term culture in IL-12 + IL-18 does not induce NK cell proliferation or death. Splenocytes were isolated from AL fed mice and cultured in IL-12 + IL-18 for 10 hours and then stained for the indicated markers. NK cells were gated as $NK1.1^{+}$ $CD3^{-}$. A) Representative flow plots depicting NK cell proliferation defined by Ki-67 expression in unstimulated (left) and stimulated (right) cells B) Flow plots depicting apoptosis of NK cells following short-term culture in unstimulated (left) or IL-12 + IL-18 stimulated cells (right). Data are representative of individual experiments containing 3-5 mice per group.

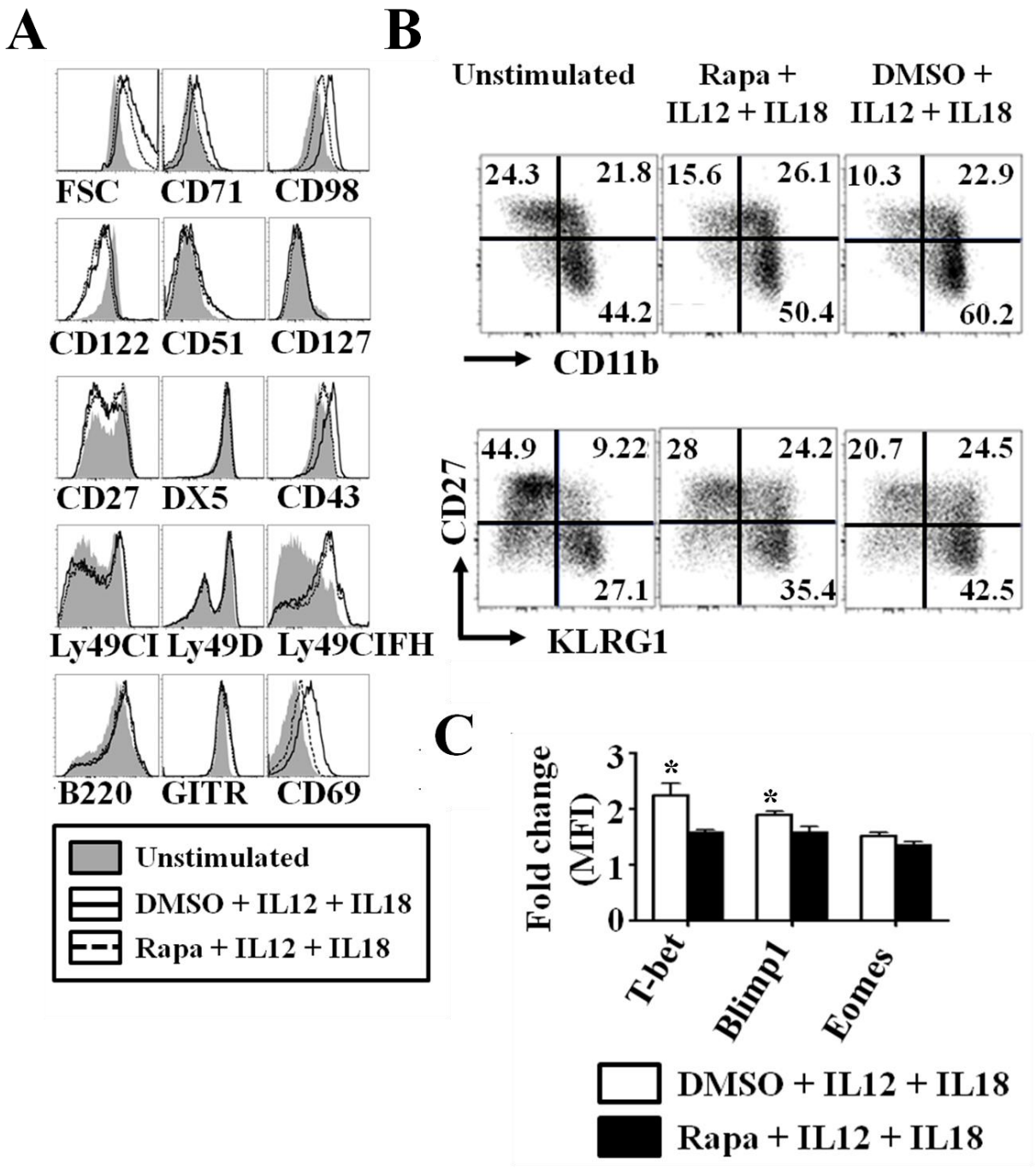

Figure 26: Influence of mTOR inhibition on NK cell phenotype and maturation during short-term culture in IL-12 + IL-18. Cells were treated with vehicle (DMSO) or rapamycin (Rapa), washed extensively and then stimulated with IL-12 + IL-18 and then stained for the indicated

(Figure 26 cont'd) markers. A) Representative histograms of cell surface markers associated with NK cell metabolism, maturation, and function. B) Flow plots depicting NK cell subset distribution defined by CD27 and CD11b (top) or CD27 and KLRG1 (bottom) in unstimulated NK cells (left), NK cells treated with rapamycin prior to stimulation with IL-12 + IL-18 (middle) and NK cells treated with vehicle prior to stimulation with IL-12 + IL-18 (right). C) Graphical summary of the influence of mTOR inhibition prior to stimulation with IL-12 + IL-18 on the expression of TFs associated with terminal maturation of NK cells, with data expressed as fold change of the MFI normalized against unstimulated cells. Flow plots and histograms are representative. Shaded grey histograms represent unstimulated cells, solid line histograms represent NK cells stimulated with vehicle prior to stimulation with IL-12 + IL-18, and dotted line histograms represent NK cells treated with rapamycin prior to stimulation with IL-12 + IL-18. Data are representative of individual experiments containing 3-5 mice per group. Experiments were repeated 2-3 times.

Function of NK cells is impaired following acute mTOR inhibition prior to short-term culture in IL-12 + IL-18

NK cells are described as both phenotypically mature and functionally mature when expressing high levels of maturation markers and are capable of robust IFN-γ production, respectively [8]. To test the influence of acute mTOR inhibition on NK cell function, NK cells were cultured in the presence of IL-12 + IL-18 or anti-NK1.1 and intracellular IFN-γ was detected by flow cytometry (**Figure 27A-B**). Acute mTOR inhibition resulted in fewer IFN-γ^+ NK cells following cytokine stimulation, however no difference was detected between samples pretreated with vehicle or rapamycin before stimulation with anti-NK1.1 (Figure 27A-B). Perhaps this is a reflection of the pathways by which these stimulants activate NK cells. Indeed, we show IL-12 + IL-18 activates NK cells in an mTOR dependent fashion, while NK1.1 ligation has been reported to result in phophorylation of ITAMs and activation of MAPK signaling [128], thus this might explain why anti-NK1.1 induced IFN-γ production was unimpaired. Furthermore, data suggest

cytokine driven IFN-γ production is impaired by mTOR inhibition while alternative pathways of NK cell activation such as the ability to respond to target cells remain unimpeded. As mTOR inhibition suppressed the maturation of NK cells *in vitro,* and mNK cells are generally thought to be the major producers of IFN-γ, it is also possible that the limited outgrowth of m NK cells due to rapamycin treatment prior to stimulation can explain the diminished IFN-γ production. NK cells perform multiple functions during response to cytokines and target cells, including producing TNF-α, GM-CSF, granzymes, and perforin, as well as degranulating [121, 346]. In our studies we only measured the ability of mTOR inhibition to suppress NK cell derived IFN-γ; it remains to be determined whether other aspects of murine NK cell function are impaired as a result of mTOR inhibition.

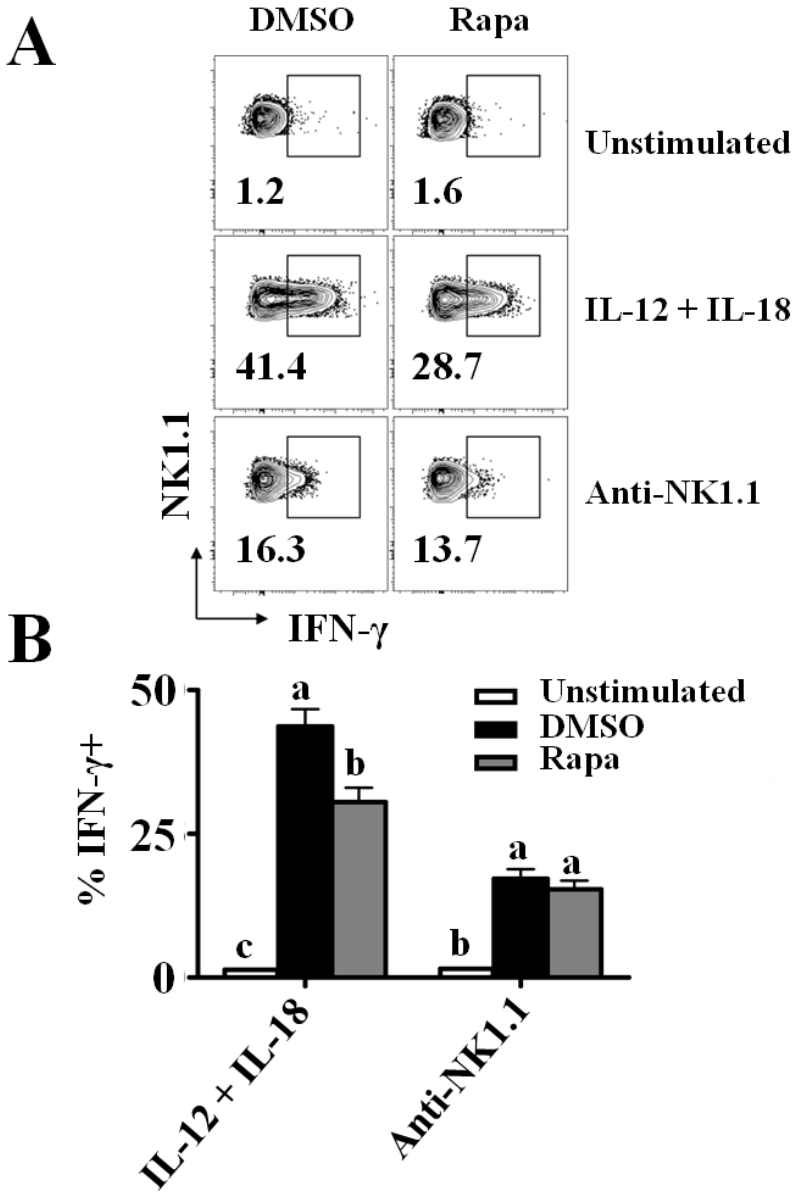

Figure 27: Brief treatment with rapamycin suppresses IL-12 + IL-18 induced IFN-γ production. Cells were treated with DMSO (left) or rapamycin (right), washed extensively and then stimulated with IL-12 + IL-18 or plate bound anti-NK1.1 in the presence of brefaldin A. A) Flow plots depicting IFN-γ production detected by intracellular flow cytometry in $NK1.1^{+}$ $CD3^{-}$ cells. B) Graphical summary of data presented in (A). Flow plots and histograms are representative. Data are representative of individual experiments containing 3-5 mice per group. Experiments were repeated 2-3 times.

CHAPTER 8:

SUMMARY AND FUTURE DIRECTIONS

By increasing our understanding of how CR alters the development and function of the immune system we gain a clearer understanding of how CR can be utilized to attenuate or worsen various disease states. This dissertation focuses mainly on the influence of CR on NK cell development and function; however this dissertation (Appendix) also provides data regarding the influence of CR on lymphopoiesis and granulopoiesis in murine BM. Despite the fact that immunological changes induced by CR are only partially characterized, the NIA has funded the CALERIE study, in which many humans will be attempting to follow a CR protocol [240]. Whether humans following a CR diet will be at an increased risk of suffering primary infections due to defects in innate immunity remains unclear at this time, although experimental evidence presented here suggests this is a possibility. Data presented herein suggests immunological changes resulting from CR alters leukocyte populations, though the mechanisms by which this is achieved have only begun to be elucidated.

Prior to the initiation of the research presented in this dissertation, our laboratory had shown that CR mice were more susceptible to influenza virus than AL mice. These data suggested that CR impairs NK cell function during influenza infection, however the application of modern immunological tools to interrogate changes to NK cells had yet to be performed. In preliminary studies, we showed that CR mice had both fewer NK cells and activated NK cells infiltrating the lungs during influenza infection compared to AL mice (Figure 6-7). Furthermore, this effect on NK cells could be recovered if CR mice were refed prior to influenza infection

(Figure 6-7). Taken together, these observations reaffirmed our theory that CR resulted in changes that impaired NK cell function during influenza infection.

Detailed investigation into the influence of CR on NK cell development was performed in which numerous aspects of NK cells were interrogated based on the field's current understanding of NK cell biology. We report a number of unique changes to NK cells resulting from a CR diet (Figure 9-11). CR alters both the development and the function of NK cells, and this is observed in classical BM derived NK cells as well as non-classical thymic derived NK cells (Figure 10-11). In CR mice, BM derived NK cells had a less mature phenotype in the periphery (Figure 10), and exhibited functional changes associated with iNK cells (Figure 13). Interestingly, although we detected changes in the maturation status of NK cells in the BM of CR mice, these changes were relatively minor and the biological significance of these observations remains in question. This led us to speculate that CR affects the peripheral homeostasis of these cells more than the developmental processes controlled in the BM. Importantly, the mature subset of NK cells that are lacking in CR mice ($CD27^{-}$ $CD11b^{+}$) are thought to be derived through peripheral processes, a possible explanation for our observations. While CR has unique suppressive effects on classical NK cell development, we observed no detrimental effects of CR on non-classical thymic NK cell development (Figure 11). This is relevant due to the functional consequence of having increased thymic NK cells and fewer classical NK cells making up the NK cell pool. We show that upon activation, the NK cell pool from CR mice has the potential to produce significantly greater amounts of inflammatory cytokines, such as TNF-α and GM-CSF (Figure 12).

The last chapter presented in this dissertation is the most mechanistically oriented. Here we investigated the direct link between metabolic signaling in NK cells from CR mice and the

development of mNK cells (Figure 22). These data show a critical role for mTOR in regulating NK cell development. Following stimulation with cytokines, as is often observed during an immune response, NK cells become terminally mature, however, if the mTOR pathway is inhibited, as we show in the case of CR mice, terminal maturation of NK cells is impaired (Figure 26). This is the first example of the relationship between metabolic signaling and NK cell development, making this data particularly important for the scientific community.

Several of the concepts presented in this dissertation are novel and expand upon our current understanding of metabolism, CR, and NK cells. However, future studies are warranted to fully understand the relationship between these three factors. For example, we show that CR results in the disruption of terminal maturation of NK cells in young adult mice, however, as CR is often used as an anti-aging paradigm, the question remains as to whether CR is capable of ameliorating any age-related defects that occur in NK cell development or function. Thymic involution resulting in decreased output of naïve T cells is known to occur during the aging process and CR is known to prevent thymic involution while also improving TCR diversity [273, 274]. Our data suggests thymic NK cell development is maintained in young adult CR mice, however it would be interesting to examine whether thymic NK cell output is maintained in advanced age and especially if CR is capable of maintaining this process during aging.

Data presented in this dissertation (Appendix) are the first to show that CR influences the developmental homeostasis of both granulocytes and lymphocytes within the BM (Figure 28), resulting in dynamic changes to the frequency (Figure 28), apoptosis (Figure 29), and maturation of B cells (Figure 29) and neutrophilic granulocytes (Figure 31). Furthermore, this dissertation provides novel data regarding the cytokine concentrations found in the BM microenvironment of CR mice in relation to AL mice (Figure 33). These factors are critical for the homeostasis and

development of hemapoietic cells and provide a potential link between CR and immunological changes resulting from dietary interventions.

We provide evidence that CR induced changes to NK cells can be reversed with refeeding of young adult CR mice. This observation prompts several lines of inquiry that remain to be investigated 1) Can refeeding of aged CR mice reverse immunological defects? 2) Can the influence of CR on lymphopoiesis and granulopoiesis in the BM be reversed with refeeding? 3) What are the precise molecular pathways by which refeeding of CR mice restores NK cell homeostasis? Indeed, this last question remains extremely important, as we have shown manipulation of metabolic signaling *in vitro* is capable of altering NK cell development using a pharmalogical inhibitor of mTOR. Identification of metabolic pathways that can be influenced by dietary paradigms is critical as these would provide potential targets for intervention. Dietary interventions represent an alternative method of treatment compared to medical therapy and have the potential to be less costly. Thus, based on this research it is possible that dietary interventions or pharmacological tools could be designed in order to suppress or activate NK cells. However, while data provided in this dissertation identifies a molecular pathway inhibited by CR and responsible for NK cell maturation, the precise biochemical events leading to the inhibition of this pathway remain elusive. Future studies utilizing adoptive transfer of CR NK cells into various transgenic mice lacking specific growth factors would be helpful in order to elucidate the pathways responsible for mTOR inhibition in CR NK cells.

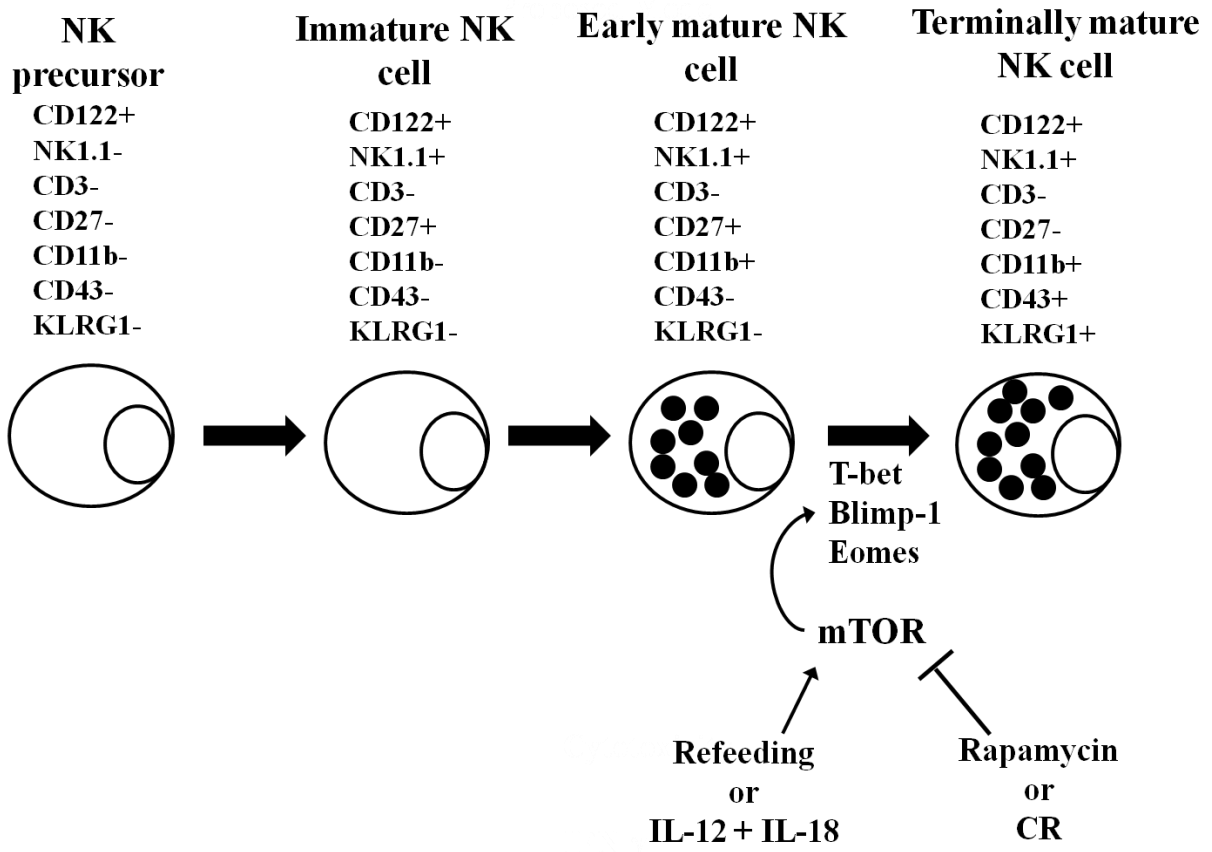

Figure 28: Proposed model for the mechanisms by which metabolic signals regulate NK cell development. Refeeding or inflammatory cytokines act as mTOR agonists and facilitate NK cell maturation while CR or pharmacological inhibition of mTOR results in the suppression of NK cell maturation.

APPENDIX

APPENDIX:

THE EFFECT OF CALORIC RESTRICTION ON BONE MARROW LYMPHOPOIESIS AND GRANULOPOIESIS

Clinthorne JF, Duriancik DM, and Gardner EM. *In preparation for submission to J Gerontology*

ABSTRACT:

There appear to be long term health benefits of CR, but few studies examine the effect of CR on immune homeostasis in the absence of age or disease. Here we describe alterations to the distribution of leukocytes within the BM of young adult C57Bl/6 mice resulting from CR. Multicolor flow cytometric analysis allowed us to determine B cell development was unaffected in the BM up to the prepro stage, after which pro and pre-B cells were found at a significantly reduced frequency and exhibited increased apoptosis in CR mice. The decrease in lymphocytes was accompanied by an increase in the proportion of monocytes, granulocytes, and mixed progenitor lineages in the BM. Within the granulocyte lineage, there was a significant increase in the percent of granulocytes that were fully differentiated mature neutrophilic granulocytes. In BM, CR altered levels of leptin and corticosterone, two hormones known to play a significant role in the development and homeostasis of lymphoid and myeloid lineages. Ultimately these changes were reflected in the periphery as C57Bl/6 mice fed a CR diet had significantly fewer B cells in the blood and spleen compared to AL mice.

INTRODUCTION:

Both aged humans and aged laboratory animals demonstrate reduced humoral immune function as a result of declining adaptive immunity. T and B cells both exhibit declining functional responses as a result of the aging process [7], which contributes to an increased risk of infection in the elderly [392]. This age related decline in immune function, also known as immune senescence, is at least partially due to the accumulation of senescent, antigen experienced, memory cells in lymphoid organs [393, 394]. As lymphoid architecture fills with senescent cells, there is little room for naïve lymphocytes in lymphoid organs and generation of lymphocytes by the BM is suppressed [395]. Reduced BM lymphopoiesis is one explanation for reduced B cell antigen repertoire in the elderly, which is thought to be partially responsible for impaired vaccination efficacy [396]. This is particularly important as elderly individuals comprise one of the major groups of individuals susceptible to severe infections, such as influenza (334). Thus, there is great interest in strategies to improve vaccination efficacy and immune function in aged populations.

One such strategy, CR, is a nutritional paradigm that is a pro-longevity dietary intervention shown to be effective in a laboratory setting. Data indicates rodents fed diets ranging from 30-70% restriction have increased median and maximal lifespan by up to approximately 50% over those fed AL diets [2, 6, 341]. Similar studies have confirmed the pro-longevity effects of CR in multiple rodent species and strains, as well as canines, and non-mammalian species [6]. Non-human primate studies of rhesus and squirrel monkeys fed a 30% CR diet suggest a comparable decrease in morbidity and mortality rates, but there are confounding data regarding the effects of CR on maximal lifespan in non-human primates [397, 398].

Studies focused on determining the mechanisms by which CR extends lifespan have revealed a significant amount of data on the effect of CR on multiple physiological systems. Immunosenescence is significantly reduced in aged mice fed a CR diet supplemented with vitamins and minerals (275). The incidence of spontaneous tumors and cancer in aged mice is also reduced by CR, as well as maintains T cell proliferation, cytokine production, and cytotoxic T lymphocyte activity [2, 6, 399]. Importantly, Effros et al. demonstrated aged CR mice exhibited a more robust antibody response to intraperotoneal injection with influenza virus than their aged matched AL fed littermates, suggesting CR may improve B cell function in the face of aging (319). Studies have also identified the utility of CR in reducing the severity of autoimmune disease in several experimental models of autoimmunity [287, 400-402]. These studies and others support the notion that metabolism and immunity are directly related, and that CR is capable of regulating the development, maintenance, and function of adaptive immune cells. However, studies investigating the mechanism(s) by which CR alters the immune system remain incomplete.

Despite the fact that there remain significant gaps in our knowledge in understanding immunological changes induced by CR, the CALERIE project has been initiated by NIA to study the efficacy of CR in humans. Thus, it is critical that we elucidate the full immunological implications of CR to determine whether CR results in any potentially detrimental immunological changes in adults. During studies performed in our laboratory describing changes to NK cells as a result of CR [309], we discovered a significant reduction in lymphocytes within the BM of CR mice. Furthermore, infection of CR mice results in increased mortality after infection which is evident as early as 3 days p.i., suggesting impaired innate immunity. These two observations combined with the lack of current knowledge regarding the

effects of CR on immunity lead us to further investigate changes to the immune system of CR mice. In this manuscript we investigated the influence of CR on the reciprocal relationship that exists between B cell lymphopoiesis and granulopoiesis in the BM [403] and changes to the local microenvironment through which CR potentially mediates this effect.

MATERIALS AND METHODS:

Animals and diets

Specific pathogen-free young adult AL male and young adult (6 mo) CR male C57Bl/6 mice were purchased from the NIA colony maintained by Charles River Laboratories (Wilmington, MA, USA). The animal use protocol for this study was approved by Michigan State University Institutional Animal Care and Use Committee (East Lansing, MI, USA). Upon arrival, mice were individually housed in micro-isolator cages in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited containment facility at Michigan State University and were acclimated at least 10-14 days prior to the initiation of each experiment. Both CR (NIH-31/NIA-fortified) and AL (NIH-31) diets were purchased from the NIA, the compositions of which have been reported in detail previously [11]. The composition of the CR diet is sufficient in protein, micronutrients and minerals, but results in restriction of total energy intake by approximately 40%. The CR regimen is designed to gradually achieve 40% restriction in mice by 3 mo, such that they were weight stable upon arrival at 6 mo. Changes in body composition, tissue and animal weight resulting from this CR protocol have been previously reported in detail [309, 341].

Cell preparation

The isolation of mononuclear cells from spleens has been described in detail [11]. Briefly, single cell suspensions were created from spleens using a dounce homogenizer and washed twice; splenocytes were lysed of RBCs using an ammonium chloride buffer and resuspended for enumeration using Trypan Blue exclusion. BM cells were flushed from the femur and tibia with RPMI 1640 using a 25 5/8 G needle and syringe, and enumerated using trypan blue exclusion. Whole blood was acquired via cardiac puncture into heperanized syringes. Whole blood and cell suspensions were kept on ice prior to staining for flow cytometry.

Flow cytometry

Cells were resuspended in FACS buffer (0.1% sodium azide, 1% fetal bovine serum (FBS), in PBS) at a concentration of $2x10^7$ cells/mL. $1\text{-}4x10^6$ cells or whole blood were incubated on ice with anti–CD32/CD16 antibody (2.4G2) (BD Bioscience, San Jose, CA, USA) to block FcγII/III receptor–mediated non-specific binding. Cell suspensions were then incubated with combinations of the following fluorochrome-conjugated antibodies (eBioscience, or BD Bioscience) at optimal concentrations: Ly6C (Allophycocyanin [AL-21]), CD31 (FITC) or (PE-Cy7 [390]), CD11b (eFluor450 or Alexa Fluor700 [M1/70]), Ly6G (PerCP)-Cy5.5 or PE [1A8]), GR-1 (PerCP-Cy5.5 [RB6-8C5]), CD3 (PerCP-Cy5.5 or Alexa Fluor700 [145-2C11]), CD24 (Biotin [M1/69]), CD43 (PE [S7]), IgM (FITC or PE-Cy7 [1141]), IgD (Allophycocyanin [11-26c.1a]), NK1.1 (PE-Cy7 or Allophycocyanin [PK136]), B220 (CD45R) (Alexa Fluor700 or PE-Cy7 [RA3-6B2]), and Annexin V (PE or FITC). Pacific Blue or PE-Cy7 conjugated streptavidin were used as secondary reagents for biotinylated antibodies. Samples were acquired on a LSR II flow cytometer (BD Biosciences) or a FACS Canto II (BD Biosciences) and analyzed using

FlowJo software package (Tree Star) following application of the appropriate compensation. Data was viewed using biexponential plotting for all parameters except for forward and side scatter parameters and DNA content analysis which were acquired using linear settings.

DNA content

Single cell suspensions were stained with lineage specific fluorochrome-conjugated antibodies, and fixed using slow-addition of -70^{o} C ethanol. Samples were incubated on ice for 60 minutes after which, fixed cells were washed with FACS buffer, and incubated for 25 minutes on ice with 7-AAD (25 ng/mL). Samples were acquired on a LSR II flow cytometer or FACS Canto II an analyzed using FlowJo software package. Cells in S/G_2/M phase were determined by analysis of the frequency of cells containing DNA greater than the G_0/G_1 peak.

In vitro apoptosis

To induce apoptosis, cells from various tissues were resuspended in single cell suspensions at 1x10^{7} cells/mL in RPMI 1640 for serum starvation and incubated at 37^{o} C for 6-10 hours [213]. After incubation cells were centrifuged and resuspended in FACS buffer for surface staining. Cells were stained on ice with lineage specific antibodies after which they were washed with Annexin V binding buffer and stained with Annexin V and 7 7-AAD according to manufacturer's protocol (BD Bioscience). Alternatively, for detection of active caspase 3, lineage phenotyped cells were fixed and permeabilized using BD cytofix/cytoperm kits according to manufactures' protocol and stained with anti-active caspase 3 (FITC [C92-605]). Samples were acquired on a FACS Canto II.

BM serum ELISAs

BM from AL and CR mice was flushed with 500µl PBS and cells were pelleted by centrifugation. The resulting supernatants were stored at -80^{o} C for further downstream application. Concentrations of growth factors in supernatants were determined by ELISA according to manufacturers' instructions. ELISAs for Flt-3L, IL-7, and SDF-1α were acquired from Raybiotech (Norcross, GA, USA). Corticosterone ELISA was acquired from AssayPro (Saint Charles, MO, USA), leptin ELISA was acquired from R&D systems (Minneapolis, MN, USA), and GM-CSF ELISA was acquired from eBioscience.

Statistics

Flow plots are representative of individual experiments. All reported values are mean ± SEM. Statistical significant was set at $p<0.05$. Normally distributed data were statistically analyzed using Student's t-test for significant differences in cell phenotype, DNA content or apoptosis, when comparing AL and CR values. All statistical analysis was performed using GraphPad Prism (GraphPad Software).

RESULTS:

CR alters proportions of immune cells in BM compared to AL fed mice

We analyzed BM cells from AL and CR mice for expression of Ly6C and CD31 (**Figure 29A)** [232]. This allows for quantification of five distinct populations including: monocytes (Ly-6C^{hi} CD31$^{+/-}$), granulocytes (Ly-6C^{int} CD31^{-}), mixed progenitors (Ly6C^{+} CD31^{+}), lymphocytes (Ly-6C^{-} CD31^{+}), and erythroblasts (Ly-6C^{-} CD31^{-}) [233]. BM from CR mice contained a

higher percentage of granulocytes, mixed progenitors and monocytes with a concomitant decrease in lymphocytes (**Figure 29B**). Erythroblast frequency was not statistically different between AL and CR BM, suggesting erythropoiesis is unaffected by CR. CR mice were smaller, likely contributing to the fact that fewer cells were harvested from femurs of CR mice (Figure 29B). This resulted in no overall difference in the number of monocytes or mixed progenitor cells, however there remained significantly more granulocytes and significantly fewer lymphocytes (**Figure 29C**) in CR BM. Thus, we chose to focus our investigation on changes to these two populations resulting from CR.

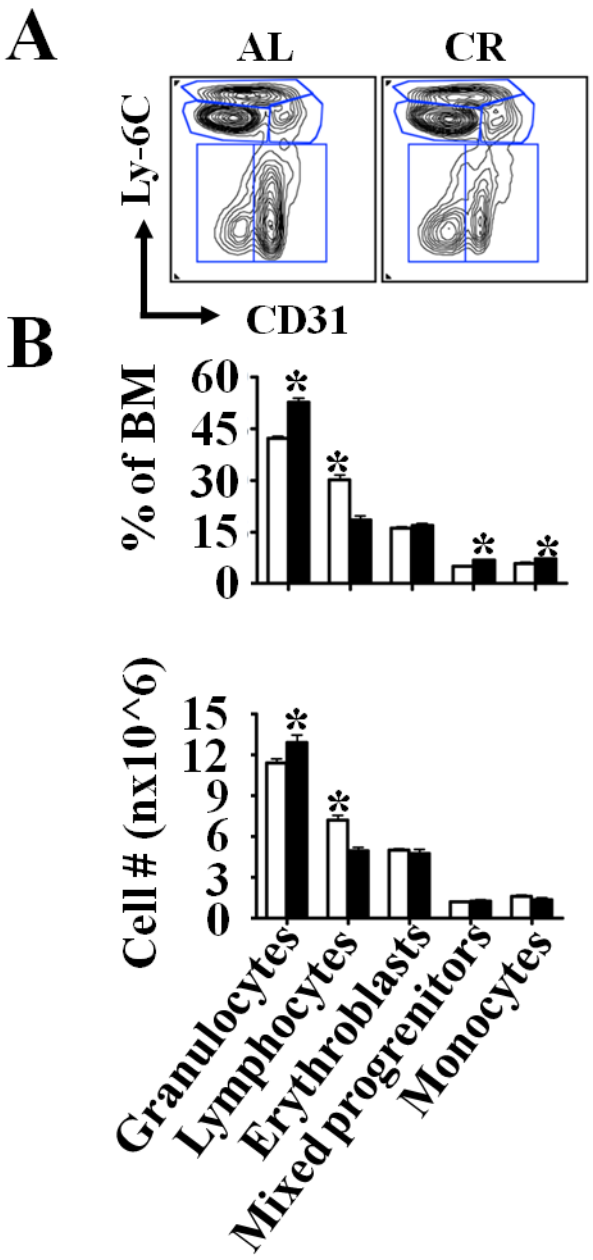

Figure 29: Hematopoietic cell distribution in the BM of AL and CR mice. A) FACS analysis of BM hematopoietic subsets using expression of CD31 and Ly-6C to classify developing cell populations. Cells were classified into subsets consisting of monocytes (Ly-6C^{hi} CD31$^{+/-}$), granulocytes (Ly-6C^{med} CD31^{-}), mixed progenitors (Ly-6C^{+} CD31^{+}), lymphocytes (Ly-6C^{-}

(Figure 29 cont'd) $CD31^+$), and erythroblasts ($Ly\text{-}6C^-$ $CD31^-$). B) Graphical summary and statistical analysis of hematopoietic populations described in (A) from BM of AL and CR mice (top) and absolute number (bottom) of cell populations in the BM of AL and CR mice. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. Data are mean ± SEM. * indicates significance, $p<0.05$ (n=3-5 mice/group/experiment). Individual experiments were repeated 3 times.

B cell development is significantly altered in the BM of CR mice.

As we observed significantly fewer lymphocytes in BM of CR mice, we investigated which lineage was affected. B cells were significantly reduced in CR mice, both as a frequency of lymphocytes (**Figure 30A**) and total cells (**Figure 30B**). B cells were also found to be significantly reduced in the spleen and blood of CR mice compared to AL mice (**Figure 30C**). Consistent with our previous report, NK cells made up a larger fraction of total BM cells [309], and for the first time we report T cells ($CD3^+$ $B220^-$ $NK1.1^-$) also made up a higher frequency of cells in CR BM compared to AL BM (Figure 29B). B220 expression can be used as an indication of B cell development [403, 404]; we observed significant alterations to the distribution of $B220^{hi}$ and $B220^{lo}$ B cells in BM of CR mice compared to AL mice (**Figure 30D**). These observations lead us to analyze the maturational progress of B cells in BM of CR mice. The majority of B cells in CR BM were mature IgM^+ IgD^+ B cells (**Figure 30E**), while IgM^+ IgD^- immature B cells were decreased by approximately 4-fold compared to AL. Our analysis revealed pro-B cells (IgM^- IgD^- $CD24^{dim}$ $CD43^+$) and pre-B cells (IgM^- IgD^- $CD24^{hi}$ $CD43^-$) were also decreased in CR mice, both as a frequency of B cells and as a frequency of

total BM cells (**Figure 30F**). The earliest developmental stage investigated, prepro-B cells, represented a comparable fraction of the total BM cells in AL and CR mice (Figure 3F).

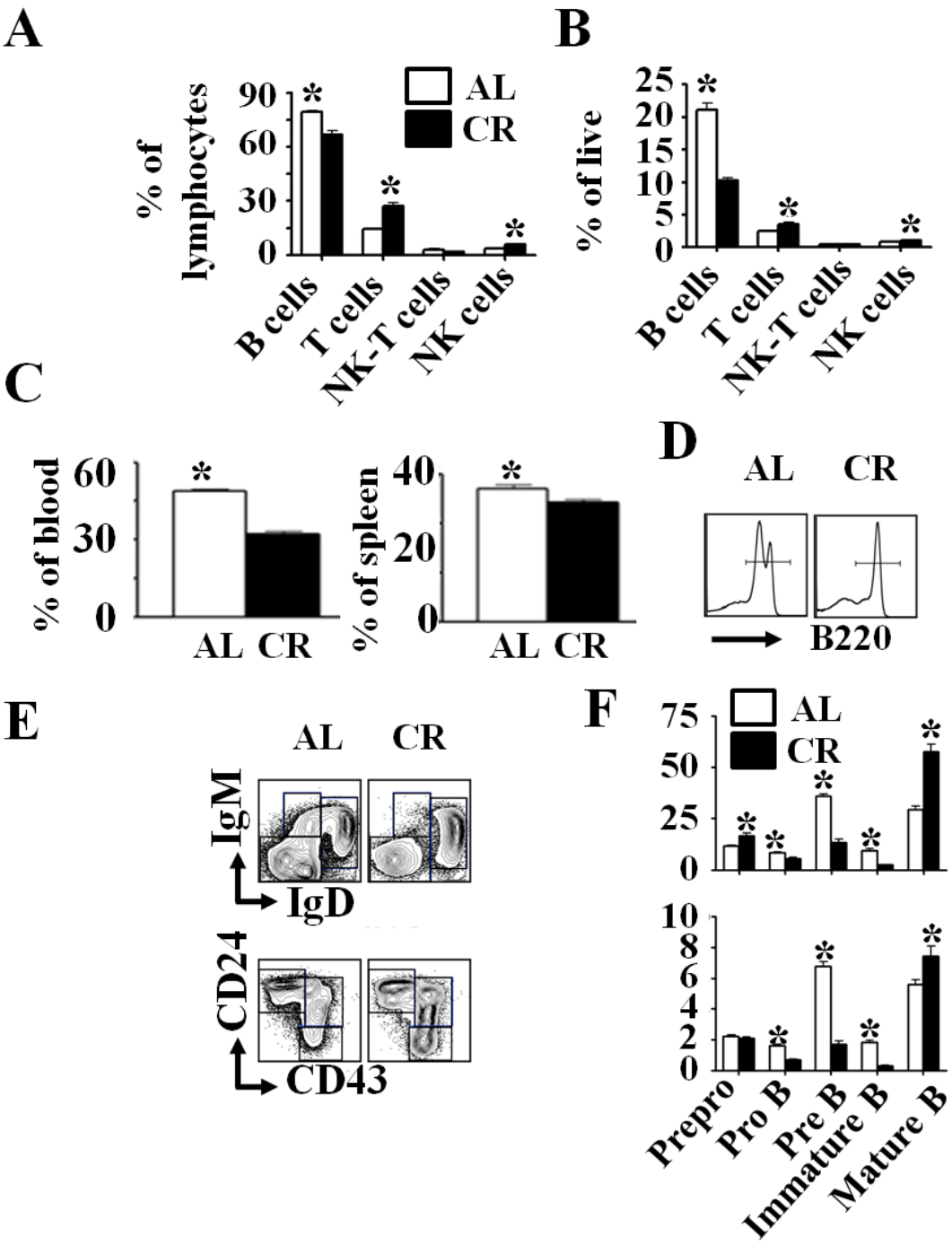

Figure 30: Flow cytometric characterization of lymphocytes from BM of AL and CR mice. A) Frequency of cells within the lymphocyte ($CD31^+$ $Ly\text{-}6C^-$) gate that were T cells ($CD3^+$ $B220^-$ $NK1.1^-$), B cells ($B220^+$ $CD3^-$ $NK1.1^-$), NK-T Cells ($NK1.1^+$ $CD3^+$), and NK cells ($NK1.1^+$

(Figure 30 cont'd) $CD3^-$). B) Frequency of total BM cells that each lymphocyte population represents. C) B cell frequency in the blood (left) and spleen (right) represented as a frequency of total $CD45^+$ cells or total splenocytes, respectively. D) Intensity of B220 expression on cells from the lymphocyte ($CD31^+$ Ly-$6C^-$) gate in BM cells from AL and CR mice. E) BM B cells gated $B220^+$ from AL and CR mice were phenotyped as mature B cells (IgM^+ IgD^+), immature B cells (IgM^+ IgD^-) (top), or pre-B cells (IgM^- IgD^- $CD24^{hi}$ $CD43^-$), pro-B cells (IgM^- IgD^- $CD24^{dim}$ $CD43^+$), and pre-pro-B (IgM^- IgD^- $CD24^-$ $CD43^+$) cells (bottom). F) Graphical summary of B cell populations in AL and CR mice represented as frequency of B cells (top) or as frequency of total BM cells (bottom).

Developing B cells exhibit increased turnover in CR mice

As lymphopoiesis is achieved through a homeostatic balance between proliferation and apoptosis, we examined whether pro-B cells ($B220^+$ $CD43^+$ IgM^- IgD^-) and pre-B cells ($B220^+$ $CD43^-$ IgM^- IgD^- $CD24^+$) from CR mice demonstrated alterations to homeostasis. The frequency of pro-B cells in mitosis ($S/G_2/M$) was not statistically different between AL and CR mice; however, the frequency of pre-B cells undergoing mitosis in CR mice was significantly greater than the frequency of pre-B cells undergoing mitosis in AL mice (**Figure 31A-B**). No difference in annexin V staining or active caspase 3 staining was observed directly *ex vivo* in the BM (data not shown). However, detection of apoptotic cells in isolated BM cells directly *ex vivo* is difficult as macrophages constantly phagocytose apoptotic bodies in the medullary cavity of

the BM [213]. Therefore, we induced apoptosis *ex vivo* by utilizing serum starvation in short term cultures. After 8-10 hour culture, we found no difference in the frequency of pro-B cells undergoing apoptosis between AL and CR mice, however, pre-B cells from CR mice were undergoing significantly higher levels of cell death (**Figure 31C-D**) following short term culture.

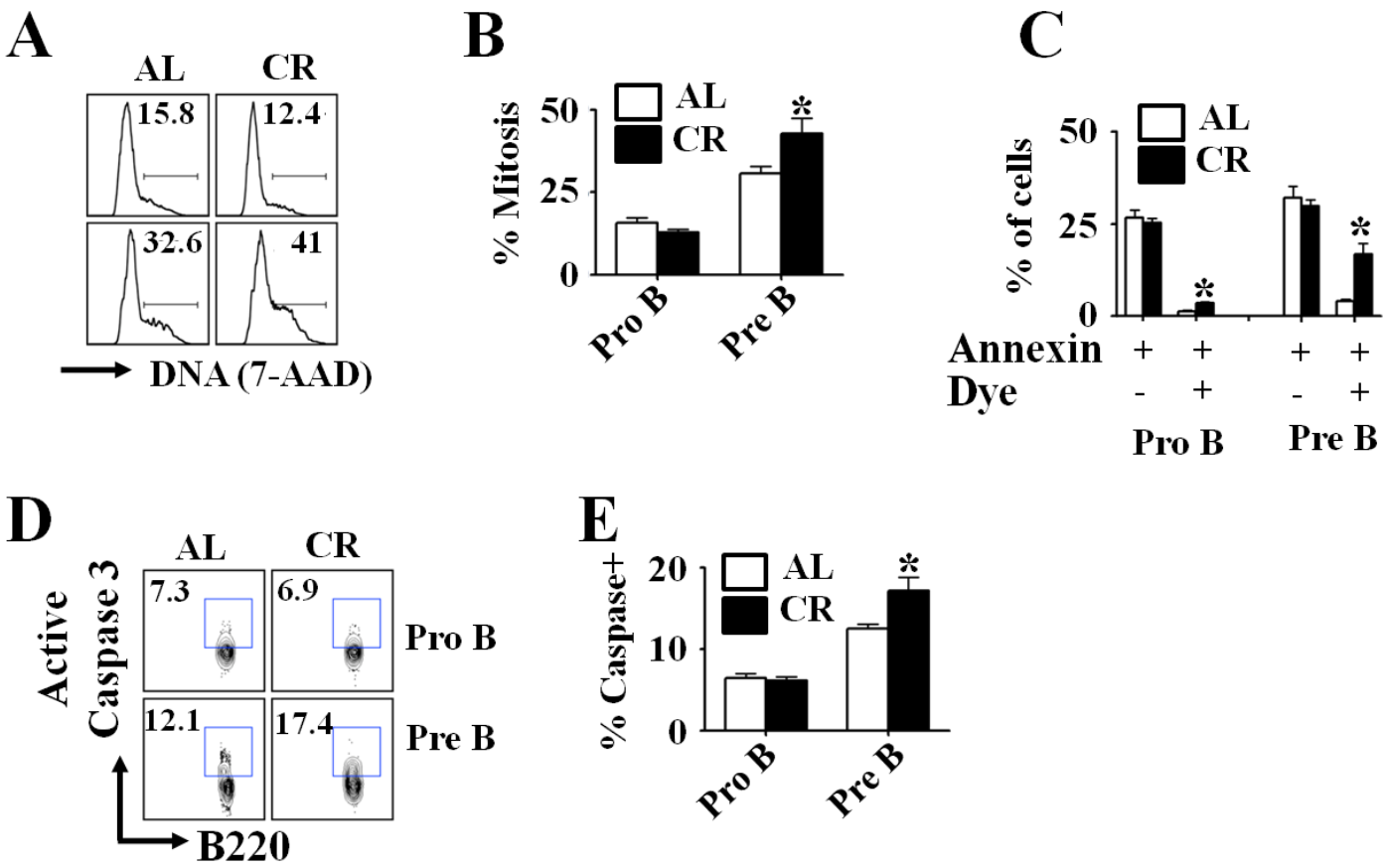

Figure 31: Quantification of proliferation and apoptosis of developing B cells in the BM of AL and CR mice. A) DNA content was determined using 7-AAD staining in pro and pre-B cells and the frequency of cells in mitotic stages ($S/G_2/M$) of the cell cycle was determined based on intensity of 7-AAD fluorescence. B) Graphical summary of the frequency of pro and pre- B cells in $S/G_2/M$

(Figure 31 cont'd) phase in BM from AL and CR mice. C-D) Following short-term culture in serum free media, B cells were phenotyped into pro and pre-B cells and the frequency of cells undergoing apoptosis from AL and CR mice was quantified using Annexin V and 7-AAD staining (C) or active caspase 3 (D) staining. Flow plots and histograms are representative. Data are means ± SEM. * indicates significance, $p<0.05$ (n=7-8 mice/group).

CR results in an increased percentage of mature granulocytes

Developing granulocytes and B cells are thought to occupy a similar niche within the BM, and through changes to the reciprocal interactions between these cell populations, a balance between granulopoiesis and lymphopoiesis is achieved [403]. BM of CR mice contained a greater portion of granulocytes and a concomitant decrease in lymphocytes within the BM of CR mice, leading us to investigate whether a specific subset of granulocytes were increased, or if the increased frequency represented an expansion of all developmental stages. Analysis of Ly-6C^{int} CD31^{-} cells for expression of Ly-6G in combination with CD11b allows for the differentiation of promyelocytes and myelocytes (Ly-6G^{-} CD11b^{+}), metamyelocytes and band cells (Ly-6G^{hi} CD11b^{lo}), and neutrophilic granulocytes (Ly-6G^{hi} CD11b^{hi}) (**Fig 32A**) [403, 405]. CR BM was found to contain significantly higher percentages of promyelocytes and myelocytes, and differentiated mature neutrophils, relative to granulocytes and total cells (**Figure 32B**). BM from CR mice also contained a reduced frequency of intermediate granulocyte progenitors; metamyelocytes and band cells**.** Metamyelocytes represented a comparable frequency of the total BM pool of AL and CR mice (Figure 32B). These changes were somewhat reflected in the periphery as when we found neutrophils represented an equal portion of CD45^{+} leukocytes in

the blood, but were significantly increased in the spleen of CR mice compared to AL mice (**Figure 32C**).

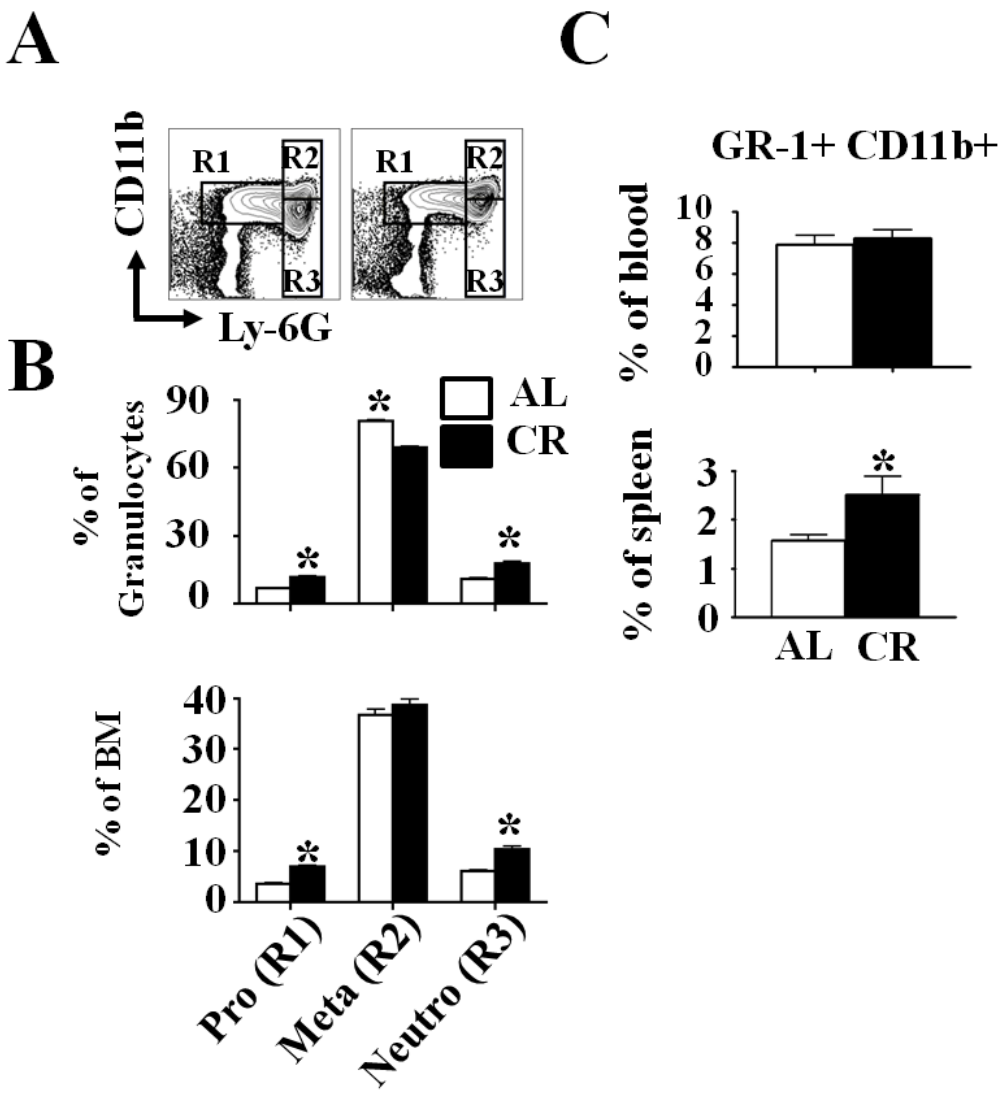

Figure 32: Developmental subsets of granulocytes in the BM of AL and CR mice. A) Granulocytes were gated Ly-6C^{int}, CD31^{-} in BM from AL and CR mice and divided into developmental stages consisting of (R1) myelocytes and promyelocytes (Ly-6G^{-} CD11b^{+}), (R2) metamyelocytes and band cells (Ly-6G^{hi} CD11b^{lo}), and (R3) neutrophilic granulocytes (Ly-6G^{hi} CD11b^{hi}). B) Graphical summary of the frequency of granulocytes in corresponding developmental stages (top), and the frequency of total BM cells that each developmental stage represents (bottom). C) Granulocyte frequency, (GR-1^{+} CD11b^{+} SSC^{hi}) in the blood (top) and

(Figure 32 cont'd) spleen (bottom) of AL and CR mice. Data are mean ± SEM. * indicates significance, $p<0.05$ (n=4-5 mice/group/experiment). Individual experiments were repeated twice.

BM granulocytes from CR mice are more resistant to apoptosis

After observing significantly more developing granulocytes in the BM of CR mice compared to the BM of AL mice, we tested whether CR alters proliferation or apoptosis of granulocytes. We found a 13% reduction in the frequency of promyelocytes and myelocytes (GR-1^{lo} $CD11b^{int}$) containing DNA content representative of $S/G_2/M$ phase in CR mice compared to AL mice (20 ± .46% vs. 23 ± .53%, respectively). No differences in the frequency of metamyelocytes and band cells or neutrophils undergoing mitosis in the BM of AL and CR mice was detected (**Figure 33A-B**). Similar to our studies on B cells, we observed no difference in apoptosis between AL and CR granulocytes directly *ex vivo* (data not shown). However, myelocytes and promyelocytes as well as mature neutrophils from CR BM were significantly more resistant to apoptosis, as fewer of these cells stained positive for indicators of cell death, Annexin V, and active caspase 3 following short term serum starvation cultures of 6 or 8 hours, respectively (**Figure 33B-C**).

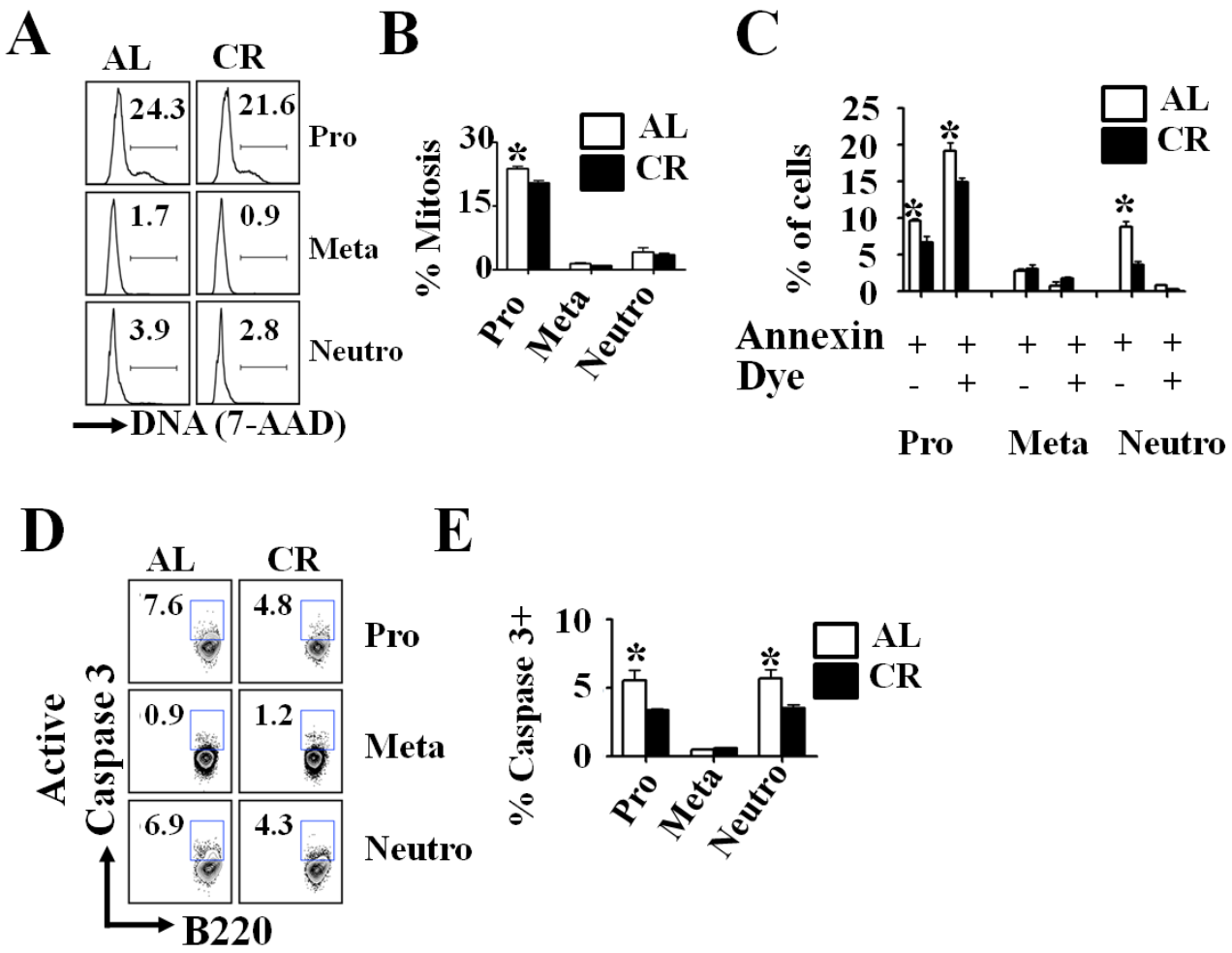

Figure 33: Proliferation and apoptosis of granulocytes in the BM of AL and CR mice**.** A) The frequency of myelocytes and promyelocytes (Pro), metamyelocytes (Meta) and neutrophilic granulocytes (Neutro) in mitotic stages ($S/G_2/M$) of the cell cycle was

(Figure 33 cont'd) determined by DNA content using intensity of 7-AAD fluorescence. B) Graphical summary of the data provided in (A). C-D) Following short-term culture in RPMI 1640, granulocytes cells were phenotyped into developmental stages and the frequency of cells undergoing apoptosis from AL and CR mice was quantified using Annexin V and 7-AAD staining (C) or active caspase 3 (D). Flow plots and histograms are representative. Data are mean ± SEM. * indicates significance, $p<0.05$ (n=7-8 mice/group).

CR alters BM microenvironment

As our data clearly indicate CR alters the balance between lymphopoiesis and granulopoiesis, we analyzed BM serum for changes in concentrations of various growth factors that could be responsible for this observation. We found IL-7 to be slightly, but significantly reduced in the BM serum from CR mice (**Figure 34**). The development of both myloid and lymphoid cells is at least partially dependent on leptin, an adipokine [232], that we found to be reduced by approximately 5-fold in the BM of CR mice (Figure 34). Corticosterone, the major endogenous glucocorticoid was also found to be approximately 2-fold higher in BM supernatants from CR mice than AL mice (7.7 ± .9 pg/ml vs. 3.9 ± .52 pg/ml, respectively). We found no change in other growth factors known to contribute to lymphopoiesis and granulopoiesis such as SDF-1α , GM-CSF, and Flt-3L, although GM-CSF trended ($p=0.056$) to be increased in the BM of CR mice (Figure 34).

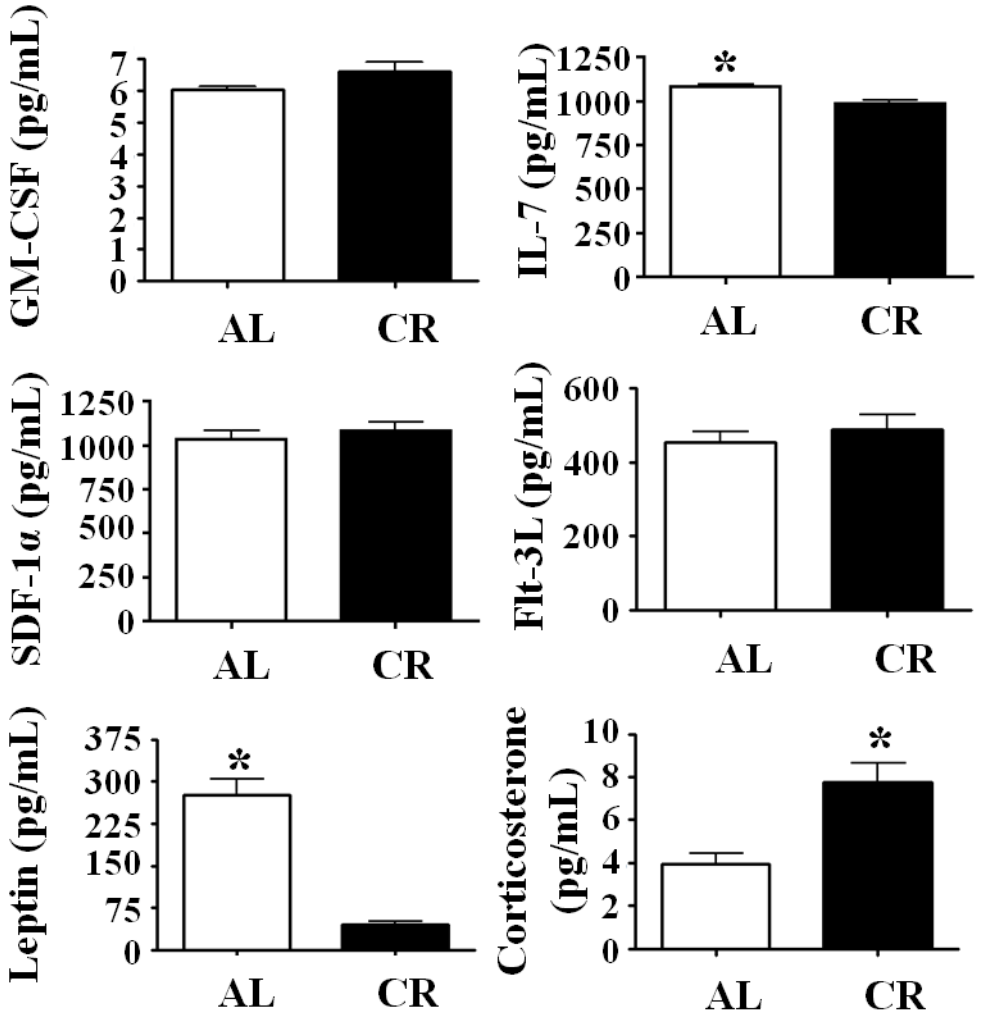

Figure 34: Characterization of BM microenvironment in AL and CR mice. BM from AL and CR mice was flushed with 500µl PBS, cells were pelleted by centrifugation, and supernatants (BM serum) stored at -80^{o} C for further downstream application. Concentrations of the indicated growth factors and cytokines in BM serum were determined by ELISA. Data are mean ± SEM. * indicates significance, $p<0.05$ (n=12-15 mice/group).

DISCUSSION:

CR influences the peripheral homeostasis of T and B cells (278), however little is known about the influence of CR on lymphopoiesis and granulopoiesis in the BM. Furthermore, CR is almost always studied in the context of aging, making it difficult to delineate the influence of CR from age related changes to the immune system that occur over the lifespan of an organism [273]. In these studies we clearly demonstrate CR, independent of age related immune senescence, suppresses B cell development in the BM and this suppression occurs during early stages of B cell development. Development of lymphoid progenitors into prepro-B cells appears unimpeded in CR mice as these cells represented a comparable frequency between CR and AL. Thus, developmental blocks in B lymphopoiesis were likely related to the observation that pro and pre-B cells are reduced in CR BM. Our data regarding the influence of CR on B cell development in the BM provide a novel link between observations regarding the influence of CR on peripheral B cell frequency and the well characterized B cell developmental process in murine BM. We also report a significant increase in granulocyte development in the BM of CR mice, an observation which, to the best of our knowledge, has never been reported before.

CR enhances humoral responses in aged rodents [313], our data must be considered in the context of age related suppression of B lymphocyte generation. Elegant studies by Keren et al show continuous depletion of B cells in the periphery results in signals to hematopoietic cells in the BM which rejuvenate B lymphopoiesis [282]. Similar parallels can be drawn between data regarding the ability of CR to inhibit immunosenescence. Senescent memory cells are deleted in peripheral tissues of CR mice, which is hypothesized to stimulate hematopoiesis and provide naïve adaptive immune cells [6]. We observed a decrease in peripheral B cells in the spleen and blood of CR mice, and increased cycling of developing B cells in the BM, perhaps in response to

increased demand for peripheral B cells. Nonetheless, we also detected increased apoptosis of developing B cells in CR BM, resulting in a continuous demand for B cells in CR mice. While the studies presented here show a decrease in developing B cells in BM of CR mice, it will be interesting to determine whether the continuous demand for B cells stimulated by CR results in enhanced B lymphopoiesis in aged CR mice compared to aged AL mice.

One of the most striking observations presented in this manuscript was that immature B cells were nearly absent in BM of CR mice. This observation, and that pre-B cells underwent enhanced apoptosis, suggests to us that pre-B cells do not survive long enough in CR BM to differentiate into immature B cells. Despite this observation, a large proportion of B cells in BM of CR mice were mature IgD^+ B cells. A similar observation has been reported in leptin deficient (*ob/ob*) mice in which pre-B cells and immature B cell numbers are severely reduced, but mature B cells represent a large fraction of B cell population in BM [232]. As memory B cells have a relatively long half-life, some capacity for self-renewal, and animals utilized in this study had been subjected to a CR diet for less than two months [406], we propose IgM^+ IgD^+ B cells in the BM represent antigen experienced, memory B cells that developed prior to the initiation of CR. Because of the dramatic impairment in B cell development induced by CR we report here, future studies should further investigate the phenotype of IgM^+ IgD^+ cells in CR BM to confirm they represent long lived memory cells.

In order to investigate potential causes for the observed changes to lymphopoiesis and granulopoiesis induced by CR, we examined the cytokine mileau in the BM microenvironment of AL and CR mice. CR without malnutrition has been demonstrated to influence multiple physiological systems; adipocyte structure and function as well as neuroendocrine function are

both influenced by CR and both are known to influence immune homeostasis [215, 407, 408]. During periods of nutritional stress the neuroendocrine system regulates hematopoiesis; in order to promote survival the organism must allocate its resources to the most critical systems [409, 410]. CR has been previously described as a nutritional stress, feedback from systemic hormones such as leptin, results in alterations to neuroendocrine function in CR mice [411, 412]. This, in turn, stimulates glucocorticoid (GC) production by the adrenal system [413]. Among the cytokines and hormones investigated, we found GCs to be increased and leptin decreased in the BM of CR mice. Here we show granulocytes in CR BM were increased and more resistant to apoptosis than granulocytes from AL BM, an observation in agreement with the known role of GCs in enhancing survival and expansion of granulocytes [405, 414].

As a major source of innate immunity, neutrophils are critical for protection from bacterial infections and some viral infections. Neutrophil dysfunction and neutropenia are often associated with increased susceptibility to infection, while neutrophilia is associated with the response to severe bacterial infections [415, 416]. During periods of stress such as starvation, neutrophils are one of the last immune populations to become compromised, highlighting the critical nature of neutrophils in providing an organism with immune defense. While others have reported no changes in peripheral neutrophil frequency as a result of CR [320], this is the first time in which the influence of CR on granulopoiesis has been examined. Our data suggest the microenvironment in CR favors the survival and maintenance of granulocytic cells, but not developing B cells, perhaps reflecting an evolutionary mechanism that provides an organism with immune defense when nutritional resources are scarce.

Several reports have identified a critical role for leptin in regulating hematopoiesis [207, 232], leading to the hypothesis that leptin acts as a metabolic signal integrating energy

availability and immune homeostasis [232, 417]. Leptin has been proposed to support B cell development and homeostasis through inhibition of B cell apoptosis [417] and our data are in agreement with this observation. Indeed, we determined pre-B cells from BM of CR mice were more susceptible to apoptosis than pre-B cells from BM of AL mice. Pre-B cells were also shown to be significantly reduced in *ob/ob* mice and expand upon leptin administration [232], supporting the hypothesis that leptin deficiency in CR contributes to reduced pre-B cells in the BM. We also detected a small, but significant decrease in the IL-7 concentrations within the BM, a cytokine which is critical for B cell proliferation and homeostasis [418, 419]. Likely, CR influences multiple systemic and local hormones and cytokines, resulting in an altered microenvironment in which immune cells develop.

The concept that a reciprocal relationship exists between developing granulocytes and B cells has been proposed over the last decade [403]. Studies indicate that this relationship can be directly influenced by inflammation, infection, stress, and obesity [232, 233, 403, 420]. Here, we show CR, a pro-longevity dietary intervention, also results in substantial changes to developing leukocytes within the BM and these changes are reflected in peripheral tissues such as blood and spleen. CR is often studied in the context of aging, yet the full extent to which CR alters the immune system independent of age has not yet been fully described [7]. Given the multitude of immunological, hormonal and metabolic changes that accompany dietary interventions such as CR, it is difficult to identify a specific pathway responsible for our observations. However, we believe as a result of CR there are alterations to multiple growth factors and cytokines in the microenvironment, resulting in changes to leukocyte homeostasis in an attempt to best protect the organism with the nutritional resources available. Future studies

are required to examine the implications of our findings on the immune response to various pathogens and disease states as well as how CR influences responsiveness to vaccination.

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