ENVIRONMENTAL IMPACT ON LIFELONG IMMUNITY: THE ROLE OF INFECTION AND NUTRITION IN HOST HOMEOSTASIS

by

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF IMMUNOBIOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2019

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

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ACKNOWLEDGMENTS

To the taxpayers who paid for this work.
To the National Institute of Health who distributed funds for this work.
To Janko who supervised this work.
To the members of the Nikolich lab who critiqued this work.
To the people who will read this work.
To the ones who told me to be quiet and get back to work.

I thank you all.

DEDICATION

For my family

Do mo theaghlach

Para mi familia

PREFACE

The beginning of wisdom is: get wisdom;

Whatever else you get, get understanding.

(Proverbs 4:7)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AdipoR – adiponectin receptor
AL – ad libitum
APC – antigen presenting cell
ASCT2 – alanine-serine-cysteine transporter 2
BAT – brown adipose tissue
BMAT – bone marrow adipose tissue
cAMP – cyclic AMP
CCL2 – chemokine ligand 2
CCR2 – C-C chemokine receptor type 2
CIITA – class II transactivator
CMV – cytomegalovirus
CR – calorie (caloric) restriction
DAMPs – damage associated molecular patterns
DR – dietary restriction
ds – double stranded
EBV – Epstein-Barr virus
eNOS – endothelial nitric oxide synthase
ERK – extracellular signal-regulated kinase
G-CSF – granulocyte colony-stimulating factor
GLUT – glucose transporter
gpCMV – guinea pig CMV
HCMV – human cytomegalovirus
IL – interleukin
ILC – innate lymphoid cell
IRS – insulin receptor substrate
JAK – Janus kinase
LAT1 – large neutral amino acid transporter 1
LepR – leptin receptor
LPS – lipopolysaccharide
MAPK - mitogen-activated protein kinases
mCMV – mouse cytomegalovirus
MHC – major histocompatibility complex
MZ – monozygotic
NF-kB – nuclear factor kappa-light-chain-enhancer of activated B cells
NK – natural killer
NLR – NOD-like receptor
OXPHOS – oxidative phosphorylation
PI3K – phosphoinositide 3-kinases
PKA – protein kinase A
PPAR – protein phosphorylation activator receptor
PRR – pattern recognition receptor
rCMV – rat cytomegalovirus
rhCMV – rhesus macaque CMV
SNS – sympathetic nervous system

STAT – signal transducer and transactivator
TAP – transporter associated with antigen processing
TCR – T cell receptor
TDEE – total daily energy expenditure
Th – T helper (type)
TLR – Toll-like receptor
TNF – tumor necrosis factor
TZDs – thiazolidinediones
VAC – viral assembly compartment
vCKRs – Viral cytokine receptors
vCKs – Viral cytokines
WAT – white adipose tissue

ABSTRACT

The impact of environmental pressures on the immune system is significant and complex. External influences contribute to immune development, cause or impact diseases, and fundamentally alter responses to future stressors. Infection and diet are two of the most profound external modifiers of host physiology. They can fundamentally alter normal biological pathways and lead to disparate phenotypes even in the context of identical genotypes, as in monozygotic twins. This dissertation will detail work that has investigated impacts of (i) lifelong infection and (ii) nutritional modulation upon host immunity, health, and lifespan. The first topic was addressed by studying the consequences of lifelong cytomegalovirus infection in the context of adipose tissue inflammation, viral persistence, and hyperglycemia. For the second, caloric restriction was used as a dietary intervention in old age and the consequences on immune cell populations across a spectrum of host tissues were studied. Obtained findings cross with the fields of immunology, gerontology, nutritional sciences, aging, virology, and endocrinology and have potentially significant and broad reaching consequences for human health.

GOALS OF THIS DISSERTATION

“Everyone has a plan until they get punched in the mouth.” – Mike Tyson

Memory is the sum of imprinted experiences and is a crucially important aspect of immunity. Experience of the environment influences the formation, maintenance, and recall responses of the innate and adaptive arms of the immune system. Factors such as age, dietary nutrition, and infectious or non-infectious disease status are of the utmost importance when interpreting data used to devise potential preventative, prophylactic or therapeutic treatments, and track clinical outcomes. Therefore, rigorous research that combines the complexities of nature and nurturing factors is of great importance to deeper understanding of immunity. The goal of this dissertation is to present research that has interrogated multiple angles of environmental pressures and to demonstrate the effects, in combination, that aging, dietary interventions, and lifelong infection can have on the immune system across the organism. Here I will highlight, specifically, research on how caloric restriction (CR) impacts the tissues of the immune system and leukocyte redistribution. I will also present an analysis of the impact of lifelong infection in a crucial endocrine organ, adipose tissue, in the context of its role in systemic metabolic health. Each chapter of this dissertation will contain relevant background information for all topics covered.

CHAPTER 1. DEFINING ENVIRONMENT AND DEFINED BY ENVIRONMENT

"…often it is not so much the kind of person a man is as the kind of situation in which he finds himself that determines how he will act." – Stanley Milgram

Nature versus nurture. Non-heritable versus heritable. Environmental impact versus germline encoded behavior. Environment, defined as the circumstances, objects, or conditions by which one is surrounded, is a significant factor in how we experience and perceive the world (Meriam-Webster). Host physiology is strongly shaped by the environment and non-heritable factors. Specifically, here, a focus will be placed on how the immune system responds after accumulation of lifelong environmental pressures and the consequences, as measured here, of these experiences. The purpose of this dissertation is to present data from studies that have interrogated the immunological consequences of nutritional and infectious challenges on lifelong immunity. This chapter will briefly introduce the current understanding of what environment means and how it interfaces with host immunity.

One of the most powerful and informative ways to study natural influences on immunological responses is an observation of monozygotic (MZ) twins. Twin studies have been of great academic interest for centuries, occupying the minds of the ancient philosopher Hippocrates of Kos and Saint Augustine [1]. However, Francis Galton is credited as being the first to suggest systematically studying twins to delineate the impacts of genetics (nature) versus external influences (nurture) [2]. Galton said of twins "that their history affords means of

distinguishing between the effects of tendencies received at birth, and of those that were imposed by the circumstances of their after lives." Nearly 150 years later, it is greatly appreciated that environmental pressure exerts tremendous power over the formation and maintenance of host immunity and other aspects of biology. This point is highlighted by a MZ twin study that analyzed 105 pairs of MZ twins for >100 immune parameters, including leukocyte cytokine production, circulating serum protein levels, and immune cell population frequencies [3]. Of these factors 77% were partially determined by non-heritable influences (> 50% of variance explained by environment) and 58% were significantly changed by a single herpesvirus, cytomegalovirus (CMV), (>80% of variance) [4], that is also a topic of this dissertation. Alternatively, to MZ twin studies are analyses of genetically similar populations such as the German American Hutterite and Amish communities. These studies provide evidence supporting the strength of environmental exposures on immune function. One particular study demonstrated that the development of innate immunity and risk of asthma depends on environmental exposure determined by disparate agricultural technologies and living conditions (due to antigen presence in dust) [5,6]. When taken together these data suggest that phenotypic variation in immune function is only partially explained by genetics, at both the identical twin and homogenous population levels, and antigenic experiences (such as environmental dust and pathogenic infections) more powerfully influence immune cell proportions and functions than genetics.

Limited ability to control the myriad variables in outbred humans still necessitates the use of well controlled and designed experiments. Utilization of animal models, therefore, remains necessary to interrogate specific environmental pressures on phenotypes of the immune system. Such studies have generated a large amount of of data on the environmental influences that have the greatest impact on molding host immunity. Synergizing these results into increasingly more complex experimental models is the next logical step in understanding host immunity. To attempt to do this, let us discuss separate effects of nutrition and of persistent infection on immunity.

Nutrition Interfacing with Environment

Nutrition is intimately connected to host immunity and immune responses. A prime example of this the activation of T cells, which is a highly metabolically coordinated event that necessitates an energetic switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis [7]. This metabolic switch sacrifices energetically efficient production of adenosine triphosphate (ATP) during OXPHOS to increase the availability of molecular precursors used in cellular proliferation and effector functions via aerobic glycolysis [8,9]. T cell proliferation during aerobic glycolysis requires glucose and amino acids, two dietarily derived substrates [10,11]. Several molecular transporters are involved in the import and export of these cellular building blocks and include amino acid transporters (such as CD98/LAT1; large neutral amino acid transporter, ASCT2; alanine-serine-cysteine transporter 2) as well as the family of glucose

transporters (GLUT). Optimal function and expression of these molecules is crucial for cellular health. Overexpression of GLUT1 on CD4 T cells results in an increased number of memory and naive T cells and T cell specific deletion results in poor cellular activation, clonal expansion, and survival [12]. Loss of CD98 prevents optimal proliferation and differentiation of CD4 and CD8 T cells during an immune challenge [13]. In addition to this lost proliferative capacity, inability to import neutral amino acids promotes the development of regulatory T cells and dampens inflammatory responses, potentially mitigating autoimmunity but at the expense of vulnerability to infection [14].

It is obvious that at the cellular level access to fuel drives host processes. Host level interventions have been investigated to alter such functions. CR and certain dietary restriction (DR) studies are examples of such attempts. These methods have been the most robust treatment in increasing host longevity and health span across multiple model organisms [15,16]. Studies have revealed several evolutionarily conserved pathways related to longevity including insulin and insulin-like growth factor (IGF) signaling pathways, amino acid signaling, target of rapamycin (TOR) S6 kinase pathways, and glucose signaling pathways such as the Ras-protein kinase A (PKA) pathway [17–19]. Although these pathways are conserved, they have functionally different consequences at the individual cellular level. Therefore, an understanding of differing dietary strategies and their consequences is necessary before adoption and recommendation of these interventions. This has resulted in the proliferation of strategies of DR and CR. Such strategies include short-term starvation, periodic fasting, diets that

mimic fasting, intermittent fasting or time-restricted fasting, macromolecule restriction (including protein, carbohydrate, or fat restrictions). The strategy highlighted here, for purposes of this dissertation (Chapter 5), is CR in the absence of malnutrition, referring to a 40% decrease in calories consumed below the total daily energy expenditure (TDEE).

Interpreting the results of CR studies, however, is increasingly complex. CR results in lifespan extension across multiple model organisms, and while we understand some molecular underpinnings underlying its effects (e.g. mTOR inhibition [15], sirtuin activation [20] and an increase in autophagy [21]), the totality of CR effects is pleiotropic and remain difficult to dissect. Prevention and delayed onset of age-related diseases suggest that the immune system may contribute to extended health span and has been interrogated utilizing, among others, rodent models. For example, lifelong adherence to CR in B6BAT6 F1 mice slows the age related accumulation of memory T cells in the blood and spleen [22] suggesting extension of the health span is, in part, improved potential to defend against pathogens. Additionally, the proliferative capacity of T cells following antigen stimulation is, likewise, maintained in old C57BL/6 mice [23]. However, the benefits of CR are not universal. Lifelong CR diet in the C57BL/6 mouse results in increased median and mean lifespans, decreased autoimmunity, and cancer incidences in both male and females [24,25]. However, this pro-longevity effect is not present in DBA/2 [26] or wild-derived laboratory bred mice [27]. These data suggest that although there are conserved pathways involved in response to CR, as described above, there are genetic

effects that alter their potential benefits. In the higher animal species, non-human primates (monkey) and humans, there are also demonstrated benefits of CR. Early monkey studies suggested that CR increased lifespan of rhesus macaques, how this experiment was done with a small number of CR monkeys and only in males [28]. Additional studies have demonstrated that the age at which CR initiates is of supreme importance, specifically in the non-human primates where CR begun in old age had no effect, and may have been detrimental [29]. Finally, humans that prescribe to a low-calorie lifestyle and those enrolled in clinical trials have demonstrated net benefits CR, including improved cardiovascular health, decreased cancer incidences, lower body fat, and low prevalence of circulating inflammatory markers [30]. However, care must be taken when interpreting these results as those who adhere to a CR diet potentially participate in other healthy lifestyle choices.

Recent studies have highlighted this need for care and suggest that the context in which an animal or individual reside is just as important as the quantity or quality of calories consumed [31]. This point is well made when interpreting the somewhat conflicting results of two major rhesus macaque CR studies. The National Institute of Aging (NIA) conducted a 23 yearlong study and [32] did not demonstrate any significant increase in longevity, although there were results suggesting improvement in health span, such as a decrease in plasma concentrations of triglycerides in male monkeys, suggesting improved liver and glucose homeostasis. Conversely, a similar study carried out at the University of Wisconsin reported increased lifespan and reduced all-cause mortality [16,33].

Although neither of these studies directly measured immune function, both groups demonstrated a slight decrease in the prevalence of cancer incidences.

Why were these factors that resulted in these studies different results? Nutritional and other factors have been invoked to explain these differing results. Limiting rhesus macaque access to food during specific times of the day or allowing unrestricted, ad libitum (AL) intake, the nutritional make-up of each diet, previous study enrollments, genetic backgrounds of both cohorts, and housing conditions (free-roaming or in specific-pathogen free style cages) have all been suggested to be the main driver for the results. Whatever the ultimate cause of the differences between these two studies, the accumulation of multiple environmental variables potentially had a dramatic effect on the presentation of specific phenotypes. Analysis of the dietary makeup of the two studies, suggested that varying caloric sources can drive differences as well. Studies that have explored the effect of caloric sources [34–36], different diet modalities [37], and alternative strategies of CR [38], all demonstrate that dietary intake can alter host homeostasis and immunity at a global level.

Impact of the Microbial Environment on Immune Development

One point that was made clear in the CR rhesus macaque study was that environmental effects outside of diet could potentially be responsible for the results. Nutrition has direct effects on immune cells and effector molecules, but it is also capable of altering the microbiome that resides on or within many, but not all, host tissues of an organism. Previous studies have suggested that the

microbial community outnumbers eukaryotic cells by a factor of 10 and represent a genome larger than endogenous human genes by 100 to one [39,40]. However, newer estimates place the cellular ratio at 1:1 possibly lowering the genetic ratio to 10 to one [41]. Regardless, microbiome still represent our ‘second self.’ In addition to the bacteria of the microbiome is the viral metagenome, which can be incorporated in the host, infect host microbiota, or persist in the host in an asymptomatic state [42,43]. Alteration of the host microbiota and viral metagenome has broad ranging health consequences, from controlling the manifestation of gastro-intestinal diseases, including Crohn’s disease and ulcerative colitis, all the way to the gut-distal changes, including metabolic dysfunction, autoimmunity, and neurological disturbances [44,45].

Another important impact of diverse microbial exposure has been highlighted by a recent study regarding the exposure of specific-pathogen free mice to wildtype, or ‘dirty,’ mice [46]. Exposing genetically inbred animals to the microbes of wild-caught mice resulted in dramatic alterations to the population dynamics of immune cells in circulation and in tissues. This study showed that specific-pathogen free mice mirrored the immune system, specifically T cell subsets, of a human newborn whereas ‘dirty’ (wild-type polymicrobial flora-exposed) mice exhibited an adult human-like immune profile. These types of experiments are critically important as they will allow an interrogation of different disease types and their therapies in the context of in the context of matured microbiota and viral metagenome, typically seen in normal, microbially exposed,

humans However, this further suggests that increasingly complex model systems are required during the design and implementation of experiments.

Environmental pressures contribute to the maturation of the immune system. Exposure to commensal bacteria and viruses, as well as to pathogenic species, shape host immunity and mature leukocyte populations from infancy to old age [47–49]. The development of strong and persisting immunological memory is the desired outcome of such microbial exposures. It is a result of an organized and appropriate response by the innate arm of the immune system that then sequentially communicates with and contributes to the activation of the adaptive immune system. This can result in lifelong protection against certain infections. One major question related to this issue in the immunological field, and of central importance to this dissertation, is how does lifelong experience of repeated antigenic stimulation alter, if at all, systemic host functions (specifically here host metabolism). Chronic and persistent infections, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), Toxoplamsa gondii, and Mycobacterium tuberculosis, profoundly influence immunity. These infections can lead to continual antigenic stimulation of the immune system and have a broad range of consequences. For some of these microbial pathogens, such as CMV, this was, in part, hypothesized to be the driving force of decreased immunological capacity and function in aged adults.

CHAPTER 2. CYTOMEGALOVIRUS

"Can you ever "solve" disease, unemployment, war, or any other societal herpes? Hell no. All you can hope for is to make them manageable enough to allow people to get on with their lives. That's not cynicism, that's maturity." – Max Brooks

CMV infects much of the world's population and has demonstrated impacts on host development, and, , has several reported associations with or direct effects on, health and immune function. CMV can alter the innate and adaptive arms of host immunity in significant ways. For the purposes of this thesis, the following characteristics of the virus are of critical importance : (i) the virus encodes many different immune evasion molecules, allowing it to become latent and persist in infected hosts for life; and (ii) CMV drives a phenomenon in both CD4 and CD8 T cells termed memory inflation, that was hypothesized to negatively impact immune function in the elderly due to spatial and energetic requirements to maintain a large pool of CMV-specific T cells. For these reasons, the Nikolich-Žugich laboratory has long been working to bring about a mechanistic understanding the impact CMV has on human health and immune responses over the lifespan. Due to the hallmark ability of this virus to hide (latency) and come out of hiding (reactivation) the holy grail of the field has been to identify anatomical locations that could harbor latent virus during its undetectable and persistent state. These questions led to the hypothesis driven work that will be discussed later (Chapter 4).

Accumulation of antigen specific T and B cells has been suggested as ‘pushing out’ and outcompeting naïve cells that have yet to experience their antigen and preventing an appropriate response to newly acquired infections [50]. Furthermore, the organization of lymphoid organs, including the thymus and lymph nodes, deteriorates with aging, thereby decreasing the number of circulating naïve T cells and increasing the demand for peripheral maintenance of lymphocytes [51,52]. The additive accumulation of cell intrinsic defects and the spatial and temporal dysregulation of innate and adaptive immune cells prevents appropriate initiation of immune responses [53–55]. Chronic and persisting pathogens have been theorized to contribute to these changes in both beneficial and deleterious ways. CMV, for instance, has been described as both harmful (overrepresentation of CMV-specific T cells) and helpful for adaptive immunity (broadening of the immune repertoire to other infections [56] potentially due to increased basal inflammation) [57]. For these reasons CMV infection across the continuum of age is a primary focus of this dissertation.

CMV Ubiquity

CMV is an ubiquitous betaherpesvirus that is estimated to asymptomatically infect 60 to 99% of the human population, depending on the population sampled [58]. Through evolutionary history the CMV family members have co-evolved with their vertebrate hosts, resulting in highly unique, and species-specific CMV adaptations [59]. Due to this specificity, while the general

structural regions of the double stranded (ds) of DNA viral genome are conserved, each species specific virus diverges by minor differences in entry, evasion, and persistence strategies [60]. Here highlighted are aspects of CMV biology, related to basic genome structure and lifecycle, cellular tropism, and known consequences of infection. When relevant, examples of species-specific features of CMV will be denoted, to include viruses specific to rhesus macaques (rhCMV), guinea pig (gpCMV), mouse (mCMV), rat (rCMV), and humans (HCMV).

Basic Structure and Replication Cycle

CMV consists of a linear dsDNA genome that is protected by a viral capsid surrounded by tegument proteins. This is encompassed by a membrane envelope that is enriched with structural viral glycoproteinsgB, gH, gL, gM, gN, and gO [61,62]. These glycoproteins are utilized during cell entry. Different combinations of these surface molecules confer CMV with a wide cellular tropism [63,64].

CMV spreads through bodily fluids such as saliva, breast milk, blood, and urine [65–67]. It is hypothesized that this is the reason for the disparity in number of seropositive individuals between developed and developing countries, where higher childhood and population density and hygiene practices are believed to contribute to increased percentage of infection in the latter. Following cellular attachment mediated by glycoproteins, gB and gH drive membrane fusion, of the viral envelope with cellular membrane, resulting viral entry into the cellular

cytosol [68] although alternative cell-dependent routes have been also described [69],. CMVs co-opt the host microtubule machinery and free capsids traffic to the nucleus where viral genome is uncoated, circularized, and viral replication begins [70,71].

Hallmark of herpesvirus family is the ability to maintain viral genome in a latent state. Initial lytic CMV infection is followed by this latent and persistent phase in which virus no longer productively replicates but lays dormant in certain cellular and tissue locations. During this time viral genome is not incorporated in host genome and is maintained at low copy number as a plasmid utilizing host histones [72,73]. In the case of HCMV, it is well established that hematopoietic progenitor cells of the bone marrow can serve as a reservoir for latent virus [74,75]. In the case of mCMV, latency follows a canonical spread and contraction [76,77]. Shortly after acute infection mCMV is detectable throughout the body, however as time passes both HCMV and mCMV localize to the lungs and salivary glands, although several other tissues harbor detectable virus [77–79]. In the salivary gland tissue, virus can be detected within the glandular epithelial cells, which secrete saliva into the salivary duct, thus providing a potential avenue for virus to spread.In both the case of HCMV and mCMV reactivation of virus has been demonstrated using ex vivo tissue explant cultures and in immune suppressed patients during tissue transplantation leading to full viral dissemination and CMV disease [80,81]. This observation suggests that during the viral lifecycle of silencing and latency, the immune system plays an active role in patrolling the sites of viral residency.

Early Immune Response and Viral Evasion

The CMVs have evolved unique strategies for immune evasion during co-evolution with the infected host. Control of CMV is a delicate balance between host immunity and viral programming that push and pull the virus into various states of activation and latency [82,83]. CMV evades both innate and adaptive immunity by preventing antigenic recognition by leukocytes and altering the cytokine environment of infected cell to alter inflammation. Control of CMV infection is largely mediated by the lymphocytes (Natural Killer (NK) cells, CD4, and CD8 T cells) after initial responses that involve monocytes and macrophages.

NK cells, as a constituent of the innate immune system, provide the host a method of rapid identification of non-self-molecules and subsequent elimination of cells without the need for previous antigenic education. Early studies in mice lacking functional NK cells, either genetically or antibody depleted, revealed their necessity for mCMV control [84–86]. which is supported by clinical data from NK cell deficient HCMV infected patients [87]. Activation of NK cells, in general, occurs via “missing self” or environmental activation pathways. “Missing self” activation occurs when the inhibitory signaling mediated by major histocompatibility molecule (MHC) class I is overcome by activation ligands [88]. This results in the activation of NK cell cytotoxic function and subsequent release of cytokines. CMV expression of a faux MHC molecule, m144, is believed to inhibit activation of NK cells by preventing missing self activation, but the

definitive mechanism of action has not yet been characterized [89]. Expression of the inhibitory Ly49 family of receptors prevents activation of NK cells. Indeed, mCMV encoded genes in the m157 locus produce a protein that interacts with the Ly49H receptor (expressed in C57BL/6 but not BALB/c, mice) and this interaction activates NK cells, leading to lysis of infected cells [90–92]. During infection and stress the direct activation of NK cells is mediated through NKG2D receptor, which recognizes DAP signaling molecules, and the NCR1 (NKp46) receptors, for which the activation mechanism is still unclear [93–95]. mCMV encodes four gene products (m138, m145, m152, and m155) that inhibit NK cells through the NKG2D receptor [96].

Monocytes are also crucial to early inflammatory responses during infection and during normal tissue homeostasis. During inflammation monocytes are mobilized to sites of challenge or injury where they contribute to inflammation through the production of tumor necrosis factor (TNF) α, nitric oxide, Interleukin (IL)-1β, lL-10, and type I interferons depending upon the context of the insult [97,98]. Monocytes extravasate into tissues through C-C chemokine receptor type 2 (CCR2) and chemokine ligand 2 (CCL2) signaling and differentiate into either monocyte derived dendritic cells and monocyte derived macrophages [99]. CMVs take advantage of these pathways through production of CC chemokine homologs, resembling host CCL2, that act to recruit myeloid cells to initial sites of infection to facilitate spread. mCMV mutants lacking the m131 locus are impaired in their ability to disseminate throughout the host [100–102].

HCMV establishes latency and persistence in CD34+ monocyte precursors cells and CD14+ patrolling monocytes [74,75,103]. Circulating monocytes provide a logical target of infection to be used as a transportation system for viral dissemination during homeostatic processes, however they present a potential bottleneck given their short half-life. CMV has evolved methods to circumvents this challenge to facilitate spread [104,105]. M45 is an anti-necroptotic mCMV protein that interacts with receptor-interacting protein (RIP) 1 and RIP3 via a RIP homotypic interaction motif and prevents cell death [106]. The mCMV product M36, a homolog to HCMV UL36, inhibits apoptosis through the caspase-8 pathway in macrophages and blocks programmed death in vivo [107]. The mCMV viral product m41 also prevents cell-mediated apoptosis by interfering with the Bcl-2 family of proteins, Bax and Bak, as demonstrated in studies using $Bak^{-/-}$ and m41 deficient mutant viruses [108]. Thereby, the CMVs extend the lifespan of patrolling and infected monocytes in order to have optimal time to replicate the early viral machinery leading to spread and release of viral progeny following maturation of monocytes to macrophages and myeloid dendritic cells after entry into tissues [109–111].

T Cell Responses and Memory Inflation

As a cellular arm of the adaptive immune system, T cells, are required for optimal control of CMV [112]. T cells recognize processed peptides derived from exogenous and endogenous proteins in the context of MHC molecules on the surfaces of infected or professional antigen presenting cells (APCs) with CD8

and CD4 T cells being restricted to MHC class I and MHC class II respectively. Each class of T cell exhibit unique T cell receptors (TCR) to recognize specific CMV peptide sequences. Recognition of a T cell's cognate peptide sequence in the context of MHC molecules, in combination with co-stimulation and cytokine cues, results in proliferation and effector functions. Expansion of effector T cells is canonically followed by a contraction to a memory population. However, this generalized understanding of T cells is subverted by CMV which results in the manifestation of unique CMV-specific T cell phenotypes.

CD8 T cells

The advent of MHCI tetramer technologies has allowed for high resolution interrogation of the T cell response against CMV [113]. Studies have also utilized overlapping peptide stimulation assays to determine the breadth of the CMV response in mouse and man revealing an enormously broad response in comparison to other viral adaptive immune responses [114,115]. These studies revealed the phenotypic complexity, but also unexpectedly high frequency, of CMV-specific memory T cell response, particularly in the CD8 lineage. Additionally, they have unveiled two different modes by which CMV stimulates CD8 T cells during infection (canonical/non-inflationary subsets vs. non-canonical/inflationary subsets) [116,117].

CD8 T cell responses against human CMV IE1 peptides, restricted by HLA-A2 and HLA-B7 can make up between 5 to 10% of all blood circulating CD8

T cells and upwards of 30% of all CD8 T cells can be specific for all CMV derived peptide in a healthy adult [113,118]. The magnitude of this response is hypothesized to be a consequence of co-evolution between virus and host and potentially a compensatory mechanism in response to CMV antagonism of T cell responses that interferes with peptide processing and presentation/pMHC complex expression. HCMV and mCMV possess multiple methods of altering presentation of MHC class I at the surface of infected cells to prevent recognition by CD8 T cells. The gene product US2 from HCMV results in the retrotranslocation of MHC class I into the cytosol whereby the complex is degraded by the proteasome, thus preventing antigen presentation. Furthermore, US6 prevents loading of peptides onto MHC molecules by antagonizing the transporter associated with antigen processing (TAP). Alternatively, mCMV protein m04 forms a complex with the MHC class I molecule in the endoplasmic reticulum and at the cell surface preventing trafficking and the mechanical association with TCR, thereby preventing T cell activation. The m06 protein subverts host cell machinery and redirects MHC molecules into the lysosome for degradation. These immune evasion mechanisms help the virus evade CD8 T cell-mediated elimination.

In addition to the significant overrepresentation of CMV specific cells the CMV-specific CD8 T cell compartment is phenotypically unique. CMV-specific CD8 T cells largely possess an effector memory phenotype, lacking lymph node-homing markers such as CD62L and CCR7 in mouse and man respectively [113,119]. This consequentially allows the circulation of CMV-specific T cells

through the blood and into the tissues, specifically into the lung and liver in both mouse and human infections [120,121]. These cells are phenotypically mature but show very little signs of cellular exhaustion (low PD-1 expression), likely because they are driven by sustained low presence of antigen [122] and not by a massive stimulation with constant and enormous number of antigens found in uncontrolled chronic infections such as HIV and HCV [113]. Following canonical resolution of primary infection, many primary T effector cells are eliminated, just as in the acute infection. However, unlike in the acute infections, the persisting low antigen burden will drive a phenomenon known as memory inflation.

Indeed, [1]one of the most obvious immunological consequences described during CMV infection is the expansion of antigen specific memory T cells with time, which was first described by Reddehase and colleagues [123], named by Klenerman “memory inflation” and since expanded upon by others [114,117,124,125]. Total HCMV-specific T cell responses in seropositive humans can be enormous, comprising on average 10% of both the CD4 and CD8 memory compartments in blood, and reaching up to 50% in certain individuals [126]. This increase in CMV specific memory T cells leads to an increase in overall number of circulating memory T cells with age, which does not occur in the absence of CMV infection [127].

At the most basic level memory inflation in response to CMV infection occurs as follows; initial lytic systemic viremia occurs over a period of two to four

[1] This section is an adapted version from “Impact(s) of CMV upon immune aging: facts and fiction. Jergovic et al. 2019.”

days followed by a significant expansion of CMV specific T cells. Following initial rounds of replication, the viral load is systemically reduced and viral replication restrained by multiple lymphocyte subpopulations such as NK cell, CD8 and CD4 T cells [128] as CMV contracts to defined anatomical locations in mouse and man. Early mouse studies showed virus localizing to the salivary gland and lungs upon resolution of primary infection [129] although later studies detected CMV genomes in spleen, bone marrow and blood of seropositive humans [77].

The cellular reservoirs of latency following initial phase of viremia have been demonstrated within multiple cell lineages but are believed to involve few cells of any type. These cells include CD34+ hematopoietic cell precursors [103,130], the CD14+ monocyte population, vascular endothelial cells and epithelial cells [131].

After initial infection and subsequent contraction of canonical effector T cells, memory inflation continues and is as follows; CD8 T cells possessing a central memory phenotype (CD44hi, CD62Lhi) traffic through the vasculature in search of cognate antigen. Upon recognition of antigen cells traffic through the vasculature and enter, what are presumed to be infected, tissues and are maintained with expression of CD69, to antagonize S1P1 receptor, and tissue specific markers such as CD103e [132]. Locations where this tissue residency of CMV specific T cells occurs have been described in the spleen and lungs of humans [133,134], as well as the salivary gland [67,76,115], lungs [77], liver [135], and most recently in our hands in the adipose tissue (Chapter 4) of mice.

CMV infection elicits a broad array of T cell responses, both phenotypically and in antigen specificity. During acute infection in the C57BL/6 mouse model it was very clearly shown that 18 epitopes elicited a response for greater than 50% of CD8 T cell splenocytes [136]. The diversity seen in this response is somewhat maintained in the inflationary phase of T cell responses with splenocytes responding against 5 of 19 peptide pools [114]. Canonical, or non-inflationary, CD8 T cells responding to CMV peptides dominantly exhibit a central memory phenotype, being CD62L+CD44+CD127+CD28+, as briefly described above. By contrast, the non-canonical, or inflationary CD8 T cells possess an effector memory phenotype being CD62-CD44+KLRG1+CD28- as reviewed in [137]. These phenotypes speak to the nature of these cells and their anatomical location, loss of CD62L expression confers the ability of T cells to enter peripheral tissues and leave lymphoid organs. In human infection phenotypic differences are also seen in CMV specific T cells, especially in that of aged adults. Analogous markers are seen on human CD8 cells bearing a CD28-CCR7-IL7R- phenotype. However, human inflationary T cells can re-express CD45RA [138] which is not expressed in mice. HCMV specific CD8 T cells in humans recognize a variety of epitopes and display effector responses to the products of 11 HCMV open reading frames (ORFs) irrespective of the age of the donor [139].

CD4 T cells

Although there is an obvious temporal hierarchy of dependence on lymphocyte subsets during control of mCMV (NK cells > CD8 T cell > CD4 T cells), [128] each T cell subset is important for a different aspect of immune surveillance and control. CD4 T cells, similarly to the CD8 T cell compartment, can make up to 9% of blood circulating CMV-specific T cells [126]. Although, CD4 T cells do not, in general, demonstrate peptide-restricted memory inflation to the extent that their CD8 counterparts do, CMV is the only known microbial pathogen that significantly expands the total CD4 effector memory cell population [140]. Furthermore, CD4 T cells specific to m09 derived peptides in the C57BL/6 mouse model display non-canonical kinetics in expansion and contraction after early infection [115]. These observations raise the question, why are CD4 and CD8 T cell responses different? One hypothesis is the temporal regulation of mCMV viral protein expression does not result in sufficient MHC class II peptide loading [141]. Also, maintenance of mCMV genomes in non-hematopoietic cells, as discussed above, may prevent the expression of viral peptide on MHC class II molecules and be lysed by CD8 T cells prior to CD4 recognition. This may explain why there have been very limited strategies describing CMV evasion of CD4 T cell specific immune surveillance. Two studies suggested that HCMV alters expression of MHC class II molecules at the surface of infected cells and APCs by inhibiting the JAK/STAT signaling pathway and repressing the activation of the class II transactivator (CIITA) [142,143]. However, it is possible that the alteration of the cytokine milieu that T cells experience during acute and

chronic phases may be the only strategy required by HCMV to subvert CD4 T cell responses as HCMV late proteins drive the activation of IL-10 producing CD4 T cells, resulting in limited inflammation [144]. Although the mechanism is still unclear, this strategy may have evolved as a type of “cease-fire” to prevent viral spread during inflammation, as utilized by the virus, and prevent autoimmune damage to the host.

Despite a lack of massive expansion during the memory phase of mCMV or CMV infection CD4 T cells still play a crucial role for driving virus into the latent phase. Mice that lack CD4 T cells, through antibody depletion or genetic knockouts, have delayed viral control which manifests as continued shedding in the salivary gland and higher frequencies of viral reactivation, which is hypothesized to be partially dependent upon CD4 T cell help [145,146]. CD4 T cells are absolutely required to control virus within the salivary gland through production of IFNγ and are dependent upon cognate antigen as opposed to CD8 T cells that are dependent upon production of TGF-β [76].

CMV in Summary

Both host and virus have accumulated numerous tools during millennia of co-evolution. This has resulted in a balance of inflammation and very limited disease manifestation during CMV infection in immunocompetent hosts [113]. The human, and mouse, immune systems dedicate an enormous amount of energy to maintaining homeostatic balance in the face of lifelong and chronic

CMV infection. In summary, CMV dissemination into a new host is characterized by lytic replication in many cells and very specifically the monocytes and endothelial cells. Patrolling monocytes enter tissues and release virus following maturation into macrophages or dendritic cells. Contraction of viral load following lytic replication is, in part, driven by activation of the innate immune system. NK cells do much of the heavy lifting in the early time points post infection and lyse infected cells when possible. CMV has evolved many strategies to antagonize and prevent NK cell mediated lysis. Balance is brought to the system when the adaptive immune system expands to kill infected cells. CD4 and CD8 T cells are required in non-redundant ways for optimal control of CMV. Temporal control of viral products is hypothesized to drive memory inflation in the CD8 T cell compartment that results in effector memory populations trafficking through the blood and into tissues as tissue resident memory T cells. During the lifespan, CMV can reactivate in response to environmental stressors, such as infection with additional pathogens, elevated stress, radiation, and immune deficiency.

Studying CMV's interplay with the innate and adaptive immune system has revealed many biological mechanisms and given a deeper understanding of basic immunity. However, several questions remain: what are the definitive locations of CMV persistence? Are all tissues treated equally during immune defense and chronic surveillance? What are the consequences, if any, of continued inflammatory responses in different tissue locations? Very specifically these questions are addressed here in the context of adipose tissue due to the theoretical susceptibility to CMV.

CHAPTER 3: ADIPOSE TISSUE

"Because I'm fat, I'm fat, come on..." – Alfred Matthew Yankovic

Adipose Tissue Basics

Adipose tissue is an all-encompassing description of the heterogenous tissue that consists of adipocytes and many other supportive cell types that make up the stromal vascular fraction (SVF) [147,148]. Adipocytes are morphologically large oval shaped cells containing large lipid droplets resulting in the peripheral displacement of their nuclei. These cells are crucially important to the metabolic function and health of their host organism [149,150]. Depending on the primary adipocyte type found in the adipose depot they are responsible for different biological functions. At a very basic level, adipocytes found in white adipose tissue (WAT) provide energy storage and molecular signaling. Alternatively brown adipocyte tissue (BAT), made up of brown or beige adipocytes, support thermogenesis, primarily for hibernating animals [151]. Adipocytes are sensitive to the metabolic requirements of their host. During periods of starvation adipocytes are broken down to prevent to breakdown of other cells, such as muscle tissue [152]. Conversely, during times of excess caloric intake they take up lipid to be stored for later use. When the homeostatic balance of energy storage and utilization is altered, such as during the onset of obesity, significant consequences occur adipocytes undergo altered secretory activity, cell death and necrosis, and generalized inflammation [153].

Adipose tissue is highly vascularized. This high level of vascularization allows for the mobilization of leukocytes in and out of adipose during tissue reorganization and maintenance. This contributes to the rapid phenotypic switches that adipose tissue undergoes during starvation, excess caloric intake, and inflammation. Early histological studies suggested that there was limited blood exchange within adipose tissue based upon the morphological appearance of adipocytes and surrounding tissue. However, thick sections revealed an extensive network of capillaries in intimate contact with adipocytes and serve as the foundation for adipose tissue development [154]. In general, WAT is organized on a branching system of vasculature that is lined by mesenchymal stem cells, adipocyte progenitors, and other perivascular cells [155,156]. Pre-adipocytes and other progenitor cells differentiate away from the vasculature further into the adipose tissue, essentially creating a conveyor belt like system of differentiation. Adipose tissue is highly dynamic and responsive to systemic changes. For example, increased dietary intake in the absence of offsetting caloric expenditure results in hypertrophy and hyperplasia of adipocytes via a signaling cascade from the vasculature and surrounding cells [157,158]. The balance of these processes and subsequent cardiovascular angiogenesis, molecular signaling, and number and size of adipocytes within fat depots, specifically of WAT depots, are of crucial importance to host health [159].

Altered homeostasis of adipocytes can result in elevated blood pressure, increased lipid uptake, dysfunctional glucose homeostasis, local and systemic inflammation, and atherosclerotic plaque development [160]. These outcomes

are dependent upon adipocyte derived cytokines, the adipokines. In addition to adipokines, adipocytes also make other cytokines, including IL-6, TNFα, and CCL2 that have far reaching consequences during leukocyte mediated inflammation [160–162].

Adipokines

Two of the best understood adipokines, which are almost exclusively produced by adipocytes, are leptin and adiponectin. These adipokines have opposing actions and their secretion is altered by several environmental influences, such as the development of obesity, infectious status, age, and weight.

Adiponectin

Adiponectin is one of the most studied and well understood adipokines ; it is sensed through Adiponectin Receptor 1 and 2 (AdiopR1 and AdiopR2) [163]. The adiponectin receptors are widely expressed within adipose tissue but are also expressed on skeletal muscle (AdipoR1) and in the liver (AdiopR2). Furthermore, both receptors have been found expressed in the brain, demonstrating the systemic reach of adipose derived signaling molecules [164]. Expression of adiponectin influences insulin sensitivity of the host through these tissues and thus links adipose tissue to systemic host function and health. Expression of adiponectin is negatively correlated with adiposity and as such has

been the focus of many studies that focus on obesity linked metabolic diseases [165].

Adiponectin signaling in the brain modifies glucose metabolism by altering glucose uptake, decreasing glucose production, altering satiety signaling and locomotor functioning [166,167]. Signaling in skeletal muscle decreases systemic triglycerides, which are thought to interfere with insulin signaling, and increase expression of molecules involved in fatty-acid oxidation thus increasing sensitivity to insulin action [168]. In addition, adiponectin acts through protein phosphorylation activator receptor-α (PPAR) in both skeletal muscle and liver to further decrease triglyceride content. Finally, adiponectin acts on the liver to lower gluconeogenesis and contributes to insulin and glucose homeostasis [169]. Outside of metabolic consequences of adiponectin, it is also an anti-inflammatory molecule. Adiponectin inhibits activation of macrophages through suppression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway and scavenger receptor expression, as well as upregulating expression of IL-10 [170]. Endothelial cells are affected by adiponectin through the cyclic AMP and protein kinase A (cAMP/PKA) to reduce adherence molecules in the vasculature, preventing leukocyte activation and extravasation, and reduce angiogenesis through the regulation of endothelial nitric oxide synthase (eNOS) [171,172].

Altogether, the actions of adiponectin suppress systemic inflammation by preventing the activation of macrophages and reducing the expression of molecules that would trigger an inflammatory response. Furthermore, action on the brain suggests that satiety signaling and alterations on nervous system

signaling contribute to host metabolism. Finally, adiponectin can contribute to increased insulin sensitivity through molecular expression and suppression in both the liver and skeletal muscle.

Leptin

Leptin is the second of the two most well understood adipokines and has actions that directly oppose adiponectin. One such example is the positive correlation between leptin and adiposity [173]. The consequences of dysfunctional leptin production and signaling have been very well described through mouse models. Dysfunctional leptin gene expression, ob, has been characterized through the ob/ob mouse model [174]. The ob/ob mouse arose due to a spontaneous nonsense mutation in the leptin gene that results in rapid weight gain, hyperglycemia, insulin insensitivity, and elevated plasma insulin concentration [175,176]. In addition to the ob/ob mouse is the db/db mouse, which arose due to a point mutation in the leptin receptor and manifests similar phenotypes to its ob/ob counterpart. These mouse models have revealed the importance of leptin in regulating body weight, adiposity, satiety, and general metabolic health.

Leptin signaling is mediated through the leptin receptor (LepR) expressed in the hypothalamus, on T cells, NK cells, neutrophils, and on vascular endothelial cells [177,178]. Leptin signaling is propagated through the Janus kinase (JAK) – signal transducer and transactivator (STAT), extracellular signal-regulated kinase (ERK) – mitogen-activated protein kinases (MAPK), and insulin

receptor substrate (IRS) – phosphoinositide 3-kinases **(**PI3K) pathways [179]. The JAK-STAT pathway appears to be the most important in the hypothalamus and seems to regulate food intake and therefore body weight [180]. The PI3K pathway contributes to the regulation of glucose metabolism and the MAPK pathway regulates triglyceride accumulation in the liver and skeletal muscles [181].

Leptin also plays an important role in the context of inflammation. The molecular structure of leptin is homologous to the class I cytokine receptor superfamily, which include IL-6 and granulocyte colony-stimulating factor (G-CSF). This led to the hypothesis that leptin plays a role in activation of the immune system. Indeed, leptin is a survival signal for neutrophils, mature NK cells, and to the thymus for maintenance of immature T lymphocytes [180,182,183]. Not surprisingly, leptin deficient mice show decreased ability of T cells to secrete cytokines compared to wild type counterparts and this may protect against autoimmunity in a CD4 helper T cell dependent manner [184].

Overall, leptin contributes to the regulation of glucose homeostasis, insulin signaling, satiety signaling, and body weight regulation. Furthermore, the immune system seems to be less able to carry out inflammatory processes when leptin or leptin signaling is lost.

Adipose Tissue Inflammation

Environmental pressures on adipose tissue contribute to the altered secretion of adipokines. One of the most powerful external pressures on adipose tissue is nutrition, as illustrated by the progression and establishment of obesity. Studies on the consequences of obesity have revealed many key mechanisms, such as cytokine and adipokine secretion described above, in which adipose tissue is involved and disease states to which adipose tissue inflammation is correlated, such as type 2 diabetes, hyperglycemia, and insulin resistance [185].

Obesity is one of the greatest public health risks that the world faces, with greater than 1 in 3 people being obese or overweight in the developed world [186]. Increased rates of overweight and obese patients has resulted in significant medical and healthcare burden due to increased prevalence of metabolic diseases [187]. Several diseases connected to dietary induced obesity, such as type 2 diabetes mellitus, nonalcoholic fatty liver disease, cancer, cardiovascular diseases, and cognitive decline, are all linked to chronic low-grade inflammation [188–190]. Obesity occurs during a sustained positive energy balance, more calories consumed than expended. This results in an increase in the size and number of adipocytes to facilitate storage of excess lipids. Ultimately an upper limit is reached, and adipocytes can no longer adequately expand, resulting in an inflammatory trigger. Several mechanisms have been proposed as being the definitive “Big Bang” event for adipose tissue inflammation, but the precise cause is still unclear and is most likely a synergy of multiple mechanisms [153].

Expansion of adipocytes leads to decreased oxygen availability resulting in hypoxic conditions. Hypoxia can result in cellular death triggering an immune cascade. Dead or necrotic adipocytes are engulfed by F4/80+ leukocytes that results in the formation of crown-like structures [191]. Infiltrating leukocytes, primarily macrophages, recognize damage-associated molecular patterns (DAMPs) released from dying cells. This results in the activation of the immune system through the NOD-like receptor (NLR) family and subsequent release of inflammatory IL-1β and IL-18 to mitigate and control local damage [192].

The immune cells present within adipose tissue during lean conditions is generally anti-inflammatory but skews towards an inflammatory state during the onset and progression of obesity. Under normal homeostatic conditions IL-33 is secreted by endothelial and progenitor cells [193], which maintains the secretion of IL-5 and IL-13 by type 2 innate lymphoid cells (ILCs) [194,195]. The secretion of IL-5 and IL-13 activates eosinophils to produce IL-4 and subsequently maintains macrophages in an M2-polarized state and promotes their production of IL-10 [196]. However, during the progression of obesity a phenotypic change occurs driving what was an anti-inflammatory T helper type 2 (Th2) environment towards an inflammatory T helper type 1 (Th1) environment. Adipocytes upregulate an NK cell activating receptor, NCR1, which results in the production of IFNγ triggering the polarization of macrophages towards an inflammatory M1 phenotype [197]. Although NK cells contribute to the polarization of macrophages in adipose tissue, it appears that the adaptive immune system infiltrates adipose prior to innate immune activation. B cells and T cells both infiltrate adipose tissue

prior to a significant accumulation of macrophages, suggesting an alteration in antigenic presentation [198,199].

That microbes can promote adipose tissue inflammation is suggested by studies demonstrating that lipopolysaccharide (LPS) and endogenous lipids can activate leukocyte subsets. Obesity increases gut permeability and results in circulating LPS from the gut microbiota [200]. Adipocytes express the pattern-recognition receptor (PRR) Toll-like receptor (TLR) 4, for which LPS is a ligand [201]. This potentially links obesity-induced ‘leaky gut’ with adipose tissue inflammation that is upstream from metabolic dysfunction. Furthermore, endogenous saturated free fatty acids are able to trigger NF-kB mediated inflammatory response through TLR2 and 4 [202,203]. This activation leads to the production of CCL2 and the infiltration of macrophages into adipose tissue. Coupled with a high density of lipid-specific CD1d-restricted T cells in C57BL/6 adipose, the connection between T cell activation and increased lipid density is clear [204–206]. However, not all lipid species are created equal. Indeed, certain lipids have demonstrably anti-inflammatory properties, such as omega-3 and -9 fatty acids. These lipids are the potential mechanisms for the anti-inflammatory properties of the Mediterranean diet (discussed in Chapter 1) [207,208].

Anti-Inflammatory Treatments for Metabolic Regulation

The definitive mechanism by which metabolic dysfunctions arise is unknown. However, the clear correlation with adipose tissue inflammation and these subsequent disease states is clear and attempts have been made to target

adipose tissue inflammation as a therapy. Obese patients with adipose tissue inflammation are statistically more likely to exhibit insulin resistance whereas obese patients with little or no adipose inflammation remain insulin sensitive in a macrophage dependent manner [209,210]. Targeting macrophage secreted cytokines has provided mixed results. Anti-TNF antibody treatment has improved glucose metabolism in some patients, but the effects have been limited in its effect as a type 2 diabetes treatment [211,212]. Targeting IL-1β, however, has been more successful in improving glucose metabolism in type 2 diabetes patients, but this mechanism appears to work through the pancreas as opposed to on adipose tissue inflammation directly [213]. Targeting nuclear factors of adipocytes has also demonstrated success in mitigating adipose tissue inflammation and subsequent metabolic dysfunction. Activation of peroxisome proliferator-activated receptor γ (PPAR-γ), a nuclear transcription factor highly expressed in adipose tissue, results in reduced plasma free fatty acids and glucose concentrations, and consequent improved insulin sensitivity. The antidiabetic drugs, thiazolidinediones (TZDs) specifically target this pathway and result in increased secretion of adiponectin and reduced IL-6 and TNFα secretion [214,215]. Finally, metformin, which can increase lifespan in mice [216], also acts on adipose tissue inflammation by inhibiting the production of reactive oxygen species and NF-kB signaling leading to a polarization of macrophages back towards the anti-inflammatory M2 phenotype [217–219].

When taken together, these data suggest that adipose tissue inflammation is linked with metabolic disease states. However, there does not appear to be

one specific inflammatory pathway that can singularly explain the entirety of altered host homeostasis. Furthermore, the interconnectedness between adipose tissue inflammation and other metabolically relevant tissues, such as the pancreas, liver, and skeletal muscle cannot be understated. Studies delineating the impacts of each individual tissue suggest that dysfunctional metabolic health is context dependent [220–222]. The definitive trigger(s) for metabolic disease states, therefore, require more investigation.

Conclusion: Fat and Unhappy

Adipose tissue is highly responsive to environmental pressures. Homeostatic health of adipose tissue involves a delicate balance of energy intake, immune cell populations, and infectious status. As discussed above, adipose tissue is awash with leukocyte subsets, inflammatory and anti-inflammatory cytokines, and adipokines. Indeed, adipose tissue is a residence to T cells, B cells, macrophages, dendritic cells, NK cells, among other cell types [160,161,223,224] and therefore potentially highly responsive to their activation. Depending upon the balance of signaling molecules and general health of the host these leukocytes can carry out healthy homeostatic processes or can be the driving force behind metabolic dysfunction and deteriorating host health. Several pathogens have been shown to infect adipose tissue, and potentially cause adipose tissue inflammation (HIV, Chagas Disease, Toxoplasma gondii) [225–228] but the consequences of these infections have yet to be fully revealed. Therefore, when these studies and observations are taken together, they

necessitate further investigation of the consequences of environmental pressures on adipose tissue and potentially that of whole host health. Specifically, these concerns are addressed in the following chapter in the context of lifelong infection of mCMV.

CHAPTER 4: LIFE-LONG CONTROL OF CYTOMEGALOVIRUS (CMV) BY T RESIDENT MEMORY CELLS IN THE ADIPOSE TISSUE RESULTS IN INFLAMMATION AND HYPERGLYCEMIA

"You can't stop him; you can only hope to contain him." – Dan Patrick

Abstract

Cytomegalovirus (CMV) is a ubiquitous herpesvirus infecting most of the world's population. CMV has been rigorously investigated for its impact on lifelong immunity and potential complications arising from lifelong infection. A rigorous adaptive immune response mounts during progression of CMV infection from acute to latent states. CD8 T cells, in large part, drive this response and have very clearly been demonstrated to take up residence in the salivary gland and lungs of infected mice during latency. However, the role of tissue resident CD8 T cells as an ongoing defense mechanism against CMV has not been studied in other anatomical locations. Therefore, we sought to identify additional locations of anti-CMV T cell residency and the physiological consequences of such a response.

Through RT-qPCR we found that mouse CMV (mCMV) infected the visceral adipose tissue and that this resulted in an expansion of leukocytes in situ. We further found, through flow cytometry, that adipose tissue became enriched in cytotoxic CD8 T cells that are specific for mCMV antigens from day 7 post infection through the lifespan of an infected animal (> 450 days post

infection) and that carry markers of tissue residence. Furthermore, we found that inflammatory cytokines are elevated alongside the expansion of CD8 T cells. Finally, we show a correlation between the inflammatory state of adipose tissue in response to mCMV infection and the development of hyperglycemia in mice. Overall, this study identifies adipose tissue as a location of viral infection leading to a sustained and lifelong adaptive immune response mediated by CD8 T cells that correlates with hyperglycemia. These data potentially provide a mechanistic link between metabolic syndrome and chronic infection.

Introduction

Cytomegalovirus (CMV) is a ubiquitous beta-herpesvirus that infects most of the worldwide population, with largest prevalence being observed in older adults [127,229–231]. Acute CMV infection is characterized by system-wide viremia after which latency and lifelong persistence is established in select cells such as CD34+ monocytes and hematopoietic progenitor cells in humans [129,232,233]. Although CMV infections are generally asymptomatic, untreated infections in utero or amongst the immunocompromised individuals can result in substantial developmental defects, pathology, and death [232,234–236]. However, in immunocompetent patients the substantial and varied (NK, CD8, CD4, and B cells) resources are mobilized to successfully control viral spread and reactivation. One well described arm of anti-CMV immunity, the CD8 T cell compartment, is heavily involved in viral control with up to 5-10% of total CD8s in the blood and secondary lymphoid tissues reactive to CMV antigens during a

primary immune response [114,237–239]. Moreover, in the course of lifelong infection, cycles of latency and reactivation drive an expansion of CD8 T cells, termed memory inflation (MI), that in some cases reaches up to 30-50% of the total memory compartment in mice and men [119,240]. The magnitude of this memory response is largely unparalleled in any other infection and for this reason CMV has been used as a model to understand the effects of MI during immune aging [127,241–244].

Studies of CMV-driven MI, viral dissemination, and persistence throughout the host have largely focused on the spleen, liver, blood, lung, and salivary glands [237,245–248]. It has become clear that the blood contains a major pool of CMV-reactive T effector memory (Tem) cells that presumably scan the vasculature as a bulwark against systemic CMV reactivation and that accumulate with age [127,249]. Tissue control of CMV has been narrowly studied in the context of the lungs and the salivary gland as these sites were shown to harbor mCMV-specific resident non-circulating T cell populations in response to latent virus. However, it remains unclear at this point whether tissue resident memory T cells are universally used to control a persistent pathogen such as CMV in situ and to what extent other tissue locations contribute to such a host defense and potential viral latency.

We therefore considered potential locations for where CMV could establish itself and hypothesized that adipose tissue, given several tissue specific properties, could offer a plausible site for infection. Adipose tissue is found at a variety of anatomical locations and consists of multiple cell types including

adipocyte progenitors, leukocytes, and stromal cells, some of which have shown susceptibility to mCMV infection [147,148]. Furthermore, the adipose tissue of mouse and man is home to a large proportion of both innate and adaptive immune cells, suggesting that adipose tissue contributes to the mounting of an effective immune response [159–161,224,250]. The immune system represented within visceral adipose tissue has been clearly linked to development of diseases of the metabolic syndrome in the context of obesity [160,161,223,224,251–254] Given the reported linkage between lifelong CMV infection and metabolic dysfunction in humans we reasoned that mCMV could potentially drive inflammation within adipose tissue that contributes to these phenotypes [244,255]. In further support of this hypothesis, adipocytes have been demonstrated to be susceptible to adenovirus and parasitic infection, raising the possibility that infection could drive insulin resistance and glucose intolerance [256–259]. Furthermore, adipose tissue of HIV/SIV infected humans/monkeys harbored latent virus even after patients were declared virus free following retroviral treatment, suggesting that adipose tissue can provide a safe haven to viruses [260].

Lifelong herpesvirus infections have been studied for their contribution to global host inflammation in the context of frailty and immune aging, but the consequences of these infections on, and the control of infection within adipose tissue have not been investigated. Given the susceptibility of individual cells within adipose tissue to CMV infection we hypothesized that mCMV could infect a cellular constituent of adipose tissue and therefore trigger in an inflammatory

immune response in situ. We show here that mCMV infects adipose tissue during early peak viremia, followed by infiltration by mCMV-specific CD8 T cells during the acute phase post infection (p.i.). The adipose T cells were specific for mCMV epitopes, with a large fraction of them possessing a Tem phenotype. The presence of inflammatory monocytes was not necessary for the mCMV-specific immune response in adipose tissue, suggesting direct viral spread to adipose tissue during initial infection. mCMV infection and the resulting anti-mCMV CD8 T cells were associated with persistent inflammation within the adipose tissue from very early points during the immune response (days three to five) through the lifespan of the infected host (greater than 450 days). Moreover, infected fat tissue exhibited a decreased production of adiponectin. Finally, an analysis of lifelong-infected animals revealed that the mCMV specific CD8 T cells were bona fide Trm (CD69+) cells that exhibit limited exchange with the vasculature based on intravenous staining. The presence of both mCMV and mCMV-specific non-circulating resident CD8 T cells in the adipose tissue for life suggests that Trm cells may be the primary mechanism by which the host controls mCMV tissue reservoirs and their association with inflammation suggest that this interaction may alter metabolic health in infected animals.

Results

mCMV Infects adipose tissue triggering in an early inflammatory immune response

mCMV was reported to infect cells in the adipose tissue [197,261,262], but the local consequences of this infection in vivo have not been well characterized. We first determined if adipose tissue was susceptible to mCMV infection by comparing both RNA (Fig 1A) and DNA (Fig 1B) extracted from total adipose 3 days (d) post infection (p.i.) to uninfected counterparts, we also analyzed the presence of viral genomes in several other tissues and visceral adipose tissue had a two log higher burden at day 3, when compared to subcutaneous adipose, liver, and spleen, likely due to route of infection (Fig. 1). Analysis of RNA transcripts revealed the presence of immediate early (IE) viral gene products, IE1, in infected but not uninfected animals (Fig. 2A). To determine if the presence of mCMV transcript resulted in an immune response, we measured leukocyte infiltration and found at 3d p.i. a significant increase in the global leukocyte population, which was driven by lymphocytes, neutrophils, monocytes, and eosinophils (as normalized per gram of adipose tissue, Fig. 2B). We further characterized the cells participating in this early, 3d p.i., adipose response by flow cytometry and found a significant expansion of NK cells (NK1.1+CD3-; Fig 3A) and an expansion, although not significant, in the macrophage population (F4/80+CD11b+; Fig 3B). Polarization of macrophages to an inflammatory, classically activated M1 phenotype (CD11c+) also trended higher

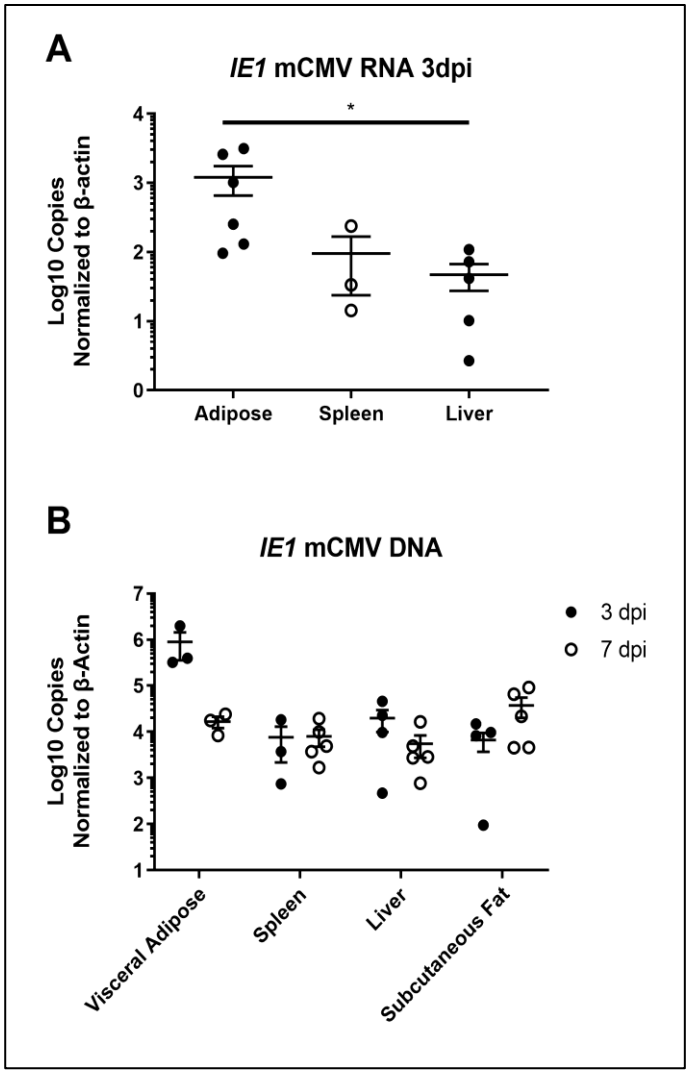

Figure 1. mCMV DNA genome is detectable across tissues from d3 and d7 p.i.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route and sacrificed at 3d and 7d p.i.. Tissues were snap frozen in Qiazol then DNA and RNA extracted. (A) Total mCMV RNA burden in visceral adipose, spleen, and livers at 3d p.i. (B) Total mCMV DNA burden in subcutaneous adipose, visceral adipose, spleens, and liver was normalized to β-actin. Uninfected animals were used to establish C_T cut off at 32. Technical duplicates were run for both RNA and DNA. Data is representative of three independent experiments. n = 3 to 6 total animals per group. Kruskal-Wallis with Dunn's multiple comparisons. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001

(Fig 3C). We next examined the cytokine content of total adipose tissue homogenate using a flow cytometry-based LegendPlex platform and found significant increases in IFNγ (p=0.004), TNFα (p=0.001), IL-1α (p=0.0007), IL-6 (p<0.0001), and CCL2 (p<0.0001) (Fig. 2C-G) with infection in the adipose tissue

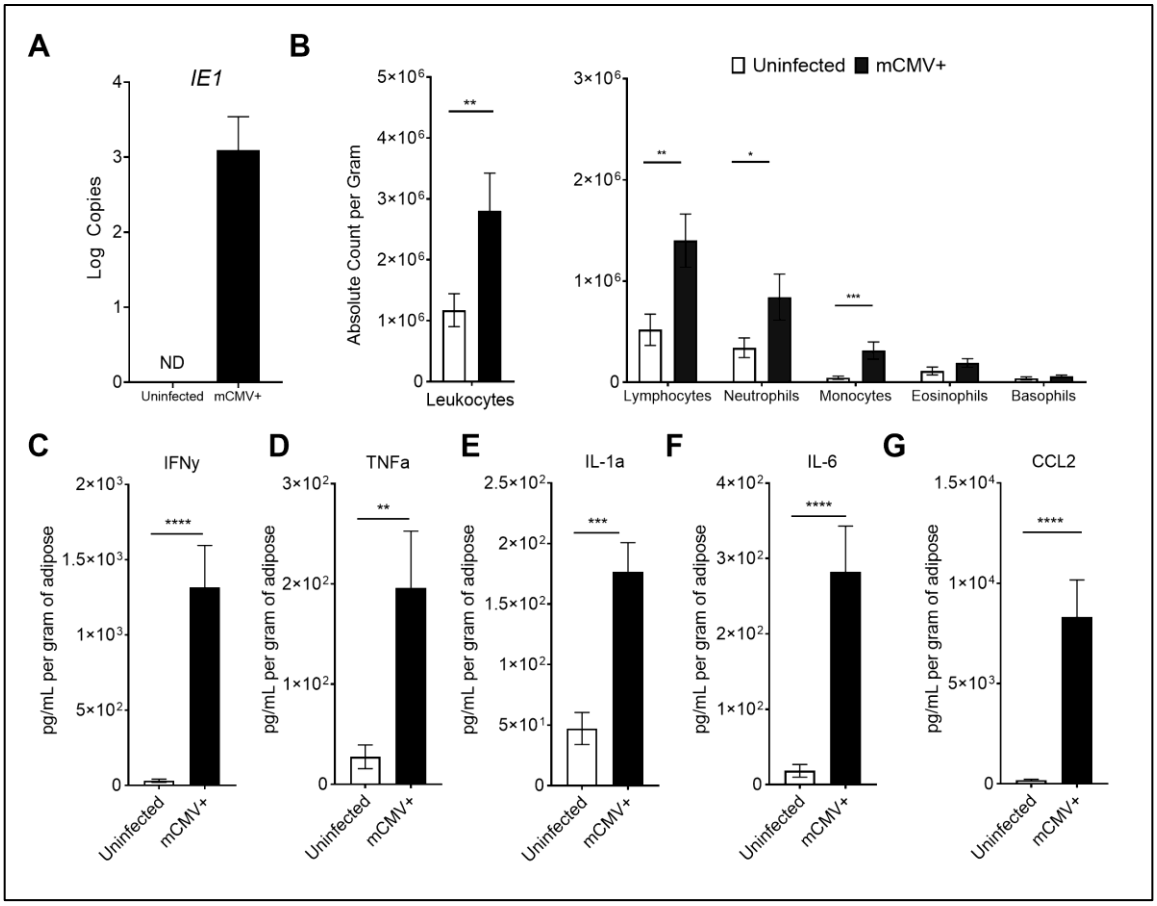

Figure 2. mCMV infects adipose tissue and triggers an immune response.

12-week-old C57BL/6J mice were i.p. injected with 10^5 pfu of mCMV. (A) Adipose tissue was collected three days post infection into QIAzol reagent and RNA extracted to detect viral gene products IE1 in 50 ng of total RNA by RT-qPCR and normalized to plasmid. (B) Three days p.i., adipose tissue was processed by collagenase D digestion and stromal vascular fraction was analyzed by hemocytometer. (C-G) Mice infected from three to five days post infection were sacrificed and total adipose homogenized to analyze by BioLegend LegendPlex 13-plex Inflammation Panel. Data are pooled results of two independent experiments. n = 10 uninfected and 10 infected animals total. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

on days 3 and 5 p.i. (days 3 and 5 are pooled in the figure). However, at this time point we did not observe any significant changes in the amounts of GM-CSF, IFNβ, IL-1β, IL-12, IL-17A, IL-23, or IL-27**.** Therefore, during the early acute timepoints of infection, we found evidence of virus transcription, cellular and cytokine immune responses in the total adipose tissue.

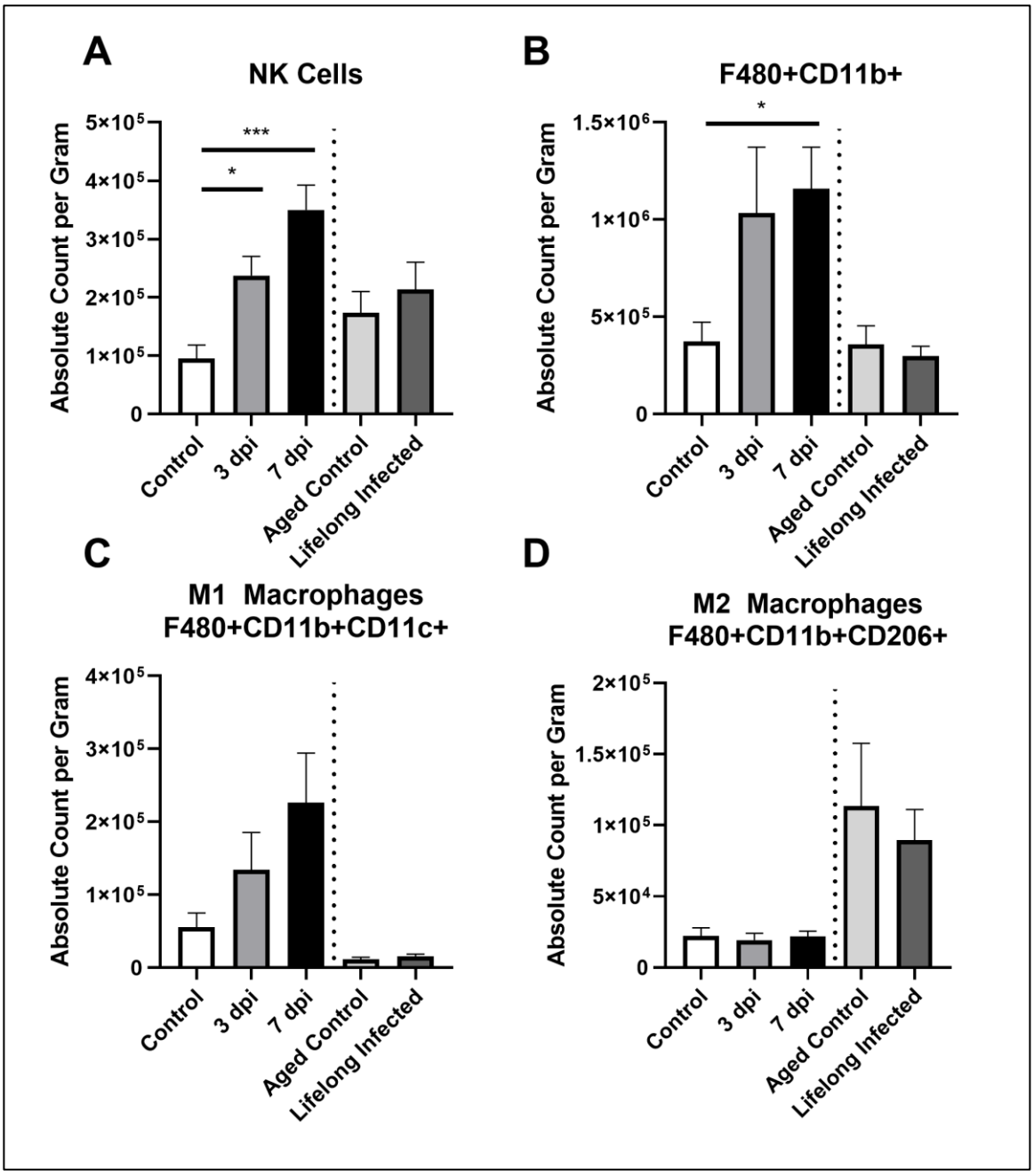

Figure 3. Adipose tissue NK cell and macrophage respond to early mCMV infection, but not chronic and lifelong infection.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route and sacrificed at 3d, 7d and > 450d p.i. Stromal vascular fraction was analyzed by flow cytometry and cell populations quantified (A) NK cells. (B) F480+CD11b+ Macrophages. (C) F480+CD11b+CD11c+ M1 Macrophages. (D) F480+CD11b+CD206+ M2 Macrophages. Data are pooled data of two independent experiments. n = 4 – 9 mice per group. Error bars represent mean ± SEM. Lifelong and aged matched control groups were analyzed by unpaired two-tailed Mann-Whitney U test.

Control, 3 dpi, and 7 dpi were analyzed by Kruskal-Wallis with Dunn's multiple comparisons. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001

mCMV-Specific CD8 T cells infiltrate adipose tissue during acute infection

Following infection, mCMV replicates systemically, leading to detectable host viremia that is resolved within days of infection. It is possible that the inflammation observed in the adipose at this early timepoint could resolve during reduction of the viral load. On 7d p.i. we were able to detect mCMV viral products (Fig. 1). Given this detection we wondered if there was a continued immune response within adipose tissue. We therefore quantified inflammation during the peak adaptive immune response at the same time, again by characterizing the cellular content of the stromal vascular fraction (SVF). The global leukocyte population of infected animals (Figure 4A, left) remained elevated, driven by lymphocytes, neutrophils, monocytes, eosinophils, and basophils (Figure 4A, right). Contributing to this expansion were NK cells which were still significantly elevated (Fig 3A). Total macrophage population became significantly expanded in infected adipose tissue at this time as well (Fig 3B). Approximately half of all leukocytes detected in adipose tissue at this time were lymphocytes, and a majority of these were CD3 T cells, with significant increases in both the CD4 and CD8 (p=0.0004) populations (Fig. 4B). Both the central memory (CD44+CD62L+; p=0.012) and effector memory (CD44+CD62L-; p=0.004) populations were significantly increased in infected animals, whereas, as expected, there was no change in the total numbers of naïve (CD44- CD62L+) T cells (Fig. 4C). Amongst the memory subsets we found roughly equivalent

numbers of memory-precursor effector cells (CD127+KLRG1-; MPECs; p=0.0028) and short-lived effector cells (CD127-KLRG1+; SLECs; p=0.0004), with both populations significantly increased in infected animals. We next asked whether these T cells were specific for mCMV antigen or were accumulating due to perhaps inflammatory stimulation in a non-specific manner. Peptide:MHC (pMHC) tetramer staining revealed a significant expansion of T cells specific for the acute, non-inflationary immunodominant epitope M45 (p=0.0004), with smaller, but also highly significant expansion of CD8 T cells specific for inflationary epitopes m38 (p=0.0004) and m139 (p=0.0004) (Fig. 4E). Given an influx of antigen specific T cells we also tested whether the cytokine milieu was still altered, and found, by LegendPlex, significant increases in the protein levels of IFNγ (Fig 5A; p=0.004) and CCL2 (Fig 5A; p=0.0005), but not of GM-CSF, IFNβ, IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-17A, IL-23, IL-27, or TNFα. In addition to these protein analyses we found several inflammatory transcripts that were significantly upregulated at this time point, including Cd3e, Ifng, Cxcr3, Ccr5, Casp1, and Adgre1, indicative of a myeloid and T lymphocyte infiltration (Fig. 6).

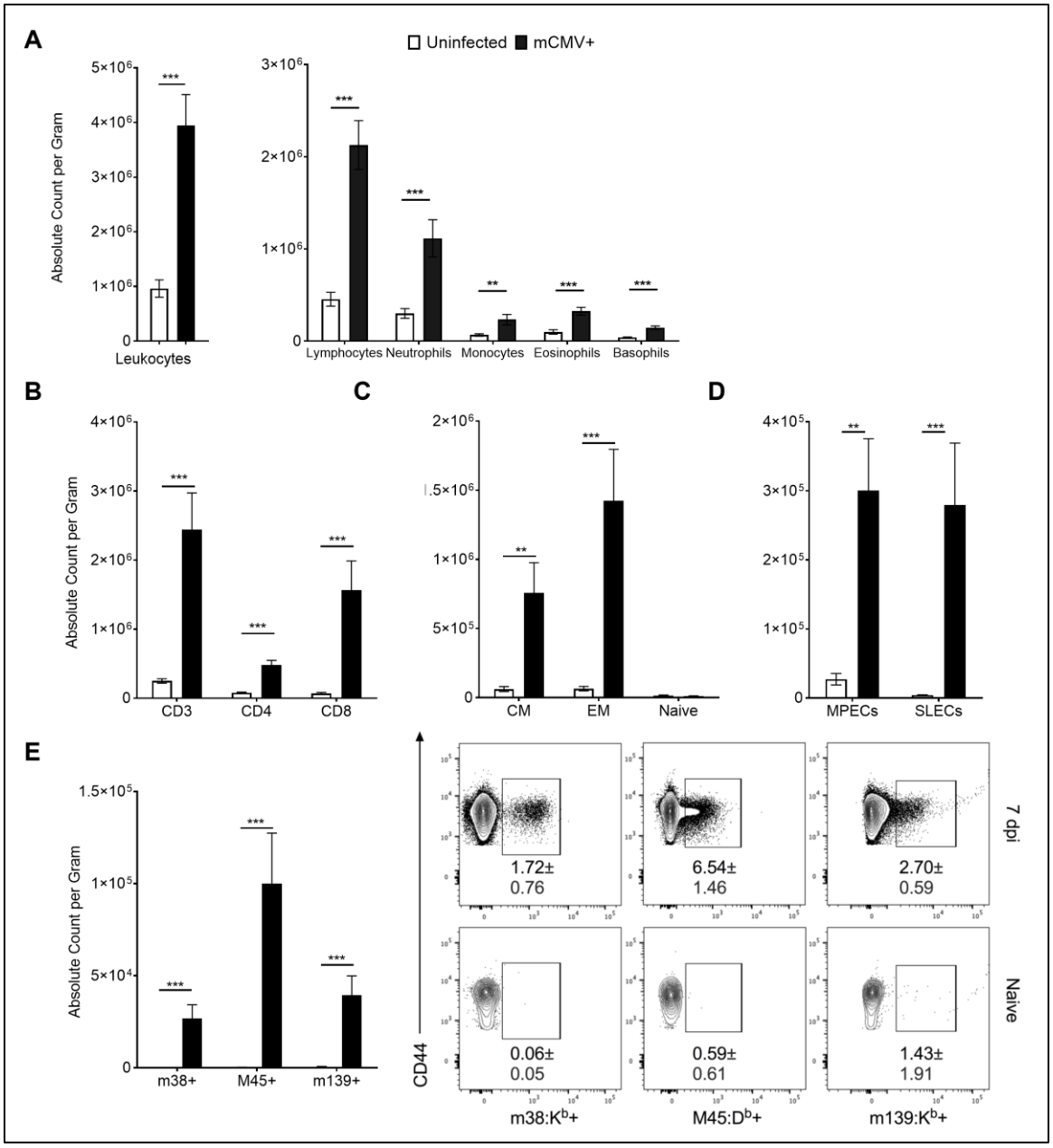

Figure 4. Adipose tissue is infiltrated by mCMV-specific T cells at 7d p.i..

12-week-old C57BL/6J mice were i.p. injected with 10^5 pfu of mCMV and sacrificed on d7 p.i.. Stromal vascular fraction was analyzed by Drew Scientific HemaVet 950 and flow cytometry. (A) Total leukocytes were quantified by hemocytometer. (B) Flow cytometry analysis was used to quantify absolute numbers per gram of adipose tissue of CD3 T cells and subsetted into CD4 or CD8 pools. (C) CD8 T cells were phenotyped based on expression of CD62L and CD44 and quantified. (D) CD44+ CD8 T cells were analyzed for expression of KLRG1 and CD127 to quantify number of MPECs and SLECs. (E) CD44+ CD8 T cells were analyzed for mCMV specificity by tetramer staining. Data are pooled results of two independent experiments. n = 9 total infected and 6 uninfected animals total. Frequencies shown in the dot plots represent SD. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

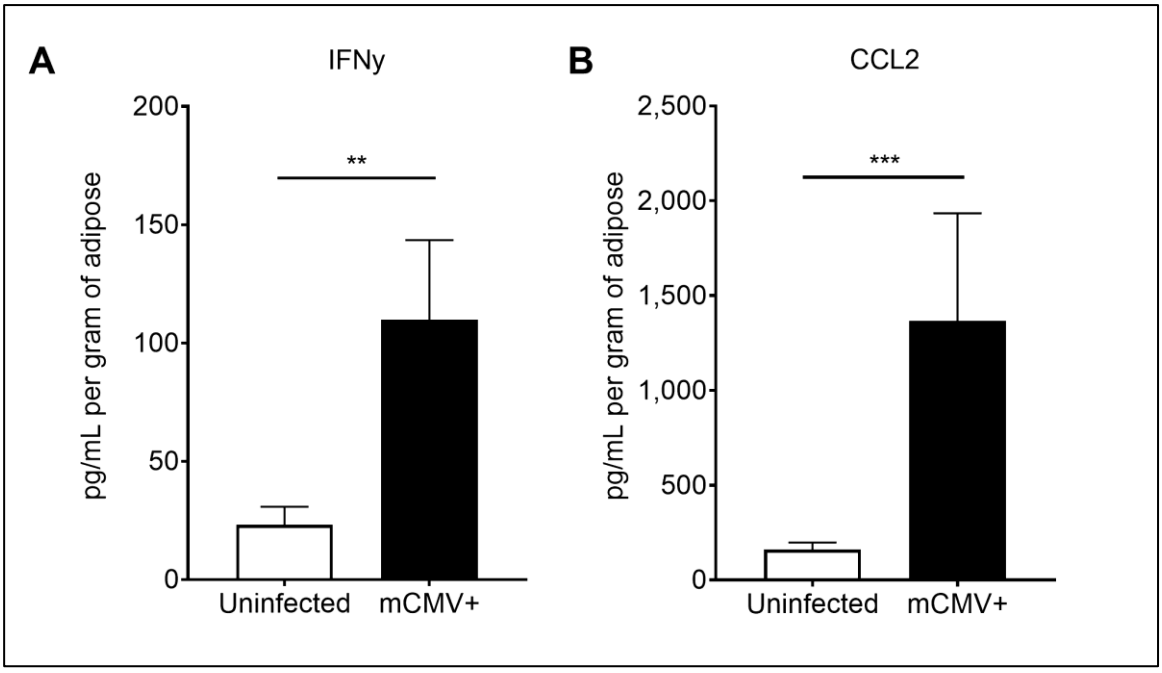

Figure 5. Acute mCMV infection alters adipose cytokine milieu.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route and sacrificed at 7d p.i.. Total adipose tissue was homogenized and analyzed by BioLegend LegendPlex for (A) IFNγ; (B) CCL2. Data are pooled results of two independent experiments. n = 10 uninfected and 10 infected animals total. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

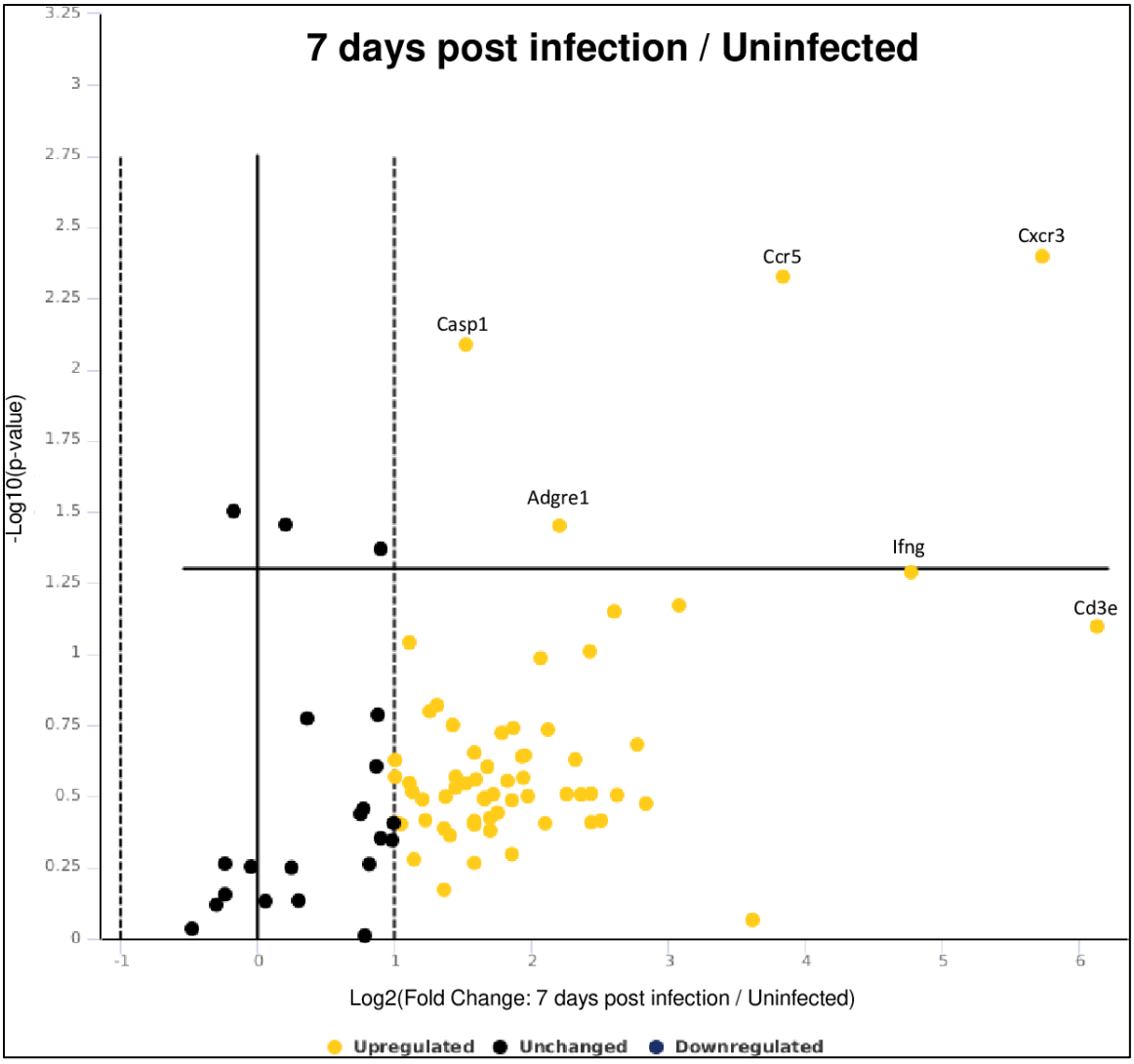

Figure 6. Inflammatory transcripts are upregulated at 7d p.i..

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route and sacrificed at 7d p.i.. Transcriptome was analyzed using RT2 Insulin Resistance Miniarray Profiler and presented as a volcano plot. All housekeeping genes were used for normalization. A total of 3 infected and 3 uninfected animals were used. A cut off of 35 cycles was set as undetectable per manufacturer's suggestions.

Adipose tissue inflammation has been demonstrated to alter adipocyte derived cytokines, adipokines, during the onset and establishment of obesity [150,263]. We therefore examined the production of two well described adipokines, leptin and adiponectin. We found that adiponectin, which is

decreased during inflammation-induced by obesity [166], was significantly decreased in infected animals (Fig 7A; p=0.0186), whereas, we found no change in the amount of leptin, which is positively correlated with body weight (Fig 7B) [264]. No significant change in total leptin, however, comes as no surprise as we saw no significant change in total epididymal fat pad or body weight during the lifespan of infection (Fig. 8). These data suggest that inflammation driven by infection and influx of immune cells can trigger a secretory response by adipocytes. Taken all together, these data indicate that mCMV infection results in a CD8 T cell response detectable in adipose tissue at 7d p.i..

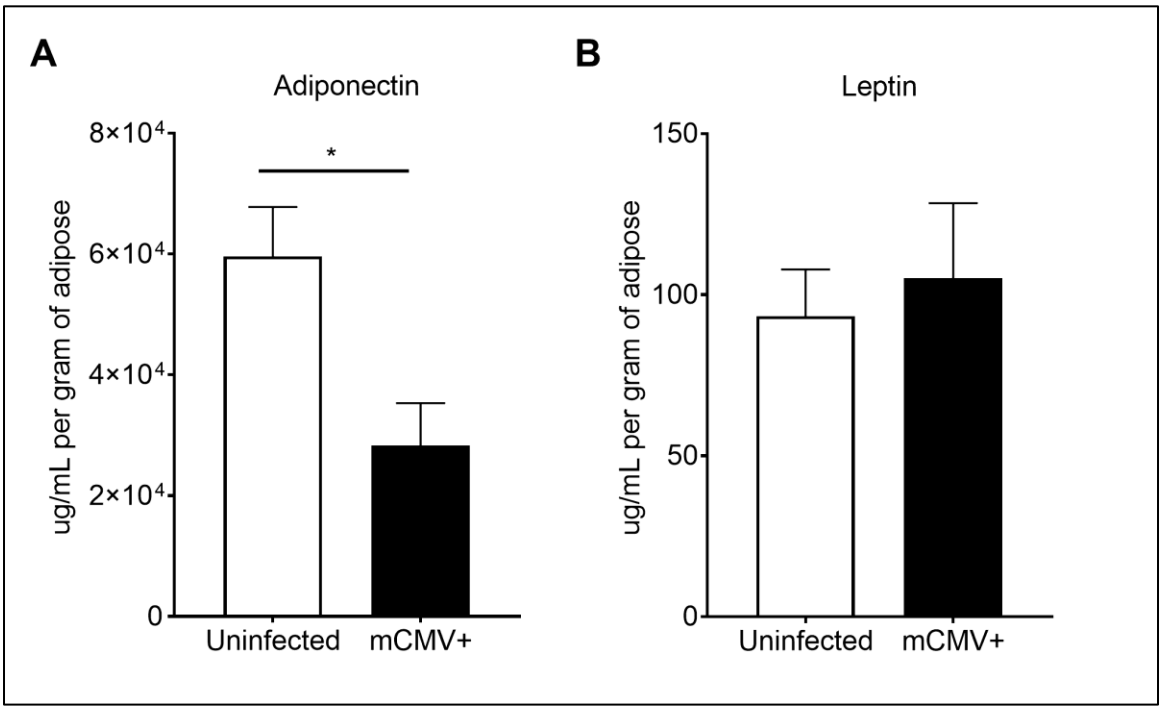

Figure 7. Acute mCMV infection alters adipose adipokine milieu

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route and sacrificed at 7d p.i.. Total adipose tissue was homogenized and analyzed by ELISA for (A) Adiponectin; and (B) Leptin. Data are pooled results of two independent experiments. n = 5 uninfected and 8 infected animals total. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

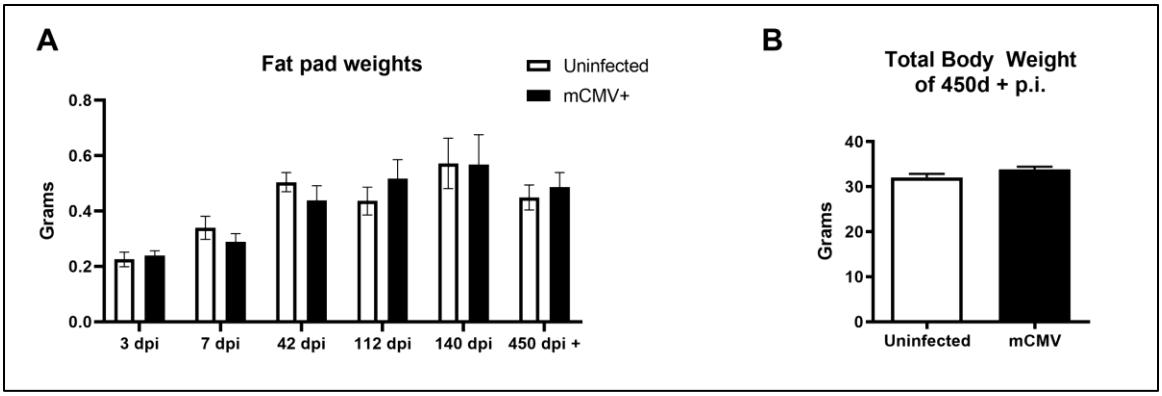

Figure 8. mCMV infection does not alter adipose tissue weight across lifespan or total body weight during lifelong infection.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route. At sacrifice times as noted through the manuscript, adipose tissue was collected and analyzed. (A) Total weight of epididymal fat pad at time of harvest. (B) Body weight of mice infected for greater than 450 days and their aged matched counterparts. Data is pooled from multiple experiments. n = 5 – 35 total animals per group.

Adipose tissue accumulates CD8+ mCMV-specific CD8 T cells regardless of the route of infection or the presence of CCR2+ cells

CCR2+ cells are believed to be the major carriers of mCMV, involved in virus dissemination [265,266]. Moreover, intraperitoneal injection, used in our experiments, may result in an indiscriminate and non-physiological distribution of the virus, including to the epididymal adipose tissue. To assess whether the virus exhibited true tropism for adipose tissue or perhaps infected the immunological constituents of adipose tissue regardless of the route of infection, we infected animals using 10^5 pfu of mCMV via the footpad (f.p.) route of infection. We considered two possibilities in which this receptor could be required 1) that CCR2 is required for homeostatic seeding of CCR2+ infected cells from the periphery into the adipose tissue, resulting in “reinfection” of adipose; or 2) that CCR2+ patrolling cells are not required to maintain antigen and therefore the presence of

T cells in situ. We also wondered whether CCR2+ cells were needed to spread the virus during normal homeostatic seeding of adipose. To that effect, we infected both C57BL/6 and CCR2$^{-/-}$ mice [267] via the f.p. route. Surprisingly, when we analyzed adipose tissue at days 3 and 7 p.i. of C57BL/6 and CCR2$^{-/-}$ mice we were unable to detect viral product in either strain of mouse, perhaps indicative of virus being below the limit of detection or unable to spread to adipose tissue through this route. However, at 7d p.i. when we analyzed adipose tissue by flow cytometry, we found a significant increase in CD3 T cell numbers driven by a significant expansion of CD8 T cell in adipose of both wildtype and CCR2$^{-/-}$ mice (Fig 9A). Of interest, we saw a very limited expansion of central memory CD8 T cells (CD62L+CD44+) in the adipose when infected through this route in both wildtype and CCR2$^{-/-}$ mice, while the significant expansion of effector memory cells (CD62L-CD44+) was not diminished (Fig. 9B). When we assayed tetramer specificity, we found a significant expansion in both acute, M45, and inflationary, m139, epitopes (Fig 9C). Finally, we analyzed the MPEC and SLEC populations and found that MPECs did not significantly expand whereas SLECs did (Fig 9D). These results suggest that mCMV infection, perhaps even at extremely low levels of viral burden, leads to the accumulation of mCMV-specific T cells in the adipose tissue regardless of the route of infection or in the presence of CCR2 (and, presumably, of CCR2+ cells). However, it should be noted that memory precursors and central memory cells appear to be insensitive to this route of infection (Fig 9B and Fig 9D).

We then wished to determine if there was a requirement for CCR2 to maintain viral-specific T cells within adipose tissue given the lack of central memory and memory precursors through f.p. infection. We therefore infected as before, via the footpad route, $CCR2^{-/-}$ and quantified the mCMV-specific T cell in adipose at an early memory timepoint post infection, greater than 30d p.i.. We found an overall diminished T cell population within the adipose of infected animals with no single T cell subset being significantly increased during infection (Fig 9E). Just as in the 7d p.i. timepoint, we saw no significant expansion of central memory CD8 T cells and found a trend of increase in the effector memory pool (Fig 9F). However, even in the absence of this expansion of global T cell populations, there was a significant increase in the mCMV M45- and m139-specific subsets (Fig 9G) as well as an expansion of both MPECs and SLECs at this time (Fig 9H).

Taken together, we conclude that the f.p. route of infection leads to undetectable viral load in adipose tissue, which results in no expansion of central memory and memory precursor CD8 T cells. Nonetheless, effector and short-lived effector populations do expand, albeit to a lesser degree than i.p.. Finally, tetramer specific T cells still arise and persist in adipose tissue regardless of the absence of CCR2 and route of infection.

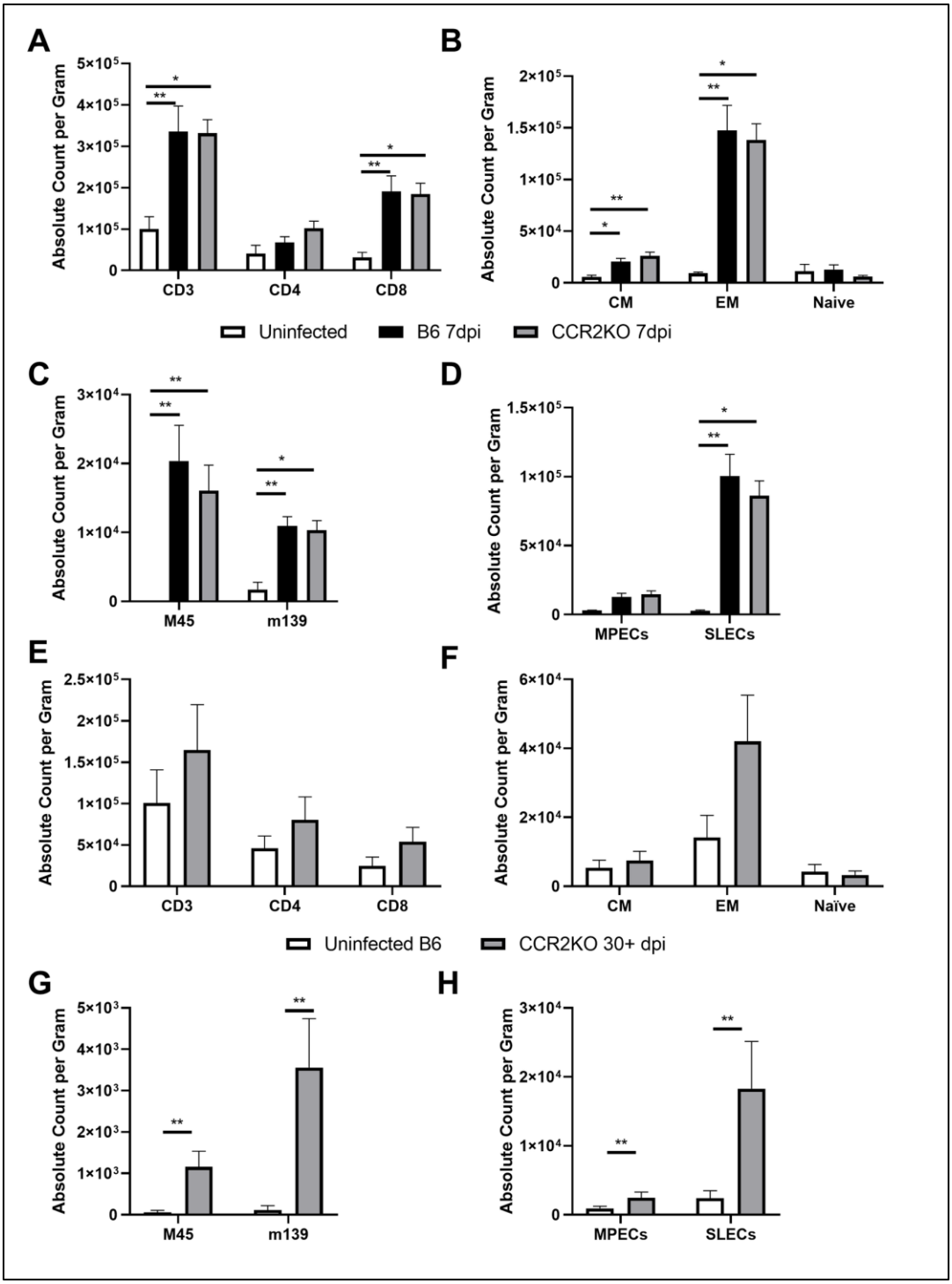

Figure 9. CD8+ mCMV-specific CD8 T cells accumulate in adipose tissue regardless of the route of infection or the presence of CCR2+ cells.

12-week-old C57BL/6J and CCR2$^{-/-}$ mice were infected with 10^5 pfu of mCMV via the footpad and sacrificed at 7d p.i.. Stromal vascular fraction was analyzed by flow cytometry. (A – D) 7d p.i. (A) Total number of T cells and subsetted into CD4 and D8 pools. (B) CD8 T cells were phenotyped based on expression of CD62L and CD44 and quantified. (C) CD44+ CD8 T cells were analyzed

for mCMV specificity by tetramer staining. (D) CD44+ CD8 T cells were analyzed for expression of KLRG1 and CD127 to quantify number of MPECs and SLECs. (E – H) 30d + p.i. (E) Total number of T cells and subsetted into CD4 and D8 pools. (F) CD8 T cells were phenotyped based on expression of CD62L and CD44 and quantified. (G) CD44+ CD8 T cells were analyzed for mCMV specificity by tetramer staining. (H) CD44+ CD8 T cells were analyzed for expression of KLRG1 and CD127 to quantify number of MPECs and SLECs. Data are pooled results of two independent experiments. (A – B) n = 5 uninfected, 10 C57BL/6J infected, 10 $CCR2^{-/-}$ infected animals total. (E – H) n = 6 uninfected and n = 6 infected. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001. 7d p.i. are tested by Kruskal-Wallis with Dunn's multiple comparisons and 30d+ p.i. by unpaired two-tailed Mann-Whitney U test.

Long-term persistence of tissue-resident mCMV-specific T cells and of inflammation in the infected adipose tissue

To assess the impact of mCMV infection in the adipose tissue over the lifespan, we analyzed the fat pads of lifelong infected animals. During the lifelong time points of infection (>450d p.i.) we were unable to detect mCMV RNA. We therefore looked at the maintenance of mCMV genomes in the adipose tissue of infected animals via qPCR. To get a better resolution on viral genome loads, we initially compared CD45- non-hematopoietic and CD11b+CD45+ myeloid cell pools after FACs sorting (Fig 10A). Since the CD45- fraction showed a trend towards a higher mCMV genome burden (Fig. 10B), we focused on these cells in subsequent kinetic analysis. mCMV genomes persisted in the adipose CD45- tissue at comparable levels from 90 to approximately 300+ days post infection, suggesting a lifelong presence of the latent and/or reactivating virus in the adipose tissue of infected animals (Fig. 10C). We next went on to characterize the immunological response at these late time points. We found that total leukocyte counts in the adipose tissue were no longer significantly elevated, showing just a trend (Fig. 12A). However, there remained a significant increase in CD3 T cells in infected animals (p=0.0407), which was entirely driven by a

robust expansion of CD8 T cells (Fig. 12B; p=0.0011), with a dominant and significant increase in Tem CD8 cells (Fig. 12C; p<0.0001), and a stronger skewing towards SLECs (p<0.0001) over MPECs (p=0.0069) compared to the acute (d7d p.i) infection (Fig. 12D). At this point NK cells and macrophage populations in infected animals mirrored that of their aged-matched counterparts (Fig. 3B), suggesting that perhaps viral control of mCMV in adipose at these later time points is more reliant upon T cells.

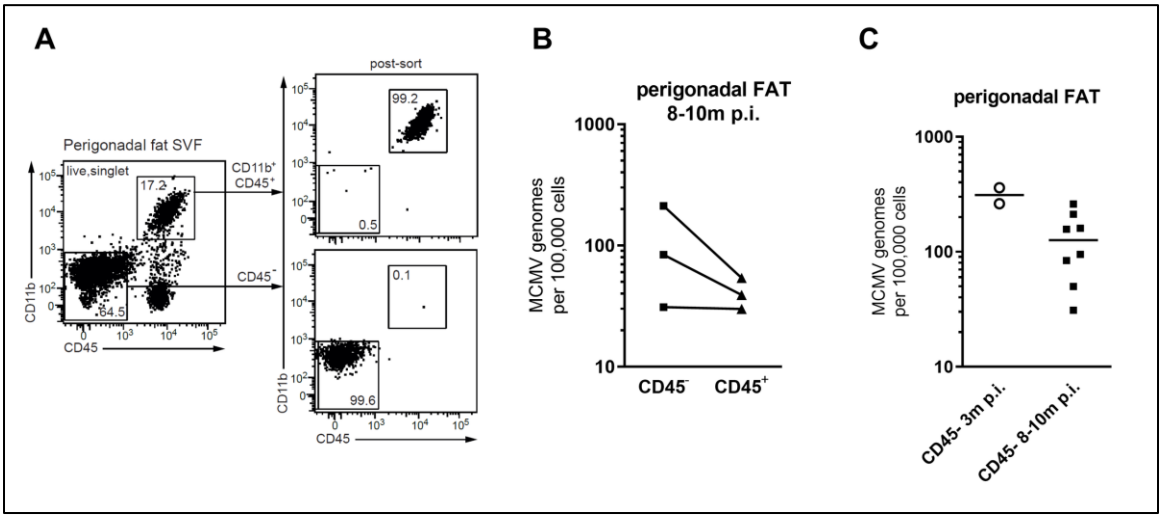

Figure 10. mCMV genomes are detectible in CD45- and CD45+ adipose tissue cells at 240d p.i..

8-week-old C57BL/6J female mice were i.p. injected with 10^6 pfu of bacterial artificial chromosome–derived mCMV (pSM3fr-MCK-2 full-length and sacrificed at 90d or at greater than 240d p.i. Perigonadal adipose tissue stromal vascular fractions were isolated and stained with antibodies and FACS-sorted into CD45- and CD45+CD11b+ subsets. (A) FAC-sort purity (B) mCMV DNA burden in CD45- vs CD45+CD11b+ subsets of visceral adipose tissue of 10 months post infected mice

To examine whether CD8 T cells in the adipose tissue were recirculating from the systemic pool, we performed two experiments. First, we analyzed expression of CD69 on CD8 T cells. This molecule, often used as a marker of immediate activation, is an antagonist of the S1P1 receptor, leading to the

retention of T cells in their specific tissue [268]. We found that 75% of all CD8 T cells expressed CD69 in infected animals, a significant increase compared to that of their uninfected counterparts (Fig. 12E; p=0.0015). We also analyzed the dual expression of CD103e, which has been used to define tissue resident cells in other tissues [268,269] and found no significant differences between its expression on CD8 T cells in infected and uninfected animals (Fig. 11). Second, to independently test whether and how many CD8 T cells in the adipose tissue may be of resident memory type, we assessed their accessibility to a systemic anti-CD45 antibody injected into the vasculature in vivo, as a measure of their vascular vs. tissue-resident location. We injected an Alexa Fluor 700 labeled anti-CD45 antibody intravenously (i.v.) into lifelong infected animals, harvested the adipose tissue 5 min later, as previously described, to determine the extent of T cell tissue residency [270]. We found that approximately 95% of all T cells in adipose tissue of infected (as well as uninfected) animals stained only with the ex vivo antibody and therefore could be classified as resident to adipose tissue (Fig. 12F).

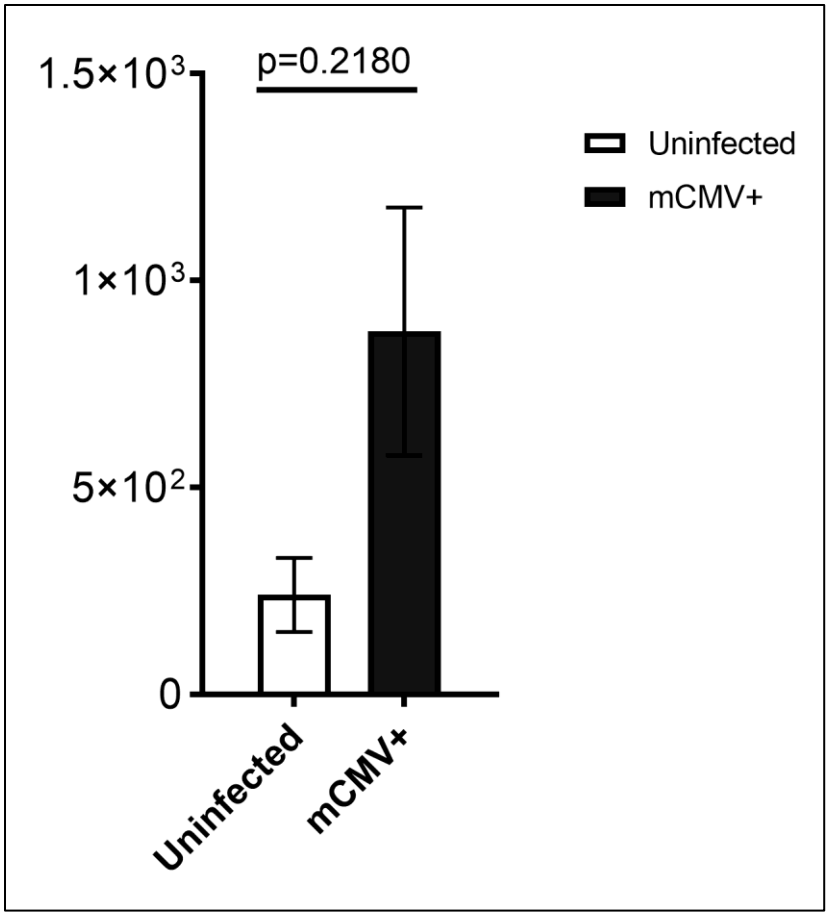

Figure 11. Dual expression of CD69 and CD103e is not significantly different between CD8 T cells in lifelong mCMV infected and uninfected adipose tissue.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route. At greater than 450d p.i. mice were sacrificed Stromal vascular fraction was analyzed by flow cytometry and cell populations quantified. Dual expression of CD69+CD103e+ CD44+ CD8 T cells were quantified. Data are pooled results of two individual experiments. n = 10 infected and n = 10 uninfected. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test within genotypes.

To ascertain that T cells in lifelong infected animals are specific for mCMV antigens, we repeated the tetramer staining as performed in earlier timepoints. We found that a majority of CD8 T cells within adipose tissue remained specific for mCMV tetramers, with an expected and significant expansion of T cells specific for the inflationary T cells epitopes, m38 ($p<0.0001$) and m139 ($p<0.0001$). A much smaller, but also significantly expanded population was

specific for the acute M45 epitope (p=0.0018), possibly indicative of recent viral

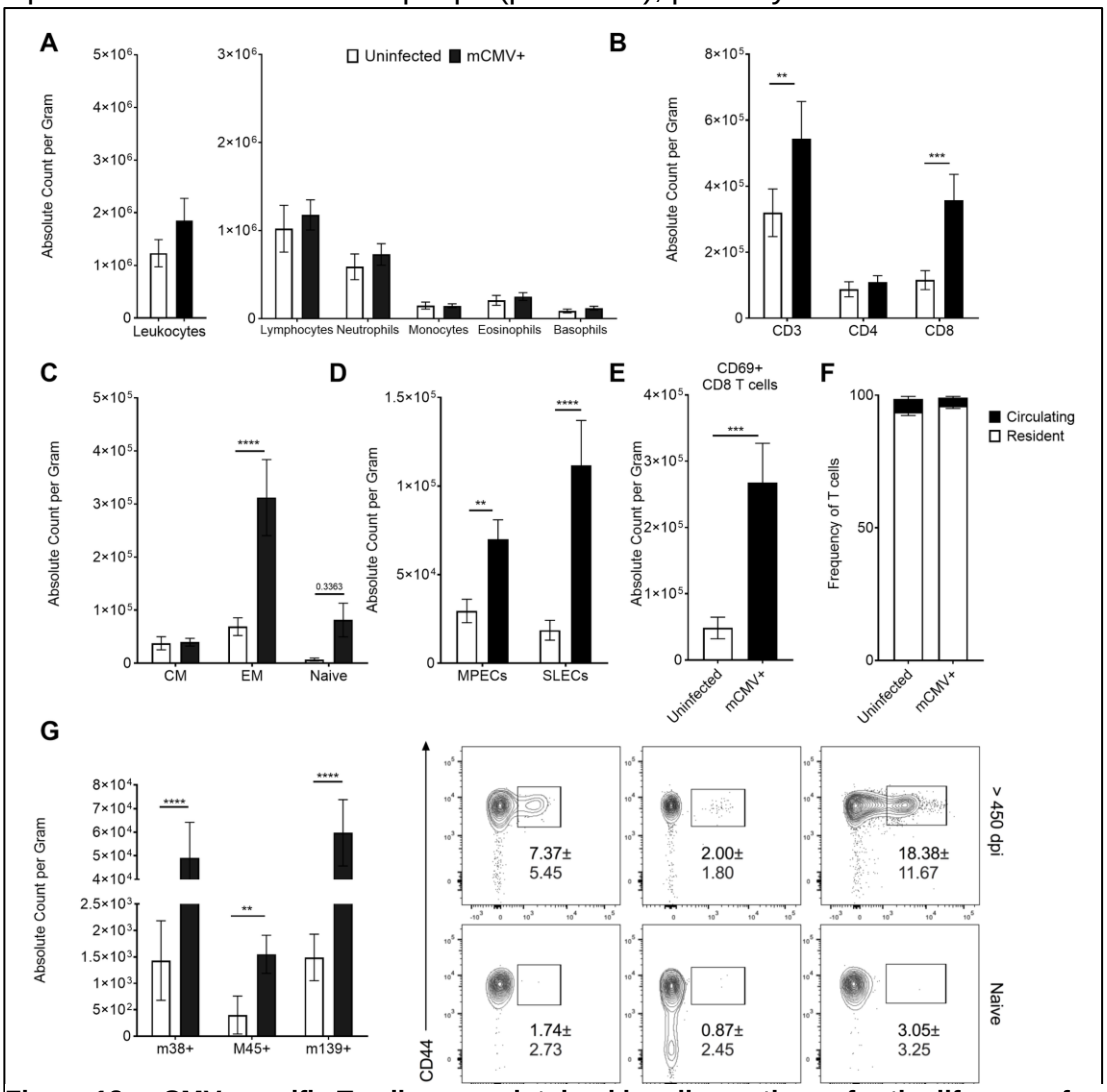

Figure 12. mCMV-specific T cells are maintained in adipose tissue for the lifespan of infection.

12-week-old C57BL/6J mice were i.p. injected with 10^5 pfu of mCMV and sacrificed >450d p.i. Stromal vascular fraction was analyzed by Drew Scientific HemaVet 950 and flow cytometry. (A) Total leukocytes were quantified by hemocytometer. (B) Flow cytometry analysis was used to quantify absolute numbers per gram of adipose tissue of CD3 T cells and gated on CD4 or CD8. (C) CD8 T cells were phenotyped based on expression of CD62L and CD44 and quantified. (D) CD44+ CD8 T cells were analyzed for expression of KLRG1 and CD127 to quantify number of MPECs and SLECs. (E) Total CD8 T cells were analyzed for surface expression of CD69. (F) Lifelong infected animals were injected i.v. with 3 µg of CD45 antibody to determine tissue residency of T cells. Frequency of *in* vivo and *ex* vivo stained animals is shown. (G) CD44+ CD8 T cells were analyzed for mCMV specificity by tetramer staining. (A-E and G) Data are pooled results of two independent experiments. n = 20 uninfected animals and 19 infected animals total. (F) Data are pooled results of two independent experiments with an n = 9 uninfected animals and n=9 infected animals total. Frequencies shown in the dot plots represent SD. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

reactivation (Fig. 12G). Taken together, these data demonstrate that mCMV-specific CD8 T cells are maintained within the adipose tissue for the lifespan of infection, as bona fide Trm cells.

The presence of phenotypically active mCMV specific T cells in adipose tissue provides evidence of a continued surveillance against mCMV. Next, we investigated whether this significant presence of mCMV-specific Trm cells within adipose tissue over the lifespan may be associated with persistent inflammation. We found that IL-23 ($p=0.0201$), IL-1α ($p=0.0071$), IFNγ ($p=0.0113$), TNFα ($p=0.0258$), CCL2 ($p=0.0083$), IL-6 ($p=0.0083$), IL-27 ($p=0.0109$), and GM-CSF ($p=0.0175$) were all significantly elevated in lifelong infected adipose tissue when compared to uninfected age matched controls (Fig. 13A-H). By contrast, IFNβ, IL-1β, IL-10, IL-17A, and IL-12 did not exhibit significant changes when compared to uninfected animals. These data indicated that adipose tissue is a site of lifelong accumulation, or maintenance, of mCMV-specific Trm cells that exhibit phenotypic evidence of recent antigenic stimulation, and that this correlates with an inflammatory cytokine response over the entire lifespan of the host.

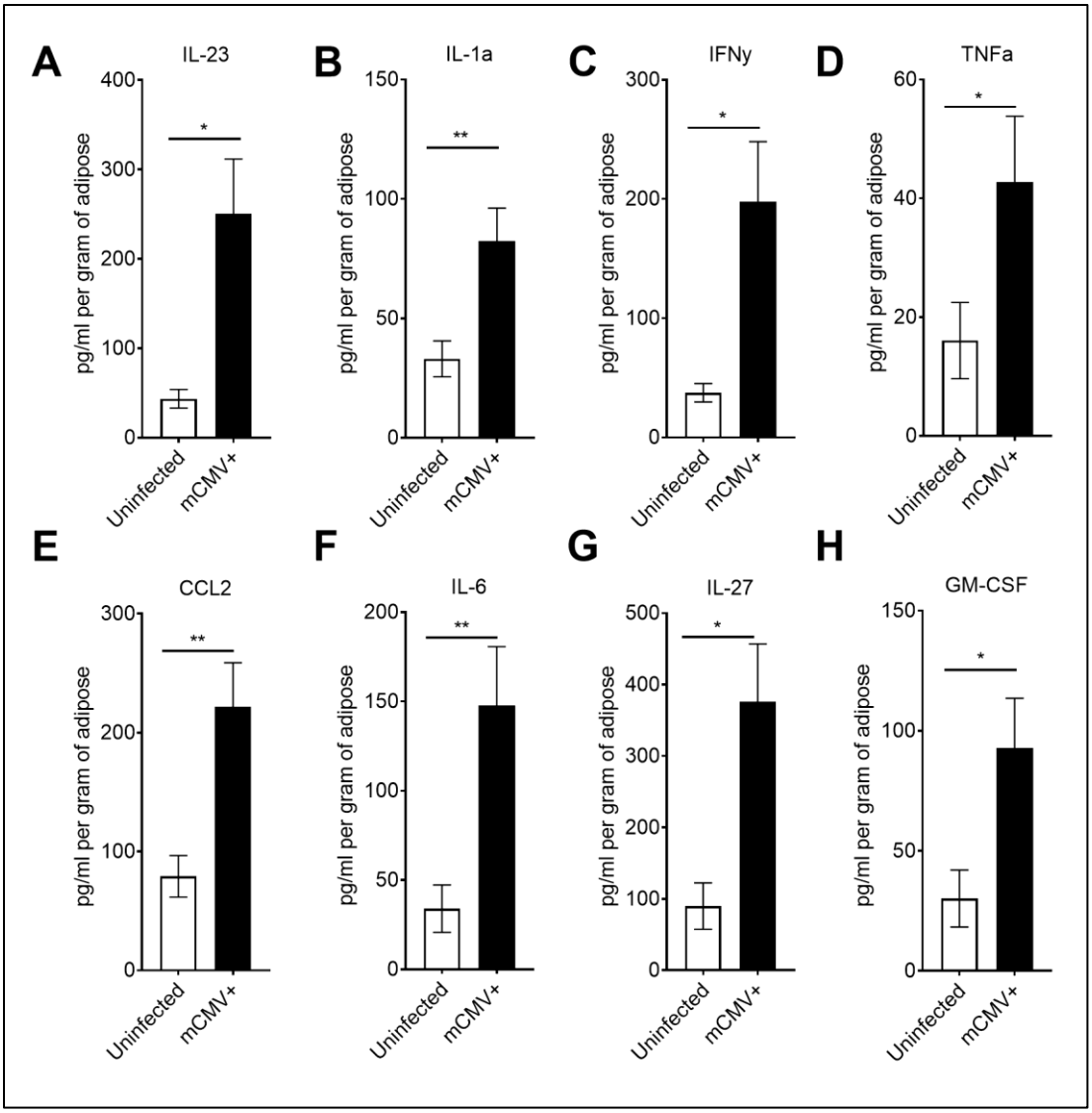

Figure 13. Lifelong mCMV infection results in inflammation in the adipose tissue.

12-week-old C57BL/6J mice were injected with 10^5 pfu of mCMV and sacrificed at greater than 450d p.i.. Total adipose tissue was homogenized and analyzed by BioLegend LegendPlex. (A) IL-23 (B) IL-1α (C) IFNγ (D) TNFα (E) CCL2 (F) IL-6 (G) IL-27 and (H) GM-CSF were all statistically increased. Data are pooled results of two independent experiments. n = 9 uninfected and 10 infected animals total. Error bars represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; **** $p \leq 0.0001$ by unpaired two-tailed Mann-Whitney U test.

Persistent mCMV infection is correlated with hyperglycemia

Adipose tissue inflammation associated with obesity has been clearly linked with multiple phenotypes of the metabolic syndrome, including glucose intolerance and insulin resistance [197,271,272]. Based on the observed increase in inflammatory cytokines and cytotoxic T cells in lifelong infected animals we hypothesized that infected mice could exhibit an altered metabolic profile. Indeed, we found that between ten- and twelve-weeks post infection there was an elevation of fasted blood glucose in infected animals (Fig. 15A) with no significant differences between infected and uninfected animals in plasma insulin levels (Fig. 15B). To determine if the hyperglycemia was correlated with increased adiposity of infected animals, we longitudinally followed mice and analyzed the weight of their fat pads and found no significant change in fat pad weights between infected and uninfected mice (Fig. 8A). When we calculated the homeostasis model assessment insulin resistance (HOMA-IR) index and found that infected animals exhibited significant elevation of this index compared to uninfected animals (Fig. 15C; $p=0.0155$). Conversely, the inverse of HOMA-IR, the insulin sensitivity index, expectedly suggested that infected animals were less sensitive to insulin than uninfected controls (Fig. 15D; $p=0.0155$). At >450 days post infection, significantly elevated levels of blood glucose were still observed in infected animals (Fig. 15E; $p=0.0006$) and this occurred in the absence of a significant increase in body weight (Fig 8B). This elevation in fasted blood glucose appeared to be dependent on mature CD8 T cells as we found no

significant differences between the fasted blood glucose of chronically infected and uninfected mice lacking beta-2-microglobulin (B2m KO) (Fig. 14).

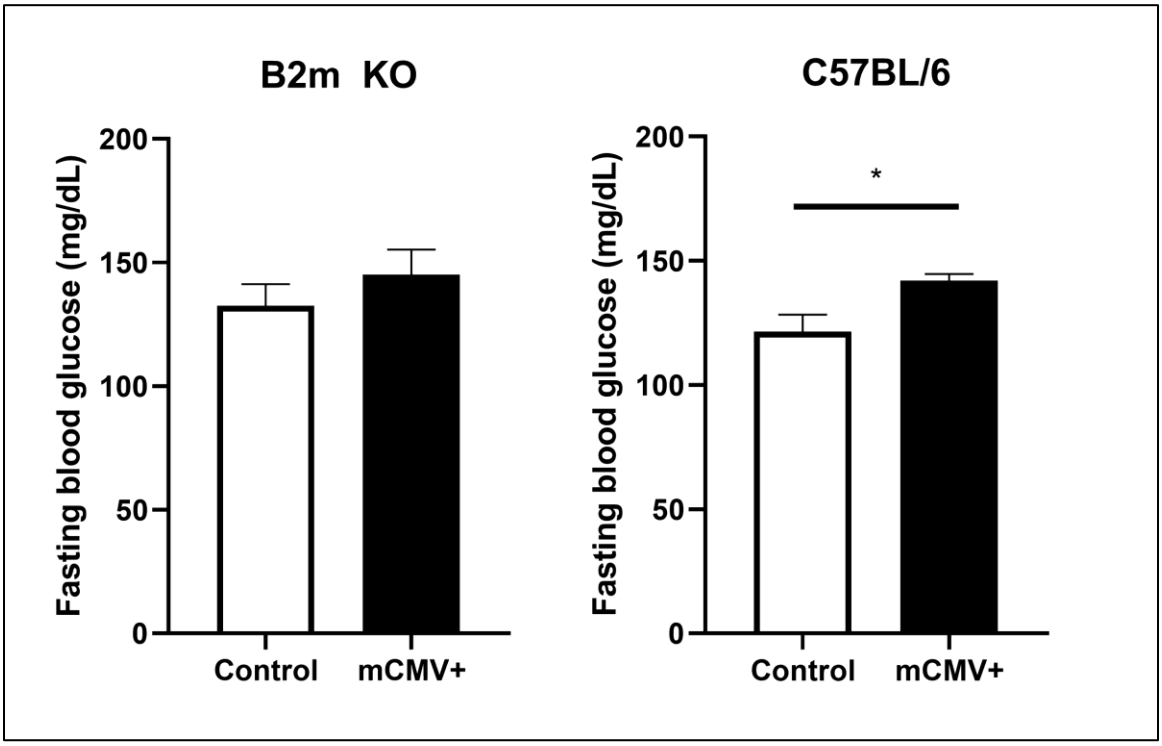

Figure 14. B2m is required for manifestation of hyperglycemia during lifelong mCMV infection.

12-week-old C57BL/6J and B2m KO mice were infected with 10^5 pfu of mCMV by the i.p. route. After greater than 300d p.i. mice were fasted for 6 hours and fasted blood glucose was analyzed. Data are representative of two individual experiments. n = 10 infected B2m and n = 9 uninfected B2m. n = 5 infected C57BL/6 and n = 4 uninfected C57BL/6. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test within genotypes.

Based on the results of the HOMA-IR analysis of mice between ten- and twelve-weeks post infection we analyzed more broadly the metabolic system of lifelong infected animals. First, we determined the extent to which infected and uninfected animals clear a bolus of glucose by i.p. challenge. We found that mCMV infected animals did not clear glucose from the blood as quickly as uninfected counterparts (Fig. 15F). We next tested whether the HOMA-IR was an accurate representation of insulin resistance in our model. Therefore, we i.p.

challenged with fast acting insulin to determine insulin sensitivity and found no significant differences between infected and uninfected animals (Fig. 16A). Finally, we wondered if elevated fasted blood glucose indicated a hyperactive gluconeogenesis driven by the liver. We therefore challenged mice with sodium pyruvate i.p. to determine liver sensitivity to infection and found no difference in gluconeogenesis in infected and uninfected animals (Fig 16B).

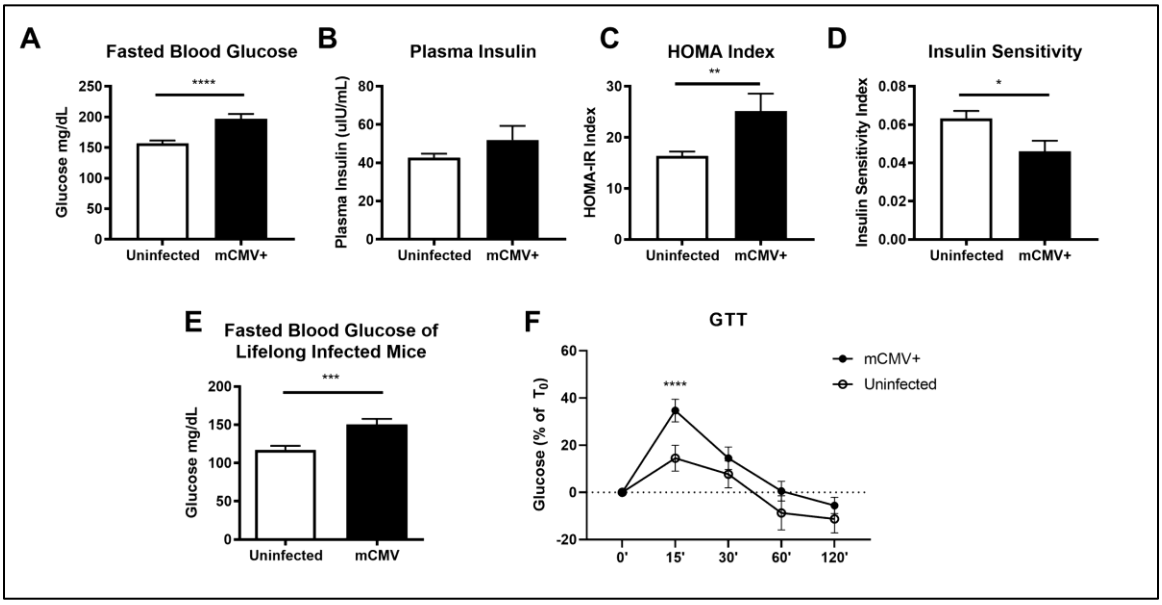

Figure 15. Chronic mCMV infection is correlated with hyperglycemia.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route. Prior to blood glucose measurements via tail nick or collection of blood for plasma via retro-ortibal bleed mice were fasted for at least 7 hours. (A) Fasted blood glucose of mice infected between 10 and 12 weeks as measured by Bayer Contour Next EZ Glucose Meter. (B) Plasma insulin concentration measurements by ELISA of mice infected between 6 and 10 weeks. (C) HOMA-IR and inverse measurement (D) Insulin sensitivity index. (E) Fasted blood glucose of lifelong (>450d p.i.) infected mice. Data are pooled results of two to four independent experiments. For the 6 to 10-week experiments there were an n = 14 uninfected and n = 10 infected animals total. For the lifelong experiments there was an n = 23 uninfected and 35 infected animals total. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

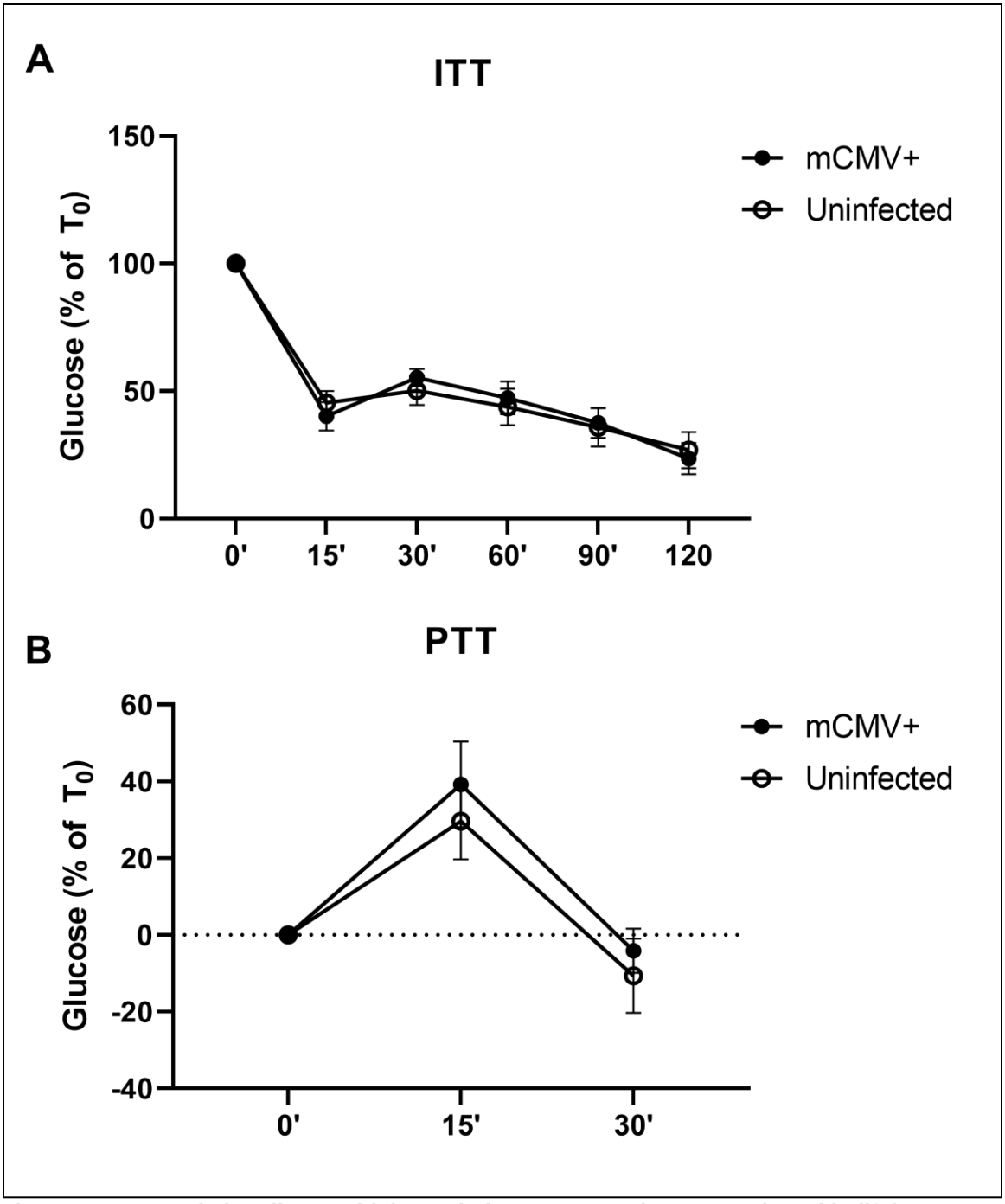

Figure 16. Systemic insulin sensitivity and gluconeogenesis are not altered in lifelong mCMV infected animals.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route. After greater than 450d p.i. mice were challenged with insulin tolerance (ITT) and pyruvate tolerance tests (PTT). (A) Percent change of fasted blood glucose compared to Time 0 after i.p. injection of 1 U / kg insulin. (B) Percent change of fasted blood glucose compared to Time 0 after i.p. injection of 2 mg / kg sodium pyruvate. Data are representative of two repeated experiments for each test. n = 18 infected and 10 uninfected animals in total.

Taken together these data are consistent with recently published work that suggests alterations in glucose tolerance and insulin sensitivity in mice acutely infected with mCMV and influenza infection of mice being fed a high fat diet [222]. When we measured adiponectin expression in adipose tissue homogenate, we expected decreased amounts of total protein as we saw in the acute time point post infection, however we found no significant change in the amount of adiponectin protein in uninfected and lifelong infected animals (Suppl. Fig 17), perhaps indicating an age related decrease in adiponectin expression that may mask changes induced by infection. Overall, we found a clear initial alteration in the glycemic profile of mCMV-infected mice following infection that appears to be driven by delayed glucose clearance in infected animals and is possibly dependent upon mature T cells. Additional studies will be required to mechanistically extend these data, one is tempted to speculate that mCMV may make animals susceptible to clinical metabolic changes pending action of other environmental stressors, including diet, as previously published, and aging in our model.

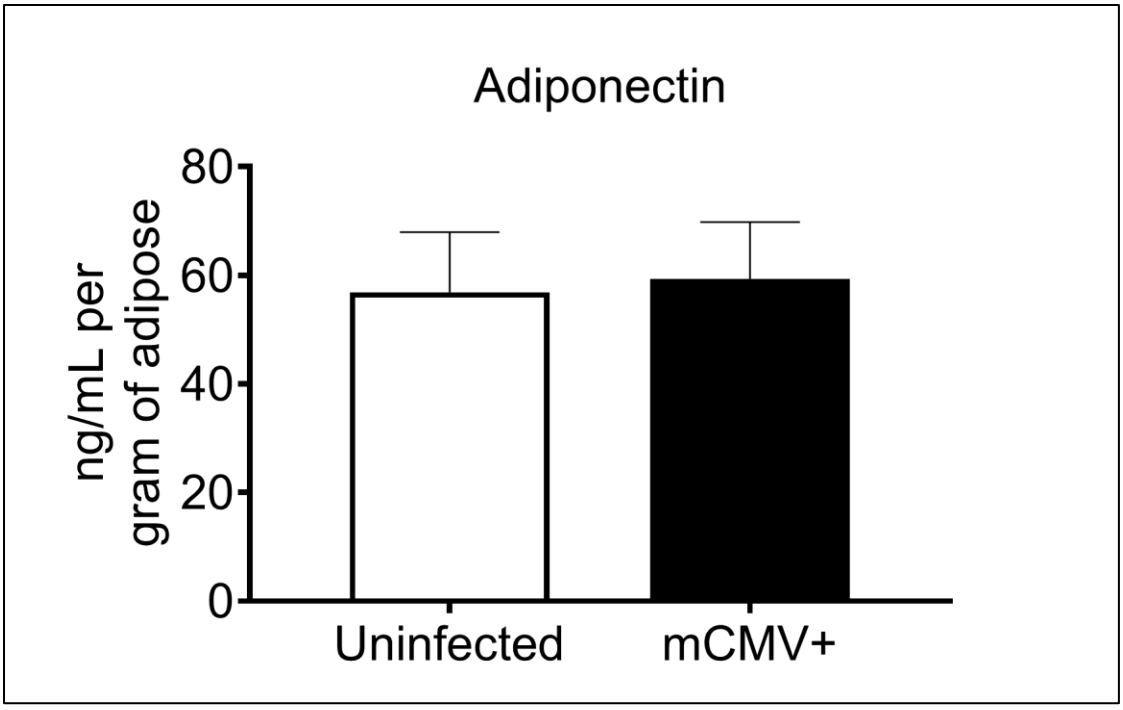

Figure 17. Lifelong mCMV infection does not alter adiponectin levels.

12-week-old C57BL/6J mice were infected with 10^5 pfu of mCMV by the i.p. route and sacrificed at >450d p.i.. Total adipose tissue was homogenized and analyzed by ELISA for Adiponectin. Data are pooled results of two independent experiments. n = 12 uninfected and 17 infected animals total. Error bars represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; **** p ≤ 0.0001 by unpaired two-tailed Mann-Whitney U test.

Discussion

CD8 T cell immunity against mCMV infection has been extensively studied in the context of inflationary memory T cell expansion in the blood, as well as in the lungs (as the port of CMV entry) and salivary gland (as the site of intense primary CMV replication). Results of these studies have suggested that the blood contains a large pool of CMV-specific circulating Tem cells, guarding against potential systemic reactivation, whereas both the site of primary entry (lungs) and extensive initial replication and shedding (salivary gland) contain Trm cells standing guard against potential reinfection (lung) and/or local reactivation (lung and salivary gland). CMV is believed to infect many cells, but to establish latency

only in very few [233,273,274]. In that context and in the context of an early and lifelong CMV infection and immunity, we know very little about CMV-specific CD8 T cell immunity and control in other tissues. For example, one fundamental question remains on whether the large systemic circulating CD8 T cell pool is responsible for the control of other potential sites of latency and reactivation.

Recent studies show that white adipose tissue is enriched in leukocytes, including a significant population of memory T cell populations even in mice housed under specific-pathogen free conditions [161,275,276]. Furthermore, it has been demonstrated that pathogen-specific T cells can arise in both mesenteric and epididymal adipose tissue following bacterial and parasitic infection [260,275]. It has also been demonstrated that murine adipose tissue can harbor infectious mCMV as demonstrated by plaque assay and microscopy during early time points post infection [222,261,277]. We show here that adipose tissue is an early site of infection which leads to generalized inflammation, maintains viral genomes for the lifetime, and possesses a sustained antigen-specific adaptive immune response. We found that mCMV-specific Tem CD8 T cells dominated the immune response early, and this response was maintained for life. Moreover, both phenotypic and functional (vascular accessibility) data were consistent with the Trm nature of fat residing CD8 T cells.

Several groups have demonstrated that mucosal tissues, such as the salivary gland and lungs, are home to non-recirculating T cells that respond to mCMV [76,77,124]. This is largely believed to be in response to mCMV utilizing mucosa as a means for spread through saliva, breast milk, urine, and vaginal

fluids [113]. Authors have suggested two potential methods that result in T cell accumulation in the lung and the salivary gland: 1) T cells primed in the periphery traffic to these locations; and/or 2) viral antigens, (even in the absence of full replication as demonstrated by experiments conducted with replication-incompetent mCMV) are presented in situ, evoking cytokine and chemokine cues that maintain memory T cells after original antigenic stimulation. We interpret our data as indicative of continual maintenance of memory T cells in situ. However, this raises two questions. First, why would adipose tissue be evolutionarily advantageous for mCMV infection? HCMV alters the lipid metabolism of infected cells [278,279] and given the high density of lipids within adipocytes it is possible that adipocytes or their progenitors and even fibroblasts could provide significant sources of lipids and therefore become prime targets for infection. Furthermore, different cells of the adipose tissue, including adipose tissue-derived stem cells [280], have been shown to be susceptible to CMV infection. Second, if viral antigens are not being presented within adipose tissue, why would the immune system divert a lifelong T cell population to this site? Other groups have suggested that the presence of memory T cells in the fat would be expected given the anatomical location of adipose tissue with respect to lymphatic organs, the gut, and the vasculature to provide clean-up for any antigenic leakage from these tissues. We show that fat-residing CMV-specific T cells are phenotypically activated, suggesting recent antigenic stimulation. That would support the hypothesis of antigenic presentation in situ, which may be supported by the PCR detection of viral gene products during the 10 months post infection period.

While, at present, we cannot formally exclude that CMV antigens may indeed leak from these proximal tissues, we consider such a possibility less likely, given the tight temporal regulation of mCMV antigen expression. An alternative possibility would be that trafficking cells harboring CMV, such as inflammatory monocytes, could potentially be continually seeding the adipose tissue and that this would help maintain the mCMV-specific Trm cells. Against that possibility, we found that CCR2$^{-/-}$ mice, infected via the footpad route, as an attempt to isolate initial replication as much as possible, also exhibited significant accumulation of mCMV-specific T cell population in the fat, suggesting either cell-free spread or a non-monocyte cell-associated virus, below our limit of detection at this time, as drivers of T cell accumulation in the adipose tissue. Based on the preponderance of evidence, we favor the scenario whereby a persistent, bona fide latency established by mCMV within adipose tissue drives the accumulation of CD8 Trm cells.

Inflammation within adipose tissue has been widely investigated for its role in the development of metabolic syndromes [272]. In our experiments, mCMV infection resulted in inflammation within adipose tissue in the absence of obesity. The influx into the adipose tissue by leukocytes and specifically CD8 Tem/rm cells could potentially alter the metabolic profile of infected mice. When we measured glucose and insulin changes in infected animals, we did not observe any changes in fasted blood glucose until animals were ten to twelve weeks post infection, by which time we did not detect any difference in systemic insulin levels. This difference was maintained in lifelong infected animals, showing a

significant elevation in the fasted blood glucose of infected animals, but with no statistical difference in the total weight, at end of life, or longitudinal differences in fat pad hypertrophy or atrophy. These observations are consistent with recent data demonstrating that the production of IFNγ in response to mCMV and influenza infection in a model of dietary-induced obesity was the “tipping” point in the manifestation of insulin resistance [222]. Furthermore, we find that neither gluconeogenesis nor reduced insulin sensitivity were responsible for elevated fasted blood glucose. Rather, we believe that infection potentially alters systemic glucose control, an issue that will require further experimentation. Thus, our finding could provide one potential mechanism to link epidemiological data in humans showing that HCMV infection increases the risk of developing atherosclerosis, insulin resistance, and other metabolic diseases [72–76]. In that scenario, we speculate that CMV infection alone could increase one’s risk for developing metabolic disorders, but that additional environmental factors are required, such as diet, other infections, and aging; to what extent this interplay is dependent upon adipose tissue remains to be established.

Our data identify adipose tissue as a potential reservoir for mCMV genomic persistence, through our detection of viral products at 10 months post infection. mCMV infection clearly leads to the continuous stimulation of antigen specific CD8 T cells that take up residency within adipose tissue, based upon phenotypic data. Trm cells are maintained for the lifetime of infection and likely contribute to an inflammatory environment within adipose tissue. These data reveal a strategy by which the adaptive immune system controls mCMV in

tissues and provide insights that could mechanistically link mCMV infection of the adipose tissue to metabolic dysfunction, that may depend on additional metabolic and environmental stressors, such as aging and diet.

Methods

Table 1. Key Resources Table for mCMV Adipose T cell methods

Reagent	Source	Identifier
Adiponectin Mouse ELISA Kit	ThermoFisher Scientific	KMP0041
anti-mouse CD103e	BioLegend	121419
anti-mouse CD127	BioLegend	135035
anti-mouse CD3	BioLegend	100229
anti-mouse CD4	BioLegend	100541
anti-mouse CD44	BioLegend	103011
anti-mouse CD62L	ThermoFisher Scientific	RM4317
anti-mouse CD69	BioLegend	104507
anti-mouse CD8	BioLegend	100733
anti-mouse KLRG1	BioLegend	138415
anti-mouse CD3	BioLegend	100216
anti-mouse CD45.2	BioLegend	109822

anti-mouse CD45.2	BioLegend	109807
anti-mouse CD11b	BioLegend	101216
Purified anti-mouse CD16/32	BioLegend	101301
LEGENDplex Mouse Inflammation Panel	BioLegend	740446
Collagenase D	Sigma-Aldrich	11088858001
RNeasy Lipid Tissue Mini Kit	Qiagen	74804
QIAzol Lysis Reagent	Qiagen	79306
RT² Profiler PCR Array Mouse Insulin Resistance	Qiagen	330231
H-2D(b) MCMV M45 985-993 HGIRNASFI	NIH Tetramer Core Facility	41184
H-2K(b) MCMV m139 419-426 TVYGFCLL	NIH Tetramer Core Facility	41186
H-2K(b) MCMV m38 316-323 SSPPMFRV	NIH Tetramer Core Facility	41185
LIVE/DEAD Fixable Aqua Dead Cell Kit	ThermoFisher Scientific	L34957
LIVE/DEAD Fixable Near-IR Dead Cell Kit	ThermoFisher Scientific	L10119
7-AAD Viability Staining Solution	BioLegend	420404

Mouse Inflammation Panel LegendPlex	BioLegend	740150
NP-40 Surfact-Amps Detergent Solution	ThermoFisher Scientific	28324
Protease Inhibitor Cocktail	Sigma-Aldrich	P8340
Mouse Leptin ELISA Kit	Sigma-Aldrich	RAB0334
Insulin Mouse ELISA Kit	ThermoFisher Scientific	EMINS
BD Cytofix/Cytoperm Fixation and Permeabilization Solution	Fisher Scientific	BDB554655
Humalog Insulin (100 U / mL)	Eli Lilly	Vet Prescribed
D-(+) Glucose	Sigma-Aldrich	G8270-25KG
Sodium Pyruvate	Lonza	13-115E

Ethics statement

Mouse studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Arizona (IACUC #08–102, PHS Assurance Number: A3248-01). Footpad injections were performed under isoflurane anesthesia. Euthanasia was performed by isoflurane overdose. Animal

experiments performed at Helmholtz Centre for Infection Research (Braunschweig, Germany) were approved by Lower Saxony State Office of Consumer Protection and Food Safety under the license number 33.9- 42502-04-14/1712.

Mice and Lifelong MCMV Infection.

Ten-week-old adult C57BL/6J and congenic CD45.1 (B6, H-2b), B2m KO, and $CCR2^{-/-}$ male mice were purchased from The Jackson Laboratory. At 12 weeks of age, adult mice were infected with 10^5 pfu of mCMV intraperitoneally or via footpad, both routes produce overlapping data, (Smith strain, originally obtained from M. Jarvis and J. Nelson, Oregon Health and Science University, Portland, OR, passage 3 on M210B4 cells. Mice were maintained under specific pathogen-free conditions in the animal facilities at the University of Arizona and at Helmholtz Centre for Infection Research (Braunschweig, Germany).

Isolation of Stromal Vascular Fraction

Animals were sacrificed by isoflurane overdose. White adipose tissue from the epididymal fat pad was excised, weighed, and cut in small pieces using forceps and scissors. Cut pieces were resuspended in DMEM containing 2 mg/ml Collagenase D (1mL solution per 0.5 grams of adipose tissue) and incubated for 30 minutes at 37 °C with shaking. Digestion suspensions were thoroughly vortexed and centrifuged at (800g for 5 min). Adipocyte fraction and liquid

interphase was sterile vacuumed away from pellet, which was resuspended in DMEM containing 5% BSA and pushed through a 70 µm nylon mesh filter to remove remaining cell debris. Cells were centrifuged and resuspended in 250 ul PBS containing 1% BSA. 50 ul of resuspension was used to calculate cell counts per gram of adipose tissue and the remaining used for flow cytometry. Numerical quantification of single cell suspensions was carried out using Drew Scientific HemaVet 950.

Real-time PCR quantification of viral RNA load in tissues

Adipose tissue was collected into a microcentrifuge tube filled with 1 mL of Qiazol and autoclaved glass beads and then snap frozen in liquid nitrogen. Samples were thawed and homogenized using a bead beater for two 2-minute cycles. RNA was extracted using Qiagen RNeasy Lipid Tissue Mini Kit per the manufacturer's protocol. Reverse transcription was carried out using Sensiscript RT Kit per manufacturer instructions. Amplification of cDNA was performed using SYBR Green Master Mix on an ABI 7300. Standard curve was generated using plasmid gifted from Wayne Yokoyama, MD, Washington University in St. Louis. Primer set were gifted by Chris Benedict, PhD, La Jolla Institute of Immunology [281]. For applications utilizing the RT2 Miniarray Profiler samples were treated as above, analyzed for RNA Integrity Number (RIN) by the University of Arizona Genetics Core Facility on an Agilent Bioanalyzer 2100. Following manufacturer protocol only samples with a RIN greater than 7 were used. Analysis was carried out through Qiagen's Data Analysis Center.

Real-time PCR quantification of viral genome load in tissues

The liver, spleen, subcutaneous fat, and perigonadal adipose tissue were harvested from mCMV infected and uninfected age matched controls. Each tissue was collected into a microcentrifuge tube containing 1 mL of Qiazol and autoclaved glass beads, and snap frozen in liquid nitrogen. Following thaw, samples were homogenized by bead beating with two 2-minute cycles. DNA was extracted from each sample per the Qiazol manufacturer's protocol. qPCR was performed using PowerUP SYBR Green Master Mix on an Applied Biosciences Step One real-time PCR system using the following cycle protocol: an initial step at 2 min 50°C followed by 95° for 10 min, followed by 40 cycles of 95° for 15 sec, 60° for 1 min. Recombinant plasmids containing IE1 and C57/BL6 β-actin were used as template to establish standard curves for quantification. The primer sequences were as follows: IE1-fw (5'- CCC TCT CCT AAC TCT CCC TTT-3') and IEI-rv (5'-TGG TGC TCT TTT CCC GTG-3'), β-actin-fw (5'-AGC TCA TTG TAG AAG GTG TGG-3') and β-actin-rv (5'-GGT GGG AAT GGG TCA GAA G-3'). Cycle 32 was set as a negative cut-off based on uninfected controls. Primer sets and recombinant plasmids were gifted by Wayne Yokoyama, MD, Washington University in St. Louis.

Real-time PCR quantification of viral genome load in FACS-purified cell subsets

8-week-old C57BL/6J female mice were i.p. injected with 10^6 pfu of bacterial artificial chromosome–derived mCMV (pSM3fr-MCK-2 full-length [282]) and

sacrificed at 90d or at greater than 240d p.i. Perigonadal adipose tissue stromal vascular fractions were isolated as described previously [283], stained with antibodies and FACS-sorted into CD45- and CD45+CD11b+ subsets. DNA was extracted using QIAamp DNA Micro Kit (QIAGEN) according to manufacturer's protocol. Real-time PCR quantification of viral genome load was performed as described previously [284] with modifications. Briefly, equivalent volumes of each DNA sample were analyzed in qPCR reactions with primer pairs specific for either the viral gene M55/gB or the mouse gene Pthrp. Reactions were set up using Fast EvaGreen qPCR master mix (Biotium, Fremont, CA) and run in a LightCycler480 (Roche, Mannheim, Germany) using the following cycling protocol: an initial step of 2 min at 95°C followed by 50 cycles of 10 s at 95°C, 20 s at 56°C, and 30 s at 72°C. Specificity of the amplicons was confirmed through melting curve analysis and by electrophoresis on agarose gels. Absence of cross-contamination was ascertained by parallel assessment of negative water controls and of DNA samples from non-infected animals. A recombinant plasmid standard containing sequences of both gB and Pthrp genes [284] was used as a template to establish standard curves for quantification. The dynamic range of the assay stretched from 101 to106 mCMV genome copies per reaction. The following primer sequences were used: gB-fw (5′-GCAGTCTAGTCGCTTTCTGC-3′) and gB-rev (5′-AAGGCGTGGACTAGCGATAA-3′); Pthrp-fw (5′-GGTATCTGCCCTCATCGTCTG-3′) and Pthrp-rev (5′-CGTTTCTTCCTCCACCATCTG-3′).

Flow Cytometry

Isolated cells were stained using flow cytometry reagents as indicated in the Key Resources Table. Dead cells (identified as 7-Amino-Actinomycin D+ or using LIVE/DEAD Fixable Dead Cell Staining Kits) and cell aggregates (identified on FSC-A versus FSC-W scatter plots) were excluded from all analyses. Cells were plated into 96-well round bottom plates (Costar). Cells were treated with FcBlock (anti-CD16/32) in PBS supplemented with 2% BSA (FACs buffer) for 10 minutes at 4 C and then surface staining antibodies, also in FACs buffer, added for an additional 45 minutes at 4 C. In experiments requiring intravascular staining animals were injected with 3 ug of anti-CD45 antibody in 50 ul of PBS retro-orbitally and waiting 5 minutes prior to sacrificing animals. After initial staining steps, cells were washed three times FACs buffer and then stained using LIVE/DEAD viability dye in PBS alone for 30 minutes at 4 C. Finally, cells were washed once with PBS and three times with FACs. Cells were fixed in BD Cytofix following manufacturers protocol and then washed three times prior to analysis. Data acquisition was performed on a custom-made, four-laser BD Fortessa flow cytometer (Becton Dickinson), and was analyzed using FlowJo software (Tree Star). Cell sorting was performed on a FACSAriaII (BD Biosciences). Gating was informed by using fluorescence minus one (FMO) controls.

Collection of adipose tissue homogenate for ELISA and BioLegend LegendPlex

Total epididymal adipose tissue was excised from infected and control animals and weighed to normalize downstream analysis per gram. Adipose was collected into 0.5% NP-40 buffer in PBS plus 1/100 protease inhibitor cocktail and homogenized using Qiagen TissueRuptor. Samples were incubated at room temperature for 30 minutes and then centrifuged at 4700 RPM for 30 minutes at 4 C. Liquid interphase was taken for downstream analysis. Adiponectin and Leptin ELISAs and BioLegend LegendPlex 13-plex Inflammation Panel analyses were carried out following manufacturer protocols.

Blood Glucose and Plasma Insulin Measurements, Glucose Tolerance Test (GTT), Insulin Tolerance Test (ITT), and Pyruvate Tolerance Test (PTT).

Prior to collection of fasted blood glucose and plasma for insulin measurements mice were fasted for at least seven hours. Blood glucose was measured by tail nick and using Bayer Counter Next EZ Glucose Meter. After glucose measurements blood was obtained via retro-orbital bleeding into EDTA treated tubes (ThermoFisher) by centrifugation for 15 minutes in 4 C at 2,000 x g. Insulin was measured using Insulin Mouse ELISA kit (ThermoFisher). HOMA-IR was calculated by multiplying fasted plasma insulin and fasted blood glucose and dividing the product by 22.5, the inverse of this result was taken to represent Insulin Sensitivity [285]. For GTT, ITT, or PTT, following fasting mice were i.p.

challenged with 1 mg/kg glucose, or 1 U/kg Humalog insulin (Eli Lilly), or 2.5 g/kg sodium pyruvate in PBS respectively and blood glucose measured as described above.

Statistical Analysis

Statistics were performed in Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Two-tailed Mann Whitney U tests with equal SD were carried out on all analyses. Significance is noted as follows throughout: ns = not significant, ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05. All error bars shown are SEM. In all cases, a bar overlies groups compared for significance and the stars as described above denote significance.

CHAPTER 5: CALORIE RESTRICTION INDUCES REVERSIBLE LYMPHOPENIA AND LYMPHOID ORGAN ATROPHY DUE TO CELL REDISTRIBUTION

“If you change your eating habits to include more whole food (beans, rice, vegetables and fruit), then you'll eat less.” – Roy Walford

Abstract

Calorie restriction (CR) without malnutrition increases life span and health span in multiple model organisms. In non-human and human primates, CR causes changes that protect against several age-related pathologies, reduces inflammation, and preserves or improves cell-mediated immunity. However, CR has also been shown to exhibit adverse effects on certain organs and systems, including the immune system, and to impact genetically different organisms of the same species differentially. Alternately, short periods of fasting followed by refeeding may result in the proliferation of bone marrow stem cells, suggesting a potential rejuvenation effect that could impact the hematopoietic compartment. However, the global consequences of CR followed by refeeding on the immune system have not been carefully investigated. Here, we show that individuals practicing long-term CR with adequate nutrition have markedly lower circulating levels of total leukocytes, neutrophils, lymphocytes, and monocytes. In 10-month-old mice, short-term CR lowered lymphocyte cellularity in multiple lymphoid tissues, but not in bone marrow, which appears to be a site of influx, or a “safe haven” for B, NK, and T cells during CR. Cellular

loss and redistribution was reversed within the first week of refeeding. Based on BrdU incorporation and Ki67 expression assays, repopulating T cells exhibited high proliferation in the refeeding group following CR. Finally, we demonstrated that the thymus was not essential for T cell repopulation following refeeding. These findings are of potential relevance to strategies to rejuvenate the immune system in mammals and warrant further investigation.

Introduction

Aging is associated with an increased risk of morbidity and mortality from infection, due to a dysfunction affecting both the innate and adaptive arms of the immune system [286]. Susceptibility to emerging pathogens and decreased efficacy of vaccines are driven in part by this age related immune decline, termed immune senescence [50,287,288]. The earliest age-related change that partially sets the stage for a subsequent immune decline is the involution of the thymus, that results in a decreased output of naïve T cells into the periphery as early as before puberty in humans [289–291]. This mandates long-term peripheral maintenance of the naïve T cell pool [52,292] that, while initially successful, also fails in the last third of life [241,292–295]. Moreover, pathogen exposure across the lifespan selectively converts antigen-specific naïve T cells into memory T cells [291], further pronouncing the relative dominance of memory lymphocytes with aging. Similar changes are seen in B-lymphocytes. In addition to these cell population changes, cell intrinsic defects, as well as decline in environmental/stromal and soluble factor-driven coordination of immune

homeostasis and immune responses, results in the exacerbation of age related immune decline [296].

Calorie restriction (CR) without malnutrition is the most robust intervention known to increase lifespan and health span in most model organisms investigated to date [16,32,286]. In humans, it causes changes that protect against multiple age-associated chronic disease, and powerfully reduces inflammation without impairing the delayed-type hypersensitivity skin response or the antibody response to vaccines [30,297,298]. However, experimental animal investigation of the consequences of CR on the immune system has revealed potentially beneficial, as well as detrimental, effects. In the mouse model, CR reduced the extent of thymic involution in old animals [23]; and in both murine and, non-human primate models, the proportion of naïve peripheral T cells was increased [299]. These observations seemingly indicate that CR could be used as a therapeutic regimen in the elderly to prevent age related immune senescence. On the other hand, studies of infectious challenge in vivo have produced cautionary results. Several groups, including ours, have demonstrated that mice living in specific pathogen-free facilities on lifelong CR regimen displayed increased mortality compared to ad libitum fed counterparts in response to sepsis [300], influenza A [301], parasitic infections [302], and West Nile virus infection [17]. These studies suggested that in the face of microbial challenge, both innate and adaptive immune responses require access to energy to optimally defend the organism. Collectively, these results suggest that it is necessary to evaluate modalities of CR that provide increased longevity and

rejuvenating effects in non-immune tissues, can be adhered to, and do not compromise the functional integrity of the immune system.

A variety of CR modalities have been reported to be associated with lymphopenia [298,303] presenting another potential adverse immune effect of CR. As this effect has remained largely unexplored, we investigated changes in numbers of a broad array of leukocytes within multiple lymphoid tissues (lymph nodes, thymus, bone marrow, blood, and spleens) during two months of CR induction. We then returned animals to ad libitum (AL) feeding and monitored alterations to populations of leukocytes within these same tissues and tested whether any of the observed changes may be due to de novo cell production or to potential cellular redistribution between peripheral reservoirs.

We here demonstrate that CR resulted in global leukopenia in both rodents and humans, which could be reversed following return to ‘normal,’ or AL feeding. We further show that, after a return to AL feeding these cells exhibited rapid proliferation driven by homeostatic and peripheral factors, without a significant input from the thymus. We discuss the results considering potential modulation of the functional ability of the immune system during aging.

Results

Caloric Restriction in Humans Results in Blood Leukopenia

We analyzed multiple health parameters of healthy volunteers practicing long-term CR with adequate nutrition for an average of 10 years (range: 3-20 yrs) and age-matched sedentary individuals consuming typical Western diets (WD)

[304]. Weight, BMI, and body fat percentages of people on CR versus WD were significantly lower in both men and women. We also measured multiple subsets of leukocytes by complete blood count (CBC) test and found that men and women practicing long-term CR have a decrease in total leukocyte count when compared to WD control subjects. These observations were then broken down to analyze neutrophils, lymphocytes, and monocytes and all subsets were significantly reduced in comparison to the WD cohort (Table 2). From these data we conclude that CR drives a reduction in total leukocyte cellularity, either by sequestration in tissues or a contraction/elimination during periods of reduced calories. As these questions cannot be ethically answered in human CR, we employed a mouse model of CR in which we induced, in a step-wise manner, a caloric deficit in 10-month-old C57BL/6 mice for approximately two-months and immediately returned mice to AL feeding for an additional two months.

Table 2. Characteristics of Human Calorie Restriction vs Western Diet Subjects

		CR Group (n = 34)	WD Group (n = 58)	P value
Age		52.7 ± 12	54.5 ± 7	ns
Sex (M/F)		29/5	41/27	
Height (m)		1.74 ± 0.1	1.73 ± 0.1	ns
Weight (kg)		57.7 ± 7.0	79.0 ± 12.1	0.0001
BMI (kg/m^2)	Men	19.0 ± 1.2	26.1 ± 2.6	0.0001
	Women	19.0 ± 2.0	26.3 ± 2.8	0.0001
Body fat (%)	Men	9.2 ± 3.1	23.9 ± 4.2	0.0001

	Women	20.5 ± 7.5	38.9 ± 5.6	0.0001
Lean mass (kg)	Men	51.9 ± 5.6	59.7 ± 8.5	0.001
	Women	35.6 ± 1.8	40.8 ± 3.6	0.001
WBC (K/cumm)	Men	3.1 ± 0.8	5.7 ± 1.5	0.0001
	Women	3.1 ± 0.3	6.2 ± 1.5	0.0001
Absolute neutrophil (K/cumm)	Men	1.7 ± 0.5	3.3 ± 1.2	0.0001
	Women	1.9 ± 0.4	3.7 ± 1.1	0.0001
Absolute lymphocyte (K/cumm)	Men	1 ± 0.2	1.7 ± .04	0.0001
	Women	0.9 ± 0.2	2.0 ± 0.5	0.0001
Absolute monocyte (K/cumm)	Men	0.27 ± 0.1	0.45 ± 0.1	0.0001
	Women	0.28 ± 0.1	0.44 ± 0.1	0.014
Values are means ± SD				

Caloric Restriction Results in Weight Loss that is Rapidly Reversed

To address the above questions in mice, we elected to initiate CR at 10 months of age. This age was chosen as it is analogous to mid adulthood in humans, based on studies showing that the effects of calorie restriction are most beneficial when initiated in early to middle adulthood [299]. Reduction in caloric intake resulted in weight loss. As our model of acute calorie restriction has not been fully characterized in mid-adulthood mice, we first sought to determine the consequences of restriction on the weight profiles of mice during and after the reduction of calories. Animals were weighed prior to feeding, at least twice a week during induction, maintenance, and cessation of CR. To calorically restrict

mice, AL food was replaced by 3-gram pellets of irradiated NIH-31 chow and administered to mice once a day. Total food intake was reduced by 40% of ad libitum diet over three weeks, which in our hands is equal to a total reduction of 1.4 grams of food. CR diet resulted in a steady, gradual, and modest daily loss of weight to no greater than 20% of starting weight, that was immediately, within one day, reversible following return to AL food access (Figure 18) [305,306].

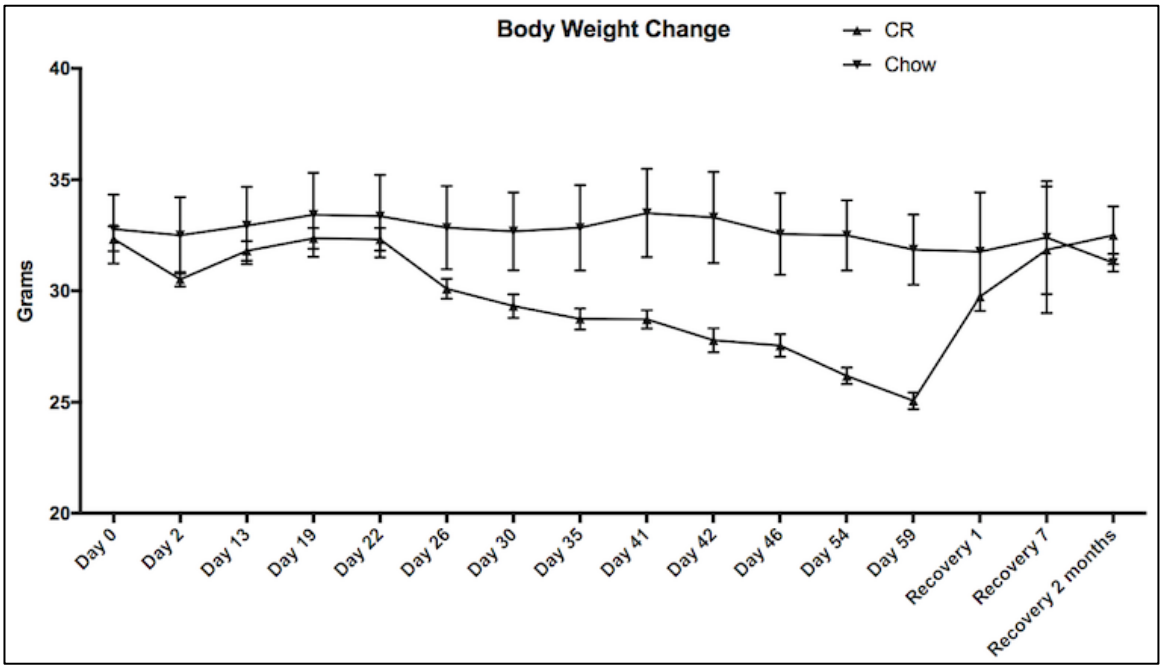

Figure 18. CR treatment results in gradual weight loss that is rapidly reversible

Two Months of Calorie Restriction Result in Lymphopenia and Lymphocyte Redistribution Across Multiple Lymphoid Organs

To determine how CR affects the distribution of cells of the immune system, we compared the effect of our two-month bout of CR to age-matched littermate AL control male C57BL/6 mice. We sought to address the basis of leukopenia observed in CR human cohort. Our primary hypothesis was that CR

induces a redistribution of leukocytes within the host, resulting in lower number of leukocytes within lymphoid tissues. We further hypothesized that this lost cellularity is reversible following return to adequate nutrition. We specifically investigated changes in the lymphocyte compartments; B cells, Natural Killer (NK) cells, and T cells, as well as of T cell subsets (Naïve and Memory, as defined by CD44 and CD62L expression), of both CR and AL fed mice across the

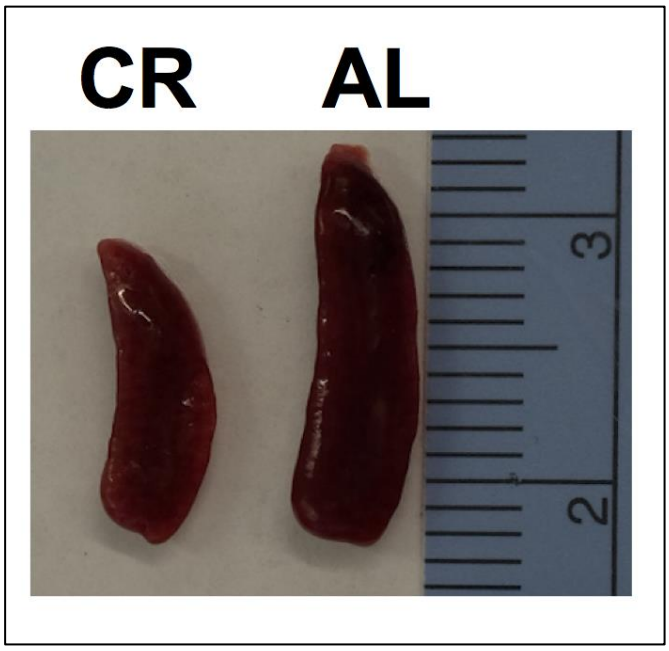

Figure 19. Splenic size decreases during CR

blood, bone marrow, spleen, and brachial and inguinal lymph nodes. We found that CR resulted in a statistically significant decrease of all three of these lymphocyte subsets across the spleen (CR mice all had spleens that were visibly smaller when compared to their AL fed counter parts (Figure 19)), blood, and pooled brachial and inguinal lymph nodes (Fig 20A-C).

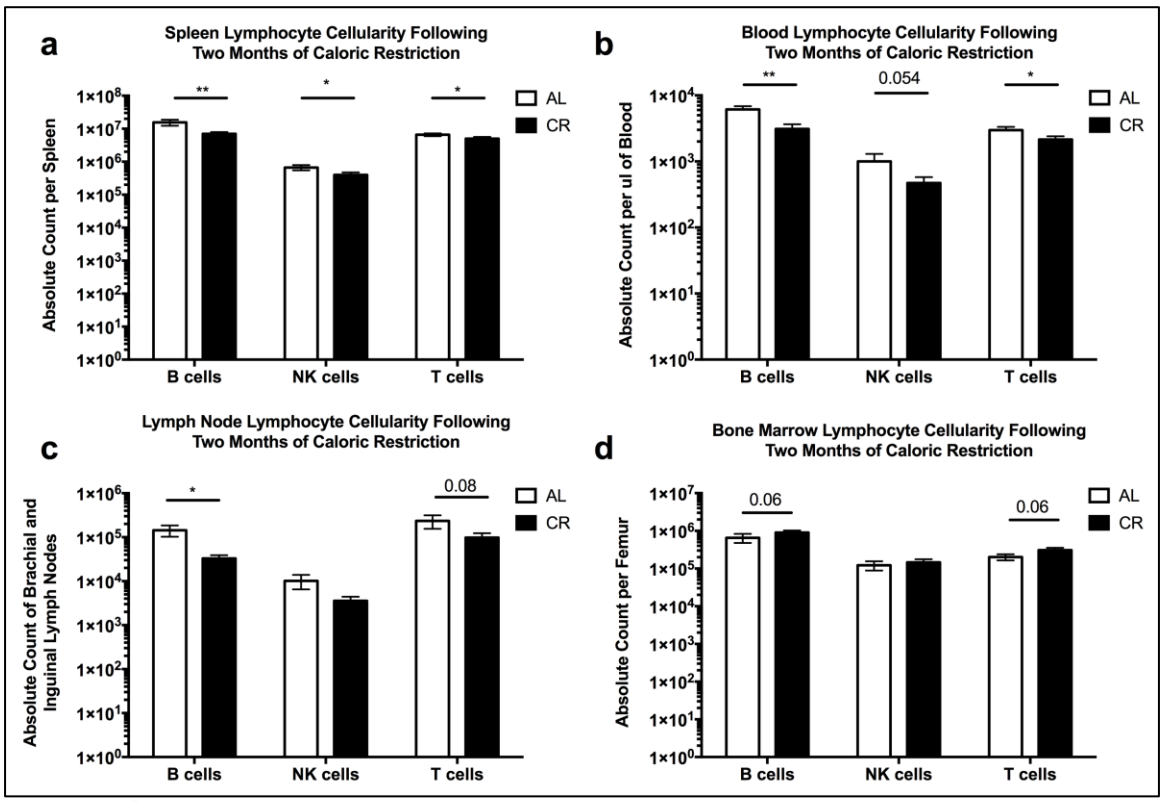

Figure 20. Lymphoid organ cellularity is decreased after caloric restriction.

Ten-month-old B6 mice were calorically restricted, as described in the “Methods,” for 2 months and lymphocyte subsets were analyzed by flow cytometry from the a spleen, b blood, c brachial and inguinal lymph nodes, and d bone marrow of the femur. Statistical differences were calculated by one-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. *P < 0.05; **P < 0.01. Data are representative of three independent experiments with 2–8 mice per treatment group

By contrast, we observed a trend of an increase in the B cells and T cells within the bone marrow of CR mice when compared to AL controls (Fig 1D), although that trend did not reach significance with the group size used in our experiments. Thus, in line with our hypothesis and modeling the results from humans, these data suggest that the induction of CR over a period of two months results in an alteration of the cellularity of multiple lymphoid tissues. The data also confirm, in our hands, that lymphoid organ size is affected by the nutritional state of an animal, as previously demonstrated by others [307–309].

Thymic Size and Cellularity is Reduced Following Two Months of Calorie Restriction

Lifelong CR has been shown to improve cellularity of the old thymus and to reduce its accumulation of adipocytes [23]. Moreover, thymic output was improved and the peripheral T cell compartment contained a higher frequency of naïve T cells in lifelong CR mice compared to AL controls [23]. Therefore, we wondered whether a two-month period of CR would induce similar changes in the thymus as seen in the lifelong model. We found significant decreases in the absolute numbers of CD4+CD8+ double positive, CD4+ and CD8+ single positive, and double negative (DN) thymocytes within the thymi of CR mice in comparison to AL fed mice (Fig 21A). Furthermore, the main subsets of DN thymocytes (DN1-4, as delineated by the expression of CD25 and CD44 [310,311]) were also universally decreased in comparison to AL fed controls (Fig 21B) and the size of the thymus of CR mice was visibly smaller than that of AL

counterparts (Figure 22). These data demonstrate that a short period of CR results in a loss of both size and cellularity of the thymus. The lost cellularity of the thymus is seen in all subsets of the αβ T lymphocyte lineage analyzed.

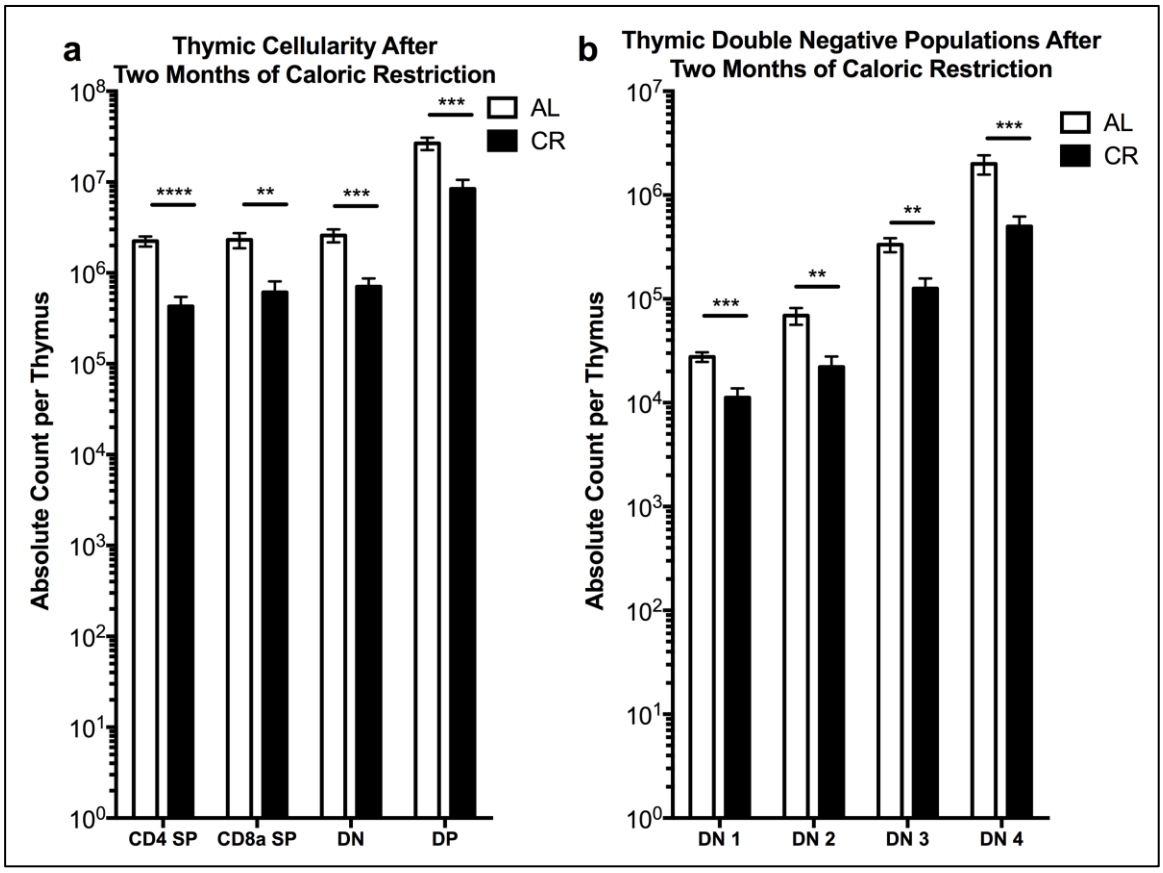

Figure 21. Thymic cellularity is decreased after caloric restriction.

Ten-month-old B6 mice treated by CR as in Fig. 1 were analyzed for thymus lymphocyte subset cellularity by flow cytometry. a Absolute counts of CD4 single positive, CD8 single positive, double negative, and CD4/CD8 double positive thymocytes. b Absolute count of CD4/CD8 double negative thymocyte subsets (DN1-4), based on CD44 and CD25 expression. Statistical differences were calculated by one-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are representative of three independent experiments with 2–8 mice per treatment group

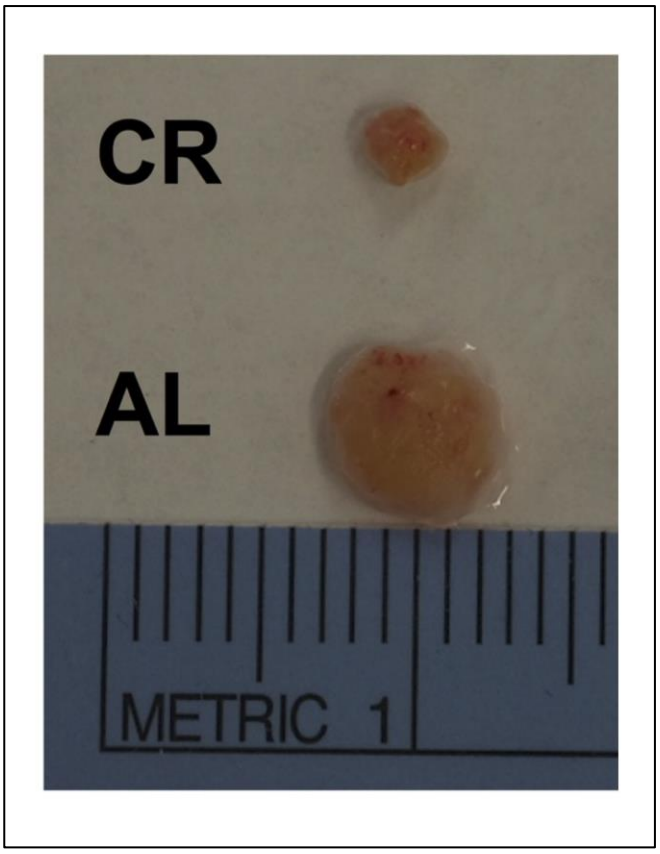

Figure 22. Thymic size decreases during CR

Return to Ad Libitum Feeding Results in Increased Blood Cellularity and T cell cycling

We next wished to determine whether the cells lost from blood and lymphoid organs were capable of rebounding back to baseline levels following a return to AL feeding. To that effect, following the completion of the two-month period of CR we returned mice to AL food. At the same time, mice were given a thymidine analog, BrdU in drinking water, to label dividing cells and thereby estimate their turnover. Following one week of refeeding and BrdU treatment, we determined the fraction of cells in the blood that had incorporated BrdU and also expressed the cell cycle marker Ki67 (actively cycling cells within the last 2-3 days of analysis) as in our prior studies [52]. After one week of CR reversal with AL refeeding (CR-RF), both CD4 and CD8 T cells in the blood rebounded to or above control levels (Fig 23A). Furthermore, both the CD4 and CD8 T cell compartments contained significantly more cells that were double positive for BrdU and Ki67 relative to the AL controls that did not undergo CR (Fig 23B). These data suggest that when mice are returned to AL feeding after CR, cells enter into cell cycle and undergo a proliferative burst as indicated by the increased number of T cells in the blood in CR-RF mice when compared to AL counterparts. To evaluate potential mechanisms of this rebound, we analyzed CD8 T cell subsets (effector memory, central memory, and naïve; based on expression of CD62L and CD44) and found that CD8 central memory (CD62L+CD44+) and naïve (CD62L+CD44-) T cells contained significantly more actively cycling (Ki67+BrdU+) cells following refeeding relative to AL controls (Fig

23C). These data are supportive of the hypothesis that during CR T lymphocyte populations contract, redistribute, and then following return to AL nutrition, undergo homeostatic proliferation, presumably to fill the void left by redistributing cells in response to homeostatic cytokines. Since CD8 naïve and central memory cells, but not CD8 effector memory cells, proliferated, we would speculate that this proliferation is most likely to be driven by IL-7 and not IL-15.

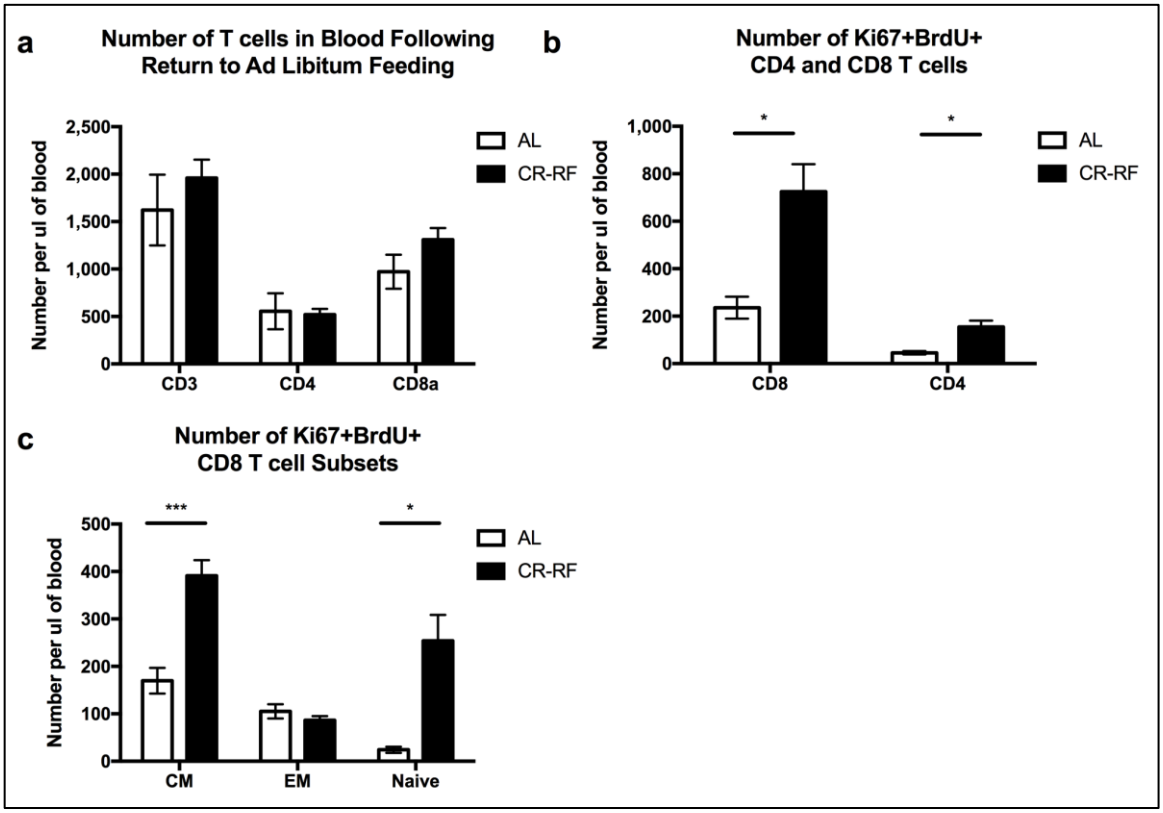

Figure 23. Peripheral T lymphocytes enter cell cycle after 1 week of refeeding.

Calorically restricted mice were returned to AL food access and treated with BrdU in their drinking water. The T cell compartment was analyzed by flow cytometry. a Absolute count of T cells per µl of blood. b Absolute count of Ki67+BrdU+ CD8a and c CD4 T cells. d–f Absolute count of Ki67+BrdU+ central memory (CD44+CD62L+), naïve (CD44−CD62L+), and effector memory (CD44+CD62L−) cells in blood. Statistical differences were calculated by two-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. *$P < 0.05$. Data are representative of two independent experiments with 3–7 mice per treatment group

Lymphoid tissue cellularity returns to AL levels 2 months following return to AL feeding

During the early time point after return to AL feeding, T cells appeared to have entered cell cycle based upon BrdU incorporation and Ki67 expression. This observation led us to ask whether organ size and cellularity lost during the period of CR return to control sizes? Mice that were reverted to AL feeding were maintained on the AL diet for 2 months and once again, we analyzed lymphocyte subsets within the blood, bone marrow, spleen, and brachial and inguinal lymph nodes. We found that absolute cell counts of B, NK, and T cells were not statistically different across tissues between AL and CR-RF animals (Fig. 24). Similar results were obtained with thymus cellularity after 2 months of refeeding. We found that CD4 single positive, double negative, and double positive thymocytes exhibited an absolute increase within the CR-RF groups when compared to AL controls (Fig. 25A). When the double negative population was analyzed further, we found a statistically significant increase in the DN1 and DN2 populations (Fig. 25B). However, we found no statistically significant differences between the DN3 and DN4 populations in CR-RF and AL mice. Therefore, following contraction in both size and cellularity during CR, lymphoid organ cellularity equilibrated at pre-intervention steady state following 2 months of AL feeding.

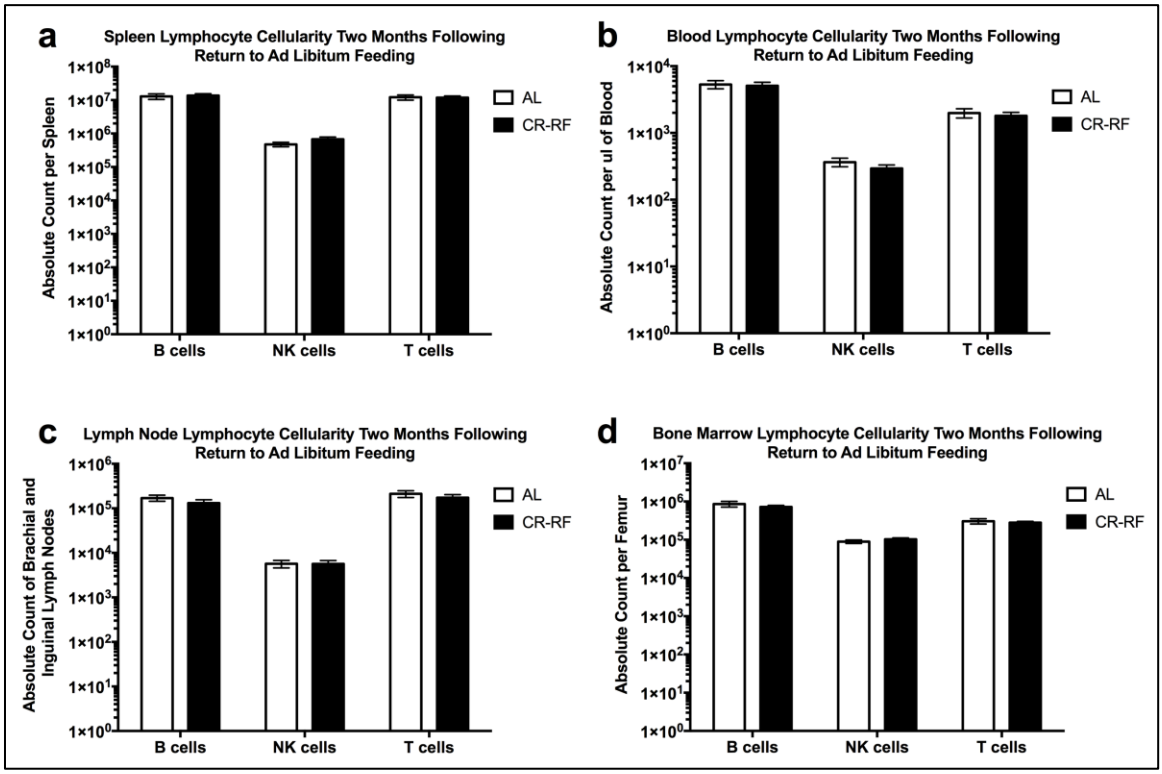

Figure 24. Lymphoid organ cellularity returns to AL levels following 2 months of refeeding.

Calorically restricted mice were maintained on AL food for 2 months after CR treatment. Lymphocyte subsets were then analyzed by flow cytometry from
the a spleen, b blood, cbrachial and inguinal lymph nodes, and d bone marrow of the femur. Statistical differences were calculated by two-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. We found no statistical difference in numbers between the groups. Data are representative of three independent experiments with 2–8 mice per treatment group

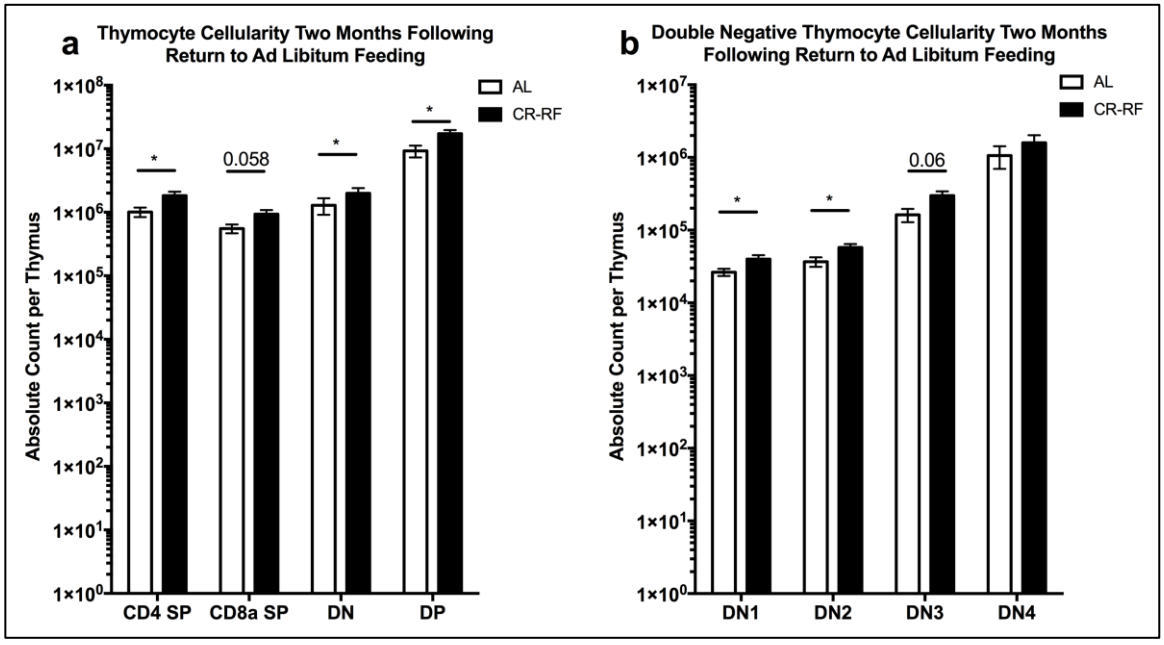

Figure 25. Thymic cellularity returns to AL levels following 2 months of refeeding.

Calorically restricted mice were maintained on AL food for 2 months after CR treatment. Thymus subsets were analyzed by flow cytometry. a Absolute counts of CD4 single positive, CD8 single positive, double negative, and CD4/CD8 double positive thymocytes. b Absolute count of CD4/CD8 double negative thymocyte subsets, based on CD44 and CD25 expression. Statistical differences were calculated by two-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. *P < 0.05. Data are representative of three independent experiments with 2–8 mice per treatment group

Thymus is dispensable for the refeeding-triggered T cell rebound after CR

Our results were consistent with the possibility that during a relatively short period of CR, lymphocytes may leave secondary lymphoid tissues, and that at least some of them, may reside in the bone marrow, so that upon refeeding they can repopulate the same lymphoid tissues to re-establish homeostasis. However, because thymus also rebounds post-refeeding, it was possible that some or even all of the rebounding cells were originating from the regenerated thymus. To formally and stringently test this possibility we performed adult thymectomy (ATX) on 3-month-old C57BL/6 mice and then aged them to 10 months of age before inducing our model of CR and compared results to sham-thymectomized (Sh-TX) littermates.

ATX mice exhibited weight loss changes in response to CR induction and return to AL feeding indistinguishable from either their sham controls or surgically intact animals from our initial experiments. Furthermore, following two-months of CR, ATX mice displayed similar losses of lymphocytes across multiple tissues. We observed a significant decrease in B cells and NK cells in the spleen, whereas T cell data trended lower but did not reach significance (Fig 26A). In the blood, B cells were significantly reduced, but NK and T cells were not (Fig 26B). Brachial and inguinal lymph nodes displayed the most significantly decreased number of B, T, and NK cells (Fig 26C). As in the case of thymus-sufficient wild-type mice there was no significant changes in the cellularity of B, T, or NK cells in the bone marrow (Fig 26D). Therefore, from these data we conclude that loss of

the thymus does not alter CR induced lymphopenia across the spleen and lymph nodes.

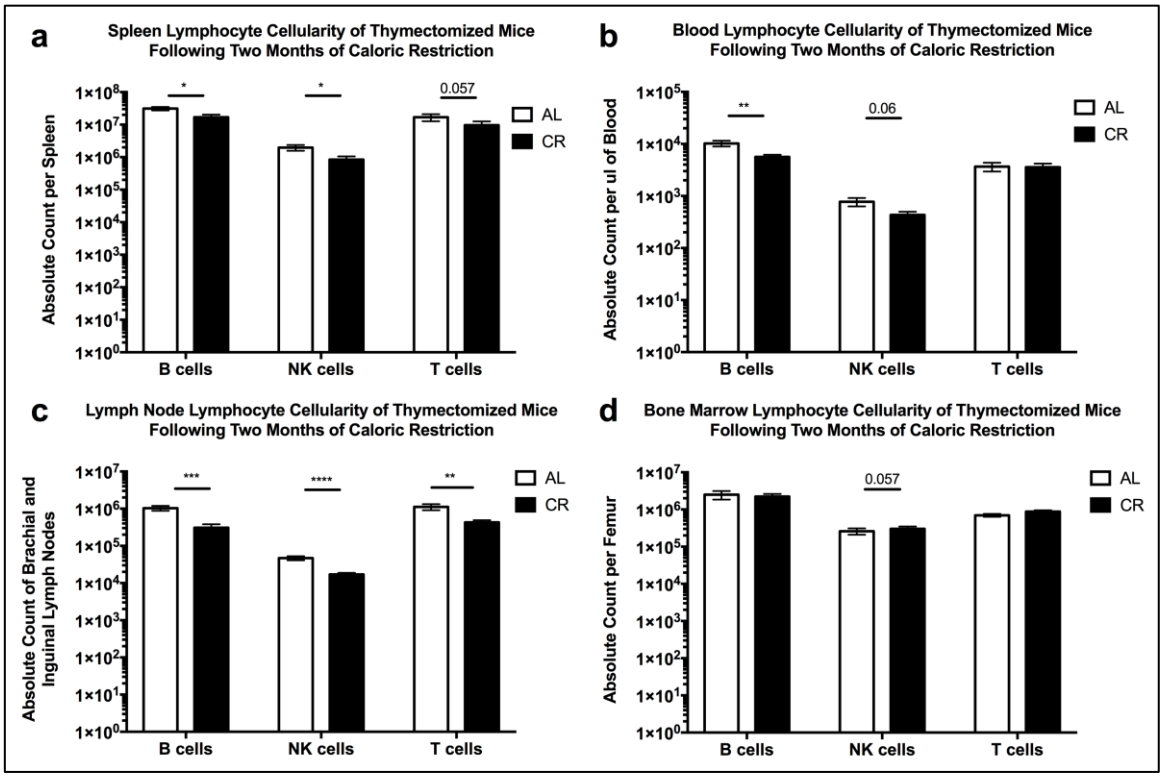

Figure 26. Thymectomized mice display CR-induced lymphopenia.

B6 mice were thymectomized in house or at the Jackson Laboratory (Bar Harbor, ME) and then subjected to 2 months of CR and lymphocyte subsets were analyzed by flow cytometry from the a spleen, b blood, c brachial and inguinal lymph nodes, and d bone marrow of the femur. Statistical differences were calculated by one-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Data are representative of two independent experiments with 4–8 mice per treatment group

We next reverted mice back to AL food. Two months post refeeding of ATX animals, we observed that B, NK, and T lymphocyte cellularity across the blood, spleen, lymph nodes, and bone marrow rebounded to pre-CR levels and was indistinguishable from the AL fed ATX controls (Fig 27). These data demonstrate that T cells return to the organs and restoration of T cell homeostasis is reestablished by two months post refeeding regardless of the

presence of the thymus. We interpret these data to suggest that T cells contract and/or redistribute to bone marrow and/or other tissues, not surveyed by our study, during periods of a caloric deficit, and ‘come out of hiding’ when nutritional supply is reestablished, independent from central (thymic) production. It remains to be seen to what extent homeostatic proliferation following refeeding is necessary for full restoration of homeostasis in this model of CR and refeeding.

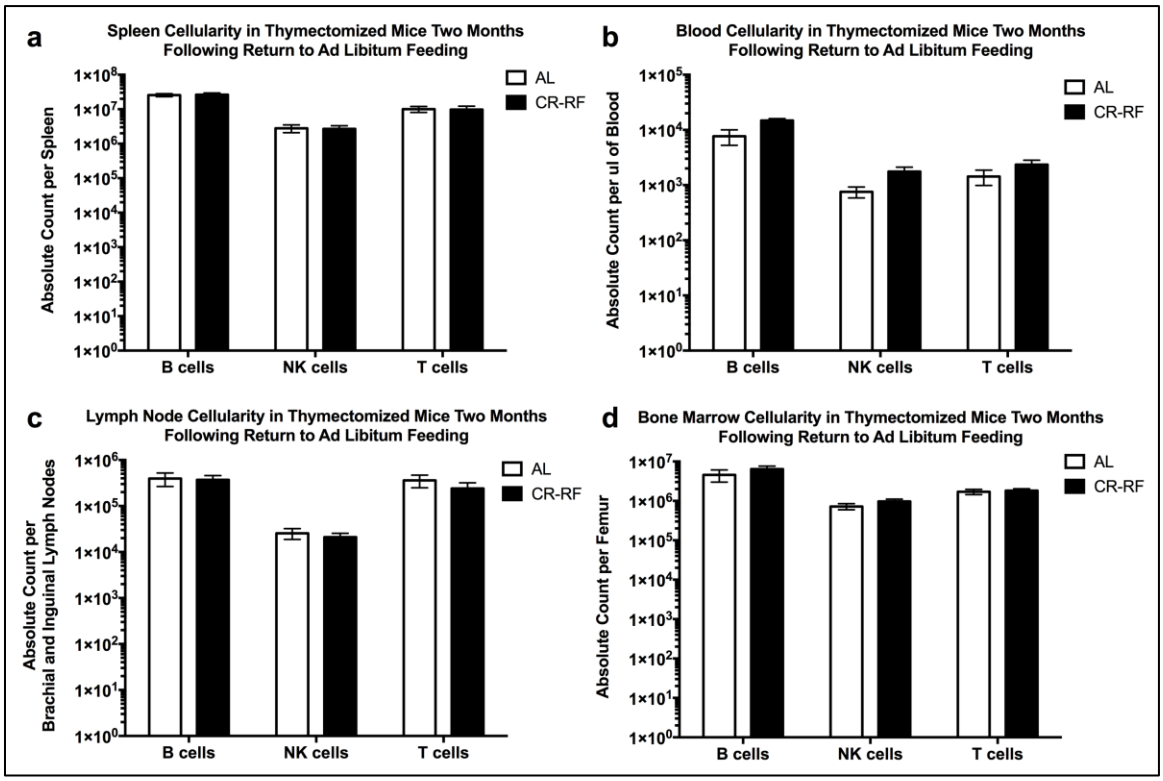

Figure 27. Lymphocyte recovery is not dependent upon the thymus.

Calorically restricted thymectomized mice treated as in Fig. 6 (return to AL food for 2 months after CR) were analyzed exactly as in Fig. 6. a Spleen. b Blood. c Brachial and inguinal lymph nodes. dBone marrow of the femur. Statistical differences were calculated by two-tailed Mann Whitney U tests by comparing cellularity to age-matched, AL fed mice. Data are representative of two independent experiments with 4–8 mice per treatment group

Discussion

In this study, we report that individuals practicing long-term CR with adequate nutrition have significant lower circulating levels of total leukocytes, neutrophils, lymphocytes and monocytes. In middle-aged 10-mo old mice, we found that during periods of caloric restriction the absolute number of lymphocytes within the blood, inguinal and brachial lymph nodes, and spleen are reduced in comparison to AL fed counterparts. By contrast, the bone marrow of CR animals appeared to be a site of influx, or a “safe haven” for B, NK, and T cells, the numbers of which were at least intact, if not elevated in bone marrow. Lymphocyte cellularity within all organs returned to AL levels as early as a week following refeeding. Repopulating T cells exhibited high proliferation in the CR-RF group, based on BrdU incorporation and Ki67 expression. Finally, we demonstrated that thymus was not essential for T cell repopulation following re-feeding.

Based on the above observations, we propose the following model of lymphocyte migration following CR and subsequent re-feeding: (1) Upon CR induction, lymphocytes leave secondary lymphoid organs (SLO) and migrate into niches such as the bone marrow, that are known to protect and maintain different lymphocyte subsets (dominantly memory T and B cells) in the course of normal homeostasis. (2) Thymus also drastically loses cellularity, because early steps in the $\alpha\beta$ T cell development (proliferation at the DN2 and DN3b/4 stages) require enormous energy expenditure. (3) Following refeeding, there is almost instantaneous redistribution of cells back to SLO from the bone marrow and perhaps other niches. (4) In addition to this redistribution, cells arriving into SLO

undergo homeostatic proliferation, presumably because the SLO environment has a relative surplus of homeostatic cytokines, chiefly IL-7, that were not used in situ due to CR-induced lymphopenia. (5) Thymus cellularity also rebounds, but thymic production of new T cells is not essential to the repopulation of SLO.

What mechanisms may be operating behind lymphocyte redistribution in the course of different phases of CR-RF? Emigration out of LN is typically mandated by downregulation of S1P1 receptor on lymphocytes, and is usually associated with acquisition of chemokine receptors and integrins such as CXCR3, CCR5 and VLA-4 [312] that target T cells to inflamed tissues. While micronutrients, and vitamins A and D, have been shown to be involved in programming of lymphocytes for residence in different tissues (gut vs. skin, etc.), so far there are no information on how a paucity of calories would influence lymphocyte migration. However, it is likely that the same mechanism is operating in the reversal of the initial redistribution and the release of lymphocytes back to the SLO, and for both of these phases, GLUT-1 or other glucose transporters, and/or amino acid transporters (like CD98) could very well be involved. An alternative, and not mutually exclusive, mechanism would have it that upon refeeding, SLO stromal elements [313] might secrete chemokines to reattract lymphocytes to SLO. Studies are currently in progress in our laboratory to discriminate between these possibilities.

An obvious question of interest in this model relates to the functional consequences of CR-induced lymphopenia on immune defense. We and others have shown that systemic sepsis [300], epithelial [301] and cutaneous [17]

infections all produce substantially higher mortality in old mice on lifelong CR. Less is known about vulnerability of mid-life animals in the course of CR induction, or for that matter, animals that are not held within specific pathogen free environments [46]. Certainly, hypocellular LN would delay initial priming and expansion of effector lymphocytes and that would have the potential to adversely impact protective immunity. This issue deserves urgent experimental attention and currently we are investigating the role of broader antigen experience in functional immunity during aging.

The power of CR in extending lifespan has been shown in many models over nearly a century. However, difficult compliance regimens, reports of uneven effects of CR in different genetic backgrounds [314] as well as reports of potential adverse effects of CR upon certain organ systems in certain strains of mice, including the immune system have all spurred intense search for other modalities of nutritional manipulation, including intermittent dieting [38,216,315–317]. In light of these efforts and our results above, it will be important to understand the impact of these manipulations on functional immunity, to validate appropriate modalities of CR, and to be able to properly manage induction phases of any dietary regimens that may adversely impact functional immunity.

Methods

Humans

Thirty-four individuals had been on CR for an average of 10 years (3-20 years). Subjects were instructed by an experienced research dietician to record all food and beverages consumed, preparation methods, and approximate portion sizes for 7 consecutive days. Food records were analyzed by using the NDS-R program (version 4.03_31), which is the Nutrition Data System for research from the Nutrition Coordinating Center at the University of Minnesota. CR subjects consumed a variety of nutrient-dense unprocessed foods (i.e. vegetables, fruits, nuts, egg whites, fish, poultry, low-fat dairy products, whole grains and beans) which supplied > 100% of the Recommended Daily Intake for all essential nutrients. Refined foods rich in empty calories, and trans-fatty acids were avoided. Energy intake was 30% lower in the CR group (1790±225 kcal/d) than in the Western diet (WD) group (n=58) (2540±358 kcal/d) ($p \leq 0.0001$). The percentage of total energy intake derived from protein, carbohydrate, fat and alcohol was 22%, 51%, 27% and 0.2%, respectively in the CR group and 17%, 48%, 34% and 1% in the WD group. Height was measured without shoes to the nearest 0.1 cm. Body weight and venous blood samples were taken after subjects had fasted for at least 12 hours. Total body fat mass and fat free mass were determined by dual-energy X-ray absorptiometry (DXA) (QDR 4500, Hologic, Waltham, MA). The human study was approved by the Human Studies Committee of Washington University School of Medicine, and all participants gave informed consent before their participation.

Mice

All mice were C57BL/6 and were purchased from Jackson Laboratories and held under specific pathogen-free conditions in the animal facility at the University of Arizona (UA) for life. All experiments were conducted by guidelines set by the UA Institutional Animal Care and Use Committee. Thymectomies were carried out in-house (cohort 1) or at the Jackson Laboratories (cohort 2), with superimposable results between the cohorts. Upon necropsy, we validated lack of thymus in all ATX animals.

Calorie Restriction

Mice were introduced to caloric restriction by first switching to the NIH-31 fortified formula in the form of 3-gram pellets. In a stepwise manner mouse were acclimated to two pellets per day to allow for acclimation to the change in food access (6 grams/mouse), followed by a second- and third-week reduction to 4.5 g/mouse and 3 g respectively. Ultimately, mice were reduced to 2.4 g a day, resulting in approximately a 40% reduction of food intake (in our hands mice averaged ~3.5 g of ad libitum chow a day) that translated to approximately 20% weight loss. This diet was maintained until approximately 20% weight loss, over a period of two months.

Leukocyte Isolation

At the end of study or at selected time points, mice were euthanized by isofluorane. Spleens, brachial and inguinal lymph nodes, and thymi were collected into ice cold RPMI and accutase treated for 30 minutes at 37 C and mechanically disassociated through 40 um nylon filters and re-suspended in RPMI supplemented with 5% fetal bovine serum (FBS). Bone marrow was obtained from femur, tibia, and fibula, by clipping the ends of the bones and pushing PBS through to dislodge marrow. Marrow was then pushed through a 40 um nylon filter with a rubber plunger of a 3 cc syringe? and re-suspended in RPMI supplemented with 5% FBS. Blood was obtained by retro-orbital bleeding from living, anesthesia-free mice. Red blood cells were hypotonically lysed. Isolated leukocytes were then quantified on a Hemavet 950 (Drew Scientific, US) and put into 96-well plates for flow cytometric staining.

Flow Cytometry (FCM)

Prior to each collection, voltages were manually calibrated to a common template using Rainbow Beads (BD Biosciences, San Jose, CA, USA), to insure accurate MFI tracking over time. Cells were treated with antibody for CD16/CD32 (BD Biosciences) and then against antibodies for CD3 (17A2), CD4 (RM4-5), CD8a (53-6.7), CD19 (6D5), Mouse I-1/I-E (M5/114.15.2), Ki67 (16A8), CD49b (DX5), CD25 (PC61) (BioLegend, US), NK1.1 (PK136), CD44 (IM7), CD62L (MEL-14) (ThermoFisher, US). Staining occurred at 4C followed by fixation and permeabilization (BD Cytofix). Fluorescence minus one (FMO) controls were

conducted to determine gating schema. Samples were run on a Fortessa Flow Cytometer equipped with four lasers and using DiVa software (BD Biosciences). Compensation and analysis were performed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistics

Statistics were performed in Prism 7.0 (GraphPad Software, La Jolla, CA, USA). During experiments analyzing the effects of CR one-tailed Mann Whitney U tests with equal SD were carried out between cell types of CR and AL groups based upon the hypothesis that CR groups would have lower values compared to AL counter parts. Two-tailed Mann Whitney U tests with equal SD were carried out during refeeding experiments. Significance is noted as follows throughout: ns = not significant, ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05. All error bars shown are SEM.

CHAPTER 6. DISCUSSION AND CONCLUSIONS

“The more I read, the more I acquire, the more certain I am that I know nothing.” – Voltaire

The purpose of this dissertation was to frame two studies regarding nutritional intervention and chronic infection as environmental pressures that have great impact on lifelong immunity. Optimal nutritional manipulation at the appropriate age windows have the potential to improve host immune function, health span, and longevity. Alternatively, immunological memory and exposure to infectious pathogens and the presence, or lack of, commensal species, can establish a beneficial level of inflammation and subsequent immune protection or chronic inflammation resulting in disease.

Environmental pressure has been used as an all-encompassing term to describe any non-germline encoded influence on the host. These pressures, as discussed, can be derived from factors that include, but are not limited to, infection, diet, the host microbiota, and the viral metagenome. The data presented in Chapters 4 and 5 explored the separate effects of CR diet and lifelong infection upon host immunity. Specifically, periods of CR induced leukocyte redistribution and reorganization and lifelong CMV infection results in a maintained inflammatory response in adipose that could dysregulate glucose metabolism. Here, will be speculated possible reasons why these phenotypes

occur and experiments that may provide deeper insight to mechanisms driving them.

Why Adipose?

A significant contribution of this dissertation is the identification of adipose tissue as a potential viral reservoir for the maintenance of mCMV genome during lifelong infection. Viral persistence has previously been established predominantly within the salivary glands and lungs [67]. From the cellular standpoint, the virus is believed to persist in hematopoietic progenitors, myeloid cells in the bone marrow (and their progeny), as well as in endothelial cells [233]. Considering the data presented in Chapter 4 of this thesis, several questions require consideration. Why is adipose tissue infected by CMV? Why does CMV infection (detectable or undetectable) result in accumulation of viral specific T cells? Why is hyperglycemia associated with this inflammatory status and is it potentially harmful?

The salivary gland and lungs seemingly offer obvious advantages to the virus, because of their clear potential to mediate viral spreading to new hosts via transmission through with bodily fluids. However, adipose tissue does not seem positioned to provide this same benefit. So why would adipose be infected? Two possibilities arise to explain the presence of virus, and therefore infection, in adipose. First, CMV in the adipose tissue could be the result of trafficking of infected monocytes into the tissue, with the viral load effectively controlled by CD8 T cells, and then infection reestablished by a new wave of infected

monocytes from peripheral tissues by continuous trafficking. mCMV genome was detected in the acute (3d p.i.; Figure 2) and chronic (10 months p.i.; Figure 10) following systemic (i.p.) infection. This would suggest that the maintenance of T cells in adipose tissue is dependent upon bona fide infection and not acting as a generalized sink for pathogen specific T cells after antigenic clearance as demonstrated in the salivary gland for mCMV [76] and for mesenteric adipose tissue for pathogens such as Toxoplasma gondii and Y. pseudotuberculosis [275]. Detection of viral genomes in mice 10 months post infection could potentially be due to infected monocytes trafficking into adipose tissue (outside-in model). Monocytes are called to sites of primary infection and reactivation through the usurpation of inflammatory responses. MCK2 derived from CMV and inflammatory molecules generated during an immune response attract monocytes, providing basis for an internal spread mechanism [265,318]. In addition, monocytes continually seed adipose tissue for the purposes of tissue remodeling under normal conditions [319]. Coupled together, these data raise the possibility that adipose tissue infection is a byproduct, but ultimately a desirable outcome for the virus, during the CMV infectious lifecycle and latency in myeloid precursors. Against this possibility, however, are the data from the infection of CCR2-KO mice (Figure 9). These mice lack circulating monocytes that would ultimately respond to MCK2 and to inflammatory cues generated by mCMV infection in the periphery. Because we have seen clear evidence for CMV-specific T cell accumulation in this model, this would argue that infection of adipose tissue is not a result of continued monocyte seeding. However, during

these experiments CMV was undetectable within bulk adipose tissue. This could be due to low, below the detection level, of true infection of the adipose tissue, an explanation we favor over the possibility that CMV-specific CD8 T cells randomly distributed to the adipose tissue and stayed there.

As an alternative explanation, to the outside-in model, it is possible that resident adipose tissue cells, such as resident leukocytes, pre-adipocytes, mesenchymal stem cells, vascular endothelial cells, and/or others are, in fact, bona fide viral reservoirs that maintain viral genome and contribute to reinfection of peripheral tissues in the host (inside-out model). Adipose tissue has inherent characteristics that would seem to provide support of the inside-out model during CMV infection. First, adipose tissue is enriched with progenitor cell populations [320]. Pre-adipocytes and adipose derived stem cells have the potential to provide the preferred progenitor type cells that maintain HCMV genomes [103]. Furthermore, CMV alters lipids in infected cells in order to produce progeny [278,321] and adipocytes are the primary lipid storing cell in the host, which could provide a benefit to infection. As discussed above mice lacking CCR2 had a maintained mCMV-specific T cell population during acute and memory time points post infection. This would suggest that, although it needs to be more stringently tested, that viral antigen was able to enter adipose tissue at some time via an alternative spread mechanism (possibly neutrophils or cell free virus). Furthermore, data from Figure 10 suggest that the mCMV genome is maintained at a higher level in CD45- compartment of adipose tissue when compared to CD45+ cells. That non-hematopoietic cells in the adipose (pre-adipocytes, stem

cells, endothelial cells, etc.) are responsible for maintaining virus would be in line with current understanding of CMV latency [277,322,323]. As these cells are truly resident to adipose tissue, this would suggest that virus is maintained in situ and potentially released upon differentiation of progenitor cells within adipose, as seen in CD14 and CD34 differentiation in tissues. The above data seemingly favor the inside-out model of viral maintenance, possibly suggesting that latently infected CD14 and CD34 monocytes in the periphery and bone marrow are infected as a result of adipose tissue viral release.

A simple experiment to test these hypotheses would be characterization of flow sorted cell populations at different time points (24 hours, 3 days, 7 days, 30+ days post infection) and determine which cells are carrying CMV genome through PCR [324]. However, this is time intensive and dependent upon very stringent isolation of cell populations. Alternatively, the use of an mCMV expressing Cre recombinase under the control of IE1, which has recently been developed (unpublished data), could reveal spread kinetics and cell dynamics. Using this virus and infecting Ai9 mice, which have tdTomato floxed into the ubiquitous ROSA locus [325], it would be possible to trace infected cells in a longitudinal fashion by flow cytometry and pinpoint what specific cells are infected at a given time. These characterization experiments will undoubtedly be affected by route of infection and therefore require multiple experiments exploring differences in route of infection [67,326–329]. Comparisons of olfactory, i.p., breast milk, salivary//oral and in utero infection will be necessary to fully understand the consequences of natural infection and if the phenotypes

described in Chapter 4 hold across all routes, given reported differences on viral load and cellular dissemination dynamics in vivo.

The above experiments will establish what cells are infected through the various routes of infection. The next logical step would be to determine the contributions of individual cell populations to the maintenance of T cells in adipose and the manifestation of hyperglycemia. There are currently no good models for depleting adipose tissue specific cells. Compounding this gap in technical ability is antibodies that do not seem to penetrate adipose tissue, as demonstrated by intravascular staining, ruling out antibody depletion. Knockouts or knockdowns of adipose tissue constituents could be used, but these may alter metabolic development and host health, making it hard to delineate mCMV-specific associated alterations in glucose metabolism. One potential strategy, in the face of these concerns, would be targeted disruption of the virus instead of host cells. Utilizing cell specific Cre recombinase expression in combination with a suicide mCMV mutant (potentially by floxing replication required gL and IE1 genes; named mCMV-flox here for descriptive purposes) could constrain viral tropism and allow for pointed, cell-specific, interrogation of the phenotypes seen here.

One specific experiment utilizing mCMV-flox would be infecting a mouse that expresses Cre recombinase under the control of apelin receptor [330–333]. Apelin receptor is expressed on the vasculature in vessels that are less than 50 μm in diameter as found in skeletal muscle, white adipose tissue, and brown adipose tissue [330]. CCR2 was not required for activation and maintenance of

anti-viral CD8 T cells within adipose tissue, suggesting that circulating monocytes were not required for viral spread. It is possible that cell free virus enters adipose tissue from the circulation via infection of endothelial cells. Given the structure of adipose, the vascular endothelial cells could be a prime target for infection and spread into adipose tissue and may act as a barrier for pathogenic entry into adipose. HCMV triggers vascular growth (angiogenesis) through several mechanisms (reviewed [334]) and may provide a method of entry and viral dissemination into adipose. One potential hypothesis is that CMV infection results in activation of the vascular endothelium to promote angiogenesis to grow potential cell targets and expand further into tissues for a boost in infection. Therefore, infecting mice that express Cre recombinase driven by Apelin receptor (Apln-Cre) with mCMV-flox should prevent complete replication of mCMV in adipose tissue endothelial cells and potentially angiogenesis into adipose tissue specifically. Following infection of Apln-Cre mice, the first step would be to determine if CMV is detected non-vascular endothelial SVF cells of adipose tissue through flow-sorted PCR. The working hypothesis here is that creating a replicative dead-end for mCMV in vascular endothelial cells should prevent further angiogenesis and subsequent spread of CMV into additional SVF cells. Next, analysis of adipose tissue SVF in order to detect mCMV-specific T cells and leukocyte infiltration, both of which are expected to be absent, would reveal if viral replication in vascular endothelial is required for lymphocyte accumulation. Finally, it is possible that infection of the SVF after endothelial cells results in the production of cytokines and subsequent activation of the immune system leading

to hyperglycemia. Therefore, Alpn-Cre mice infected with mCMV-flox may not present these phenotypes. This would suggest that the vascular endothelial cells within adipose tissue are the so-called gate keepers of systemic inflammation.

A second contribution of this dissertation is the identification of a sustained CD8 T cell response in adipose tissue that could potentially link correlative studies of HCMV infection with the manifestation of cardiovascular diseases that have included high blood pressure and type 2 diabetes [335–337]. CMV infection, in our hands, was accompanied by hyperglycemia, in a time dependent manner. It remains unclear as to why hyperglycemia would occur in response to CMV infection. Anti-viral induced interferon response has been linked to decreased insulin sensitivity during acute infection, but dependent upon skeletal muscle [338]. Furthermore, ‘stress’ induced hyperglycemia has been suggested as an evolutionarily acquired strategy to improve immune effector functions [339]. Perhaps elevated fasted blood glucose levels have a simple explanation. T cells require glucose to carry out their effector functions, the presence of a chronic pathogen requires a prolonged T cell response to maintain host health, therefore the host requires elevated glucose to feed T cell reactions. In order to test if this is a physiologically conserved mechanism, a side-by-side comparison of blood glucose during other chronic herpesvirus infections or acute infections with other viruses could be carried out.

It should be noted that the C57BL/6 mouse strain is inherently Th1 skewed in comparison to BALB/c mice that are slanted towards Th2 immune responses. Moreover, the C57BL/6 strain is genetically predisposed to increase

their body weight and trunk adiposity during their lifespan, possibly predisposing them towards increased fasted blood glucose during chronic inflammation. Male mice average approximately 25 grams in adulthood and increase to 45 grams in old age with a normally distributed increase in visceral adipose tissue. These genetic factors could have potential consequences for metabolism and antiviral defense and it would be of value to carry out these experiments in different genetic backgrounds, such as mice of the Collaborative Cross [340]. The key observations of Chapter 4 suggest the hypothesis: lifelong cytomegalovirus infection elevates the risk for developing hyperglycemia but require additional stressors that could be linked to genetic background, dietary make up, and other behavior. This might, in part, explain why studies regarding HCMV infection suggest a role for gender, racial, and socioeconomic factors when analyzing CMV correlated metabolic dysregulation [341].

A third contribution of this section of the dissertation is the revelation of the role infection plays within adipose tissue. Obesity rates, and increased adiposity, in the Westernized world continue to increase along with accompanied metabolic perturbances such as type 2 diabetes and hyperglycemia. Therefore, it is important to consider what interconnectedness, if any, there is between chronic infection and adipose tissue inflammation. A few pathogens have been identified as infecting adipose tissue to date including, Chagas Disease [225], adenovirus 36 [228], and HIV [260], and several pathogens are known to induce an adipose tissue T cell response [275]. The data here suggest that CMV fall in the former category given the detection of mCMV genome in chronic time points post

infection. It is reasonable to believe that infection and memory T cell accumulation could have potential consequences on adipose tissue inflammation in conjunction with lifestyle choices. The data presented in Chapter 4 represent an advancement in our understanding of the consequences of lifelong CMV infection and chronic adipose tissue inflammation. It is very important to take caution when interpreting these data, because at this point this is neither a claim that CMV infection results in the metabolic syndrome nor that hyperglycemia associated with infection is ultimately deleterious to host health.

The metabolic syndrome is a state in which three of the following are present: high blood pressure, high blood sugar levels, excess fat around the waist, elevated triglycerides, low levels of good cholesterol [342,343]. Blood pressure was not analyzed in this study, but it is reportedly elevated in CMV infected humans [335]. Blood sugar levels were elevated in this study (Figure 15), but there was no alteration in adiposity of the mice when compared to uninfected controls (Figure 8). Triglycerides were elevated in the blood plasma of infected animals but there was no change in total cholesterol (Figure 16). These phenotypes fall short of the three required criteria to claim the onset of metabolic syndrome. Therefore, this study would benefit from more intensive metabolic assays that include an analysis of blood pressure and a determination of the types of triglycerides and cholesterol in infected plasma through mass spectrometry.

Although this study does not conclude that lifelong mCMV infection results in the manifestation of the metabolic syndrome it, at the very least, suggests an

increased risk for developing hyperglycemia, or perhaps increased lower limit of blood glucose, that is correlated with adipose tissue inflammation in response to a communicable pathogen. Abrogation of this inflammation through the experiments described above could potentially reveal the mechanistic trigger of such a phenotype, if dependent upon adipose tissue leukocyte responses.

Feeding Immunity?

CR and certain methods DR are linked to decreased inflammation and in certain cases increased health span and longevity, reduced adverse effects during cancer chemotherapy, and potential cognitive regeneration [344,345]. It is possible that these consequences are dependent upon decreased immune activity and function because of decreased peripheral quantity of leukocytes and ability to activate in the face of diminished cellular fuel sources (decreased glucose, amino acid, etc. availability) (Table 2 and Figure 21). Therefore, it is necessary to more fully understand these potential defects (decreased immune cell activity and number) and to reverse them in order to reap the benefits of CR (increased longevity and health span). Chapter 6 is an attempt to address these issues and further characterize a modality of CR in the context of the middle age of a mouse.

The significance of this work in the context of the CR and DR literature is the expansion of the physiological effects that this specific dietary method has on immune cell mobilization and reorganization. Perhaps one of the most unique and interesting phenotypes described in this work is the sequestration or

protection of leukocyte subsets in the bone marrow (Figure 21). Is the bone marrow truly protective or is it a site of influx and protection during periods of short-term starvation? Short-term starvation experiments have demonstrated a phenotypic change in bone marrow adipose tissue (BMAT). Paradoxically, these adipocytes increase in number during periods of starvation whereas their peripheral counterparts diminish [346–348]. This raises the question, why are adipocytes broken down into energy in peripheral tissues whereas these bone marrow residents increase in number? Furthermore, is increased bone marrow adipogenesis during starvation linked to leukocyte protection and/or mobilization to bone marrow? One potential explanation could involve the β-adrenergic receptor pathway. During periods of starvation increased sympathetic nervous system (SNS) signaling through norepinephrine and epinephrine have been suggested to activate β-adrenergic signaling pathways and lead to increased adiposity in the bone marrow. Blocking this pathway results in decreased bone marrow adiposity during periods of short-term starvation [349]. Furthermore, blocking β-adrenergic signaling prevented downregulation of glucose transporters and maintained glucose influx in activated tumor infiltrating CD8 T cells [350]. Therefore, mice subjected to CR in this model should be treated with β-adrenergic agonists to see if survival of bone marrow leukocytes is maintained. The expectation would be that β-adrenergic agonism would prevents the accumulation of adipocytes and potential energetic fuel for cells that are sequestered to bone marrow during starving period. Taken together, this could suggest that any lymphocytes within bone marrow, or those that traffic there,

would be metabolically suppressed and perhaps not require much nutritional resources. In combination with increased adipogenesis of BMAT, this could provide sufficient survival signals and cellular building blocks for long-term maintenance of T cell subsets in bone marrow upon CR.

This study did not address the consequences of leukocyte reorganization on functional immunity during periods of starvation and during the period of refeeding (Figure 24). Furthermore, this study did not measure any consequences on the health span or lifespan of the treated mice. Therefore, an obvious and reasonably straightforward experiment would be to induce the two-month period of CR, followed by refeeding of two months and measuring any alterations lifespan of mice and make measures of frailty (plasma IL-6 levels, fur graying, body fat accumulation, and gait). One of the major drawbacks of the CR and DR methods studied to date are the deleterious effects to adaptive immunity [15,300]. Therefore, after employing this two-month fasted and two-month refed model, one would want to measure immune function - cytokine production following PMA/Ionoymcin stimulation, and response to acute challenge with Listeria monocytogenes as a model pathogen at day 0, day 7, and two months post refeeding - during initial starvation and at different points of the refeeding stage (day 7 and two months post). Infection with Listeria monoytogenes as the model antigen would provide insight into a largely CD8 T cell mediated response [351]. Comparing the T cell response to infectious challenge at different times during the refeeding phase would be insightful as the consequences of clonal expansion and cytokine production during these phases would be markedly

different, with the expectation that cells at day 7 post refeeding period would be better responders to bacteria than at day 0. Such experiments could provide guidance to patient undergoing their own fasting or using CR modalities during the manifestation of disease.

It is obvious that there is a lot of room for further investigation of the nutritional impact and the restriction of nutrition on the function of immunity and health implemented by different CR or specific DR modalities. Furthermore, given the breadth of different types of caloric sources, access to food, and general population health, it becomes necessary to understand the impact of various dietary modalities in research. It will be of interest to explore to what extent these complex and real-life diets impact immune cell migration and function in defense against microbial pathogens.

EPILOGUE

“There's no limit to how complicated things can get, on account of one thing always leading to another.” — E.B. White

REFERENCES

1. Byrne B. Historical perspectives on twins and twin research. In: Conversations in Twins Research, Twins Research Australia [Internet]. 2018 [cited 12 Feb 2019]. Available: https://www.twins.org.au/research/tools-and-resources/125-conversation-in-twin-research/371-historical-perspectives-on-twins-and-twin-research
2. Galton F. The history of twins, as a criterion of the relative powers of nature and nurture. 1875;12: 566–576. Available: https://genepi.qimr.edu.au/staff/nick_pdf/classics/1875_galton_heredity_566-576.pdf
3. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJL, Furman D, et al. Variation in the human immune system is largely driven by non-heritable influences. Cell. Elsevier; 2015;160: 37–47. doi:10.1016/j.cell.2014.12.020
4. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJL, Furman D, et al. Variation in the human immune system is largely driven by non-heritable influences. Cell. Elsevier Inc.; 2015;160: 37–47. doi:10.1016/j.cell.2014.12.020
5. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. N Engl J Med. Massachusetts Medical Society; 2016;375: 411–421. doi:10.1056/NEJMoa1508749
6. Ober C, Sperling AI, von Mutius E, Vercelli D. Immune development and environment: lessons from Amish and Hutterite children. Curr Opin Immunol. NIH Public Access; 2017;48: 51–60. doi:10.1016/j.coi.2017.08.003
7. Pearce EL. Metabolism in T cell activation and differentiation. Curr Opin Immunol. 2010;22: 314–320. doi:10.1016/j.coi.2010.01.018
8. Fox CJ, Hammerman PS, Thompson CB. Fuel feeds function: energy metabolism and the T-cell response. Nat Rev Immunol. 2005;5: 844–852. doi:10.1038/nri1710
9. Michalek RD, Rathmell JC. The metabolic life and times of a T-cell. Immunol Rev. NIH Public Access; 2010;236: 190–202. doi:10.1111/j.1600-065X.2010.00911.x
10. Bröer S, Bröer A. Amino acid homeostasis and signalling in mammalian cells and organisms. Biochem J. Portland Press Ltd; 2017;474: 1935–1963. doi:10.1042/BCJ20160822
11. Galili G, Amir R, Fernie AR. The Regulation of Essential Amino Acid Synthesis and Accumulation in Plants. Annu Rev Plant Biol. Annual Reviews ; 2016;67: 153–178. doi:10.1146/annurev-arplant-043015-112213
12. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, et al. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. Cell Metab. NIH Public Access; 2014;20: 61–72. doi:10.1016/j.cmet.2014.05.004
13. Cantor J, Slepak M, Ege N, Chang JT, Ginsberg MH. Loss of T Cell CD98

H Chain Specifically Ablates T Cell Clonal Expansion and Protects from Autoimmunity. J Immunol. 2011;187: 851–860. doi:10.4049/jimmunol.1100002

14. Poffenberger MC, Jones RG. Amino Acids Fuel T Cell-Mediated Inflammation. Immunity. Cell Press; 2014;40: 635–637. doi:10.1016/J.IMMUNI.2014.04.017
15. Goldberg EL, Romero-Aleshire MJ, Renkema KR, Ventevogel MS, Chew WM, Uhrlaub JL, et al. Lifespan-extending caloric restriction or mTOR inhibition impair adaptive immunity of old mice by distinct mechanisms. Aging Cell. Wiley-Blackwell; 2015;14: 130–8. doi:10.1111/acel.12280
16. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun. 2014;5: 3557. doi:10.1038/ncomms4557
17. Goldberg EL, Romero-Aleshire MJ, Renkema KR, Ventevogel MS, Chew WM, Uhrlaub JL, et al. Lifespan-extending caloric restriction or mTOR inhibition impair adaptive immunity of old mice by distinct mechanisms. Aging Cell. 2015;14: 130–138. doi:10.1111/acel.12280
18. Wei M, Fabrizio P, Hu J, Ge H, Cheng C, Li L, et al. Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet. Public Library of Science; 2008;4: e13. doi:10.1371/journal.pgen.0040013
19. Katic M, Kahn CR. The role of insulin and IGF-1 signaling in longevity. C Cell Mol Life Sci. Birkhäuser-Verlag; 2005;62: 320–343. doi:10.1007/s00018-004-4297-y
20. Guarente L. Calorie restriction and sirtuins revisited. Genes Dev. 2013;27: 2072–2085. doi:10.1101/gad.227439.113
21. Bagherniya M, Butler AE, Barreto GE, Sahebkar A. The effect of fasting or calorie restriction on autophagy induction: A review of the literature. Ageing Res Rev. Elsevier; 2018;47: 183–197. doi:10.1016/J.ARR.2018.08.004
22. Chen J, Astle C, Harrison D. Delayed immune aging in diet-restricted B6CBAT6 F1 mice is associated with preservation of naive T cells. Fac Res 1990 - 1999. 1998; Available: https://mouseion.jax.org/stfb1990_1999/1122
23. Yang H, Youm Y-H, Dixit VD. Inhibition of thymic adipogenesis by caloric restriction is coupled with reduction in age-related thymic involution. J Immunol. 2009;183: 3040–3052. doi:10.4049/jimmunol.0900562
24. Weindruch R, Walford RL. Dietary restriction in mice beginning at 1 year of age: effect on life-span and spontaneous cancer incidence. Science. American Association for the Advancement of Science; 1982;215: 1415–8. doi:10.1126/SCIENCE.7063854
25. Turturro A, Duffy P, Hass B, Kodell R, Hart R. Survival Characteristics and Age-Adjusted Disease Incidences in C57BL/6 Mice Fed a Commonly Used Cereal-Based Diet Modulated by Dietary Restriction. Journals Gerontol Ser A Biol Sci Med Sci. Narnia; 2002;57: B379–B389. doi:10.1093/gerona/57.11.B379

26. FORSTER MJ, MORRIS P, SOHAL RS. Genotype and age influence the effect of caloric intake on mortality in mice. FASEB J. 2003;17: 690–692. doi:10.1096/fj.02-0533fje
27. Harper JM, Leathers CW, Austad SN. Does caloric restriction extend life in wild mice? Aging Cell. 2006;5: 441–449. doi:10.1111/j.1474-9726.2006.00236.x
28. Bodkin NL, Alexander TM, Ortmeyer HK, Johnson E, Hansen BC. Mortality and Morbidity in Laboratory-maintained Rhesus Monkeys and Effects of Long-term Dietary Restriction. Journals Gerontol Ser A Biol Sci Med Sci. Narnia; 2003;58: B212–B219. doi:10.1093/gerona/58.3.B212
29. Means LW, Higgins JL, Fernandez TJ. Mid-life onset of dietary restriction extends life and prolongs cognitive functioning. Physiol Behav. 1993;54: 503–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/8415944
30. Most J, Tosti V, Redman LM, Fontana L. Calorie restriction in humans: An update. Ageing Res Rev. 2017;39: 36–45. doi:10.1016/j.arr.2016.08.005
31. Hall KD, Bemis T, Brychta R, Chen KY, Courville A, Crayner EJ, et al. Calorie for Calorie, Dietary Fat Restriction Results in More Body Fat Loss than Carbohydrate Restriction in People with Obesity. Cell Metab. Elsevier; 2015;22: 427–436. doi:10.1016/j.cmet.2015.07.021
32. Mattison J a., Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, et al. Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature. Nature Publishing Group; 2012;489: 318–321. doi:10.1038/nature11432
33. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, et al. Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys. Science (80-). 2009;325: 201–204. doi:10.1126/science.1173635
34. Hall KD, Bemis T, Brychta R, Chen KY, Courville A, Crayner EJ, et al. Calorie for Calorie, Dietary Fat Restriction Results in More Body Fat Loss than Carbohydrate Restriction in People with Obesity. Cell Metab. Elsevier; 2015;22: 427–436. doi:10.1016/j.cmet.2015.07.021
35. Levine ME, Suarez JA, Brandhorst S, Balasubramanian P, Cheng C-W, Madia F, et al. Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. Cell Metab. Elsevier; 2014;19: 407–17. doi:10.1016/j.cmet.2014.02.006
36. Mirzaei H, Suarez JA, Longo VD. Protein and amino acid restriction, aging and disease: from yeast to humans. Trends Endocrinol Metab. Elsevier; 2014;25: 558–66. doi:10.1016/j.tem.2014.07.002
37. Goldberg EL, Asher JL, Molony RD, Shaw AC, Zeiss CJ, Wang C, et al. β-Hydroxybutyrate Deactivates Neutrophil NLRP3 Inflammasome to Relieve Gout Flares. Cell Rep. Elsevier; 2017;18: 2077–2087. doi:10.1016/j.celrep.2017.02.004
38. Brandhorst S, Choi IY, Wei M, Cheng CW, Sedrakyan S, Navarrete G, et al. A Periodic Diet that Mimics Fasting Promotes Multi-System Regeneration, Enhanced Cognitive Performance, and Healthspan. Cell

Metab. 2015;22: 86–99. doi:10.1016/j.cmet.2015.05.012
39. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. Europe PMC Funders; 2010;464: 59–65. doi:10.1038/nature08821
40. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. Genome Res. Cold Spring Harbor Laboratory Press; 2009;19: 1141–52. doi:10.1101/gr.085464.108
41. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. Public Library of Science; 2016;14: e1002533. doi:10.1371/journal.pbio.1002533
42. Wylie KM, Weinstock GM, Storch GA. Emerging view of the human virome. Transl Res. NIH Public Access; 2012;160: 283–90. doi:10.1016/j.trsl.2012.03.006
43. Lim ES, Zhou Y, Zhao G, Bauer IK, Droit L, Ndao IM, et al. Early life dynamics of the human gut virome and bacterial microbiome in infants. Nat Med. NIH Public Access; 2015;21: 1228–34. doi:10.1038/nm.3950
44. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011;474: 327–36. doi:10.1038/nature10213
45. Lankelma JM, Nieuwdorp M, De Vos WM, Wiersinga WJ. The gut microbiota in internal medicine: implications for health and disease [Internet]. Available: www.metahit.eu
46. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. Nature. Nature Publishing Group; 2016;532: 512–516. doi:10.1038/nature17655
47. Macpherson AJ, de Agüero MG, Ganal-Vonarburg SC. How nutrition and the maternal microbiota shape the neonatal immune system. Nat Rev Immunol. Nature Publishing Group; 2017;17: 508–517. doi:10.1038/nri.2017.58
48. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. Science. American Association for the Advancement of Science; 2016;352: 539–44. doi:10.1126/science.aad9378
49. Round JL, Mazmanian SK. The gut microbiome shapes intestinal immune responses during health and disease. Nat Rev Immunol. NIH Public Access; 2009;9: 313. doi:10.1038/NRI2515
50. Nikolich-Žugich J. Aging of the T cell compartment in mice and humans: from no naive expectations to foggy memories. J Immunol. NIH Public Access; 2014;193: 2622–9. doi:10.4049/jimmunol.1401174
51. Thompson HL, Smithey MJ, Uhrlaub JL, Jeftić I, Jergović M, White SE, et al. Lymph nodes as barriers to T-cell rejuvenation in aging mice and nonhuman primates. Aging Cell. 2019;18: e12865. doi:10.1111/acel.12865
52. Cicin-Sain L, Messaoudi I, Park B, Currier N, Planer S, Fischer M, et al.

Dramatic increase in naive T cell turnover is linked to loss of naive T cells from old primates. Proc Natl Acad Sci. 2007;104: 19960–19965. doi:10.1073/pnas.0705905104
53. Haynes L, Swain SL. Aged-related shifts in T cell homeostasis lead to intrinsic T cell defects. Semin Immunol. Academic Press; 2012;24: 350–355. doi:10.1016/J.SMIM.2012.04.001
54. Jergović M, Smithey MJ, Nikolich-Žugich J. Intrinsic and extrinsic contributors to defective CD8+ T cell responses with aging. Exp Gerontol. Pergamon; 2018;105: 140–145. doi:10.1016/J.EXGER.2018.01.011
55. Jiang J, Fisher EM, Murasko DM. Intrinsic defects in CD8 T cells with aging contribute to impaired primary antiviral responses. Exp Gerontol. NIH Public Access; 2013;48: 579–86. doi:10.1016/j.exger.2013.02.027
56. Smithey MJ, Venturi V, Davenport MP, Buntzman AS, Vincent BG, Frelinger JA, et al. Lifelong CMV infection improves immune defense in old mice by broadening the mobilized TCR repertoire against third-party infection. Proc Natl Acad Sci. 2018;115. doi:10.1073/pnas.1719451115
57. Furman D, Jojic V, Sharma S, Shen-Orr SS, L. Angel CJ, Onengut-Gumuscu S, et al. Cytomegalovirus infection enhances the immune response to influenza. Sci Transl Med. 2015;7: 281ra43-281ra43. Available: http://stm.sciencemag.org/content/7/281/281ra43.abstract
58. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol. 2010;20: 202–13. doi:10.1002/rmv.655
59. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, et al. Genetic content of wild-type human cytomegalovirus. J Gen Virol. 2004;85: 1301–1312. doi:10.1099/vir.0.79888-0
60. Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, Chee MS, et al. The DNA sequence of the human cytomegalovirus genome. DNA Seq. 1991;2: 1–12. Available: http://www.ncbi.nlm.nih.gov/pubmed/1666311
61. Connolly SA, Jackson JO, Jardetzky TS, Longnecker R. Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol. 2011;9: 369–381. doi:10.1038/nrmicro2548
62. Spear PG, Longnecker R. Herpesvirus entry: an update. J Virol. 2003;77: 10179–85. doi:10.1128/jvi.77.19.10179-10185.2003
63. Feire AL, Koss H, Compton T. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc Natl Acad Sci U S A. 2004;101: 15470–5. doi:10.1073/pnas.0406821101
64. Scrivano L, Sinzger C, Nitschko H, Koszinowski UH, Adler B. HCMV spread and cell tropism are determined by distinct virus populations. PLoS Pathog. Public Library of Science; 2011;7: e1001256. doi:10.1371/journal.ppat.1001256
65. Vochem M, Hamprecht K, Jahn G, Speer CP. Transmission of cytomegalovirus to preterm infants through breast milk. Pediatr Infect Dis J. 1998;17: 53–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/9469396
66. Lanzieri TM, Dollard SC, Josephson CD, Schmid DS, Bialek SR. Breast

Milk-Acquired Cytomegalovirus Infection and Disease in VLBW and Premature Infants. Pediatrics. 2013;131: e1937–e1945. doi:10.1542/peds.2013-0076

67. Farrell HE, Lawler C, Tan CSE, MacDonald K, Bruce K, Mach M, et al. Murine Cytomegalovirus Exploits Olfaction To Enter New Hosts. MBio. American Society for Microbiology; 2016;7: e00251-16. doi:10.1128/mbio.00251-16
68. Vanarsdall AL, Johnson DC. Human cytomegalovirus entry into cells. Curr Opin Virol. NIH Public Access; 2012;2: 37–42. doi:10.1016/j.coviro.2012.01.001
69. Wang D, Yu Q-C, Schröer J, Murphy E, Shenk T. Human cytomegalovirus uses two distinct pathways to enter retinal pigmented epithelial cells. Proc Natl Acad Sci U S A. National Academy of Sciences; 2007;104: 20037–42. doi:10.1073/pnas.0709704104
70. Jean Beltran PM, Cristea IM. The life cycle and pathogenesis of human cytomegalovirus infection: lessons from proteomics. Expert Rev Proteomics. NIH Public Access; 2014;11: 697–711. doi:10.1586/14789450.2014.971116
71. Compton T, Feire A. Early events in human cytomegalovirus infection [Internet]. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge University Press; 2007. Available: http://www.ncbi.nlm.nih.gov/pubmed/21348067
72. Murphy JC, Fischle W, Verdin E, Sinclair JH. Control of cytomegalovirus lytic gene expression by histone acetylation. EMBO J. European Molecular Biology Organization; 2002;21: 1112–20. doi:10.1093/emboj/21.5.1112
73. Davison AJ. Comparative analysis of the genomes [Internet]. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge University Press; 2007. Available: http://www.ncbi.nlm.nih.gov/pubmed/21348122
74. Cheng S, Caviness K, Buehler J, Smithey M, Nikolich-Žugich J, Goodrum F. Transcriptome-wide characterization of human cytomegalovirus in natural infection and experimental latency. Proc Natl Acad Sci U S A. National Academy of Sciences; 2017;114: E10586–E10595. doi:10.1073/pnas.1710522114
75. Larsson S, Söderberg-Nauclér C, Wang FZ, Möller E. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. Transfusion. 1998;38: 271–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/9563407
76. Thom JT, Weber TC, Walton M, Torti N. The Salivary Gland Acts as a Sink for Tissue-Resident Memory CD8 + T Cells, Facilitating Protection from Local Cytomegalovirus Infection. Cell Rep. 2015;13. doi:10.1016/j.celrep.2015.09.082
77. Gordon CL, Miron M, Thome JJC, Matsuoka N, Weiner J, Rak MA, et al. Tissue reservoirs of antiviral T cell immunity in persistent human CMV infection. J Exp Med. 2017;214: 651–667. doi:10.1084/jem.20160758

78. Vliegen I, Herngreen S, Grauls G, Bruggeman C, Stassen F. Improved detection and quantification of mouse cytomegalovirus by real-time PCR. Virus Res. Elsevier; 2003;98: 17–25. doi:10.1016/J.VIRUSRES.2003.08.009
79. Brune W, Hengel H, Koszinowski UH. A mouse model for cytomegalovirus infection. Curr Protoc Immunol. 2001;Chapter 19: Unit 19.7. doi:10.1002/0471142735.im1907s43
80. Kercher L, Mitchell BM. Persisting Murine Cytomegalovirus Can Reactivate and Has Unique Transcriptional Activity in Ocular Tissue. J Virol. 2002;76: 9165–9175. doi:10.1128/JVI.76.18.9165-9175.2002
81. Azevedo LS, Pierrotti LC, Abdala E, Costa SF, Strabelli TMV, Campos SV, et al. Cytomegalovirus infection in transplant recipients. Clinics (Sao Paulo). Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo; 2015;70: 515–23. doi:10.6061/clinics/2015(07)09
82. Barton ES, White DW, Cathelyn JS, Brett-McClellan KA, Engle M, Diamond MS, et al. Herpesvirus latency confers symbiotic protection from bacterial infection. Nature. 2007;447: 326–329. doi:10.1038/nature05762
83. Böhm V, Seckert CK, Simon CO, Thomas D, Renzaho A, Gendig D, et al. Immune evasion proteins enhance cytomegalovirus latency in the lungs. J Virol. 2009;83: 10293–8. doi:10.1128/JVI.01143-09
84. Shellam GR, Allan JE, Papadimitriou JM, Bancroft GJ. Increased susceptibility to cytomegalovirus infection in beige mutant mice. Proc Natl Acad Sci U S A. National Academy of Sciences; 1981;78: 5104–8. doi:10.1073/pnas.78.8.5104
85. Bukowski J, Woda B, virology RW-J of, 1984 undefined. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. Am Soc Microbiol. Available: https://jvi.asm.org/content/52/1/119.short
86. Bukowski J, Warner J, … GD-J of E, 1985 undefined. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. jem.rupress.org. Available: http://jem.rupress.org/content/161/1/40.abstract
87. Biron CA, Byron KS, Sullivan JL. Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells. N Engl J Med. 1989;320: 1731–1735. doi:10.1056/NEJM198906293202605
88. Kärre K. Natural killer cell recognition of missing self. Nat Immunol. 2008;9: 477–480. doi:10.1038/ni0508-477
89. Farrell HE, Vally H, Lynch DM, Fleming P, Shellam GR, Scalzo AA, et al. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. Nature. 1997;386: 510–514. doi:10.1038/386510a0
90. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL. Direct Recognition of Cytomegalovirus by Activating and Inhibitory NK Cell Receptors. Science (80-). 2002;296: 1323–1326. doi:10.1126/science.1070884
91. Adams EJ, Juo ZS, Venook RT, Boulanger MJ, Arase H, Lanier LL, et al. Structural elucidation of the m157 mouse cytomegalovirus ligand for Ly49 natural killer cell receptors. Proc Natl Acad Sci U S A. National Academy of Sciences; 2007;104: 10128. doi:10.1073/PNAS.0703735104
92. Bubić I, Wagner M, Krmpotić A, Saulig T, Kim S, Yokoyama WM, et al.

Gain of virulence caused by loss of a gene in murine cytomegalovirus. J Virol. 2004;78: 7536–44. doi:10.1128/JVI.78.14.7536-7544.2004
93. Koch J, Steinle A, Watzl C, Mandelboim O. Activating natural cytotoxicity receptors of natural killer cells in cancer and infection. Trends Immunol. 2013;34: 182–191. doi:10.1016/j.it.2013.01.003
94. Jelenčić V, Šestan M, Kavazović I, Lenartić M, Marinović S, Holmes TD, et al. NK cell receptor NKG2D sets activation threshold for the NCR1 receptor early in NK cell development. Nat Immunol. Nature Publishing Group; 2018;19: 1083–1092. doi:10.1038/s41590-018-0209-9
95. Hadad U, Thauland TJ, Martinez OM, Butte MJ, Porgador A, Krams SM. NKp46 Clusters at the Immune Synapse and Regulates NK Cell Polarization. Front Immunol. Frontiers Media SA; 2015;6: 495. doi:10.3389/fimmu.2015.00495
96. Jonjić S, Polić B, Krmpotić A. Viral inhibitors of NKG2D ligands: Friends or foes of immune surveillance? Eur J Immunol. 2008;38: 2952–2956. doi:10.1002/eji.200838823
97. Serbina N V., Jia T, Hohl TM, Pamer EG. Monocyte-Mediated Defense Against Microbial Pathogens. Annu Rev Immunol. 2008;26: 421–452. doi:10.1146/annurev.immunol.26.021607.090326
98. Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol. 2009;10: 1200–1207. doi:10.1038/ni.1792
99. Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. Nature Publishing Group; 2011;475: 222–225. doi:10.1038/nature10138
100. Noda S, Aguirre SA, Bitmansour A, Brown JM, Sparer TE, Huang J, et al. Cytomegalovirus MCK-2 controls mobilization and recruitment of myeloid progenitor cells to facilitate dissemination. Blood. American Society of Hematology; 2006;107: 30–8. doi:10.1182/blood-2005-05-1833
101. Saederup N, Aguirre SA, Sparer TE, Bouley DM, Mocarski ES. Murine cytomegalovirus CC chemokine homolog MCK-2 (m131-129) is a determinant of dissemination that increases inflammation at initial sites of infection. J Virol. 2001;75: 9966–76. doi:10.1128/JVI.75.20.9966-9976.2001
102. Saederup N, Lin Y c., Dairaghi DJ, Schall TJ, Mocarski ES. Cytomegalovirus-encoded beta chemokine promotes monocyte-associated viremia in the host. Proc Natl Acad Sci. 1999;96: 10881–10886. doi:10.1073/pnas.96.19.10881
103. Hahn G, Jores R, Mocarski ES. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proc Natl Acad Sci. 1998;95: 3937–3942. doi:10.1073/pnas.95.7.3937
104. Frascaroli G, Varani S, Moepps B, Sinzger C, Landini MP, Mertens T. Human Cytomegalovirus Subverts the Functions of Monocytes, Impairing Chemokine-Mediated Migration and Leukocyte Recruitment. J Virol.

2006;80: 7578–7589. doi:10.1128/JVI.02421-05
105. Rice GP, Schrier RD, Oldstone MB. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. Proc Natl Acad Sci. 1984;81: 6134–6138. doi:10.1073/pnas.81.19.6134
106. Upton JW, Kaiser WJ, Mocarski ES. Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2008;283: 16966–70. doi:10.1074/jbc.C800051200
107. Ebermann L, Ruzsics Z, Guzmán CA, Rooijen N van, Casalegno-Garduño R, Koszinowski U, et al. Block of Death-Receptor Apoptosis Protects Mouse Cytomegalovirus from Macrophages and Is a Determinant of Virulence in Immunodeficient Hosts. PLoS Pathog. Public Library of Science; 2012;8: e1003062. doi:10.1371/JOURNAL.PPAT.1003062
108. Fleming P, Kvansakul M, Voigt V, Kile BT, Kluck RM, Huang DCS, et al. MCMV-mediated inhibition of the pro-apoptotic Bak protein is required for optimal in vivo replication. PLoS Pathog. Public Library of Science; 2013;9: e1003192. doi:10.1371/journal.ppat.1003192
109. Sinzger C, Eberhardt K, Cavignac Y, Weinstock C, Kessler T, Jahn G, et al. Macrophage cultures are susceptible to lytic productive infection by endothelial-cell-propagated human cytomegalovirus strains and present viral IE1 protein to CD4+ T cells despite late downregulation of MHC class II molecules. J Gen Virol. 2006;87: 1853–1862. doi:10.1099/vir.0.81595-0
110. Riegler S, Sinzger C, Brossart P, Hebart H, Einsele H, Jahn G. Monocyte-derived dendritic cells are permissive to the complete replicative cycle of human cytomegalovirus. J Gen Virol. 2000;81: 393–399. doi:10.1099/0022-1317-81-2-393
111. Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C, Yurochko AD. Human cytomegalovirus (HCMV) infection of endothelial cells promotes naive monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. J Virol. 2006;80: 11539–55. doi:10.1128/JVI.01016-06
112. Polić B, Hengel H, Krmpotić A, Trgovcich J, Pavić I, Lučin P, et al. Hierarchical and Redundant Lymphocyte Subset Control Precludes Cytomegalovirus Replication during Latent Infection. J Exp Med. 1998;188: 1047–1054. doi:10.1084/jem.188.6.1047
113. Klenerman P, Oxenius A. T cell responses to cytomegalovirus. Nat Rev Immunol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2016;advance on. doi:10.1038/nri.2016.38
114. Munks MW, Cho KS, Pinto AK, Sierro S, Klenerman P, Hill AB. Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. J Immunol. 2006;177: 450–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/16785542
115. Arens R, Wang P, Sidney J, Loewendorf A, Sette A, Schoenberger SP, et al. Cutting edge: murine cytomegalovirus induces a polyfunctional CD4 T

cell response. J Immunol. NIH Public Access; 2008;180: 6472–6. Available: http://www.ncbi.nlm.nih.gov/pubmed/18453564
116. Snyder CM, Cho KS, Bonnett EL, van Dommelen S, Shellam GR, Hill AB. Memory Inflation during Chronic Viral Infection Is Maintained by Continuous Production of Short-Lived, Functional T Cells. Immunity. 2008;29: 650–659. doi:10.1016/j.immuni.2008.07.017
117. Komatsu H, Sierro S, V Cuero A, Klenerman P. Population analysis of antiviral T cell responses using MHC class I-peptide tetramers. Clin Exp Immunol. 2003;134: 9–12. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1808834&tool=pmcentrez&rendertype=abstract
118. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4 $^{+}$ and CD8 $^{+}$ T cells dominate the memory compartments of exposed subjects. J Exp Med. 2005;202: 673–685. doi:10.1084/jem.20050882
119. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, et al. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J Immunol. 2003;170: 2022–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/12574372
120. Akulian JA, Pipeling MR, John ER, Orens JB, Lechtzin N, McDyer JF. High-Quality CMV-Specific CD4 $^{+}$ Memory Is Enriched in the Lung Allograft and Is Associated With Mucosal Viral Control. Am J Transplant. 2013;13: 146–156. doi:10.1111/j.1600-6143.2012.04282.x
121. Ward SM, Jonsson JR, Sierro S, Clouston AD, Lucas M, Vargas AL, et al. Virus-specific CD8+ T lymphocytes within the normal human liver. Eur J Immunol. 2004;34: 1526–1531. doi:10.1002/eji.200324275
122. Hertoghs KML, Moerland PD, van Stijn A, Remmerswaal EBM, Yong SL, van de Berg PJEJ, et al. Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. J Clin Invest. American Society for Clinical Investigation; 2010;120: 4077–90. doi:10.1172/JCI42758
123. Holtappels R, Pahl-Seibert M-F, Thomas D, Reddehase MJ. Enrichment of Immediate-Early 1 (m123/pp89) Peptide-Specific CD8 T Cells in a Pulmonary CD62Llo Memory-Effector Cell Pool during Latent Murine Cytomegalovirus Infection of the Lungs. J Virol. 2000;74: 11495–11503. doi:10.1128/JVI.74.24.11495-11503.2000
124. Morabito KM, Ruckwardt TJ, Bar-Haim E, Nair D, Moin SM, Redwood AJ, et al. Memory Inflation Drives Tissue-Resident Memory CD8+ T Cell Maintenance in the Lung After Intranasal Vaccination With Murine Cytomegalovirus. Front Immunol. Frontiers; 2018;9: 1861. doi:10.3389/fimmu.2018.01861
125. Smith CJ, Turula H, Snyder CM. Systemic hematogenous maintenance of memory inflation by MCMV infection. PLoS Pathog. Public Library of Science; 2014;10: e1004233. doi:10.1371/journal.ppat.1004233
126. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4 $^{+}$ and CD8 $^{+}$ T cells dominate the memory compartments of exposed subjects. J Exp Med.

2005;202: 673–685. doi:10.1084/jem.20050882
127. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, et al. Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. J Immunol. NIH Public Access; 2014;192: 2143–55. doi:10.4049/jimmunol.1301721
128. Polic B, Hengel H, Krmpotic A, Trgovcich J, Pavic I, Luccaronin P, et al. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. J Exp Med. 1998;188: 1047–1054. doi:10.1084/jem.188.6.1047
129. Reddehase MJ, Podlech J, Grzimek NK. A. Mouse models of cytomegalovirus latency: overview. J Clin Virol. Elsevier; 2002;25 Suppl 2: S23-36. doi:10.1016/S1386-6532(02)00087-2
130. Mendelson M, Monard S, Sissons P, Sinclair J. Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. J Gen Virol. Microbiology Society; 1996;77: 3099–3102. doi:10.1099/0022-1317-77-12-3099
131. Bentz GL, Yurochko AD. Human CMV infection of endothelial cells induces an angiogenic response through viral binding to EGF receptor and 1 and 3 integrins. 2008;105.
132. Thom JT, Oxenius A. Tissue-resident memory T cells in cytomegalovirus infection. Curr Opin Virol. Elsevier; 2016;16: 63–69. doi:10.1016/J.COVIRO.2016.01.014
133. Turner DL, Bickham KL, Thome JJ, Kim CY, D'Ovidio F, Wherry EJ, et al. Lung niches for the generation and maintenance of tissue-resident memory T cells. Mucosal Immunol. Nature Publishing Group; 2014;7: 501–510. doi:10.1038/mi.2013.67
134. van Aalderen MC, Remmerswaal EBM, ten Berge IJM, van Lier RAW. Blood and beyond: Properties of circulating and tissue-resident human virus-specific αβ CD8 $^{+}$ T cells. Eur J Immunol. John Wiley & Sons, Ltd; 2014;44: 934–944. doi:10.1002/eji.201344269
135. Popovic B, Golemac M, Podlech J, Zeleznjak J, Bilic-Zulle L, Lukic ML, et al. IL-33/ST2 pathway drives regulatory T cell dependent suppression of liver damage upon cytomegalovirus infection. Benedict CA, editor. PLOS Pathog. Public Library of Science; 2017;13: e1006345. doi:10.1371/journal.ppat.1006345
136. Munks MW, Gold MC, Zajac AL, Doom CM, Morello CS, Spector DH, et al. Genome-wide analysis reveals a highly diverse CD8 T cell response to murine cytomegalovirus. J Immunol. American Association of Immunologists; 2006;176: 3760–6. doi:10.4049/JIMMUNOL.176.6.3760
137. O'Hara GA, Welten SPM, Klenerman P, Arens R. Memory T cell inflation: understanding cause and effect. Trends Immunol. 2012;33: 84–90. doi:10.1016/j.it.2011.11.005
138. Derhovanessian E, Larbi A, Pawelec G. Biomarkers of human immunosenescence: impact of Cytomegalovirus infection. Curr Opin Immunol. 2009;21: 440–445. doi:10.1016/j.coi.2009.05.012
139. Jackson SE, Mason GM, Okecha G, Sissons JGP, Wills MR. Diverse

Specificities, Phenotypes, and Antiviral Activities of Cytomegalovirus-Specific CD8+ T Cells. J Virol. American Society for Microbiology Journals; 2014;88: 10894–10908. doi:10.1128/JVI.01477-14

140. Pourgheysari B, Khan N, Best D, Bruton R, Nayak L, Moss PAH. The Cytomegalovirus-Specific CD4+ T-Cell Response Expands with Age and Markedly Alters the CD4+ T-Cell Repertoire. J Virol. 2007;81: 7759–7765. doi:10.1128/JVI.01262-06
141. Snyder CM, Cho KS, Bonnett EL, Allan JE, Hill AB. Sustained CD8+ T Cell Memory Inflation after Infection with a Single-Cycle Cytomegalovirus. Harty JT, editor. PLoS Pathog. 2011;7: e1002295. doi:10.1371/journal.ppat.1002295
142. Le Roy E, Mühlethaler-Mottet A, Davrinche C, Mach B, Davignon JL. Escape of human cytomegalovirus from HLA-DR-restricted CD4(+) T-cell response is mediated by repression of gamma interferon-induced class II transactivator expression. J Virol. 1999;73: 6582–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/10400755
143. Miller DM, Rahill BM, Boss JM, Lairmore MD, Durbin JE, Waldman JW, et al. Human Cytomegalovirus Inhibits Major Histocompatibility Complex Class II Expression By Disruption of the Jak/Stat Pathway. J Exp Med. 1998;187: 675–683. doi:10.1084/jem.187.5.675
144. Mason GM, Jackson S, Okecha G, Poole E, Sissons JGP, Sinclair J, et al. Human Cytomegalovirus Latency-Associated Proteins Elicit Immune-Suppressive IL-10 Producing CD4+ T Cells. Rooney CM, editor. PLoS Pathog. 2013;9: e1003635. doi:10.1371/journal.ppat.1003635
145. Jonjic S, Mutter W, Weiland F, Reddehase MJ, Koszinowski UH. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4+ T lymphocytes. J Exp Med. 1989;169: 1199–1212. doi:10.1084/jem.169.4.1199
146. Walton SM, Mandaric S, Torti N, Zimmermann A, Hengel H, Oxenius A. Absence of Cross-Presenting Cells in the Salivary Gland and Viral Immune Evasion Confine Cytomegalovirus Immune Control to Effector CD4 T Cells. Früh K, editor. PLoS Pathog. 2011;7: e1002214. doi:10.1371/journal.ppat.1002214
147. Trayhurn P, Beattie JH. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc Nutr Soc. 2001;60: 329–39. Available: http://www.ncbi.nlm.nih.gov/pubmed/11681807
148. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. Diabetes. 2006;55: 1537–45. doi:10.2337/db06-0263
149. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature. NIH Public Access; 2006;444: 847–53. doi:10.1038/nature05483
150. Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol. 2005;115: 911–919. doi:10.1016/j.jaci.2005.02.023
151. Townsend K, Tseng Y-H. Brown adipose tissue: Recent insights into development, metabolic function and therapeutic potential. Adipocyte. Taylor & Francis; 2012;1: 13–24. doi:10.4161/adip.18951

152. Duncan RE, Ahmadian M, Jaworski K, Sarkadi-Nagy E, Sul HS. Regulation of Lipolysis in Adipocytes. Annu Rev Nutr. NIH Public Access; 2007;27: 79. doi:10.1146/ANNUREV.NUTR.27.061406.093734
153. Wensveen FM, Valentić S, Šestan M, Wensveen TT, Polić B. The “Big Bang” in obese fat: events initiating obesity-induced adipose tissue inflammation. Eur J Immunol. 2015;45: 2446–2456. doi:10.1002/eji.201545502
154. Slavin BG. The morphology of adipose tissue. New Perspect Adipose Tissue. Butterworth-Heinemann; 1985; 23–43. doi:10.1016/B978-0-408-10857-7.50007-5
155. Berry DC, Stenesen D, Zeve D, Graff JM. The developmental origins of adipose tissue. Development. Company of Biologists; 2013;140: 3939–49. doi:10.1242/dev.080549
156. Nakajima I, Aso H, Yamaguchi T, Ozutsumi K. Adipose tissue extracellular matrix: newly organized by adipocytes during differentiation. Differentiation. Elsevier; 1998;63: 193–200. doi:10.1111/J.1432-0436.1998.00193.X
157. Jo J, Gavrilova O, Pack S, Jou W, Mullen S, Sumner AE, et al. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. PLoS Comput Biol. Public Library of Science; 2009;5: e1000324. doi:10.1371/journal.pcbi.1000324
158. Muir LA, Neeley CK, Meyer KA, Baker NA, Brosius AM, Washabaugh AR, et al. Adipose tissue fibrosis, hypertrophy, and hyperplasia: Correlations with diabetes in human obesity. Obesity. John Wiley & Sons, Ltd; 2016;24: 597–605. doi:10.1002/oby.21377
159. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. Cell. 2014;156: 20–44. doi:10.1016/j.cell.2013.12.012
160. Grant RW, Dixit VD. Adipose tissue as an immunological organ. Obesity (Silver Spring). 2015;23: 512–8. doi:10.1002/oby.21003
161. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med. 2009;15: 930–9. doi:10.1038/nm.2002
162. Després J-P, Lemieux I. Abdominal obesity and metabolic syndrome. Nature. 2006;444: 881–887. doi:10.1038/nature05488
163. Ye R, Scherer PE. Adiponectin, driver or passenger on the road to insulin sensitivity? Mol Metab. Elsevier; 2013;2: 133–141. doi:10.1016/J.MOLMET.2013.04.001
164. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature. Nature Publishing Group; 2003;423: 762–769. doi:10.1038/nature01705
165. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, et al. Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. Biochem Biophys Res Commun. Academic Press; 1999;257: 79–83. doi:10.1006/BBRC.1999.0255
166. Achari A, Jain S. Adiponectin, a Therapeutic Target for Obesity, Diabetes,

and Endothelial Dysfunction. Int J Mol Sci. Multidisciplinary Digital Publishing Institute; 2017;18: 1321. doi:10.3390/ijms18061321
167. Wang X, Buechler N, Yoza B, McCall C, Vachharajani V. Adiponectin treatment attenuates inflammatory response during early sepsis in obese mice. J Inflamm Res. Dove Press; 2016;Volume 9: 167–174. doi:10.2147/JIR.S119021
168. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med. Nature Publishing Group; 2001;7: 941–946. doi:10.1038/90984
169. Combs TP, Berg AH, Obici S, Scherer PE, Rossetti L. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. J Clin Invest. American Society for Clinical Investigation; 2001;108: 1875–81. doi:10.1172/JCI14120
170. Ohashi K, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, et al. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2010;285: 6153–60. doi:10.1074/jbc.M109.088708
171. Bråkenhielm E, Veitonmäki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, et al. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. Proc Natl Acad Sci U S A. National Academy of Sciences; 2004;101: 2476–81. doi:10.1073/pnas.0308671100
172. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, et al. Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2003;278: 2461–8. doi:10.1074/jbc.M209033200
173. Al Maskari MY, Alnaqdy AA. Correlation between Serum Leptin Levels, Body Mass Index and Obesity in Omanis. Sultan Qaboos Univ Med J. Sultan Qaboos University; 2006;6: 27–31. Available: http://www.ncbi.nlm.nih.gov/pubmed/21748132
174. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994;372: 425–432. doi:10.1038/372425a0
175. Siegmund B, Lehr HA, Fantuzzi G. Leptin: a pivotal mediator of intestinal inflammation in mice. Gastroenterology. 2002;122: 2011–25. Available: http://www.ncbi.nlm.nih.gov/pubmed/12055606
176. Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol. 2000;68: 437–46. Available: http://www.ncbi.nlm.nih.gov/pubmed/11037963
177. Leshan RL, Björnholm M, Münzberg H, Myers MG. Leptin Receptor Signaling and Action in the Central Nervous System. Obesity. John Wiley & Sons, Ltd; 2006;14: 208S-212S. doi:10.1038/oby.2006.310
178. Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, et al. Identification and expression cloning of a leptin receptor, OB-R. Cell. Cell

Press; 1995;83: 1263–1271. doi:10.1016/0092-8674(95)90151-5
179. Yadav A, Kataria MA, Saini V, Yadav A. Role of leptin and adiponectin in insulin resistance. Clin Chim Acta. Elsevier; 2013;417: 80–84. doi:10.1016/J.CCA.2012.12.007
180. Cava A La, Matarese G. The weight of leptin in immunity. Nat Rev Immunol. Nature Publishing Group; 2004;4: 371–379. doi:10.1038/nri1350
181. Denroche HC, Huynh FK, Kieffer TJ. The role of leptin in glucose homeostasis. J Diabetes Investig. Wiley-Blackwell; 2012;3: 115–29. doi:10.1111/j.2040-1124.2012.00203.x
182. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature. 1998;394: 897–901. doi:10.1038/29795
183. De Rosa V, Procaccini C, Calì G, Pirozzi G, Fontana S, Zappacosta S, et al. A Key Role of Leptin in the Control of Regulatory T Cell Proliferation. Immunity. 2007;26: 241–255. doi:10.1016/j.immuni.2007.01.011
184. Hasenkrug KJ. The leptin connection: regulatory T cells and autoimmunity. Immunity. Elsevier; 2007;26: 143–5. doi:10.1016/j.immuni.2007.02.002
185. Reilly SM, Saltiel AR. Adapting to obesity with adipose tissue inflammation. Nat Rev Endocrinol. Nature Publishing Group; 2017;13: 633–643. doi:10.1038/nrendo.2017.90
186. Arroyo-Johnson C, Mincey KD. Obesity Epidemiology Worldwide. Gastroenterol Clin North Am. 2016;45: 571–579. doi:10.1016/j.gtc.2016.07.012
187. Wang Y, Beydoun MA, Liang L, Caballero B, Kumanyika SK. Will all Americans become overweight or obese? estimating the progression and cost of the US obesity epidemic. Obesity (Silver Spring). 2008;16: 2323–30. doi:10.1038/oby.2008.351
188. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444: 860–867. doi:10.1038/nature05485
189. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. J Clin Invest. 2011;121: 2111–2117. doi:10.1172/JCI57132
190. McNelis JC, Olefsky JM. Macrophages, immunity, and metabolic disease. Immunity. Elsevier; 2014;41: 36–48. doi:10.1016/j.immuni.2014.05.010
191. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005;46: 2347–2355. doi:10.1194/jlr.M500294-JLR200
192. Vandanmagsar B, Youm Y-H, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med. 2011;17: 179–188. doi:10.1038/nm.2279
193. Spallanzani RG, Zemmour D, Xiao T, Jayewickreme T, Li C, Bryce PJ, et al. Distinct immunocyte-promoting and adipocyte-generating stromal components coordinate adipose tissue immune and metabolic tenors. Sci Immunol. Science Immunology; 2019;4: eaaw3658. doi:10.1126/sciimmunol.aaw3658

194. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+ lymphoid cells. Nature. 2010;463: 540–544. doi:10.1038/nature08636
195. Molofsky AB, Nussbaum JC, Liang H-E, Van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. J Exp Med. 2013;210: 535–549. doi:10.1084/jem.20121964
196. Fujisaka S, Usui I, Bukhari A, Ikutani M, Oya T, Kanatani Y, et al. Regulatory Mechanisms for Adipose Tissue M1 and M2 Macrophages in Diet-Induced Obese Mice. Diabetes. 2009;58: 2574–2582. doi:10.2337/db08-1475
197. Wensveen FM, Jelenc V, Valentić S, Šestan M, Wensveen TT, Theurich S, et al. NK cells link obesity-induced adipose stress to inflammation and insulin resistance. 2015;16. doi:10.1038/ni.3120
198. Duffaut C, Galitzky J, Lafontan M, Bouloumié A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochem Biophys Res Commun. 2009;384: 482–5. doi:10.1016/j.bbrc.2009.05.002
199. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med. 2009;15: 914–920. doi:10.1038/nm.1964
200. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A. 2005;102: 11070–5. doi:10.1073/pnas.0504978102
201. Saad MJA, Santos A, Prada PO. Linking Gut Microbiota and Inflammation to Obesity and Insulin Resistance. Physiology. 2016;31: 283–293. doi:10.1152/physiol.00041.2015
202. Nguyen MTA, Favelyukis S, Nguyen A-K, Reichart D, Scott PA, Jenn A, et al. A Subpopulation of Macrophages Infiltrates Hypertrophic Adipose Tissue and Is Activated by Free Fatty Acids via Toll-like Receptors 2 and 4 and JNK-dependent Pathways. J Biol Chem. 2007;282: 35279–35292. doi:10.1074/jbc.M706762200
203. Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L, et al. Saturated Fatty Acid Activates but Polyunsaturated Fatty Acid Inhibits Toll-like Receptor 2 Dimerized with Toll-like Receptor 6 or 1. J Biol Chem. 2004;279: 16971–16979. doi:10.1074/jbc.M312990200
204. Gadola SD, Karadimitris A, Zaccai NR, Salio M, Dulphy N, Shepherd D, et al. Generation of CD1 tetramers as a tool to monitor glycolipid-specific T cells. Philos Trans R Soc Lond B Biol Sci. 2003;358: 875–7. doi:10.1098/rstb.2003.1267
205. Avci FY, Li X, Tsuji M, Kasper DL. Carbohydrates and T cells: a sweet twosome. Semin Immunol. 2013;25: 146–51. doi:10.1016/j.smim.2013.05.005
206. Sidobre S, Kronenberg M. CD1 tetramers: a powerful tool for the analysis

of glycolipid-reactive T cells. J Immunol Methods. 2002;268: 107–21. Available: http://www.ncbi.nlm.nih.gov/pubmed/12213347
207. Scoditti E, Massaro M, Carluccio MA, Pellegrino M, Wabitsch M, Calabriso N, et al. Additive Regulation of Adiponectin Expression by the Mediterranean Diet Olive Oil Components Oleic Acid and Hydroxytyrosol in Human Adipocytes. Papa S, editor. PLoS One. 2015;10: e0128218. doi:10.1371/journal.pone.0128218
208. Estruch R, Ros E, Salas-Salvadó J, Covas M-I, Corella D, Arós F, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. N Engl J Med. Massachusetts Medical Society; 2018;378: e34. doi:10.1056/NEJMoa1800389
209. Hardy OT, Perugini RA, Nicoloro SM, Gallagher-Dorval K, Puri V, Straubhaar J, et al. Body mass index-independent inflammation in omental adipose tissue associated with insulin resistance in morbid obesity. Surg Obes Relat Dis. 2011;7: 60–67. doi:10.1016/j.soard.2010.05.013
210. Klöting N, Fasshauer M, Dietrich A, Kovacs P, Schön MR, Kern M, et al. Insulin-sensitive obesity. Am J Physiol Metab. 2010;299: E506–E515. doi:10.1152/ajpendo.00586.2009
211. Ofei F, Hurel S, Newkirk J, Sopwith M, Taylor R. Effects of an Engineered Human Anti-TNF- Antibody (CDP571) on Insulin Sensitivity and Glycemic Control in Patients With NIDDM. Diabetes. 1996;45: 881–885. doi:10.2337/diab.45.7.881
212. Solomon DH, Massarotti E, Garg R, Liu J, Canning C, Schneeweiss S. Association Between Disease-Modifying Antirheumatic Drugs and Diabetes Risk in Patients With Rheumatoid Arthritis and Psoriasis. JAMA. 2011;305: 2525. doi:10.1001/jama.2011.878
213. Sloan-Lancaster J, Abu-Raddad E, Polzer J, Miller JW, Scherer JC, De Gaetano A, et al. Double-Blind, Randomized Study Evaluating the Glycemic and Anti-inflammatory Effects of Subcutaneous LY2189102, a Neutralizing IL-1 Antibody, in Patients With Type 2 Diabetes. Diabetes Care. 2013;36: 2239–2246. doi:10.2337/dc12-1835
214. Greenberg AS. The expanding scope of the metabolic syndrome and implications for the management of cardiovascular risk in type 2 diabetes with particular focus on the emerging role of the thiazolidinediones. J Diabetes Complications. 17: 218–28. Available: http://www.ncbi.nlm.nih.gov/pubmed/12810246
215. Bouskila M, Pajvani UB, Scherer PE. Adiponectin: a relevant player in PPARγ-agonist-mediated improvements in hepatic insulin sensitivity? Int J Obes. Nature Publishing Group; 2005;29: S17–S23. doi:10.1038/sj.ijo.0802908
216. Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, et al. Metformin improves healthspan and lifespan in mice. Nat Commun. 2013;4: 2192. doi:10.1038/ncomms3192
217. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, et al. Metformin inhibits mitochondrial complex I of cancer cells to

reduce tumorigenesis. Elife. 2014;3: e02242. doi:10.7554/eLife.02242
218. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, et al. Metformin Inhibits Proinflammatory Responses and Nuclear Factor-κB in Human Vascular Wall Cells. Arterioscler Thromb Vasc Biol. 2006;26: 611–617. doi:10.1161/01.ATV.0000201938.78044.75
219. Chawla A. Control of Macrophage Activation and Function by PPARs. Circ Res. 2010;106: 1559–1569. doi:10.1161/CIRCRESAHA.110.216523
220. Unger RH, Zhou Y-T, Orci L. Regulation of fatty acid homeostasis in cells: Novel role of leptin. Proc Natl Acad Sci. 1999;96: 2327–2332. doi:10.1073/pnas.96.5.2327
221. Tomas E, Tsao T-S, Saha AK, Murrey HE, Zhang C c., Itani SI, et al. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: Acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. Proc Natl Acad Sci. 2002;99: 16309–16313. doi:10.1073/pnas.222657499
222. Sestan M, Marinovi S, Kavazovi I, Konrad D, Wensveen FM, Poli B. Virus-Induced Interferon-γ Causes Insulin Resistance in Skeletal Muscle and Derails Glycemic Control in Obesity. Immunity. 2018;49: 164–177. doi:10.1016/j.immuni.2018.05.005
223. Ferrante AW. The immune cells in adipose tissue. Diabetes Obes Metab. 2013;15 Suppl 3: 34–8. doi:10.1111/dom.12154
224. Schäffler A, Schölmerich J. Innate immunity and adipose tissue biology. Trends Immunol. 2010;31: 228–35. doi:10.1016/j.it.2010.03.001
225. Matos Ferreira AV, Segatto M, Menezes Z, Macedo AM, Gelape C, de Oliveira Andrade L, et al. Evidence for Trypanosoma cruzi in adipose tissue in human chronic Chagas disease. Microbes Infect. 2011;13: 1002–1005. doi:10.1016/j.micinf.2011.06.002
226. Couturier J, Suliburk JW, Brown JM, Luke DJ, Agarwal N, Yu X, et al. Human adipose tissue as a reservoir for memory CD4+ T cells and HIV. AIDS. 2015;29: 667–674. doi:10.1097/QAD.0000000000000599
227. Tanowitz HB, Jelicks LA, Machado FS, Esper L, Qi X, Desruisseaux MS, et al. Adipose tissue, diabetes and Chagas disease. Adv Parasitol. 2011;76: 235–50. doi:10.1016/B978-0-12-385895-5.00010-4
228. Ponterio E, Cangemi R, Mariani S, Casella G, De Cesare A, Trovato FM, et al. Adenovirus 36 DNA in human adipose tissue. Int J Obes. Nature Publishing Group; 2015;39: 1761–1764. doi:10.1038/ijo.2015.163
229. Cannon MJ, Schmid DS, Hyde TB. Review of cytomegalovirus seroprevalence and demographic characteristics associated with. Rev Med Virol. 2010;20(4): 202–213. doi:10.1002/rmv
230. Almanzar G, Schwaiger S, Jenewein B, Keller M, Herndler-Brandstetter D, Würzner R, et al. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. J Virol. 2005;79: 3675–83. doi:10.1128/JVI.79.6.3675-3683.2005
231. Chidrawar S, Khan N, Wei W, McLarnon a, Smith N, Nayak L, et al. Cytomegalovirus-seropositivity has a profound influence on the magnitude

of major lymphoid subsets within healthy individuals. Clin Exp Immunol. 2009;155: 423–32. doi:10.1111/j.1365-2249.2008.03785.x
232. Loewendorf A, Benedict CA. Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything. J Intern Med. 2010;267: 483–501. doi:10.1111/j.1365-2796.2010.02220.x
233. Goodrum F. Human Cytomegalovirus Latency: Approaching the Gordian Knot. Annu Rev Virol. 2016;3: 333–357. doi:10.1146/annurev-virology-110615-042422
234. Vassallo J, Huguet F, Brousset P. “In situ” detection of human cytomegalovirus infection of bone marrow in a patient previously treated for B-prolymphocytic leukaemia. J Clin Pathol. 2007;60: 839–40. doi:10.1136/jcp.2005.033936
235. Swanson EC, Schleiss MR. Congenital cytomegalovirus infection: new prospects for prevention and therapy. Pediatr Clin North Am. 2013;60: 335–49. doi:10.1016/j.pcl.2012.12.008
236. Faber DW, Wiley CA, Lynn GB, Gross JG, Freeman WR. Role of HIV and CMV in the pathogenesis of retinitis and retinal vasculopathy in AIDS patients. Invest Ophthalmol Vis Sci. 1992;33: 2345–53. Available: http://europepmc.org/abstract/med/1321796
237. Lidehall AK, Sund F, Lundberg T, Eriksson B-M, Tötterman TH, Korsgren O. T cell control of primary and latent cytomegalovirus infections in healthy subjects. J Clin Immunol. 2005;25: 473–81. doi:10.1007/s10875-005-5372-8
238. La Rosa C, Diamond DJ. The immune response to human CMV. Future Virol. 2012;7: 279–293. doi:10.2217/fvl.12.8
239. Wang GC, Dash P, McCullers JA, Doherty PC, Thomas PG. T Cell Receptor αβ Diversity Inversely Correlates with Pathogen-Specific Antibody Levels in Human Cytomegalovirus Infection. Sci Transl Med . 2012;4: 128ra42-128ra42. doi:10.1126/scitranslmed.3003647
240. Sylwester AW. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med. 2005;202: 673–685. doi:10.1084/jem.20050882
241. Vescovini R, Fagnoni FF, Telera AR, Bucci L, Pedrazzoni M, Magalini F, et al. Naïve and memory CD8 T cell pool homeostasis in advanced aging: impact of age and of antigen-specific responses to cytomegalovirus. Age (Dordr). 2014;36: 625–40. doi:10.1007/s11357-013-9594-z
242. Whiting CC, Siebert J, Newman AM, Du H-W, Alizadeh AA, Goronzy J, et al. Large-Scale and Comprehensive Immune Profiling and Functional Analysis of Normal Human Aging. PLoS One. Public Library of Science; 2015;10: e0133627. doi:10.1371/journal.pone.0133627
243. Smithey MJ, Li G, Venturi V, Davenport MP, Nikolich-Zugich J, Nikolich-Žugich J. Lifelong persistent viral infection alters the naive T cell pool, impairing CD8 T cell immunity in late life. J Immunol. 2012;189: 5356–66. doi:10.4049/jimmunol.1201867
244. Looney RJ, Falsey a, Campbell D, Torres a, Kolassa J, Brower C, et al. Role of cytomegalovirus in the T cell changes seen in elderly individuals.

Clin Immunol. 1999;90: 213–9. doi:10.1006/clim.1998.4638
245. Sims S, Bolinger B, Klenerman P. Increasing inflationary T-cell responses following transient depletion of MCMV-specific memory T cells. Eur J Immunol. 2015;45: 113–8. doi:10.1002/eji.201445016
246. Rowe W, Hartley J, Cramblett H, Mastrota F. Detection of human salivary gland virus in the mouth and urine of children. Am J Hyg. 1958;67: 57–65. Available: http://www.ncbi.nlm.nih.gov/pubmed/13508653
247. Zanghellini F, Boppana SB, Emery VC, Griffiths PD, Pass RF. Asymptomatic primary cytomegalovirus infection: virologic and immunologic features. J Infect Dis. 1999;180: 702–7. doi:10.1086/314939
248. Kurz SK, Rapp M, Steffens H-P, Grzimek NKA, Schmalz S, Reddehase MJ. Focal Transcriptional Activity of Murine Cytomegalovirus during Latency in the Lungs. J Virol. 1999;73: 482–494. Available: http://jvi.asm.org/content/73/1/482.abstract?ijkey=32a35ee30bdb4c775f798c6277cadba197eedaec&keytype2=tf_ipsecsha
249. de Jongste AHC, de Graaf MT, van den Broek PDM, Kraan J, Sillevis Smitt PAE, Gratama JW. Effector memory and late memory T cells accumulate in the blood of CMV-carrying individuals but not in their cerebrospinal fluid. Cytom Part B Clin Cytom. Wiley-Blackwell; 2013;84B: 218–221. doi:10.1002/cyto.b.21073
250. Osborn O, Olefsky JM. The cellular and signaling networks linking the immune system and metabolism in disease. Nat Med. Nature Publishing Group; 2012;18: 363–74. doi:10.1038/nm.2627
251. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011;11: 85–97. doi:10.1038/nri2921
252. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011;11: 98–107. doi:10.1038/nri2925
253. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest. American Society for Clinical Investigation; 2007;117: 175–84. doi:10.1172/JCI29881
254. Mathis D, Shoelson SE. Immunometabolism: an emerging frontier. Nat Rev Immunol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2011;11: 81. doi:10.1038/nri2922
255. Firth C, Harrison R, Ritchie S, Wardlaw J, Ferro CJ, Starr JM, et al. Cytomegalovirus infection is associated with an increase in systolic blood pressure in older individuals. QJM. Oxford University Press; 2016;109: 595–600. doi:10.1093/qjmed/hcw026
256. Rogers PM, Mashtalir N, Rathod MA, Dubuisson O, Wang Z, Dasuri K, et al. Metabolically favorable remodeling of human adipose tissue by human adenovirus type 36. Diabetes. 2008;57: 2321–31. doi:10.2337/db07-1311
257. Brestoff JR, Artis D. Immune Regulation of Metabolic Homeostasis in Health and Disease. Cell. 2015;161: 146–160.

doi:10.1016/j.cell.2015.02.022
258. Milner K-L, Jenkins AB, Trenell M, Tid-Ang J, Samocha-Bonet D, Weltman M, et al. Eradicating hepatitis C virus ameliorates insulin resistance without change in adipose depots. J Viral Hepat. 2014;21: 325–32. doi:10.1111/jvh.12143
259. Nagajyothi F, Desruisseaux MS, Weiss LM, Chua S, Albanese C, Machado FS, et al. Chagas disease, adipose tissue and the metabolic syndrome. Memórias do Inst Oswaldo Cruz. 2009;104 Suppl: 219–25. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3008359&tool=pmcentrez&rendertype=abstract
260. Damouche A, Lazure T, Avettand-Fènoël V, Huot N, Dejucq-Rainsford N, Satie A-P, et al. Adipose Tissue Is a Neglected Viral Reservoir and an Inflammatory Site during Chronic HIV and SIV Infection. PLoS Pathog. Public Library of Science; 2015;11: e1005153. doi:10.1371/journal.ppat.1005153
261. Price P, Eddy KS, Papadimitriou JM, Robertson TA, Shellam GR. Cytomegalovirus infection of adipose tissues induces steatitis in adult mice. Int J Exp Pathol. Wiley-Blackwell; 1990;71: 557–71. Available: http://www.ncbi.nlm.nih.gov/pubmed/2169300
262. Sacher T, Andrassy J, Kalnins A, Dölken L, Jordan S, Podlech J, et al. Shedding Light on the Elusive Role of Endothelial Cells in Cytomegalovirus Dissemination. Nelson JA, editor. Public Library of Science; 2011;7. doi:10.1371/journal.ppat.1002366
263. Kang YE, Kim JM, Joung KH, Lee JH, You BR, Choi MJ, et al. The Roles of Adipokines, Proinflammatory Cytokines, and Adipose Tissue Macrophages in Obesity-Associated Insulin Resistance in Modest Obesity and Early Metabolic Dysfunction. López Lluch G, editor. PLoS One. Public Library of Science; 2016;11: e0154003. doi:10.1371/journal.pone.0154003
264. Wheatley KE, Nogueira LM, Perkins SN, Hursting SD. Differential effects of calorie restriction and exercise on the adipose transcriptome in diet-induced obese mice. J Obes. Hindawi; 2011;2011: 265417. doi:10.1155/2011/265417
265. Daley-Bauer LP, Roback LJ, Wynn GM, Mocarski ES. Cytomegalovirus Hijacks CX3CR1hi Patrolling Monocytes as Immune-Privileged Vehicles for Dissemination in Mice. Cell Host Microbe. Cell Press; 2014;15: 351–362. doi:10.1016/J.CHOM.2014.02.002
266. Stoddart CA, Cardin RD, Boname JM, Manning WC, Abenes GB, Mocarski ES. Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. J Virol. American Society for Microbiology Journals; 1994;68: 6243–53. Available: http://www.ncbi.nlm.nih.gov/pubmed/8083964
267. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese R V, Broxmeyer HE, et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J Clin Invest. 1997;100: 2552–2561. doi:10.1172/JCI119798
268. Schenkel JM, Masopust D. Tissue-Resident Memory T Cells. Immunity.

Cell Press; 2014;41: 886–897. doi:10.1016/J.IMMUNI.2014.12.007
269. Shin H, Iwasaki A. Tissue-resident memory T cells. Immunol Rev. NIH Public Access; 2013;255: 165–81. doi:10.1111/imr.12087
270. Anderson KG, Mayer-Barber K, Sung H, Beura L, James BR, Taylor JJ, et al. Intravascular staining for discrimination of vascular and tissue leukocytes. Nat Protoc. NIH Public Access; 2014;9: 209–22. doi:10.1038/nprot.2014.005
271. Teng K-T, Chang C-Y, Chang LF, Nesaretnam K. Modulation of obesity-induced inflammation by dietary fats: mechanisms and clinical evidence. Nutr J. BioMed Central; 2014;13: 12. doi:10.1186/1475-2891-13-12
272. Mathis D. Immunological goings-on in visceral adipose tissue. Cell Metab. Elsevier; 2013;17: 851–9. doi:10.1016/j.cmet.2013.05.008
273. Slobedman B, Mocarski ES. Mechanisms modulating immune clearance during human cytomegalovirus latency. doi:10.1073/pnas.1212245109
274. Kim JH, Collins-Mcmillen D, Buehler JC, Goodrum FD, Yurochko AD. Human Cytomegalovirus Requires Epidermal Growth Factor Receptor Signaling To Enter and Initiate the Early Steps in the Establishment of Latency in CD34+ Human Progenitor Cells. 2016; doi:10.1128/JVI.01206-16
275. Han S-J, Glatman Zaretsky A, Andrade-Oliveira V, Collins N, Dzutsev A, Shaik J, et al. White Adipose Tissue Is a Reservoir for Memory T Cells and Promotes Protective Memory Responses to Infection. Immunity. Elsevier; 2017;47: 1154-1168.e6. doi:10.1016/j.immuni.2017.11.009
276. Kolodin D, van Panhuys N, Li C, Magnuson AM, Cipolletta D, Miller CM, et al. Antigen- and cytokine-driven accumulation of regulatory T cells in visceral adipose tissue of lean mice. Cell Metab. Elsevier; 2015;21: 543–57. doi:10.1016/j.cmet.2015.03.005
277. Sacher T, Andrassy J, Kalnins A, Dö Lken L, Jordan S. Shedding Light on the Elusive Role of Endothelial Cells in Cytomegalovirus Dissemination. PLoS Pathog. 2011;7: 1002366. doi:10.1371/journal.ppat.1002366
278. Purdy JG, Shenk T, Rabinowitz JD. Fatty acid elongase 7 catalyzes lipidome remodeling essential for human cytomegalovirus replication. Cell Rep. NIH Public Access; 2015;10: 1375–85. doi:10.1016/j.celrep.2015.02.003
279. Yu Y, Maguire TG, Alwine JC. Human Cytomegalovirus Infection Induces Adipocyte-Like Lipogenesis through Activation of Sterol Regulatory Element Binding Protein 1. J Virol. 2012;86: 2942–2949. doi:10.1128/JVI.06467-11
280. Zwezdaryk KJ, Ferris MB, Strong AL, Morris CA, Bunnell BA, Dhurandhar N V, et al. Human cytomegalovirus infection of human adipose-derived stromal/stem cells restricts differentiation along the adipogenic lineage. Adipocyte. Taylor & Francis; 2016;5: 53–64. doi:10.1080/21623945.2015.1119957
281. Verma S, Wang Q, Chodaczek G, Benedict CA. Lymphoid-tissue stromal cells coordinate innate defense to cytomegalovirus. J Virol. American Society for Microbiology; 2013;87: 6201–10. doi:10.1128/JVI.00113-13

282. Jordan S, Krause J, Prager A, Mitrovic M, Jonjic S, Koszinowski UH, et al. Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced growth in salivary Glands due to a fixed mutation of MCK-2. J Virol. 2011;85: 10346–53. doi:10.1128/JVI.00545-11
283. Sitnik KM, Wendland K, Weishaupt H, Uronen-Hansson H, White AJ, Anderson G, et al. Context-Dependent Development of Lymphoid Stroma from Adult CD34+ Adventitial Progenitors. Cell Rep. Cell Press; 2016;14: 2375–2388. doi:10.1016/J.CELREP.2016.02.033
284. Simon CO, Seckert CK, Dreis D, Reddehase MJ, Grzimek NKA. Role for tumor necrosis factor alpha in murine cytomegalovirus transcriptional reactivation in latently infected lungs. J Virol. American Society for Microbiology (ASM); 2005;79: 326–40. doi:10.1128/JVI.79.1.326-340.2005
285. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care. American Diabetes Association; 2004;27: 1487–95. doi:10.2337/DIACARE.27.6.1487
286. Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010;328: 321–326. doi:10.1126/science.1172539
287. Parmigiani A, Alcaide ML, Freguja R, Pallikkuth S, Frasca D, Fischl M a., et al. Impaired antibody response to influenza vaccine in HIV-infected and uninfected aging women is associated with immune activation and inflammation. PLoS One. 2013;8: e79816. doi:10.1371/journal.pone.0079816
288. Ortman BJM, Velkoff VA, Hogan H. An Aging Nation : The Older Population in the United States. Curr Popul Reports. 2014;1964.
289. Koup RA, Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature. Nature Publishing Group; 1998;396: 690–695. doi:10.1038/25374
290. Sempowski GD, Gooding ME, Liao HX, Le PT, Haynes BF. T cell receptor excision circle assessment of thymopoiesis in aging mice. Mol Immunol. 2002;38: 841–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/11922942
291. Rudd BD, Venturi V, Li G, Samadder P, Ertelt JM, Way SS, et al. Nonrandom attrition of the naive CD8+ T-cell pool with aging governed by T-cell receptor:pMHC interactions. Proc Natl Acad Sci U S A. 2011;108: 13694–13699. doi:10.1073/pnas.1107594108
292. Naylor K, Li G, Vallejo AN, Lee W-W, Koetz K, Bryl E, et al. The influence of age on T cell generation and TCR diversity. J Immunol. 2005;174: 7446–7452. doi:10.4049/jimmunol.174.11.7446
293. Ferrando-Martínez S, Ruiz-Mateos E, Hernández A, Gutiérrez E, Rodríguez-Méndez M del M, Ordoñez A, et al. Age-related deregulation of naive T cell homeostasis in elderly humans. Age (Omaha). Springer Netherlands; 2011;33: 197–207. doi:10.1007/s11357-010-9170-8
294. Ferrando-Martínez S, Romero-Sánchez MC, Solana R, Delgado J, de la Rosa R, Muñoz-Fernández MÁ, et al. Thymic function failure and C-reactive protein levels are independent predictors of all-cause mortality in healthy elderly humans. Age (Omaha). Springer Netherlands; 2013;35:

251–259. doi:10.1007/s11357-011-9341-2
295. Rezzani R, Nardo L, Favero G, Peroni M, Rodella LF. Thymus and aging: morphological, radiological, and functional overview. Age (Omaha). Springer Netherlands; 2014;36: 313–351. doi:10.1007/s11357-013-9564-5
296. Becklund BR, Purton JF, Ramsey C, Favre S, Vogt TK, Martin CE, et al. The aged lymphoid tissue environment fails to support naïve T cell homeostasis. Sci Rep. 2016;6: 30842. doi:10.1038/srep30842
297. Heilbronn LK, Ravussin E. Calorie restriction and aging: review of the literature and implications for studies in humans. Am J Clin Nutr. 2003;78: 361–369. Available: http://ajcn.nutrition.org/content/78/3/361.short
298. Meydani SN, Das SK, Pieper CF, Lewis MR, Klein S, Dixit VD, et al. Long-term moderate calorie restriction inhibits inflammation without impairing cell-mediated immunity: a randomized controlled trial in non-obese humans. Aging (Albany NY). Impact Journals, LLC; 2016;8: 1416–31. doi:10.18632/aging.100994
299. Messaoudi I, Warner J, Fischer M, Park B, Hill B, Mattison J, et al. Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates. Proc Natl Acad Sci. National Acad Sciences; 2006;103: 19448–19453. doi:10.1073/pnas.0606661103
300. Sun D, Muthukumar AR, Lawrence RA, Fernandes G. Effects of calorie restriction on polymicrobial peritonitis induced by cecum ligation and puncture in young C57BL/6 mice. Clin Diagn Lab Immunol. American Society for Microbiology; 2001;8: 1003–11. doi:10.1128/CDLI.8.5.1003-1011.2001
301. Gardner EM. Caloric restriction decreases survival of aged mice in response to primary influenza infection. J Gerontol A Biol Sci Med Sci. 2005;60: 688–94. Available: http://www.ncbi.nlm.nih.gov/pubmed/15983169
302. Kristan DM. Chronic calorie restriction increases susceptibility of laboratory mice (Mus musculus) to a primary intestinal parasite infection. Aging Cell. 2007;6: 817–25. doi:10.1111/j.1474-9726.2007.00345.x
303. Weindruch R, Lane MA, Ingram DK, Ershler WB, Roth GS. Dietary restriction in rhesus monkeys: lymphopenia and reduced mitogen-induced proliferation in peripheral blood mononuclear cells. Aging (Milano). 1997;9: 304–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/9359942
304. Fryar CD, Carroll MD, Ogden CL. Prevalence of Overweight, Obesity, and Extreme Obesity Among Adults: United States, Trends 1960–1962 Through 2009–2010. 2012; Available: https://www.cdc.gov/nchs/data/hestat/obesity_adult_09_10/obesity_adult_09_10.pdf
305. Hambly C, Rauw W, Speakman JR. Mice that gorged during dietary restriction increased foraging related behaviors and differed in their macronutrient preference when released from restriction. 2015; doi:10.7717/peerj.1091
306. Mahoney LB, Denny CA, Seyfried TN. Caloric restriction in C57BL/6J mice mimics therapeutic fasting in humans. Lipids Health Dis. BioMed Central;

2006;5: 13. doi:10.1186/1476-511X-5-13
307. Wing EJ, Magee DM, Barczynski LK. Acute starvation in mice reduces the number of T cells and suppresses the development of T-cell-mediated immunity. Immunology. Wiley-Blackwell; 1988;63: 677–82. Available: http://www.ncbi.nlm.nih.gov/pubmed/3259207
308. Weindruch R, Sohal RS. Caloric Intake and Aging. N Engl J Med. NIH Public Access; 1997;337: 986–94. doi:10.1056/NEJM199710023371407
309. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Simmons H a, Kemnitz JW, et al. recommendations. Ongoing full genome sequenc- ing will monitor for the possibility of future reas- sortment events (39). Science (80-). 2009; 201–204.
310. Nikolić-Žugić J. Phenotypic and functional stages in the intrathymic development of αβ T cells. Immunol Today. Elsevier Current Trends; 1991;12: 65–70. doi:10.1016/0167-5699(91)90160-U
311. Godfrey DI, Kennedy J, Suda T, Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol. 1993;150: 4244–52. Available: http://www.ncbi.nlm.nih.gov/pubmed/8387091
312. Nolz JC, Starbeck-Miller GR, Harty JT. Naive, effector and memory CD8 T-cell trafficking: parallels and distinctions. Immunotherapy. NIH Public Access; 2011;3: 1223–33. doi:10.2217/imt.11.100
313. Thompson HL, Smithey MJ, Surh CD, Nikolich-Žugich J. Functional and Homeostatic Impact of Age-Related Changes in Lymph Node Stroma. Front Immunol. Frontiers; 2017;8: 706. doi:10.3389/fimmu.2017.00706
314. Liao C-Y, Rikke BA, Johnson TE, Diaz V, Nelson JF. Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening. Aging Cell. 2010;9: 92–95. doi:10.1111/j.1474-9726.2009.00533.x
315. Cheng CW, Adams GB, Perin L, Wei M, Zhou X, Lam BS, et al. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell- based regeneration and reverse immunosuppression. Cell Stem Cell. Elsevier Inc.; 2014;14: 810–823. doi:10.1016/j.stem.2014.04.014
316. Johnson SC, Rabinovitch PS, Kaeberlein M. mTOR is a key modulator of ageing and age-related disease. Nature. 2013;493: 338–45. doi:10.1038/nature11861
317. Ehninger D, Neff F, Xie K. Longevity, aging and rapamycin. Cell Mol Life Sci. Springer; 2014;71: 4325–46. doi:10.1007/s00018-014-1677-1
318. Stahl FR, Keyser KA, Heller K, Bischoff Y, Halle S, Wagner K, et al. Mck2-dependent infection of alveolar macrophages promotes replication of MCMV in nodular inflammatory foci of the neonatal lung. Mucosal Immunol. 2015;8: 57–67. doi:10.1038/mi.2014.42
319. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. Nature Publishing Group; 2013;496: 445–455. doi:10.1038/nature12034
320. Gimble JM, Katz AJ, Bunnell BA. Adipose-Derived Stem Cells for

Regenerative Medicine. Circ Res. 2007;100: 1249–1260. doi:10.1161/01.RES.0000265074.83288.09
321. Purdy JG, Xi Y, Harwood S, Wise L. Human Cytomegalovirus Remodeling of Lipid Metabolism Requires pUL37x1. bioRxiv. Cold Spring Harbor Laboratory; 2019; 526228. doi:10.1101/526228
322. Seckert CK, Schader SI, Ebert S, Thomas D, Freitag K, Renzaho A, et al. Antigen-presenting cells of haematopoietic origin prime cytomegalovirus-specific CD8 T-cells but are not sufficient for driving memory inflation during viral latency. J Gen Virol. 2011;92: 1994–2005. doi:10.1099/vir.0.031815-0
323. Torti N, Walton SM, Brocker T, Rülicke T, Oxenius A. Non-Hematopoietic Cells in Lymph Nodes Drive Memory CD8 T Cell Inflation during Murine Cytomegalovirus Infection. Hill AB, editor. PLoS Pathog. 2011;7: e1002313. doi:10.1371/journal.ppat.1002313
324. Majka SM, Miller HL, Helm KM, Acosta AS, Childs CR, Kong R, et al. Analysis and isolation of adipocytes by flow cytometry. Methods Enzymol. 2014;537: 281–96. doi:10.1016/B978-0-12-411619-1.00015-X
325. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. 2010;13: 133–140. doi:10.1038/nn.2467
326. Daley-Bauer LP, Roback LJ, Wynn GM, Mocarski ES. Cytomegalovirus Hijacks CX3CR1hi Patrolling Monocytes as Immune-Privileged Vehicles for Dissemination in Mice. Cell Host Microbe. 2014;15: 351–362. doi:10.1016/j.chom.2014.02.002
327. Collins TM, Quirk MR, Jordan MC. Biphasic viremia and viral gene expression in leukocytes during acute cytomegalovirus infection of mice. J Virol. 1994;68: 6305–11. Available: http://www.ncbi.nlm.nih.gov/pubmed/8083970
328. Farrell HE, Bruce K, Lawler C, Oliveira M, Cardin R, Davis-Poynter N, et al. Murine Cytomegalovirus Spreads by Dendritic Cell Recirculation. Bennink JR, editor. MBio. 2017;8. doi:10.1128/mBio.01264-17
329. Hsu KM, Pratt JR, Akers WJ, Achilefu SI, Yokoyama WM. Murine cytomegalovirus displays selective infection of cells within hours after systemic administration. J Gen Virol. 2009;90: 33–43. doi:10.1099/vir.0.006668-0
330. Hwangbo C, Wu J, Papangeli I, Adachi T, Sharma B, Park S, et al. Endothelial APLNR regulates tissue fatty acid uptake and is essential for apelin's glucose-lowering effects. Sci Transl Med. American Association for the Advancement of Science; 2017;9: eaad4000. doi:10.1126/scitranslmed.aad4000
331. Payne S, De Val S, Neal A. Endothelial-Specific Cre Mouse Models. Arterioscler Thromb Vasc Biol. Lippincott Williams & WilkinsHagerstown, MD; 2018;38: 2550–2561. doi:10.1161/ATVBAHA.118.309669
332. Chen HI, Sharma B, Akerberg BN, Numi HJ, Kivelä R, Saharinen P, et al. The sinus venosus contributes to coronary vasculature through VEGFC-

stimulated angiogenesis. Development. Oxford University Press for The Company of Biologists Limited; 2014;141: 4500–12. doi:10.1242/dev.113639
333. Pi J, Cheng Y, Sun H, Chen X, Zhuang T, Liu J, et al. Apln-CreERT:mT/mG reporter mice as a tool for sprouting angiogenesis study. BMC Ophthalmol. BioMed Central; 2017;17: 163. doi:10.1186/s12886-017-0556-6
334. Caposio P, Orloff SL, Streblow DN. The role of cytomegalovirus in angiogenesis. Virus Res. NIH Public Access; 2011;157: 204–11. doi:10.1016/j.virusres.2010.09.011
335. Chanouzas D, Dyall L, Nightingale P, Ferro C, Moss P, Morgan MD, et al. Valaciclovir to prevent Cytomegalovirus mediated adverse modulation of the immune system in ANCA-associated vasculitis (CANVAS): study protocol for a randomised controlled trial. Trials. BioMed Central; 2016;17: 338. doi:10.1186/s13063-016-1482-2
336. Chen S, de Craen AJ, Raz Y, Derhovanessian E, Vossen AC, Rudi WG, et al. Cytomegalovirus seropositivity is associated with glucose regulation in the oldest old. Results from the Leiden 85-plus Study. Immun Ageing. BioMed Central; 2012;9: 18. doi:10.1186/1742-4933-9-18
337. Hsich E, Zhou YF, Paigen B, Johnson TM, Burnett MS, Epstein SE. Cytomegalovirus infection increases development of atherosclerosis in Apolipoprotein-E knockout mice. Atherosclerosis. 2001;156: 23–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/11368993
338. Šestan M, Marinović S, Kavazović I, Cekinović Đ, Wueest S, Turk Wensveen T, et al. Virus-Induced Interferon-γ Causes Insulin Resistance in Skeletal Muscle and Derails Glycemic Control in Obesity. Immunity. Elsevier; 2018;49: 164-177.e6. doi:10.1016/j.immuni.2018.05.005
339. Marik PE, Bellomo R. Stress hyperglycemia: an essential survival response! Crit Care. BioMed Central; 2013;17: 305. doi:10.1186/cc12514
340. Chesler EJ, Miller DR, Branstetter LR, Galloway LD, Jackson BL, Philip VM, et al. The Collaborative Cross at Oak Ridge National Laboratory: developing a powerful resource for systems genetics. Mamm Genome. NIH Public Access; 2008;19: 382–9. doi:10.1007/s00335-008-9135-8
341. Fleck-Derderian S, McClellan W, Wojcicki JM. The association between cytomegalovirus infection, obesity, and metabolic syndrome in U.S. adult females. Obesity. 2017;25: 626–633. doi:10.1002/oby.21764
342. Levine TB, Levine AB. Metabolic Syndrome and Cardiovascular Disease [Internet]. John Wiley & Sons; 2012. Available: https://books.google.com/books?id=sl4KJOgnHj4C&pgis=1
343. Sherling DH, Perumareddi P, Hennekens CH. Metabolic Syndrome. J Cardiovasc Pharmacol Ther. SAGE PublicationsSage CA: Los Angeles, CA; 2017;22: 365–367. doi:10.1177/1074248416686187
344. Choi IY, Lee C, Longo VD. Nutrition and fasting mimicking diets in the prevention and treatment of autoimmune diseases and immunosenescence. Mol Cell Endocrinol. NIH Public Access; 2017;455: 4–12. doi:10.1016/j.mce.2017.01.042

345. Brandhorst S, Choi IY, Wei M, Cheng CW, Sedrakyan S, Navarrete G, et al. A Periodic Diet that Mimics Fasting Promotes Multi-System Regeneration, Enhanced Cognitive Performance, and Healthspan. Cell Metab. 2015;22: 86–99. doi:10.1016/j.cmet.2015.05.012
346. Devlin MJ. Why does starvation make bones fat? Am J Hum Biol. NIH Public Access; 2011;23: 577–85. doi:10.1002/ajhb.21202
347. Bathija A, Davis S, Trubowitz S. Bone marrow adipose tissue: Response to acute starvation. Am J Hematol. John Wiley & Sons, Ltd; 1979;6: 191–198. doi:10.1002/ajh.2830060303
348. Devlin MJ, Cloutier AM, Thomas NA, Panus DA, Lotinun S, Pinz I, et al. Caloric restriction leads to high marrow adiposity and low bone mass in growing mice. J Bone Miner Res. John Wiley & Sons, Ltd; 2010;25: 2078–2088. doi:10.1002/jbmr.82
349. Baek K, Park H-J, Hwang HR, Baek J-H. Propranolol attenuates calorie restriction- and high calorie diet-induced bone marrow adiposity. BMB Rep. Korean Society for Biochemistry and Molecular Biology; 2014;47: 587–92. doi:10.5483/BMBREP.2014.47.10.176
350. Qiao G, Bucsek MJ, Winder NM, Chen M, Giridharan · Thejaswini, Olejniczak SH, et al. β-Adrenergic signaling blocks murine CD8 + T-cell metabolic reprogramming during activation: a mechanism for immunosuppression by adrenergic stress. Cancer Immunol Immunother. 2019;68: 11–22. doi:10.1007/s00262-018-2243-8
351. Khan SH, Badovinac VP. Listeria monocytogenes: a model pathogen to study antigen-specific memory CD8 T cell responses. Semin Immunopathol. NIH Public Access; 2015;37: 301–10. doi:10.1007/s00281-015-0477-5