THESIS AND ABSTRACT

The Role of Autophagy in CD8$^+$ T cell Immunity

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, University of Oxford

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Abstract

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CD8$^+$ T cells form a crucial arm of the adaptive immune system and act as sentinels against infection and cancer. The role of autophagy, a major lysosomal degradation pathway, in T cell biology is currently limited. Here, we show that autophagy is required for naïve CD8$^+$ T cell homeostasis. When the essential autophagy gene *Atg7* is deleted in the T cell lineage (T-Atg7$^{-/-}$ mice), mice develop lymphopaenia leading to increased CD8$^+$ T cell homeostatic proliferation. When CD8$^+$ T cells encounter antigen, they undergo an initial expansion before contracting, leaving behind a population of long-lived memory cells that provide long-lasting immunity. How CD8$^+$ T cells utilise autophagy when responding to infection is currently unknown. We demonstrate that autophagy is dispensable for CD8$^+$ T cell expansion, but loss of *Atg7* results in the failure to establish CD8$^+$ T cell memory to influenza and MCMV infection. Interestingly, CD8$^+$ T cell memory becomes defective in old age, although the cellular mechanism for this is largely unknown. Here, aged mice were shown to exhibit defective CD8$^+$ T cell expansion and memory formation to immunisation. Autophagy levels were diminished in antigen-specific CD8$^+$ T cells from aged mice and the CD8$^+$ T cell response of old mice to vaccination could be rejuvenated in an autophagy-dependent manner using the compound spermidine. These results suggest that targeting of the autophagy pathway might be beneficial as a route to effective T cell modulation and enhanced immunity to infection and vaccination.

Author's Publications

Puleston, D.J., Zhang, H., Powell, T. J. Lipina, E.Watson, A. S., Sitbon, M., Sims, S., Cerundolo, V., Townsend, A.R.M., Klenerman, P. and Simon, A. K. Autophagy is a critical regulator of memory $CD8^+$ T cell formation. *eLife* 2014;3:e03706 (2014).

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Majid Jabir, Lee Hopkins, Neil Ritchie, Ihsan Ullah, Hannah Bayes, Dong Li, Panagiotis Tourlomousis, Alison Lupton, **Daniel Puleston**, Anna Katharina Simon, and Thomas Evans. Mitochondrial Damage Contributes to *Pseudomonas aeruginosa* Activation of the Inflammasome and Is Down-Regulated By Autophagy. *Autophagy, **11**(1): 166-182* (2015).

Alexander Watson, Thomas Riffelmacher Amanda Stranks, Shivani Khadwala, Michael Lutterop, Petter Woll, Kanchan Phadwal, Monika Mortensen,

Daniel Puleston, David Ferguson, Elizabeth Soilleux, Sten Eirik Jacobsen, Anna Katharina Simon. Deficient autophagy ties haematopoietic stem cell dysfunction to myeloid dysplasia and acute myeloid leukemia. *Cell Death and Discovery (In Press)*.

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Science has been one of the few sustained passions in my life that I have carried with me from early childhood into adulthood. If I were not a scientist in real life, I very much doubt I would spend any less of my time pondering over the mysteries of nature. Therefore, turning this hobby into a career represents somewhat of a dream job. Thankfully, my DPhil has lived up to many of my preconceptions as to what real science is like, although that's not to say I did not suffer my defeats in the 11th hour of 12 hour experiments. I have been very fortunate to receive my training at arguably the best university in the world and my time as a student at Oxford is everything I had hoped it would be and more.

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Abbreviations

Ab	Antibody
ALFY	Autophagy-linked FYVE protein
AMP	Adenosine mono-phosphate
AMPK	AMP-activated protein kinase
APC	Antigen Presenting Cell
Atg	Autophagy-related gene
ATP	Adenosine triphosphate
BCG	Bacillus Calmette–Guérin
Bcl-2	B cell lymphoma 2
Blimp-1	B lymphocyte-induced maturation protein-1
BM	Bone Marrow
BrdU	5-bromo-2-deoxyuridine
CDK	Cyclin-dependent kinase
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cells
DN	Double negative
DP	Double positive
dsRNA	double stranded ribonucleic acid
EOMES	Eomesodermin
ER	Endoplastic reticulum
ESX-1	ESAT-6 secretion system
ETP	Early T lineage progenitor
FA	Fatty acid
FACS	Fluorescent-activated cell sorting
FADD	Fas-Associated protein with Death Domain
FAO	Fatty acid oxidation
FIP200	FAK-family interacting protein of 200 kDa
FOXO	Forkhead box class O
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GLUT-1	Glucose transporter-1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HSCs	Haematopoietic stem cells
HSV	Herpes simplex virus
HTLV	Human T cell leukaemia virus
IFN	Interferon
IGF	Insulin growth factor
IGFR	Insulin Growth Factor Receptor
IL	Interleukin
IRF4	Interferon regulatory factor 4

i.v	Intravenous
KLRG1	killer-cell lectin-like receptor G1
LAMP-2A	Lysosome-associated membrane protein type 2A
Lck	lymphocyte-specific protein tyrosine kinase
LC3	Microtubule-associated protein 1 light chain 3
LCMV	lymphocytic choriomeningitis virus
LIP	Lymphopaenia-induced proliferation
LIR	LC3-interaction region
LPS	Lipopolysaccharide
MCMV	Murine cytomegalovirus
MDA-5	Melanoma Differentiation-Associated protein 5
MDP	Muramyldipeptide
MHC	Major histocompatibility complex
MIICs	MHC class II compartments
MP	Memory phenotype
MPEC	Memory precursor effector cell
mRNA	Messenger ribonucleic acid
mTOR	Mammalian Target of Rapamycin
NAC	N-acetyl cysteine
NIX	NIP3-Like Protein X
NBR1	Neighbor Of BRCA1 Gene 1
NDP52	nuclear dot protein 52 kDa
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain-containing protein
NP	Nucleoprotein
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol myristate acetate
RHEB	Ras homolog enriched in brain
PolyI:C	polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
Rab	Ras-related protein
RAG	Recombination-activating genes
RLR	RIG-like receptor
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
PC	Plasma cells
PD-1	Programmed cell death 1
SCID	Severe combined immunodeficiency
SIV	Simian immunodeficiency virus
SLEC	Short-lived effector cell
SLO	Secondary lymphoid organ
SP	Single positive

Spd	Spermidine
SRC	Spare respiratory capacity
STING	Stimulator of interferon genes
S6K	S6 kinase
TAP	Transporter associated with antigen processing
TCA	Tricarboxylic acid
T_{CM}	Central memory T cell
TCR	T cell receptor
TECs	Thymus epithelial cells
T_{eff}	Effector T cell
T_{EM}	Effector memory T cell
Th	T helper
TIM-3	T cell immunoglobulin and mucin domain
TLR	Toll-like receptor
T_{mem}	memory T cell
TNF	Tumour necrosis factor
Tor	Target of Rapamycin
TRAF6	TNF receptor associated factor 6
T_{RM}	Resident memory T cell
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
ULK1	unc-51 like autophagy activating kinase 1
VPS	Vacuolar protein sorting (family)
WIPI-1/2	WD-repeat domain phosphoinositide-interacting-1/2
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1

Chapter 1: General Introduction

The autophagy pathway and its role in immunity

1. Introduction

1.1. An introduction to autophagy

Cellular homeostasis demands an exquisite balance between synthetic pathways important for cell growth and catabolic processes able to provide essential building blocks and remove unwanted cytosolic components. Eukaryotic cells have evolved two separate mechanisms for the breakdown of intracellular constituents: the proteasomal and autophagy/lysosomal pathways. While the proteasome degrades small, short-lived proteins, autophagy may target large protein aggregates and entire organelles for destruction through their delivery to the lysosome. Its importance is highlighted by its ubiquitous and conserved nature throughout the tree of life, found in nearly identical form from yeast to humans [1] while parallel pathways have also been observed in plants [2] [3].

The term autophagy describes several pathways that differ in their method of delivery to the lysosome – the major catabolic factory in eukaryotic cells. During microautophagy, cytosolic components are degraded through direct invagination of the lysosomal membrane which then pinches off into the lysosomal lumen for degradation [4]. This form of autophagy, thus far only described in yeast, permits maintenance of organelle size, membrane homeostasis, and cell survival during episodes of starvation. Alternatively, chaperone-mediated autophagy is a highly selective process that targets cytosolic proteins directly to lysosomes via the chaperone molecule Hsc70. Target proteins contain a motif recognised by Hsc70 that directs proteins to LAMP-2A that facilitates entry to the lysosome [4]. Classical

macroautophagy (herein referred to as autophagy) involves the formation of an isolation membrane around non-selective areas of the cytoplasm or specific organelles. This isolation membrane is then elongated to form a double-membraned vesicle termed an autophagosome. Autophagosomes subsequently fuse with lysosomes to form the terminal digestive compartment – the autolysosome. Here, the luminal content of the autophagosome is exposed to the low pH and proteases of the lysosome and the degraded contents are recycled back to the cytoplasm through the action of lysosomal membrane permeases [5] (Figure 1.1). Classical macroautophagy will be the major focus of this thesis.

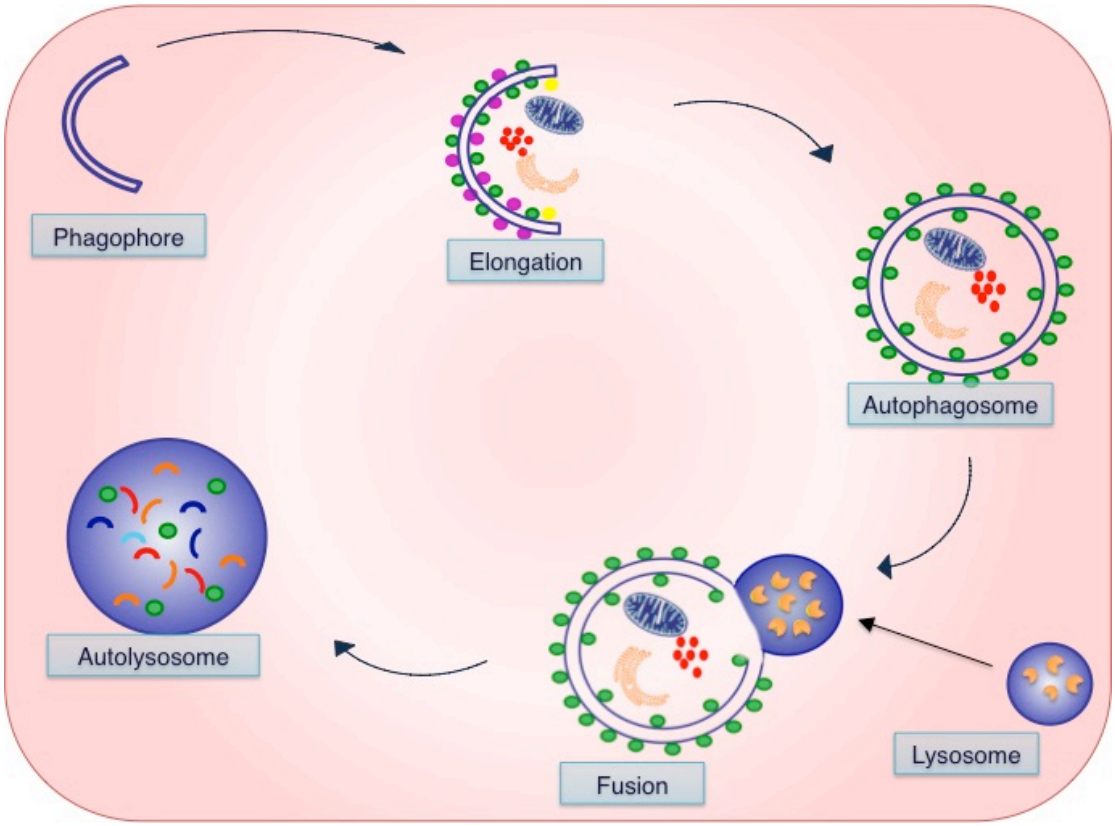


Figure 1.1 *Schematic overview of macroautophagy. During autophagy, cytoplasmic constituents are enclosed in an isolation membrane that is elongated until a double membrane vesicle called an autophagosome is formed. Autophagosomes fuse with lysosomes to form autolysosomes where breakdown of the vesicle contents takes place along with the autophagosome inner membrane.*

When originally described in the 1950s, autophagy was seen as a non-selective bulk degradation pathway activated in response to cell stress and for a while was considered a cell death pathway [6] [7]. However, since the turn of the century a large number of more nuanced functions for the autophagic machinery have come to light and the pathway is now accepted as one mostly promoting cell survival. When one considers its omnipresent nature in a vast number of cell types across an army of eukaryotic species, it is perhaps unsurprising that this ancient process has been implicated in healthy physiology ranging from cell development and differentiation [8,9] to ageing [10] [11] and senescence [12,13].

1.2. Regulation of autophagy

Autophagy is characterized by the large number of regulatory pathways that govern its activity. This complexity reflects sensitivity to a variety of stimuli able to modulate autophagy stretching from nutrient starvation, hypoxia and oxidative stress, to external stimuli such as growth factors and inflammatory cytokines [14-16]. However, many of these factors converge on a common target upstream of the molecular machinery involved in the formation of the autophagosome. The kinase mammalian target of rapamycin (mTOR) occupies this position, acting as a central hub for autophagy modulation (Figure 1.2). mTOR activation relies on several inputs including amino acids, ATP, and insulin growth factor (IGF) receptor signalling [17]. In the presence of growth factor signalling, activated class I phosphoinositide 3-kinase drives activation of Akt. Akt phosphorylates and inhibits the TSC1-TSC2 complex leading to activation of RHEB that in turn stimulates mTOR [18]. Active

mTOR drives protein synthesis and cell growth through S6K and 4E-BP1 [19], while inhibiting catabolism and autophagy by inhibiting the phosphorylation of ULK1, an autophagy initiation factor [20]. Amino acid deprivation results in mTOR inactivation and autophagy induction, as does mTOR inhibition through pharmacological agents such as rapamycin [21].

In addition to class I and III PI3K activity, mTOR can be regulated by cellular energy changes through the AMP-activated protein kinase (AMPK) pathway [22]. AMPK is a sensor of the AMP/ATP ratio within the cell and, during conditions of low energy or metabolic stress, activates the TSC1-TSC2 complex, inhibiting mTOR or directly activating autophagy by phosphorylation of ULK1 and the autophagy protein Beclin 1 [22] (Figure 1.2).

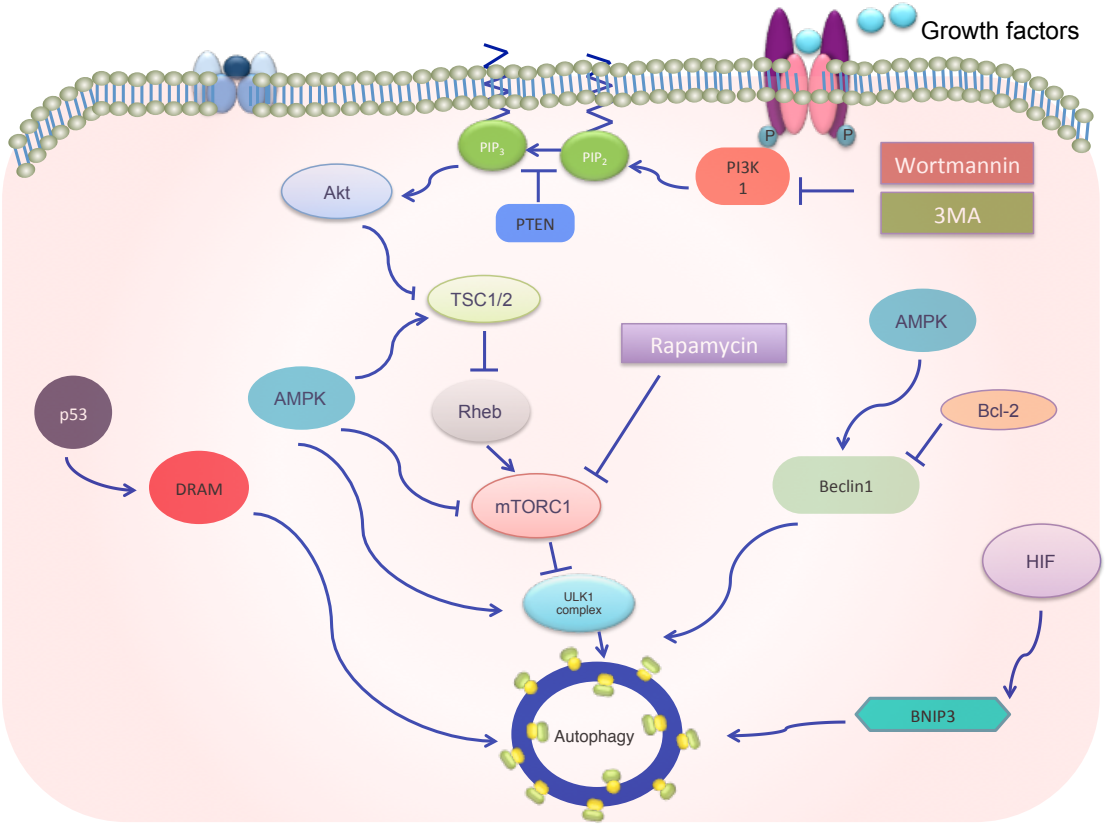


Figure 1.2 *Signalling pathways regulating autophagy. The bulk of autophagy regulation is derived from growth factor receptor signalling. In the presence of nutrients, activated PI3K activates Akt through the production of PIP_3. In turn, Akt activates the TSC1/2 complex*

that activates mTOR through RHEB inhibition. mTOR acts as the primary negative regulator of autophagy and its activation leads to inhibition of the ULK1 complex, preventing autophagy initiation. mTOR inhibitors such as AMPK and the pharmacological agent rapamycin therefore act as positive regulators of autophagy. AMPK achieves this either through direct inhibition of mTOR or through activation of the upstream TSC1/2 complex. AMPK may also positively regulate autophagy through activation of the autophagy-initiating factor Beclin 1. p53 and hypoxia inducible factor (HIF) may also act as positive regulators of autophagy. Pharmacological inhibitors of autophagy such as wortmannin and 3-methyladenine (3MA) function through inhibition of upstream PI3Ks preventing the early stages of autophagy initiation.

1.3. The mammalian autophagy pathway in detail

1.3.1. The autophagy machinery

Little was known about the molecular mechanisms of autophagy until genetic screens in yeast identified approximately 30 autophagy-related genes (Atg) whose products are required for autophagy [23,24]. Since that time, human homologues have been described for many of the yeast Atgs, providing support at the molecular level for the highly conserved nature of autophagy. The Atg proteins are essential for autophagosome formation and are referred to as the core molecular machinery. The core machinery may be divided into four major functional groups: (1) the ULK1-Atg13-FIP200 kinase complex involved in autophagy initiation; (2) the class III PI3K complex consisting of Vps34, Vps15, Beclin1 and Atg14 also important for initiation [25]; (3) two ubiquitin conjugation systems involving LC3 (yeast Atg8), Atg3, Atg5, Atg7, Atg12, and Atg16L1 that are required for elongation of the autophagosomal membrane; and (4) Atg9 and its cycling systems that provide membrane for the growing autophagosome [5].

1.3.2. Autophagosome formation

The core event in autophagy is the formation of the autophagosome. Unlike other membrane trafficking events where transport vesicles are formed from budding donor organelle membranes, as in the endocytic pathway, autophagosomes are formed in the cytoplasm *de novo* by the expansion of a flattened membraned sac known as the phagophore (also known as the isolation membrane). During autophagy initiation, the phagophore elongates and curves until its ends merge to form an autophagosome, enveloping portions of the cytoplasm in the process [26]. How does this take place at the molecular level?

Initiation of autophagy requires stepwise recruitment of autophagy proteins into multi-protein kinase complexes. The primary initiation complex in mammals includes ULK1 (mammalian Atg1 homolog) [27], Atg13 and FIP200 (mammalian Atg17 homolog) [28]. This initial complex activates a PI3K complex that contains Vps34, Vps15 and Beclin1 (mammalian Atg6). This second complex is required for nucleation of the phagophore. Activation of the PI3K complex leads to production of PI3P that is subsequently incorporated into the nascent isolation membrane (Figure 1.3). The presence of PI3P allows for the recruitment of the PI3P-binding effectors WIPI-1 and WIPI-2 (WD-repeat domain phosphoinositide-interacting-1/2). The binding of WIPI-1 and WIPI-2 permits the formation of a *bone fide* phagophore that may then be elongated by other components of the autophagy machinery [5]. The membrane

components required for phagophore and autophagosome formation are recruited under the control of Atg9 [29] along with binding partners Atg2 and WIPI1/2, which are thought to aid Atg9 recruitment to the growing isolation membrane [30]. The membrane provided by Atg9 for autophagosome formation is thought to originate from various sources, including mitochondria, Golgi, the plasma membrane and the endoplasmic reticulum (ER) [31] [23] [32].

Once initiated, phagophore elongation requires the action of two conjugation systems (Figure 1.3). In one, the hydrolysis of ATP by Atg7 leads to activation of Atg12. Subsequently, activated Atg12 is transferred to Atg10 to form an Atg12-Atg10 thioester. Finally, Atg12 is transferred to the target protein Atg5 to form an Atg12-Atg5 conjugate. Atg5 is then further bound noncovalently to Atg16L1 to form the Atg12-Atg5-Atg16L1 complex [18] (Figure 1.3). Once formed, the Atg12-Atg5-Atg16L1 complex is able to bind to the growing autophagosomal membrane where it is thought to be important for regulating curvature of the forming autophagosome [18]. In the second conjugation system, microtubule-associated protein 1 light chain 3 (MAP-LC3, also known as LC3, mammalian Atg8) is conjugated to the membrane lipid phosphatidylethanolamine (PE) (Figure 1.3). Newly synthesised LC3 is proteolytically cleaved by the cysteine protease Atg4. The exposed glycine as a result of this reaction facilitates the binding of LC3 to Atg7. Activated LC3 is then transferred to Atg3, that with the assistance of the Atg12-Atg5-Atg16L1 complex enables the conjugation of LC3 to PE to create its lipidated form, known as LC3-II [18] [33]. In this setting, the Atg12-Atg5-Atg16L1 complex also

specifies the site of LC3 lipidation. Once formed, LC3-II is studded into the isolation membrane and is proposed to play a scaffolding role in membrane growth [34]. LC3-II is distributed symmetrically on both sides of the isolation membrane with the LC3-II that resides on the outer facing membrane is released from the fully formed autophagosome by a second Atg4-dependent cleavage. LC3-II found on the inner membrane remains inside the vesicle and is delivered to the lysosome where it is degraded [35] [36]. The Atg12-Atg5-Atg16L1 also dissociates from the autophagosomal membrane upon vesicle completion.

Interestingly, mammalian cells have six functional LC3 homologs of yeast Atg8, including MAP1LC3A, MAP1LC3B, MAP1LC3C, GABARAP, GABARAPL1, GABARAPL2 (GATE-16) and while the precise functions of each is still to be determined, it appears they may be preferentially processed by different Atg4 homologs [37].

Completed autophagosomes migrate from the cell periphery toward the centriole through the action of microtubules and the dynein motor complex that bring them into proximity with lysosomes [26]. Here, autophagosomes may also fuse with late endosomes to form amphisomes prior to fusion with lysosomes [38] [39]. Fusion with endosomes is believed to provide nascent autophagosomes with machinery that is required for lysosome fusion. Fusion of the amphisome or autophagosome with the lysosome is a Rab7-dependent event [40] and forms the terminal autophagic digestive compartment – the autolysosome. Here, the luminal contents are degraded and recycled back to

the cytosol through lysosomal membrane permeases.

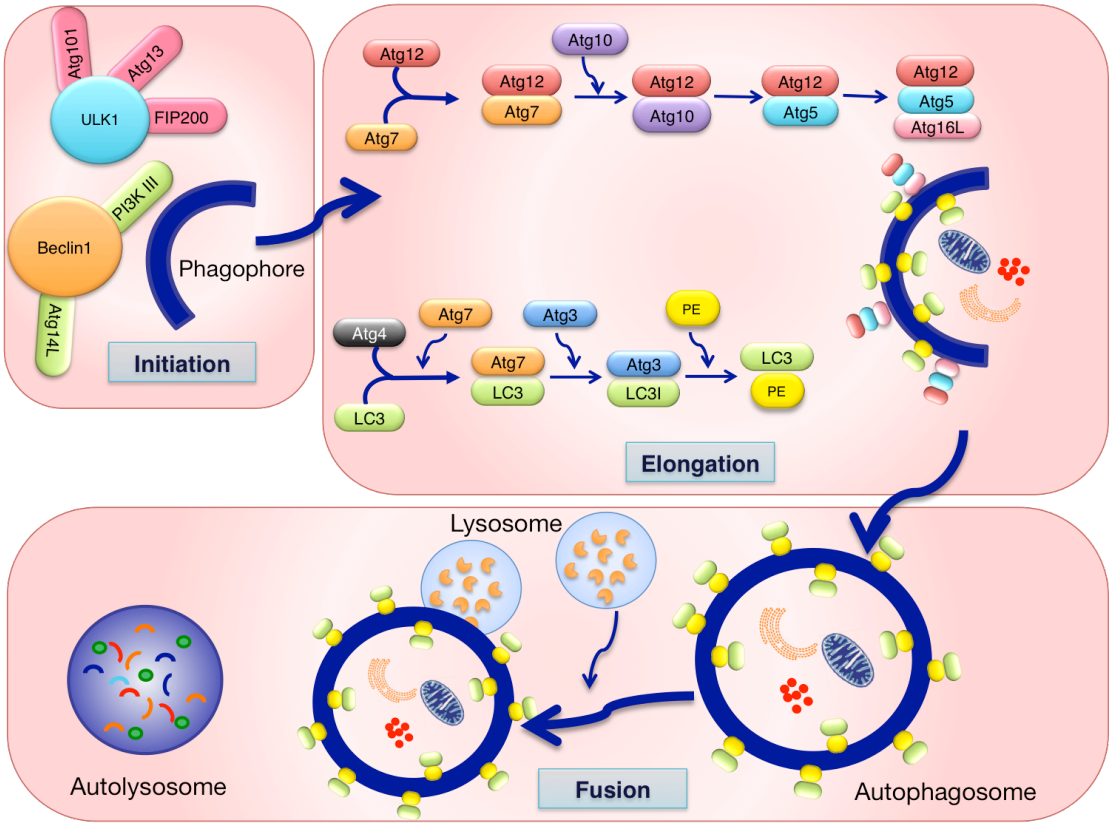


Figure 1.3 *Autophagosome initiation and formation. Autophagy initiation begins with the formation of the ULK1 complex that contains FIP200, Atg13 and Atg101. This complex in turn activates a second complex consisting of PI3Ks, Beclin 1 and Atg14L that facilitates phagophore nucleation. Phagophore elongation is under the control of two ubiquitin-like conjugation systems. In one, Atg12 is activated by Atg7 before being conjugated to Atg10. Atg10 then facilitates the conjugation of Atg12 to Atg5 which is finally non-covalently linked to Atg16L. In the second conjugation pathway, LC3 is cleaved by Atg4 enabling its binding to Atg7 which activates it. Activated LC3 can then bind to Atg3 which results in the conjugation of LC3 to phospoethanolamine (PE) to from lipidated LC3 (LC3-II). The conjugation end products LC3-II and Atg5-Atg12-Atg16L facilitate phagophore elongation to form the autophagosome. This double-membraned compartment then fuses with a lysosome to form the autolysosome where the luminal constituents are degraded.*

1.4. Targets of autophagy

Although autophagy is often considered to be a non-selective process engulfing bulk portions of the cytoplasm, in many settings autophagy can act in a highly prejudiced manner. In this context, autophagy can target damaged

or surplus organelles for degradation, including peroxisomes (pexophagy) [41], ribosomes (ribophagy) [42] and lipid droplets [43]. Another well studied form is mitophagy, the degradation of mitochondria in response to damage, developmental cues [9], oxidative stress, and hypoxia [44]. Autophagy also has a significant role in degradation of accumulated cytosolic proteins that may be superfluous, damaged, or misfolded [45]. In each example of selective autophagy, organelle and protein substrates are targeted through a set of adaptor molecules that link the target to the autophagy machinery [46]. These adaptors include p62 [47], NBR1 [48], and ALFY [49] and all function through their capacity to bind ubiquitin. p62 and NBR1 can also bind to LC3, thus providing a direct link between ubiquitin-tagged proteins and organelles and key autophagy machinery facilitating their degradation [50]. Similarly, the adaptor NIX (BNIP3L) can simultaneously bind LC3 and mitochondrial membrane proteins through its C-terminal domain [51]. Therefore, a sizeable array of molecular adaptors populate the cytosol able to target damaged or unwanted components to the lysosome via recruitment of the autophagy apparatus.

1.5. Outcomes of autophagy

One of the primary objectives of autophagy is to recycle amino acids from intracellular constituents during times of starvation and cell stress. The recycled amino acids are then thought to be used in three ways. Firstly, during extended periods of starvation when carbohydrate stores have been consumed, gluconeogenesis occurs in the liver to generate further glucose, a process which requires amino acids [52]. Secondly, amino acids are processed

through the tricarboxylic acid (TCA) cycle into energy. Finally, amino acids generated through autophagy can be used for protein synthesis, which is vital for cellular adaption during starvation periods [52].

Additionally, autophagy is chiefly in charge of the removal of potentially harmful excess components or waste with deleterious consequences if this task is not performed optimally. For example, a failure to control mitochondrial number and quality results in an accumulation of damaged mitochondria resulting in altered metabolism, increased reactive oxygen species, and inhibited maturation of erythrocytes and other hematopoietic cells [9,53]. Similarly, deletion of Atgs or p62 causes protein aggregates to accumulate in the cytoplasm [54], which have been implicated in aging, neurological disease [55], and tumorigenesis [56]. Thus, autophagy is a key survival pathway and its absence drives a plethora of abnormalities that often results in severe cellular malfunction or disease.

Autophagy in the Immune system

Functionally, autophagy was first described as a cellular response in rats to numerous stressors such as potassium deficiency and starvation [57]. However, there is now good reason to believe that autophagy forms the most ancient of immune defences. In the early evolution of eukaryotic cells its action was likely pivotal in protecting the cytosol from invading foreign organisms. An evolutionary hangover of this primitive immune function can perhaps be observed in the effective removal of mitochondria by autophagy. Mitochondria are organelles that have evolved from a *Rickettsia*-like α-

protobacterium that at one time were foreign to the cytoplasm of eukaryotic cells [58]. With the onset of complex life and intricate vertebrate immune systems, autophagy has evolved a multifaceted role becoming a *bona fide* regulator and effector of the immune system (Figure 1.4).

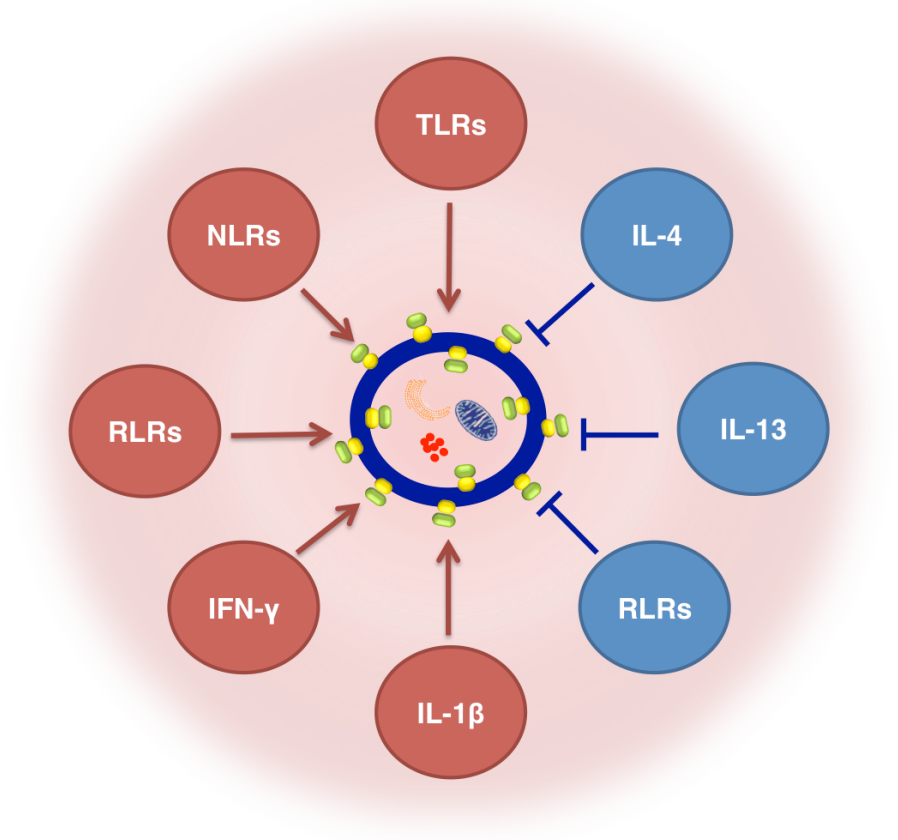


Figure 1.4 *Regulation of autophagy by the immune system. Autophagy is under the control of a host of immune factors suggesting its role as an immune effector mechanism. Autophagy lies downstream of multiple TLRs and is also induced by NLR signalling in the presence of bacteria. During viral infection, RLRs may initiate autophagy, although evidence exists that this pathway also exerts a negative influence on autophagy induction. The cytokines IFN-γ and IL-1β can also augment autophagy while IL-4 and IL-13 inhibit it, aligning autophagy with T_H1-mediated immunity.*

1.6. Autophagy in innate immunity

1.6.1. Autophagy and pattern recognition receptors

Pattern recognition receptors (PRRs) comprise a group of innate receptors responsible for detection of invading pathogens through the recognition of motifs specific to the foreign microbe. These motifs are collectively termed pathogen-associated molecular patterns (PAMPs) and stimulate a myriad of effector responses when bound to their cognate PRR [59]. Toll-like receptors (TLRs) are a group of PRRs strongly connected to the autophagy pathway. TLR4 is able to induce autophagy in murine macrophages following stimulation with lipopolysaccharide (LPS) [60]. Similarly, LPS stimulation of TLR4 was shown to increase the clearance of *Mycobacterium tuberculosis* by autophagy [61]. Induction of autophagy by TLR9 following stimulation with CpG-rich DNA has also been reported [62] [63]. Autophagy not only acts downstream of TLR signalling, but can also play a role in facilitating recognition of PAMPS by TLRs. Lee *et al*. showed that autophagy was able to deliver viral ligands to TLR7 in plasmacytoid dendritic cells (pDCs) following vesicular stomatitis virus and Sendai virus infection resulting in type I IFN production [64].

NOD-like receptors (NLRs) are a group of innate receptors responsible for intracellular bacteria sensing and represent a second class of PRRs with links to autophagy. Activation of the NLRs nucleotide-binding oligomerisation domain 1 (NOD1) and NOD2 in mice leads to recruitment of Atg16L1 at the plasma membrane to the site entry of invading *Shigella flexneri* and *Listeria monocytogenes*, resulting in their sequestration in autophagosomes and

subsequent destruction [65]. In humans, the treatment of DCs with the NOD2 ligand muramyldipeptide (MDP) has been shown to induce autophagy and this effect was lost in MDP-treated DCs from Crohn's disease patients with a polymorphism in NOD2 [66].

Autophagy can also act downstream of virus-sensing pathways mediated by RIG-like receptors (RLRs). Treatment with the dsRNA mimic polyinosine-polycytidylic acid (PolyIC), acting through the RLR MDA-5, is able to induce autophagy in melanoma cells resulting in autophagy-dependent cell death [67]. The relationship of RLRs with autophagy is notable as multiple reports document the negative regulation of RLRs by autophagy. For example, *Atg5*-deficient murine macrophages have increased RLR signalling mediated by enhanced reactive oxygen species (ROS) production owing to an increase in mitochondrial volume [68]. Thus, autophagy exhibits a complex regulatory role in the context of infection-sensing pathways via PRRs, acting in some cases to mediate the effective sensing and removal of intracellular bacteria, but can function in a negative regulatory capacity in the setting of viral recognition through RLRs.

1.6.2. Autophagy and bacteria handling

Like in the case of the selective targeting of organelles by autophagy, the uptake of cytosolic bacteria (xenophagy) is mediated by the adaptor protein p62 that recognises polyubquitin tags on bacteria and links them to LC3-positive autophagosomes through its LC3-interaction region (LIR) [58] [69]. In this way, the intracellular pathogen *L. monocytogenes* was found to be targeted

to autophagosomes. When present in the cytosol, polyubiquitin-tagged *L. monocytogenes* is recognised by p62, which in turn binds LC3, coating the bacteria in polyubiquitin-p62-LC3 that permits *L. monocytogenes'* engulfment by autophagosomes [69]. The importance of autophagy in the removal of this specialised cytosolic pathogen is highlighted by the action of three Listeria-derived factors: listeriolysin, phospholipase C, and actin polymerisation protein A that all inhibit autophagy, facilitating *L. monocytogenes* prolonged survival in the cytosol [69] [70].

Furthermore, *M. tuberculosis*, [71] [72] Salmonella [73], and *H. pylori*-containing phagosomes [74] have all been shown to fuse or be directly engulfed by autophagosomes offering an alternative method for the destruction of intracellular bacteria by autophagy. In the case of *M. tuberculosis* infection, the bacterial ESX-1 secretion system initiates permeabilisation of the phagosomal membrane that exposes bacterial DNA to components of the cytosolic DNA pathway including STING. STING is responsible for the ubiquitin-tagging of bacteria that ultimately directs the bacteria to the autophagy machinery via the adaptors p62 and NDP52 [75].

1.6.3. Cytokines and autophagy

As previously discussed, autophagy is invoked during infection following the detection of PAMPs by PRRs. However, autophagy is also under the control of cytokines and immunologically relevant cell surface receptors. The T_H1 cytokine IFN-γ is a potent inducer of autophagy while the T_H2 cytokines IL-4 and IL-13 have been shown to have inhibitory effects. This observation

suggests a role for autophagy as an effector arm of T_H1-mediated immunity and may partly explain why T_H1 cytokines afford protection against intracellular bacteria [58]. Autophagy also has a role in the biogenesis and secretion of various pro-inflammatory cytokines. *Atg16L1*-deficient macrophages exhibit enhanced IL-1β and IL-18 secretion following stimulation with LPS [76]. Human PBMCs with a Crohn's disease-associated *Atg16L1* variant display a similar phenotype, with increased IL-1β secretion in response to MDP stimulation [77]. Interestingly, IL-1β can itself induce autophagy in macrophages suggesting that it might regulate its own production through autophagy [78]. Production of multiple other cytokines are enhanced in the absence of autophagy, increases in IL-1β, IL-12, IL-17 and CXCL1 were all observed in *Atg5*$^{fl/fl}$ *LysM-Cre*$^{+}$ mice compared to wildtype controls in response to *M. tuberculosis* infection [79].

1.7. Autophagy in adaptive immunity

1.7.1. Autophagy and antigen presentation

MHC Class I Presentation

In the presence of infection T cells recognise foreign antigen presented in the context of major histocompatibility (MHC) molecules. Antigen presentation refers to the pathways involved in the effective delivery of antigens to MHC molecules, often requiring a complex interplay between intracellular factors and compartments. $CD8^+$ T cells recognise peptides presented in the context of MHC class I molecules expressed on the surface of all nucleated cells. MHC class I molecules present antigen derived from various intracellular sources

such as viral proteins, endogenous tumour antigens and cytoplasmic and nuclear self-antigens [80]. During MHC Class I presentation, antigens are processed by the proteasome into peptides before being transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) where they are loaded onto MHC Class I molecules prior to cell surface expression via the Golgi. Limited evidence exists that autophagy plays a role in the conventional MHC class I pathway (Figure 1.5). Schmid *et al*. could not demonstrate improved MHC class I presentation of a viral epitope when it was conjugated to LC3, a mechanism that was able to enhance the presentation of MHC class II antigens [81]. Multiple other studies have also failed to report a requirement for autophagy in MHC class I presentation [82] [83] [84]. However, inhibition of autophagy was found to decrease MHC class I surface expression in B16 murine melanoma cells and subsequent tumour cell cytolysis by $CD8^+$ T cells [85]. Autophagy has been implicated in an alternative pathway of MHC class I presentation that exists in DCs and macrophages, termed cross-presentation. Cross-presentation provides the means to present extracellular-sourced antigens on MHC class I molecules that would normally be routed through the MHC class II pathway in order to induce a $CD4^+$ T cell response. Via this mechanism $CD8^+$ T cells may respond to exogenous antigens and phagocytosed material [80]. Tumour-specific $CD8^+$ T cell responses have been enhanced using dendritic cells that had phagocytosed tumour cells treated with rapamycin or starvation in order to induce autophagy [86]. Immunisation of mice with purified tumour-derived autophagosomes could also induce the tumour specific T cell response.

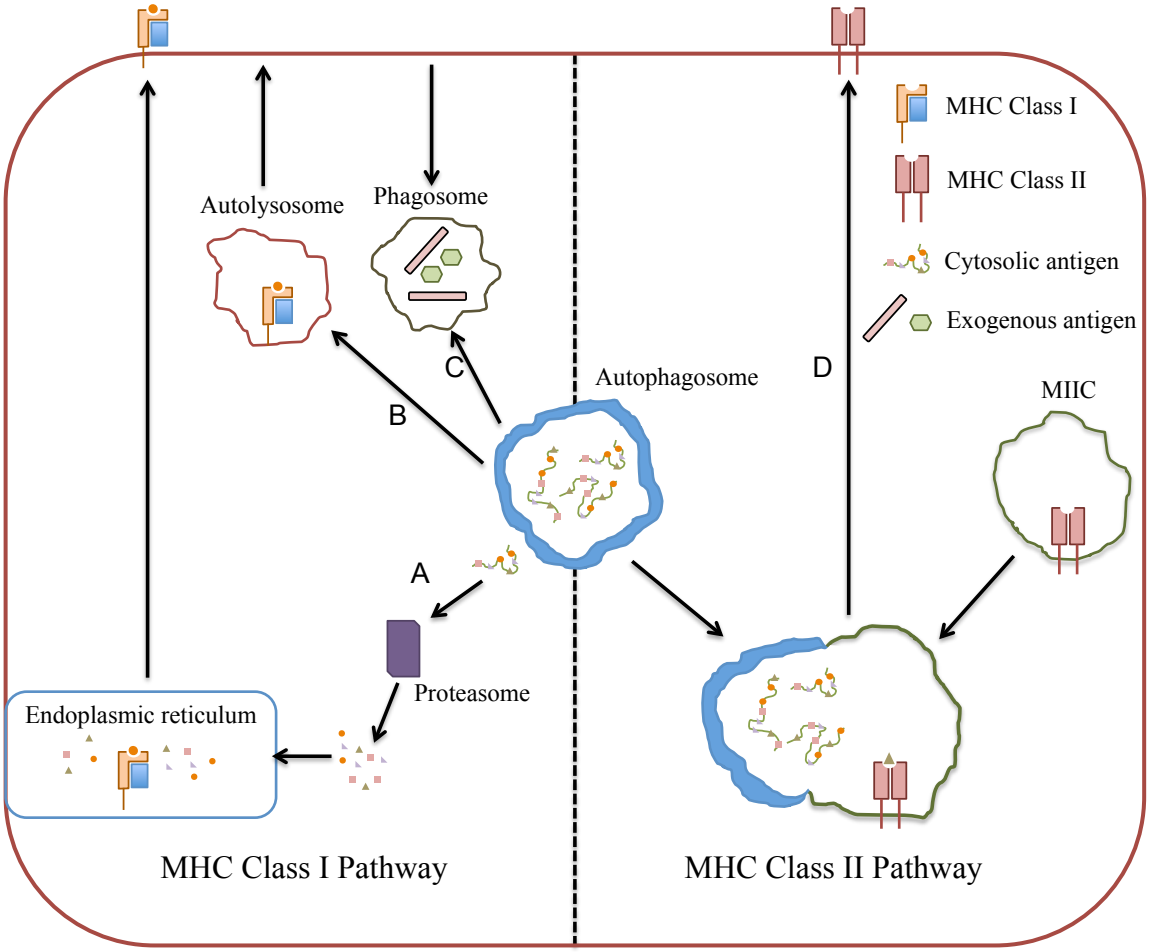


Figure 1.5 *Autophagy and antigen presenting pathways. The role of autophagy in MHC class I antigen presentation is controversial, but is postulated to occur through a number of potential mechanisms. Antigen may escape from autophagosomes into the cytosol where it can then be processed via the conventional MHC class I pathway that involves degradation by the proteasome prior to peptide loading in the ER* ***(A)****. MHC class I molecules may also be loaded in autolysosomes themselves before trafficking to the cell surface* ***(B)****. For cross-presentation, autophagosomes may intersect with phagosomes bearing phagocytosed exogenous antigen that can then be routed into the MHC class I pathway in order to prime CD8⁺ T cells* ***(C)****. During MHC class II presentation, autophagosomes regularly fuse with MHC Class II loading compartments, thereby acting as a system for the delivery of cytosolic antigens to the MHC class II pathway* ***(D)****.*

MHC Class II Presentation

CD4⁺ T cells recognise antigen in the context of MHC class II molecules expressed on professional antigen presenting cells (APC) and epithelial cells [81]. Unlike endogenous MHC class I peptides that are generated by

proteasomes in the cytosol, exogenous antigens are processed in endolysosomal compartments where they are degraded by lysosomal proteases and loaded onto MHC class II molecules that have trafficked from the ER to MHC class II compartments (MIICs). Because of the prominent role of the lysosomal system in MHC class II presentation, many studies have linked autophagy to this form of antigen presentation. Traditionally, MHC class II antigens were believed to be sourced from the extracellular space following phagocytosis by APCs. However, autophagy may comprise a significant source of MHC class II antigens derived from intracellular sources through the delivery of material to the lysosome. In primary monocyte-derived DCs, autophagosomes have been shown to frequently fuse with MHC class II compartments (MIICs) (Figure 1.5) where more than 50% of MIICs were observed to receive input from autophagosomes [81]. Furthermore, starvation-induced autophagy in human B lymphoblastoid cell lines could enhance the presentation of intracellular antigens on MHC class II molecules [87]. Use of starvation and rapamycin in macrophages and DCs can also increase MHC class II expression of mycobacterial antigens following phagocytosis of BCG [88]. In this setting, antigens were found colocalised with $LC3^+$ autophagosomes, suggesting that autophagy may capture antigens following their escape from the phagosome and deliver them to the lysosome to prime $CD4^+$ T cells. Similarly, conjugation of the influenza matrix protein 1 (MP1) to LC3 was able to enhance the priming of antigen-specific $CD4^+$ T cells [81]; while mice with a DC-specific deletion of *Atg5* have impaired $CD4^+$ T cell priming in response to herpes simplex virus infection [82]. The intersection

of autophagy with antigen processing pathways may be even closer than originally presumed.

A recent study reported the formation of autophagosome-like structures emanating from MIICs in DCs. These structures contain the molecular machinery involved in antigen processing as well as the autophagosome markers LC3 and Atg16L1 [89]. These observations offer the interesting prospect of APCs carrying out a specialised form of autophagy specific to the setting of antigen presentation, but add further complexity to the role autophagy plays in the delivery of antigens to MHC molecules. What is clear is that autophagy provides a prominent source of antigens that can be routed into antigen processing pathways through the fusion of autophagosomes with antigen loading compartments. As such, autophagy can be thought of as a key regulator of T cell priming by influencing the repertoire of epitopes presented and thereby the nature and intensity of the ensuing T cell response.

1.7.2. Autophagy in B cells

With use of *Atg5*$^{-/-}$ *Rag1*$^{-/-}$ chimeric mice and mice with a B cell-specific deletion of *Atg5* it was shown that autophagy-deficient B cell progenitors fail to transition between pro- and pre-B cell stages in the bone marrow, suggesting a requirement for autophagy in B cell development [90]. Like T cells, B cells are also severely affected by the absence of autophagy in the periphery. For example, deletion of *Atg7* in the haematopoietic system results in reduced numbers of peripheral B cells [9], while mice lacking *Atg5* in the B cell lineage show a particular requirement for autophagy in B-1a B cell homeostasis [90 91].

More recently, the role of autophagy in B cells responding to antigen has been elucidated. In response to LPS stimulation *ex vivo*, *Atg5*-deficient B cells could effectively differentiate into plasma cells (PCs), exhibited normal proliferation, cell enlargement and CD138 expression (a marker of PCs in mice). However, $Atg5^{-/-}$ PCs displayed increased intracellular immunoglobulin (Ig) content and IgM production *in vitro* in response to LPS. The absence of autophagy was shown to increase ER stress leading to an increase in Blimp-1 expression, which subsequently drives IgH expression and ultimately increased immunoglobulin production. However, when $Atg5^{flox/flox}$ *CD19-Cre* mice were immunised with a pneumococcal vaccine the antibody response *in vivo* was significantly decreased. The enhanced antibody production observed *in vitro* was negated by a requirement for autophagy in PC viability *in vivo* [91]. Autophagy is also essential for memory B cell maintenance. Mice with a B cell-specific deletion of *Atg7* exhibited normal primary antibody responses to influenza infection, but failed to generate protective secondary antibody responses upon reinfection [92]. The current status of knowledge regarding autophagy in T cells will be summarised in following chapters.

1.8. Thesis Summary: The role of autophagy in $CD8^+$ T cell immunity

Previous work in our lab has outlined an important role for autophagy in multiple areas of the haematopoietic system. At the level of haematopoietic stem cells (HSCs), loss of the essential autophagy gene *Atg7* results in defective HSC function [93] while more recent work has shown a requirement for autophagy in the differentiation of various progenitors downstream of

HSCs (Watson *et al*, submitted). In the immune system, autophagy seems to be prevalent in the innate arm with roles ranging from bacterial handling [72], cytokine control [66 58] and cell differentiation (Stranks *et al*, In Press). However, relatively little is known about how autophagy might be important for adaptive responses in lymphocytes. In T cells, autophagy has been shown to be an important player in naïve T cell homeostasis, but how it is important for responses to antigen, if at all, remains a mystery. This thesis will go on to detail a crucial role for autophagy in the $CD8^+$ T cell response to viral infection. Using a murine model in which *Atg7* is deleted specifically in T cells, the data presented herein will show how autophagy is dispensable for effector $CD8^+$ T cell responses, but an absolute requirement for the formation of memory $CD8^+$ T cells. Experiments will show that in the absence of autophagy, defective mitochondrial homeostasis and altered metabolism in antigen-specific $CD8^+$ T cells drives this failure to form the memory $CD8^+$ T cell pool. In recent years, autophagy has become increasingly linked to aging and longevity [94], although its involvement in immune aging (termed immunosenescence) is unknown. Here, data will show how autophagy levels fall in $CD8^+$ T cells from aged mice, providing a link between autophagy and poor T cell memory formation in the elderly. Most importantly, the subdued T cell response to vaccination normally observed in elderly mice can be corrected when aged mice are treated with the autophagy-inducing compound spermidine. Therefore, this thesis provides a cell-intrinsic explanation for poor T cell immunity in the aged, and offers novel immuno-modulatory compounds with the potential to improve vaccine responses in older populations.

Chapter 2: Materials & Methods

2. Materials & Methods

2.1. Mice and breeding

All mice were bred and housed in the animal facilities at Biomedical Services Oxford. Mice were maintained in filter top cages under SPF conditions and fed *ad libitum*. *Atg7*$^{flox/flox}$ mice were obtained from M. Komatsu and *Vav-iCre* mice were kindly gifted by D. Kioussis, London. Vav-Atg7$^{-/-}$ mice were obtained by breeding *Atg7*$^{flox/flox}$ mice with *Vav-iCre* mice. Wildtype controls for each experiment were taken from *Atg7*$^{flox/flox}$; *Cre*$^{-}$ or *Atg7*$^{flox/wt}$; *Cre*$^{-}$ littermate controls of the experimental *Atg7*$^{flox/flox}$; *Cre*$^{+}$ mice. *Atg5*$^{flox/flox}$ mice were kindly gifted by N. Mizushima and were crossed to *Vav-iCre* mice to form Vav-Atg5$^{-/-}$. *Atg5*$^{flox/flox}$ *Cre*$^{-}$ and *Atg5*$^{flox/wt}$ *Cre*$^{-}$ were used as wildtype controls. *CD4-Cre* mice were obtained from F. Powrie and were crossed to *Atg7*$^{flox/flox}$ mice to obtain T-Atg7$^{-/-}$ (*Atg7*$^{flox/flox}$ *Cre*$^{+}$) and heterozygous *Atg7*$^{flox/Wt}$ *Cre*$^{+}$ mice. *Atg7*$^{flox/flox}$ *CD4-Cre*$^{-}$ mice were used as littermate wildtype controls. C57BL/6 SJL CD45.1^{+} mice were bred in, and obtained from, Department of Biomedical Services, University of Oxford. Aged mice and young controls were purchased from Charles River, UK. All mice were bred on a C57BL/6 background and all procedures were carried out in accordance with Home Office Animals (Scientific Procedures) Act 1986 and as allowed under personal and project licences (PIL: 60/12270; PPL:30/2809).

2.2. Genotyping

DNA Extraction

Mice were identified and genotyped by ear tissue samples. Small tissue

sections were digested for 2 hrs at 55° C in lysis buffer (5mM EDTA, 0.1 M Tris-HCl, 0.2% SDS, 0.2 M NaCl and 7.8 mg/mL proteinase K [Sigma]) and solids pelleted by centrifugation (16000 x g, 10 min). Supernatant was transferred to a clean Eppendorf tube and DNA precipitated using an equal volume of isopropanol and gentle mixing by inversion. The resultant precipitate was pelleted (16000 x g, 10 min) and washed in 70% ethanol. Following air-drying, the DNA was dissolved in 100µL MilliQ water and incubated at 65° C for 30 min to aid suspension. DNA samples were stored at -20° C.

PCR Reactions

PCR reactions for the *Atg7 flox* & *wt*, *Vav-iCre*, and *CD4-Cre* alleles were carried out in a total volume of 20 µL, using MyTaq™ Red Mix combined PCR mix (Applied Biosystems, USA) under the same thermocycling conditions. Primers for the *Cre* and *Atg7 flox* transgenes were as follows:

Vav-iCre PCR

iCre-Fwd: 5′-AGATGCCAGGACATCAGGAACCTG-3′

iCre-Rev: 5′-ATCAGCCACACCAGACACAGAGATC-3′

Atg7 Flox and *Wt* PCR

(WT fwd Primer) 752: 5′-CCATGCTGATGGCTAATGTCTC-3′

(Flox fwd Primer) 753: 5′-CTGCAGGAATTCGATATCATAACTTCG-3′

(Reverse Primer) Atg7-14R: 5′-GTCCAGAGTCCGGTCTCTGGTTG-3′

CD4-Cre PCR

CD4-Cre-Fwd 5′-CGATGCAACGAGTGATGAGG-3′
CD4-Cre-Rev 5′-GCATTGCTGTCACTTGGTCGT-3′

DNA for *Atg7 flox* and *Wt*, *Vav-iCre*, and *CD4-Cre* PCRs were amplified under the following conditions:

1. *95° C, 1 min*
2. *95° C, 15 sec*
3. *55° C, 15 sec*
4. *72° C, 10 sec*
5. *30× cycles from step 2*
6. *Hold at 4° C*

Atg5 flox and *Wt* PCR

Atg5 flox and *Wt* PCR reactions were performed using Roche Taq DNA polymerase (courtesy of Dr Alec Watson). Wt and flox genotyping was performed separately: Wt PCR used primers 1&2, flox PCR used 2&3.

1: 5′-GAATATGAAGGCACACCCCTGAAATG-3′,

2:5′-GTACTGCAT-AATGGTTTAACTCTTGC-3′,

3:5′ACAACGTCGAGCACAGCTGCGCAAGG-3′

DNA for Atg5 Flox and Wt PCR was amplified under the following $CD8^+$ conditions:

1. *95° C, 3 min*
2. *95° C, 30 sec*
3. *64.8° C, 30 sec*
4. *72° C, 1 min*
5. *34× cycles from step 2*
6. *72° C for 10 min*
7. *Hold at 4° C*

Amplified DNA was analysed on a 2% agarose gel (Invitrogen), prepared in 50 mL 1× TBE buffer (45 mM Tris-Borate, 1mM EDTA, stained with 5µL

SYBR® Safe DNA Gel Stain (Life Technologies). Gels were run at 70 V for ~35 min until the bands were sufficiently separated.

2.3. Primary Cell Preparation

Bone marrow

The hind limbs of mice were collected in aseptic conditions, cleaned of tissue and fur and rinsed in 70% ethanol before being transferred into PBS/5% foetal calf serum (FCS). The bones were crushed in a sterile mortar and pestle in PBS/5% FCS, passed through a 70 μm cell strainer (BD Falcon) and then resuspended in 1X Red blood cell lysis (RBC) lysis buffer (eBioscience) for 5 minutes.

Splenocytes, lymph nodes, and salivary glands

Spleens, lymph nodes, and salivary glands were collected in aseptic conditions and transferred to PBS/5%FCS. A single cell suspension was created by gentle disruption through a 70 μM filter using the blunt end of a syringe, with frequent rinses of PBS/5% FCS (approximately 20 mL). After centrifugation (300 x g), cells were incubated for 5 min in 1x RBC Lysis Buffer (eBioscience), washed in PBS/5%FCS and filtered once more through a 70 μM filter.

Lungs

Lungs were first perfused by cutting the descending aorta and forcing 20 mL of PBS through the left ventricle of the heart until the lungs were devoid of blood. Lungs were then harvested and collected into PBS/5%FCS before

digestion in 2mg/ml collagenase D (Merck), 2% BSA for 45 minutes at 37°C. A single cell suspension was then created by gentle disruption through a 70 μM filter using the blunt end of a syringe, with frequent rinses of PBS/5% FCS (approximately 20 mL). After centrifugation (300 x g), cells were incubated for 5 min in 1x RBC Lysis Buffer (eBioscience), washed in PBS/5%FCS and filtered once more through a 70 μM filter.

Liver

The liver was first perfused as described for the lungs. After harvesting, a single cell suspension was made as for spleens and lymph nodes. T cells were obtained by centrifugation over a Ficoll-Hypaque gradient and subsequently harvested by pipetting.

Blood

Peripheral blood was obtained from the lateral tail vein of live animals and collected in heparin-coated tubes (Microvette 300, Sarstedt) to avoid coagulation. Blood was then transferred to RBS lysis-containing Eppendorfs and incubated for 10 minutes at RT. PBS/5%FCS was added to stop the reaction before centrifugation (300 x g , 5 mins). Cells were resuspended in PBS/5%FCS before transfer to a 96 well round bottom plate. After centrifugation (300 x g, 5 mins), cells were once more incubated in RBS lysis buffer for 5 minutes before the reaction was halted with PBS/5%FCS.

MACS enrichment

Magnetic cell labelling was used to negatively enrich splenocytes and lymph nodes for untouched $CD8^+$ T cells. Organs were collected as described above,

pelleted by centrifugation (300 x g, 10 min), and the supernatant discarded. The cell pellet was re-suspended in 40 µL of MACS buffer (PBS pH 7.2, 0.5% BSA and 2 mM EDTA) per 10^7 cells to which 10 µL of biotinylated antibody cocktail per 10^7 cells was added to remove all lineage cells other than $CD8^+$, and incubated for 5 min at 4° C. This was followed by 20 µL of anti-biotin MicroBeads (Miltenyi Biotech) per 10^7 cells, and further 10 min incubation at 4° C. The cells were then washed and centrifuged (300 x g, 10 minutes) in 2mL of buffer, the supernatant was removed and discarded, and the cell pellet was suspended in 3 mL of buffer. A LS separation column (Miltenyi Biotech) was placed in the separation magnet and rinsed with 3 mL of MACS buffer prior to the addition of the cell suspension to the column. After the cells had passed through the column, the column was rinsed 3x with 3 mL MACS buffer and the eluate containing the unlabelled $CD8^+$ T cell fraction collected and kept on ice. The isolated cell fraction was then assessed for purity of $CD8^+$ T cells by flow cytometry.

2.4. qPCR

RNA extraction

Purified $CD8^+$ T cells were pelleted (16000 x g, 15 min) and supernatant discarded before re-suspension in 1 mL Trizol reagent (Invitrogen) per 3×10^6 cells. Cells were allowed to lyse for 2 hrs or overnight. 200 µL of chloroform/isoamyl (49:1) (Fluka) was added to each tube and mixed by inversion. Phases were separated by centrifugation (16000 x g, 15 min, 4° C) and the aqueous phase transferred to a fresh Eppendorf tube before precipitation with 500 µL of isopropanol. Following 15 min of incubation

at room temperature, RNA was pelleted (16000 x g, 15 min, 4° C) and washed in 200 µL ice cold 70% ethanol. The pellet was allowed to air dry, prior to re-suspension in 10 – 20 µL DEPC-H_2O (Invitrogen). RNA concentration and quality was determined using a NanoDrop spectrophotometer (Thermo Scientific USA). Samples were stored at -80° C.

Reverse Transcription

RNA was reverse transcribed to cDNA using a High Capacity RNA to cDNA kit (Applied Biosystems). All reactions were completed in 20µL (9µL RNA, 1µL RT Enzyme Mix [20x] and 10µL RT Buffer Mix [2x]) per tube. Reactions were incubated at 37° C for 1 hr, followed by 5 min at 95° C, and finished with 10 min at 4° C. Transcribed cDNA was stored at -20° C until analysis by qPCR.

Real-time qPCR

The comparative Ct ($\Delta\Delta C_T$) method was used to evaluate relative gene expression in wildtype and *Atg7*$^{-/-}$ $CD8^+$ T cells normalised to *gapdh* and *hprt*. Validated TaqMan primer probes (Applied Biosystems) were used for all reactions (Table 1). Quantitative PCR reactions were carried out in a total of 20 µL (9 µL reverse transcribed cDNA, 10 µL TaqMan® Gene Expression Mix [Applied Biosystems] and 1µL of the relevant 20× TaqMan probe) in 96 well MicroAmp® reaction plates (Applied Biosystems) sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). Reactions were completed in standard mode of a 7500 Fast Real-time PCR system (Applied Biosystems). A list of TaqMan probes used for q-PCR can be seen in Table 1.

Cycling conditions were as follows:

1. *50° C, 2 min*
2. *95° C, 10 min*
3. *95°, 15 sec*
4. *60° C, 1 min*
5. *40× cycles from step 3.*

Gene	Probe ID
Atg5	Mm00504340_m1
Atg7	Mm00512209_m1
Atg9	Mm01264428_m1
Atg10	Mm00470550_m1
Atg12	Mm00503201_m1
beclin1	Mm0051717_m1
Map1lc3a	Mm00458724_m1
hprt	Mm01545399_m1
gapdh	Mm99999915_g1
$p16^{INK4A}$	Mm00494449_m1
$p21^{Cip1a}$	Mm04205640_g1

Table 1. *List of TaqMan Probes used during qPCR (all mouse)*

2.5. Western blot

Purified primary $CD8^+$ T cells (5×10^6) were lysed on ice using 100 µl 1 × NP-40 Lysis Buffer. Protein concentration in the supernatant was measured using BCA Protein Assay Kit (Thermo Scientific), and reducing Laemmli Sample Buffer was added to make protein samples for SDS-PAGE. Proteins of 30–50

μg per lane were separated on 16% SDS-PAGE and transferred to PVDF membrane (Millipore). After blocking with 5% skimmed milk, the membrane was blotted using the following primary antibodies: LC3 (L8918, Sigma) (1:1000), GAPDH (Millipore) (1:10000), pS6 (Cell Signaling Technology) (1:5000). For S6 blotting, membranes were blotted with anti-pS6 first, then stripped for re-blot using primary antibody against S6 (Cell Signaling Technologies) (1:1000). IRDye secondary antibodies were bought from LI-COR and used at 1:15000. Western blots were performed with kind help from Mr Hanlin Zhang.

2.6. Flow cytometry

Surface Staining

After obtaining a single celled suspension from the desired organ or tissue, cells were transferred to a round bottom 96 well plate and centrifuged (300 x g, 5 minutes). The pellet was then resuspended in PBS containing the viability dye Live/Dead Violet (Life Technologies) for 10 minutes in the dark at room temperature (RT) to exclude dead cells. After washing with PBS/5% FCS, cells were resuspended in PBS/2% FCS (containing 5mM EDTA [FACS buffer]) containing a cocktail of antibodies relevant to the desired cell surface markers. Fc block (anti-CD16/32) was typically added to the antibody mix to minimise non-specific staining. Surface antibody staining was performed at 4°C for 20 mins in the dark. A list of all surface antibodies utilised and their working concentrations can be seen in Table 2. Following incubation, cells were washed with FACS buffer and immediately analysed on a flow cytometer unless intracellular staining was required (see below).

MHC Class I monomers were generated as previously described [95] by Dr Stuart Sims of Professor Paul Klenerman's lab and Dr John-Paul Dukes of Professor Vincenzo Cerundolo's lab. MHC-Class I monomers were stored at −80°C. Biotinylated monomers were tetramerised with Streptavidin PE or APC at the right concentration to achieve a 1:1 ratio with biotin binding sites and added in 1/10th volumes waiting 10 min between additions. Tetramerised complexes were stored at 4°C. Peptide sequences for MCMV tetramers were as follows: m45 ^{985}HGIRNASFI^{993}, H-2Db-restricted; m38 ^{316}SSPPMFRV^{323}, H-2Kb-restricted; IE3 ^{416}RALEYKNL^{423}, H-2Kb-restricted. The peptide sequence for the influenza tetramer was as follows: NP ^{366}ASNENMETM^{374}, H-2Db-restricted. Cells were always stained with tetramers prior to surface antibody staining in PBS/2% FCS at 37°C for 15 min.

Intracellular Staining

Intracellular staining always took place proceeding surface marker staining. For **cytosolic markers**, cells were fixed with 100 µL Fixation buffer (eBioscience) and incubated in the dark for 20 min at RT. Cells were permeabilised with 100 µL of 1x Permeabilisation buffer (eBioscience) diluted in distilled water and pelleted by centrifugation at 300 x g for 5 min. The cells then were re-suspended in the intracellular antibody mix diluted in permeabilisation buffer and incubated for 30 min in the dark at RT. Subsequently, cells were washed twice with 100 µL of permeabilisation buffer prior to re-suspension in 300 µL of FACS buffer for analysis.

For **nuclear markers** such as EOMES and IRF4, cells were incubated with FoxP3 permeabilisation buffer (eBioscience) for 45 minutes at RT. Cells

were then washed in standard 1x Permeabilisation before nuclear antibody staining in 1x Permeabilisation buffer for 60 mins at RT in the dark. Cells were subsequently washed twice in 1x Permeabilisation buffer before resuspension in FACS buffer and analysis.

For **intracellular cytokine staining**, splenocytes were stimulated with 10 ng/mL Phorbol myristate acetate (PMA) and 1 μg/mL ionomycin (both Sigma Aldrich) for 6 hours at 37°C in complete media (R10: RPMI 1640, 10% FCS, 2 mM L-Glutamine, 100 U/mL Penicillin, all Invitrogen) in the presence of the protein transport inhibitor GolgiStop (4 μL per 6 mL media, BD Biosciences). As a control, cells were left unstimulated. Following stimulation, cells were stained with anti-TNF-α and anti-IFN-γ as performed for typical cytosolic markers described above.

Antibody (Clone)	**Fluorochrome (working dilution)**	**Company**
CD8 (Ly2)	PE (1:400), PE-Cy7 (1:300)	eBioscience
CD8 (53-6.7)	FITC (1:200), eF450 (1:300), PE-Cy7 (1:300)	eBioscience
CD4 (GK1.5)	PE (1:450), FITC (1:150), APC (1:400)	eBioscience
TCRβ (H57-597)	FITC (1:200), PE (1:350), PE-Cy7 (1:300), APC (1:400)	eBioscience
CD3 (145-2C11	eF450 (1:250), APC (1:350)	eBioscience
CD62L (MEL-14)	FITC (1:300), PE-Cy7 (1:250)	eBioscience
CD44 (IM7)	FITC (1:200), PE-Cy7 (1:300)	eBioscience
KLRG1 (2F1)	FITC (1:100), PE (1:300)	eBioscience

IRF4 (3E4)	FITC (1:100)	eBioscience
EOMES (Dan11mag)	PE-Cy7 (1:150)	eBioscience
CD127 (A7R34)	FITC (1:150), PE (1:200), eF450 (1:200)	eBioscience
Annexin V	PE-Cy 7 (1:80)	eBioscience
NK1.1 (PK136)	PE (1:200)	eBioscience
CD11b (M1/70)	eF450 (1:200)	eBioscience
CD11c (N418)	eF450 (1:200)	eBioscience
CD19 eBio1D3	eF450 (1:200)	eBioscience
B220 (RA3-6B2)	eF450 (1:300)	eBioscience
TCRγδ (eBioGL3)	eF450 (1:300)	eBioscience
Streptavidin	PE (1:250)	eBioscience
Ki-67 (SolA15)	eF450 (1:300)	eBioscience
CD24 (M1/69)	APC (1:300)	eBioscience
CD16/32 (93)	Purified Fc Block	eBioscience
CD45.2 (104)	eF450 (1:200)	eBioscience/Biolegend
PD-1 (29F.1A12)	PE-Cy7 (1:300)	Biolegend
PD-1 (RMP1-30)	PE (1:350)	Biolegend
TIM-3 (B8.2C12)	PE (1:300), APC (1:350)	Biolegend
Bcl-2 (BCL/10C4)	PE (1:200)	Biolegend
Glut1 (q)	PerCP (1:90)	R&D Systems
IL-15Rα (BAF551)	Biotinylated (1:150)	R&D Systems
Caspase-3 (C92-605)	FITC (1:150)	BD Bioscience
TNFα (TN3-19.12)	APC (1:200)	BD Bioscience
IFN-γ (XMg1.2)	FITC (1:80)	eBioscience

***Table 2**. List of antibodies used for flow cytometry.*

Autophagy Detection

Autophagy detection by flow cytometry was measured using the CytoID Autophagy Detection Kit (Enzo Life Sciences). Splenocytes and cells isolated from the lung were first stained with CytoID (1:1000) according to the manufacturer's instructions, prior to tetramer and surface antibody staining. Autophagy was also measured using flow cytometry by quantifying LC3-II mean fluorescence intensity using the FlowCellect Autophagy LC3 Antibody-based Assay Kit (FCCH100171, Merk-Millipore, MA, USA) according to the manufacture's instructions and always following cell surface antibody

staining. Use of this kit includes a step where cytosolic LC3-I is washed from the cell, leaving only membrane bound LC3-II prior to staining.

Autophagy was also measured through LC3 puncta determination using **ImageStream** (Amnis imaging flow cytometer), a technique previously used to determine autophagic flux [13]. To determine LC3 spot count, cells were stained for LC3-II (following cell surface staining) using the FlowCellect Autophagy LC3 Antibody-based Assay Kit (FCCH100171, Merk-Millipore) according to the manufacture's instructions. Before LC3 detection, to assess autophagic flux, cells were incubated for 2 hrs with the autophagy flux inhibitor provided by the kit specified and according to the manufacturer's instructions. LC3 spot count was quantified using Ideas software (Amnis), which contains a specialised objective spot counting feature. Images collected were all at x60 magnification. Samples were run with the aid of Dr Alec Watson, Dr Kanchan Phadwal, and Ms Isabel Panse.

BrdU Incorporation

To investigate cell cycle, mice were pulsed with 1 mg BrdU by intraperitoneal (i.p) injection and culled 3 days later. BrdU uptake was assessed using the BrdU Flow Cytometry Kit (BD Biosciences, FITC Conjugate) as per the manufacturers instructions and following cell surface staining.

Mitochondrial Staining

For mitochondrial investigation, cells were stained with MitoTracker Green (Life Technologies) to assess mitochondrial load at 150 nM in PBS/2% FCS for 30 minutes at 37°C. Cells were then washed twice with FACS buffer. To

analyse mitochondrial superoxide production, cells were stained with MitoSOX Red (Life Technologies) at 5 μM in PBS/2% FCS for 30 minutes at 37°C and then processed as described for MitoTracker Green. Mitochondrial dye staining always proceeded tetramer and surface marker staining and following incubation cells were analysed immediately on a flow cytometer.

Cell Death Analysis

For apoptosis detection, splenocytes were stained with m45-tetramer and surface antibody as described above and then stained with Live/Dead Fixable Violet Dead Cell Stain Kit (Life Technologies) as stated before. Cells were then stained with Annexin V PE-Cy7 (eBioscience) in Annexin V binding buffer at RT for 15 mins. Apoptotic cells were determined as Live/Dead cell dye negative (live cells), Annexin V positive.

Metabolic Analysis

GLUT-1 was measured through binding to its ligand, the receptor-binding domain (RBD) of a recombinant glycoprotein from the human T lymphotrophic virus (HTLV) fused to eGFP (HRBD-eGFP) [96]. Staining of GLUT-1 through HRBD-eGFP (a kind gift from Dr Mark Sitbon)(1:100) was achieved as described for standard surface marker staining. To identify neutral lipids, cells were stained with 500 ng/mL Bodipy 493/593 (Life Technologies) in PBS for 10 mins at 37°C following surface tetramer and antibody staining. Cells were then washed twice with FACS buffer prior to analysis. To measure uptake of Bodipy-conjugated palmitate (Bodipy FL C_{16}; Life Technologies), cells were incubated for 30 minutes at 37°C with 1 μM

Bodipy FL C_{16} in R10 complete media, then washed twice prior to analysis. To assess glucose uptake, cells were stained with the fluorescent glucose analogue 2-NBDG (Life Technologies) at 50 µg/ml for 60 mins at 37°C following surface staining.

Absolute Cell Counts

Absolute Cell counts from blood were calculated with BD TruCount tubes (BD Bioscience) according to the manufacturer's instructions. To calculate cell numbers from tissues, whole organs were counted and then used to back calculate absolute cell number of a given population from the population frequency obtained during FACS analysis.

Instrumentation

All flow cytometry experiments were performed on a Cyan (Beckman Coulter) instrument. FACS sorts were performed on Aria II (BD) with aid of Dr Craig Waugh and Mr Kevin Smith. Data were analysed with FlowJo 9.1 for Mac (Tree Star, Inc).

2.7. Bone marrow chimera

Bone marrow (BM) cells were extracted from the hind limbs (as described above) of a single 8-week old wildtype, T-Atg7$^{-/-}$ (both CD45.2^{+}), and C57BL/6 SJL mouse (CD45.1^{+}). After erythrocyte lysis, 3 x 10^6 wildtype or T-Atg7$^{-/-}$ BM cells were added to 3 x 10^6 SJL CD45.1^{+} BM cells (1:1 CD45.2^{+} : CD45.1^{+}) in a total volume of 200 µl PBS. Two hours after lethal irradiation (450 cGy twice, 4 hr apart), the 1:1 BM mix was injected intravenously (i.v)

into C57BL/6 SJL CD45.1$^+$ recipients. After 8 weeks of reconstitution, mixed BM chimera was immunized with MCMV or PR8 influenza, or left unimmunized. WT and T-Atg7$^{-/-}$ mice were always used as controls.

For *Atg5$^{flox/flox}$ Mx1-Cre* (PolyI:C inducible) chimeras, BM was extracted from the hind limbs of an 8-week old *Atg5$^{flox/flox}$ Mx1-Cre$^-$*, *Atg5$^{flox/flox}$ Mx1-Cre$^+$* (both CD45.2$^+$), and C57BL/6 SJL mouse (CD45.1$^+$). BM was transplanted into lethally irradiated SJL CD45.1$^+$ hosts as described above. After 8 weeks of reconstitution, mice were immunized with 0.005 HAU PR8 or left naïve, on days 30, 32, and 34 mice were injected i.p with 250 µg Poly:IC in 250 µL saline to induce expression of *Mx1-Cre*. Control mice were injected with 250 µL PBS alone.

2.8. Immunizations

For **MCMV**, mice were immunized i.v with 1 x 10^6 p.f.u MCMV in 100 µl (Smith strain ATCC: VR194). Unimmunized controls were injected i.v with 100 µl PBS. For **influenza infections**, mice were administered intra-nasally with either A/PR/8/34 influenza (PR8, H1N1 Cambridge strain) or X31 (H3N2) influenza at the stated dose in 50 µl viral dilution media (VDM; DMEM, 0.1% BSA, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine; all from Sigma Aldrich). Mice were anaesthetized with isofluorane and droplets of VDM containing virus were applied to the nares until the total 50 µl was inhaled. Unimmunized mice were always used as controls (50 µl of VDM alone). **For influenza vaccination**, mice were immunized with the H1N1 vaccine S-Flu [97]. S-Flu is derived from a

pseudotyped influenza virus (PR8) and is incapable of replication due to inactivation of the vRNA encoding haemagglutinin (HA). This pseudotyped virus can synthesise all vRNA, express all the viral proteins except for HA, but cannot spread from cell to cell. Therefore, S-Flu infects as efficiently as wildtype influenza to produce a contained infection complete with intracellular expression of conserved core proteins. To vaccinate, mice were immunized with 32 HAU S-Flu using the influenza infection protocol described above. Unvaccinated mice were used as controls in all influenza vaccine experiments. In unvaccinated mice that received X31 challenge, a combination of weight loss and clinical score was used as a humane endpoint

2.9. Influenza viral titres

Determination of the 50% tissue culture infective dose (TCID50) was performed on MDCK-SIAT1 cells in a 96-well flat-bottom plate performed by serial dilution of lung homogenates onto 3 x 10^4 MDCK-SIAT1 cells followed by incubation for 72 hrs at 37°C. Virus was then detected by haemagglutination, where 50 µl 1% (vol/vol) human erythrocytes (adjusted so that a 1:2 dilution gave an optical density at 600 nm) was added to 50 µl of the serially diluted lung homogenates/MDCK-SIAT1 supernatant in a 96-well V-bottom plate. Haemagglutination was analyzed by the loss of teardrop formation after tilting the plate. TCID50 was then calculated as described by [98].

2.10. Oral *in vivo* spermidine treatment

Mice were administered spermidine (Sigma Aldrich) in the drinking water at a concentration of 5 mM from a 1 M aqueous stock. Spermidine-containing

drinking water was replenished every 2–3 days.

2.11. Statistics

Statistics were calculated using GraphPad Prism software. The specific statistical test applied to each experiment and the values for significance are given in the figure legends. All data for all experiments are presented as the mean ± the standard error of the mean, unless otherwise stated. All data are also representative of single experiments, not from pooled independent experiments, unless otherwise stated.

Chapter 3: Introduction

Autophagy is required for naïve CD8⁺ T cell homeostasis

3. Introduction

Severe combined immunodeficiency patients perhaps provide the ultimate insight into the importance of T lymphocytes in nature. These individuals possess mutations in genes crucial for T cell development or survival leading to a lack of these cells in the periphery. This is not a situation compatible with life and SCID patients require bone marrow transplantation to survive past their formative years. This condition sheds light on the essential requirement for T cells in surviving threats from the local environment. The huge diversity in T cell receptor (TCR) specificity generated during T cell development affords each given individual with at least a fighting chance of outliving whatever microbial threat nature may provide [99] [100] [101] [102]. T cells manifest a huge array of effector functions including the orchestration of innate immune function; directing antibody responses; regulating the duration of inflammation and the killing of virally infected host cells. Their potency demands strict regulation of both function and number so that the best intentions do not spill over into immune pathology. This regulation begins in the thymus where T cells strongly reactive to self are killed long before they are let loose into the peripheral T cell pool. Regulation is ever-present out in the periphery, too. Here, T cell number is tightly observed through the action of various cytokines and important cell-cell contacts mediated via the TCR with MHC. The default setting for a T cell is death, not survival, therefore T cell viability is an actively maintained process and one that shall be introduced in regards to the naive compartment in the coming passages. What is currently known about autophagy and T cell homeostasis in the

steady state will also be touched upon, before data is presented that hopefully expands our knowledge as to how naïve T cells require autophagy for their maintenance.

3.1. T cell development

The aim of T cell development is the large-scale production of a diverse set of naïve T cells able to respond to foreign antigen, and to a limited degree, self, for the purposes of immune surveillance. This complex process takes place in the thymus where progenitors from the bone marrow go through a stringent selection process until suitable, mature T lymphocytes are formed. Early T lineage progenitors (ETPs) enter the thymus at the cortical-medullary junction and are termed double-negative (DN) due to the absence of CD4 or CD8 co-receptor expression [103]. This DN progenitor subset may be further divided based on the expression of CD44 and CD25 into DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$), and DN4 ($CD44^-$ $CD25^-$) with cells maturing in sequential fashion through each stage [104,105]. Lymphoid restriction is not necessary for entry into the thymus with ETPs displaying multipotent potential. At DN1, cells may still form T cells, B cell, NK cells, macrophages and DCs [106-108]. After entering the thymus, cells eventually arrive at the subcapsular epithelium at the DN3 stage. Thymus epithelial cells (TECs) constitute the major component of the thymic stroma credited with providing the signals and microenvironment necessary for thymocyte maturation [109,110]. A combination of cell-to-cell contact and soluble factors provided by TECs and other accessory cells such as DCs provide the relevant signals for T cell development. While in contact with TECs at the subcapsular epithelium, γ,

δ, and β loci rearrangement occurs in DN3 thymocytes, chiefly through the action of recombination-activating genes (RAG) 1/2. At this point, commitment to the T cell lineage is confirmed while αβ and γδ subsets diverge [111]. Successful pairing of the rearranged TCRβ chain with the pre-TCRα (pTα) results in progression to DN4 followed by a round of proliferation and upregulation of CD4 and CD8 to reach the double-positive (DP) stage. DP thymocytes reside in the cortex and it is here that TCRα rearrangement occurs in the quiescent DP population [103]. Successful pairing of αβ chains to form a MHC-restricted TCR terminates α loci recombination, leaving cells subject to one of three cell fates: death by neglect, positive selection, or negative selection. Death by neglect occurs following the failure of a clonotypic αβ TCR to engage MHC-peptide ligands [112]. The generation of a MHC-restricted receptor from random αβ pairs occurs relatively infrequently (less than 5%) despite an inherent propensity for αβ TCRs to bind MHC [113]. Thus, the vast majority of DP thymocytes fail to undergo positive selection as they do not receive the signals necessary for survival. Thymocytes with a MHC-restricted TCR with low affinity for self-peptide-MHC will be positively selected and allowed to enter the peripheral repertoire [103]. Conversely, thymocytes bearing high-affinity TCRs for self will be deleted through targeted apoptosis. This is the basis of central tolerance [114]. The small fraction of cells that achieve selection downregulate one of the co-receptors to become mature, single-positive CD4⁺ or CD8⁺ T cells and exit the thymus to join the naïve T cell pool [112].

3.2. Naïve T cell homeostasis

Thymic selection permits a miniscule fraction of immature thymocytes with low affinity for self-peptide-MHC to differentiate into mature T cells. Newly generated T cells exit the thymus into the pool of naïve T cells that recirculate within the limits of the peripheral lymphoid tissues [115]. In young animals most T cells are naïve, typified by low expression of CD44 in mice, and have a broad repertoire capable of recognizing virtually all foreign antigens from the environment. Naive T cells activated as a consequence of antigen stimulation will eventually depart the naïve pool and enter the memory compartment, bearing high levels of CD44 expression [116]. While T cells principally respond to foreign antigen, it is important they maintain a low degree of self-reactivity. Although below the threshold able to cause autoimmunity, this reactivity is essential in mediating T cell survival and can even enhance TCR sensitivity to foreign antigens [117]. The survival and composition of the mature T cell pool is governed by a complex interplay of a multitude of homeostatic mechanisms with the naïve and memory compartments regulated independently of one another [118]. Originally, the long-lived nature of naïve T cells lead many to believe that these cells possessed intrinsic survival mechanisms. However, more recent data suggests that homeostatic signals are largely a consequence of contact with self-peptide-MHC complexes and members of the common gamma chain (γ_c) family of cytokines, especially IL-7 (Figure 3.1).

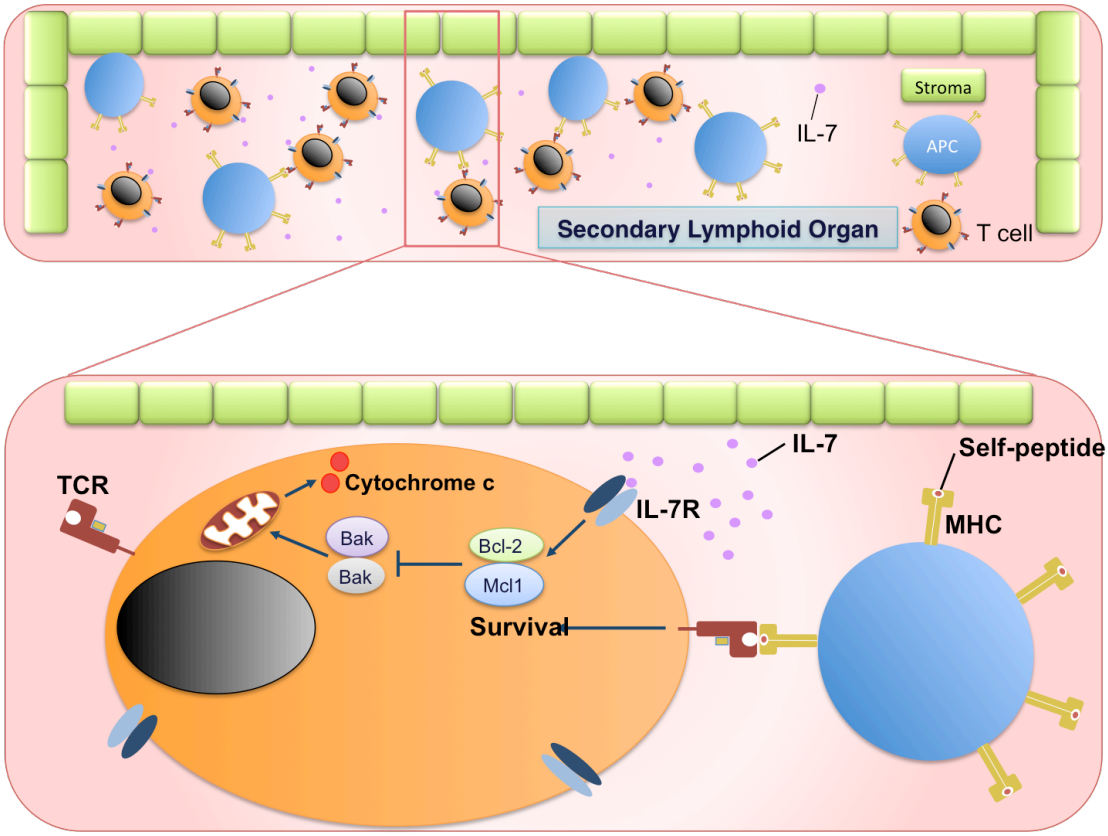


Figure 3.1 *Signals important for T cell homeostasis. Peripheral T cell survival is chiefly dependent on IL-7 and low-level TCR signalling. In secondary lymphoid organs, IL-7 secreted by local stromal cells induces expression of anti-apoptotic Bcl-2 and Mcl1 through IL-7R that maintains T cell survival. These signals work in synchrony with survival signals provided by the TCR upon contact with APCs bearing self-peptide in the context of MHC.*

3.2.1. IL-7 in the control of naïve T cell homeostasis

The essential role of IL-7 in T cell viability was discovered after adoptive transfer of T cells into *IL-7*-deficient hosts or injection of IL-7 mAb into normal mice severely affected T cell survival [119] [120] [121] [122] [116]. The quantity of available IL-7 in secondary lymphoid organs appears to regulate the size of the naïve T cell pool as overexpression of IL-7 leads to an increase in the naïve compartment [116] [123] [124]. IL-7 signals through the IL-7R, composed of IL-7Rα (CD127) and gamma chain (γ_c). CD127 is constitutively expressed on naïve T

cells maintaining a constant sensitivity to IL-7 and is downregulated only during T cell expansion. Similar to genetic ablation of IL-7, CD127 deletion also causes severe T cell deficiency in both mice and humans [125]. IL-7 mediates T cell survival through the induction of anti-apoptotic proteins Bcl-2 and Mcl1 (Figure 3.1). These inhibit the pro-death effectors Bax and Bak, which induce cytochrome C release from mitochondria resulting in cell death [126] (Figure 3.1). Indeed, *bcl-2* and *mcl1* knockout in T cells leads to a severely depleted naïve T cell pool [127] [128] [129] [130].

Besides IL-7, other γ_c cytokines can also mediate homeostatic effects on naïve T cells. IL-15 has been demonstrated to play an essential role in supporting the homeostasis of naïve CD8⁺ T cells. *il-15*-deficient mice possess roughly half the number of naïve CD8⁺ T cells as do wildtype mice [131,132]. Similarly, *il-15rα* null mice were markedly lymphopaenic despite grossly normal T cell development [133]. The role for the IL-15-IL-15R axis in naïve T cell homeostasis appears to be in supporting the homing of cells to secondary lymphoid organs where viability is then maintained through the sensing of locally produced IL-7.

3.2.2. TCR signalling in Naïve T cell homeostasis

While soluble factors are crucial for maintaining the survival and size of the naïve T cell population, interactions between MHC and the TCR are also important. This paradigm became apparent following observations that T cell lifespan was significantly shortened in the absence of contact with MHC [134,135]. Supporting evidence came from studies that abrogated TCR expression or that of the downstream kinases Fyn and Lck, resulted in altered survival

for both naïve CD4⁺ and CD8⁺ T cells [136-138]. Access to self-peptide-expressing MHC is an important determinant for T cell survival [139,140]. TCR transgenic T cells adoptively transferred in large numbers into normal syngeneic hosts display a shortened lifespan due to severe competition of this clonal population in access to the relevant cognate self-peptide-expressing MHC. When the same TCR transgenic T cells were transferred in low numbers, similar to the average precursor frequency for other clones, normal naïve T cell survival was observed [141]. Early ideas about T cell development suggested that self-reactive clones are deleted during thymic selection. We now appreciate that TCR tuning in the thymus ensures that a low amount of self-reactivity remains and this is important for maintaining homeostasis in the periphery. A delicate balance has evolved where the interaction between TCR and self-peptide-MHC complexes induces only low-level tonic signalling that is strong enough to promote survival, but weak enough so as not to send the cell into cycle. That being said, instances do occur where these interactions drive proliferation in the naïve compartment, termed homeostatic proliferation, one that is normally thought of as quiescent. In addition to thymic output, homeostatic proliferation is the second major mechanism for the production of T cells. In humans, homeostatic proliferation is a common event in both the naïve and memory compartment, although turnover of naïve T cells, with a half-life of approximately one year, is not a regular occurrence [142]. Homeostatic proliferation makes a significant contribution to the naïve repertoire in neonates with up to 50% of all T cells produced in this manner. In aged individuals, auto-proliferation becomes prominent once

more as a source of T cells as thymic production rapidly declines [142]. In mice, homeostatic turnover is not a major feature of the T cell pool unless during times of lymphopaenia, when proliferation is induced to return T cell numbers back to base-line levels.

3.3. Autophagy and T cells

Although relatively small cells with limited cytoplasm, T cells have been extensively shown to perform autophagy and express autophagy genes [143-146]. Autophagy is carried out constitutively to low levels in murine and human CD4⁺ and CD8⁺ T cells and can be induced following TCR stimulation *in vitro* [144,146]. A number of genetic model systems have been employed to investigate the role of autophagy in T cells *in vivo*. In both *Atg5*$^{-/-}$ foetal liver chimeras and *Atg7*$^{-/-}$ *Lck-Cre* mice, autophagy-deficient T cells develop normally in the thymus, although there is a reduction in overall thymic cellularity [53,146]. However, the effect of losing autophagy in the peripheral T cell compartment is much more pronounced. There is a significant reduction in T cell numbers in the spleen and lymph nodes when *Atg5*$^{-/-}$, *Atg7*$^{-/-}$, *Atg3*$^{-/-}$, and *Vps34*$^{-/-}$ are deleted in T cells [53,145-147]. Both *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ T cells also fail to proliferate effectively following TCR cross-linking *in vitro*. These defects appear to stem from an inability of autophagy-deficient T cells to regulate organelle quality.

3.4. Chapter Summary: Autophagy is required for CD8⁺ T cell homeostasis

To investigate autophagy in the T cell lineage, we employed a murine model in which the essential autophagy gene *Atg7* is deleted in T cells under the control of the *CD4* promoter (T-Atg7$^{-/-}$ mice). In this chapter, the

phenotype of T-Atg7$^{-/-}$ mice in the steady state will be presented, revealing a requirement for autophagy in the control of naïve CD8$^+$ T cell homeostasis. Confirming the work of others, observations will show how autophagy is dispensable for T cell development in the thymus, but its loss severely compromises CD8$^+$ T cell viability in the periphery resulting in lymphopaenia. In addition to other studies, experiments will show how the resulting lymphopaenia induces large-scale homeostatic proliferation in CD8$^+$ T cells, resulting in the acquisition of a memory phenotype accompanied by signs of exhaustion, terminal differentiation and senescence.

Chapter 3: Results

Autophagy is required for naïve CD8⁺ T cell homeostasis

Results

3.5. Autophagy is deleted in the T cell lineage of T-Atg7$^{-/-}$ mice

In order to study the role of autophagy in CD8⁺ T cells *in vivo*, *Atg7$^{flox/flox}$* mice were crossed to mice expressing *cre recombinase* under the control of the *CD4* promoter. The resulting mice, herein termed T-Atg7$^{-/-}$, are *Atg7*-deficient in both *CD4*- and *CD8*-expressing T cells, owing to the fact that all T cells go through a CD4⁺ CD8⁺ double positive stage in the thymus [103]. To confirm that the presence of *CD4-Cre* and floxed *Atg7* alleles in T-Atg7$^{-/-}$ mice led to loss of *Atg7* in the T cell lineage, mRNA was extracted from FACS-sorted T cells from wildtype and T-Atg7$^{-/-}$ mice. The level of *Atg7* mRNA was quantified by real-time PCR (q-PCR) where the comparative Ct (ΔΔCt) method was used to determine gene expression. T cells from T-Atg7$^{-/-}$ mice showed a near complete ablation of *Atg7* gene expression compared to T cells from wildtype mice (Figure 3.2a). Similarly, Atg7 protein levels were significantly reduced when assessed by western blot in purified CD8⁺ T cells from T-Atg7$^{-/-}$ mice, relative to CD8⁺ T cells from WT mice (Figure 3.2b).

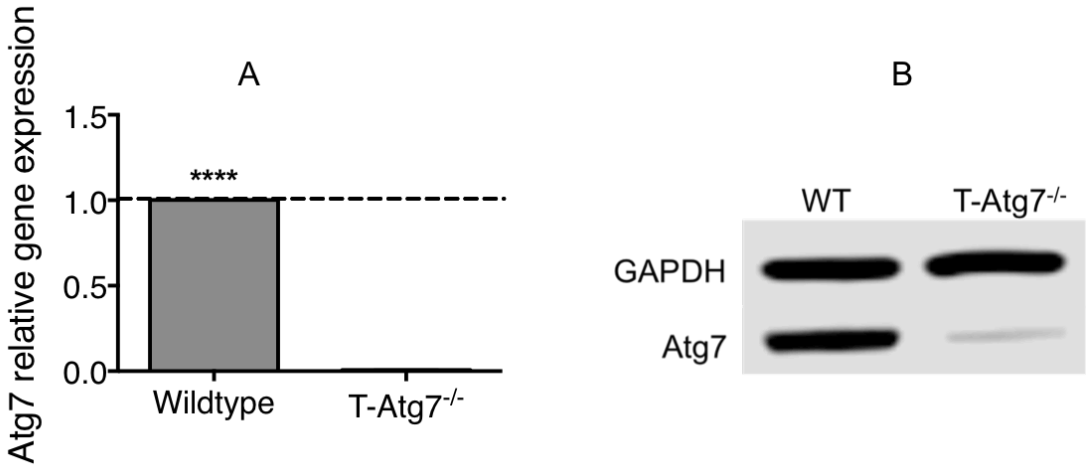


Figure 3.2 *Loss Atg7 expression in T cells from T-Atg7-/- mice.* ***a)*** *Atg7 gene expression from FACS-purified T cells was determined by q-PCR and the ΔΔCt method, values were normalised to average gapdh expression and compared to wildtype values for relative expression. For FACS-sorting, cells were isolated from the spleen based on CD8 and TCR expression. Bar graph shows Atg7 expression in T cells from T-Atg7-/- mice relative to T cells from WT mice. ****p<0.0001 by Student's test (n=4, pooled).* ***b)*** *Western blot for Atg7 protein expression. Protein was purified from MACS sorted CD8+ T cells from WT and T-Atg7-/- mice. GAPDH was used as a loading control (n=6, pooled). Western blot courtesy of Hanlin Zhang.*

3.6. Loss of *Atg7* results in ablated autophagy function

While *Atg7* is efficiently excised in T cells from T-Atg7$^{-/-}$ mice, it was important to demonstrate that this impacts upon functional autophagy levels. The archetypal method for the determination of autophagy levels is to measure levels of the autophagy protein LC3, commonly performed through western blot [148]. LC3 is found in two forms in the cytosol, an unlipidated form, termed LC3-I, or a lipidated version, known as LC3-II. The LC3-II form is inserted into autophagosomal membranes and mediates autophagosome formation [18]. Thus, measuring the conversion of LC3-I to LC3-II is a way to detect autophagy (the dogma being that more LC3-II equates to higher autophagy levels). It is also possible to determine the rate of autophagy, commonly referred to as the 'flux'. This is achieved through the addition of lysosomal inhibitors that prevent fusion of autophagosomes with lysosomes, therefore causing the accumulation of LC3-II/autophagosomes. Differences in LC3-II levels in the presence or absence of lysosomal inhibitors defines the rate of autophagic flux [148].

LC3-II could not be detected in purified *Atg7*$^{-/-}$ CD8^{+} T cells, as opposed to their wildtype counterparts. In the presence of the lysosomal inhibitor

bafilomycin, wildtype CD8⁺ T cells accumulated LC3-II, suggestive of active autophagy in these cells. However, no such accumulation was observed in *Atg7*$^{-/-}$ CD8⁺ T cells, indicative of abolished autophagy levels in the absence of *Atg7* (Figure 3.3).

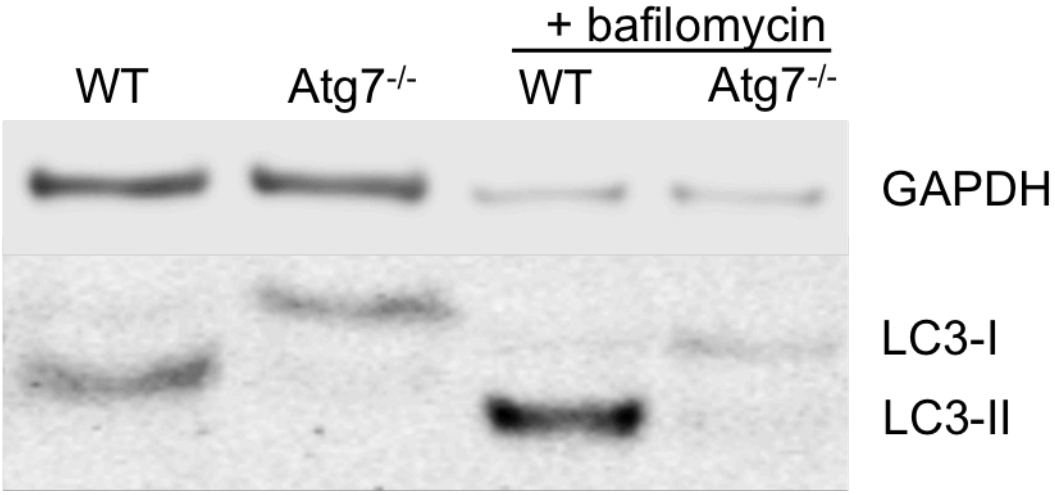


Figure 3.3 *Autophagy is abolished in Atg7*$^{-/-}$ *CD8⁺ T cells. MACS-purified CD8⁺ T cells from WT and T-Atg7*$^{-/-}$ *mice were untreated or treated with bafilomycin for 6 hours before whole protein extraction and western blot for LC3. GAPDH was used as a loading control (n=7 pooled mice). Protein levels were reduced for the bafilomycin-treated samples, as to not saturate the LC3-II band. Experiment performed with Hanlin Zhang.*

Similarly, LC3 accumulation could not be detected in *Atg7*$^{-/-}$ CD8⁺ T cells using the imaging flow cytometer, ImageStream. Through ImageStream, it is possible to visualise and quantify LC3 puncta in differing cell subsets using cell surface markers. A significantly decreased number of LC3 punctae was observed on a per cell basis in *Atg7*$^{-/-}$ CD8⁺ T cells compared to wildtype T cells (Figure 3.4). When treating with a flux inhibitor to assess autophagy flux/induction, LC3 puncta was also significantly lower in *Atg7*$^{-/-}$ CD8⁺ T cells (Figure 3.4), confirming that both basal autophagy and autophagy induction is critically impaired in the absence of *Atg7*.

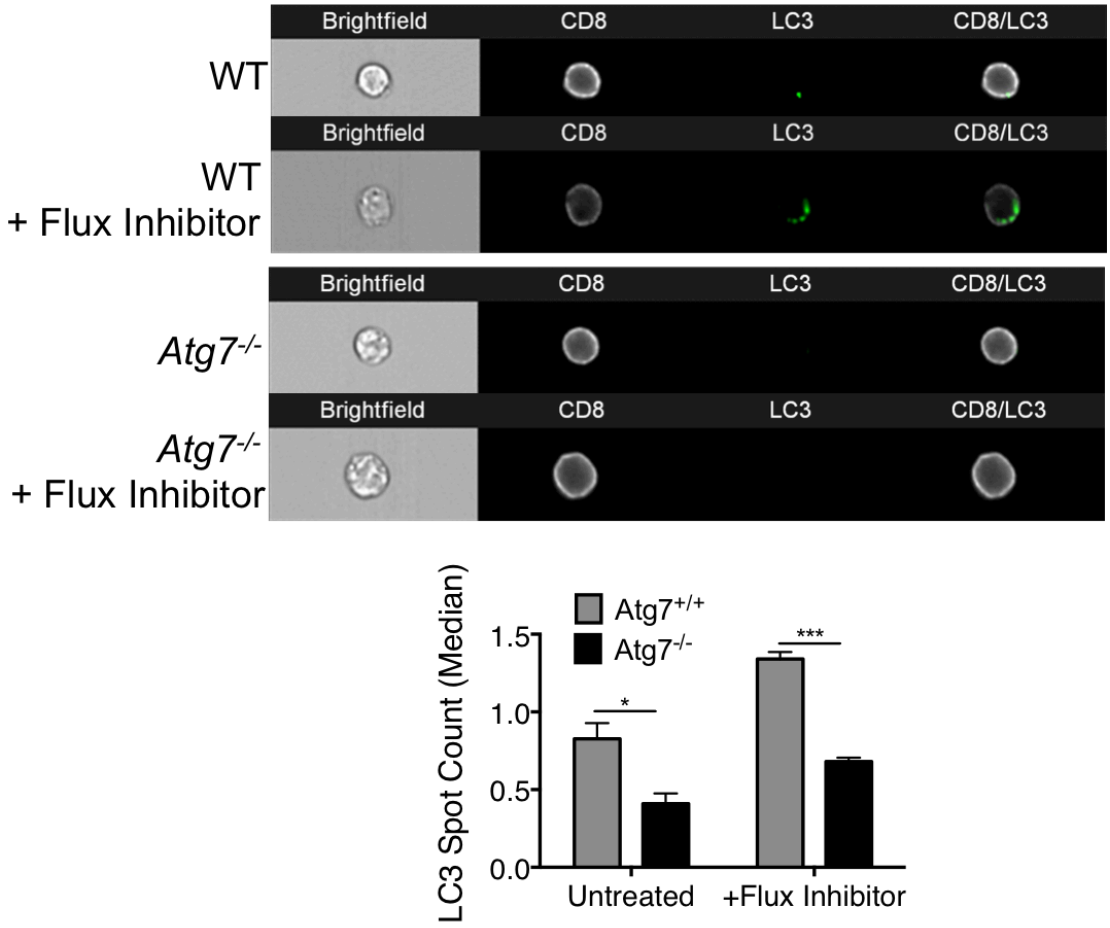


Figure 3.4 *Impaired autophagy in Atg7-/- CD8+ T cells. Splenocytes from WT and T-Atg7-/- mice were untreated or treated for 2 hours with an autophagy flux inhibitor before staining for relevant cell surface markers and LC3-II. LC3 spot count was assessed using ImageStream. Upper panels depict example LC3 puncta images (x60 magnification). The quantification in the lower panel is gated on CD8+ T cells. *p=0.0132, ***p=0.0002 as determined by Student t-test (n=4). With the kind help of Isabel Panse for analysis.*

3.7. T cell development is normal in the absence of autophagy

In T-Atg7$^{-/-}$ mice, *Atg7* is excised at the double positive stage of development when *CD4* is first expressed in the thymus. Thus, this is not a model to study the early stages of T cell development. During maturation, thymocytes proceed through a number of developmental stages defined by the expression of CD44 and CD25: DN1, CD44^{+} CD25^{-}; DN2, CD44^{+} CD25^{+}; DN3, CD44^{-}

CD25⁺; and DN4, CD44⁻ CD25⁻. Here, β loci gene rearrangements occur before cells proceed to positive and negative selection at the CD4⁺ CD8⁺ double positive stage. Selected T cells then downregulate either the CD4 or CD8 co-receptor and exit the thymus as mature T cells [103]. To study these early stages of T cell development, a model in which *Atg7* is excised under the control of the *Vav* promoter was utilised. Termed Vav-Atg7$^{-/-}$, these mice excise *Atg7* in all haematopoietic cells as early as the haematopoietic stem cell level [9 93]. While overall thymus cellularity is decreased in Vav-Atg7$^{-/-}$ mice (Figure 3.5a), thymocytes proceed normally through the early stages of maturation, with the exception of DN1 where the frequency of cells found at this stage is significantly lower in Vav-Atg7$^{-/-}$ mice (Figure 3.5b).

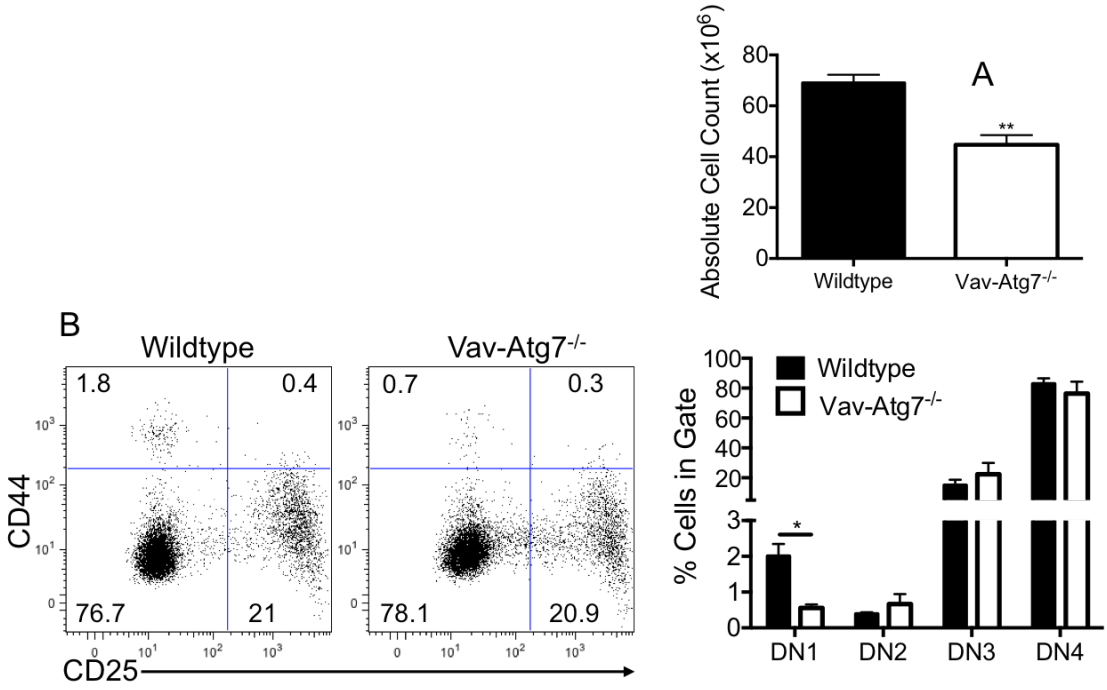


Figure 3.5 *Thymus cellularity and DN1-DN4 in Vav-Atg7$^{-/-}$ mice.* ***a)*** *Thymus cellularity in 5 week old WT and Vav-Atg7$^{-/-}$ mice, **p=0.0053 (n=4) by Student t-test.* ***b)*** *DN1-DN4 thymocyte development as determined by CD25 and CD44 expression on lineage-negative cells in 5 week old WT and Vav-Atg7$^{-/-}$ mice as assessed by flow cytometry. Example dot plots are shown, p*=0.0498 by Mann-Whitney U-test (n=4).*

The role of autophagy in the latter phases of T cell maturation, from double positive thymocytes through to the production of mature T lymphocytes, was assessed in T-Atg7$^{-/-}$ mice. Thymus cellularity was normal in this strain (data not shown), as was the frequency and cell number of CD4⁺ CD8⁺ DP cells, mature CD4⁺ SP T cells, and mature CD8⁺ SP T cells (Figure 3.6), suggesting autophagy is dispensable in T cells for their development in the thymus.

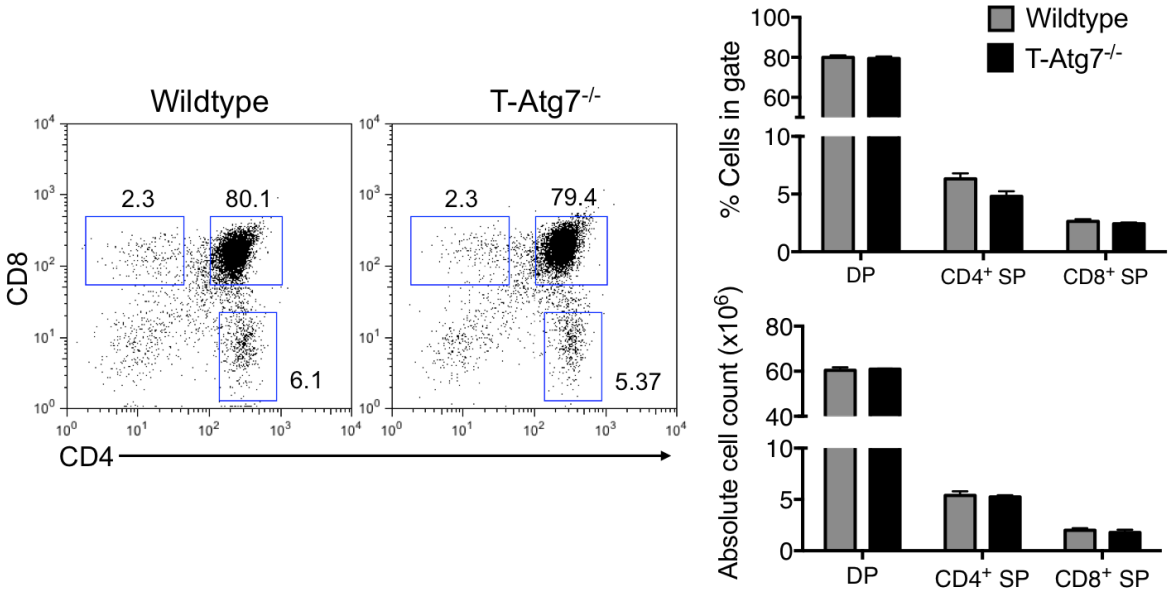


Figure 3.6 *T cell development in T-Atg7$^{-/-}$ mice. The number and frequency of DP thymocytes, CD4⁺ and CD8⁺ T cells from the thymi of 5 week old WT and T-Atg7$^{-/-}$ were counted and analysed by flow cytometry. Representative dot plots are shown (n=5).*

The maturation status of both CD4⁺ and CD8⁺ T cells in thymus may also be determined by expression of the surface marker CD24 [149]. Fully mature T cells downregulate CD24 before exiting the thymus for the periphery. A similar proportion of T cells lacking CD24 expression was observed in the thymi of wildtype and T-Atg7$^{-/-}$ mice, suggesting *Atg7$^{-/-}$* T cells exit the thymus as

normally differentiated, mature T cells. (Figure 3.7).

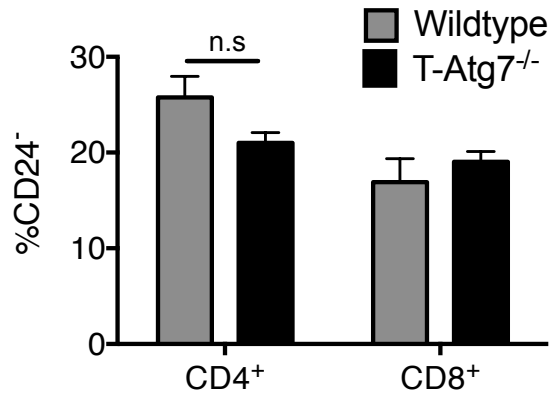


Figure 3.7 *Thymus maturation status of single-positive T cells in T-Atg7$^{-/-}$ mice. CD4^{+} and CD8^{+} T cells from the thymi 6 week old WT and T-Atg7$^{-/-}$ mice were assessed for CD24 expression by flow cytometry (n=4).*

3.8. T-Atg7$^{-/-}$ mice are lymphopaenic in the periphery

Next, the peripheral T cell compartment of T-Atg7$^{-/-}$ mice was assessed. A significant decrease in both CD4^{+} and CD8^{+} T cell frequency was observed in the blood of T-Atg7$^{-/-}$ mice at six weeks of age compared to wildtype controls (Figure 3.8a). A similar situation was found in the lymph nodes where absence of *Atg7* led to significantly diminished frequency and number of CD4^{+} and CD8^{+} T cells relative to wildtype mice (Figure 3.8b). These observations also held true for other lymphoid organs such as the spleen and bone marrow (data not shown). In both the blood and the lymph nodes, mice heterozygous for *Atg7* in the T cell lineage (T-Atg7$^{+/-}$) displayed a phenotype identical to that of wildtype mice, suggesting that loss of a single *Atg7* allele is not deleterious to T cell survival (Figure 3.8a,b).

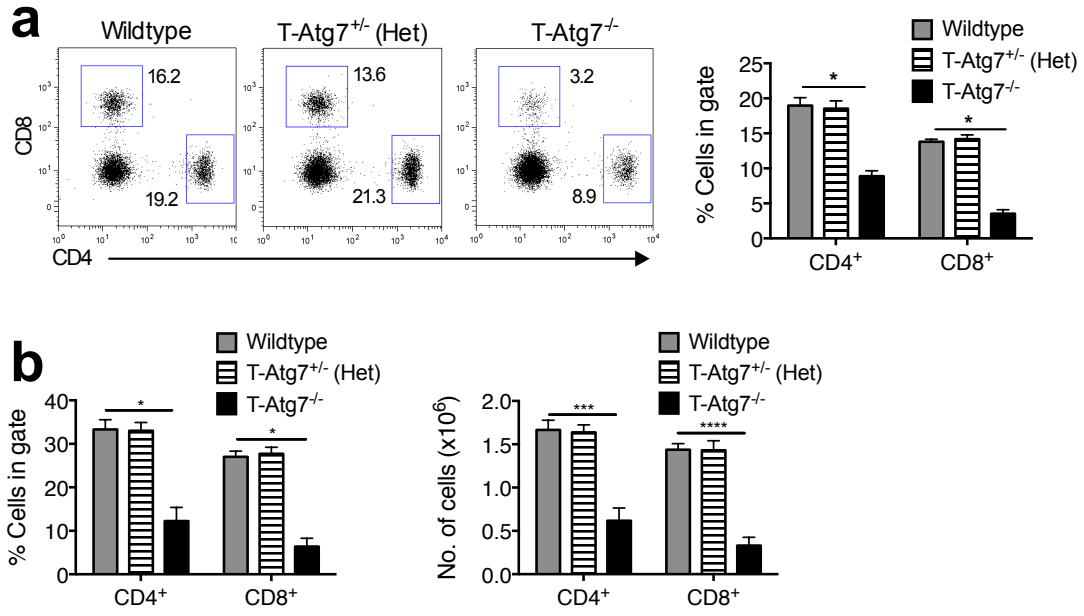


Figure 3.8 *T-Atg7-/- mice are lymphopaenic in the periphery.* ***a)*** *Flow cytometric analysis of CD4⁺ and CD8⁺ T cell frequency in the blood of wildtype, heterozygous (T-Atg7+/-), and T-Atg7-/- mice. Representative dot plots are shown. p*<0.05 by Mann-Whitney U-test (n=4).* ***b)*** *Frequency and absolute counts of T cells in lymph nodes from wildtype, T-Atg7+/- and T-Atg7-/- mice. Absolute counts were calculated from whole counting of the inguinal lymph nodes (n=4).* *p*<0.05 by Mann-Whitney U-test (n=4), p***=0.0002 by Student t-test (n=4), p****<0.0001 by Student t-test (n=4)*

T cell tracking in the blood reveals that the lymphopaenia observed in T-Atg7-/- mice is a permanent fixture, with the T cell compartment remaining in a depleted state for at least the first 62 weeks of life (Figure 3.9). However, relative to wildtype mice, this lymphopaenia does not significantly worsen over time. (Figure 3.9).

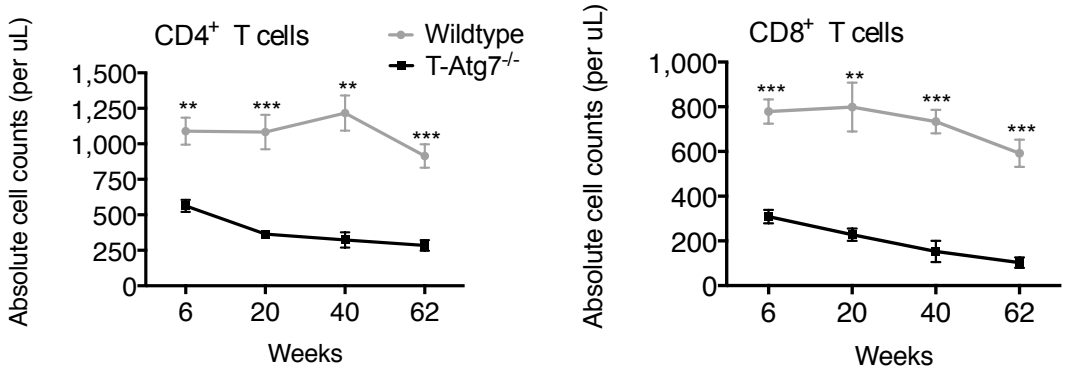


Figure 3.9 *Absolute counts of CD4⁺ and CD8⁺ T cells in the blood over time in WT and T-Atg7-/- mice. T cell counts were calculated using BD TruCount tubes. Statistics – Student t-test (n=4).*

While the peripheral T cell compartment of T-Atg7$^{-/-}$ mice is significantly perturbed, the counts of other cell populations, such as B cells (Figure 3.10a) and NK cells (Figure 3.10b), remain normal, confirming the T cell-specific nature and phenotype of this model.

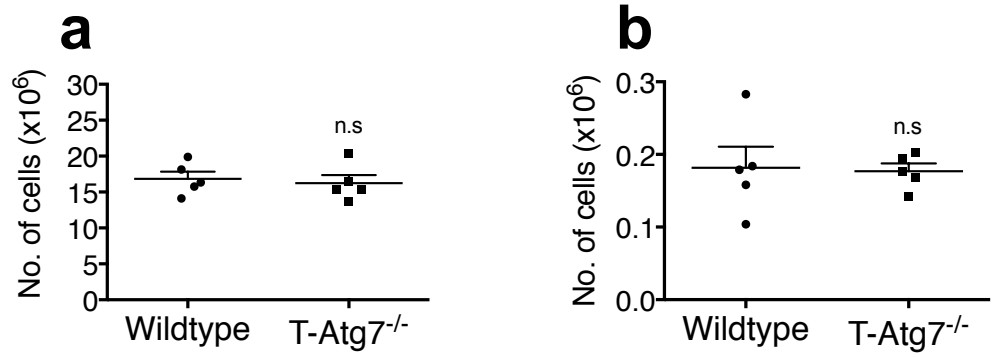


Figure 3.10 *Non-T cell populations are unaffected in number in T-Atg7-/- mice.* ***a)*** *Absolute counts of CD19⁺ B lymphocytes in the spleen of 6 week old wildtype and T-Atg7-/- mice as determined by flow cytometry (n=5).* ***b)*** *Absolute counts of NK1.1⁺ NK cells in the spleen of wildtype and T-Atg7-/- mice (n=5).*

3.9. The survival defect of *Atg7*$^{-/-}$ CD8⁺ T cells is cell intrinsic

Why does the loss of autophagy lead to T cell deficiency in T-Atg7$^{-/-}$ mice? Perhaps because *Atg7* is deleted in *CD4*-expressing cells, which also include macrophages and dendritic cells, interactions with antigen presenting cells required for T cell homeostasis are altered in a negative fashion. To address this, mixed bone marrow chimeras were employed so that *Atg7*$^{-/-}$ CD8⁺ T cells could be analysed in a normal, replete T cell environment alongside wildtype APCs. Bone marrow from either wildtype or T-Atg7$^{-/-}$ mice (both CD45.2) was mixed 1:1 with bone marrow from SJL CD45.1 mice. The mixed bone marrow was then transplanted into lethally irradiated CD45.1 hosts, which went untouched for 8 weeks to allow for reconstitution of the haematopoietic system (Figure 3.11).

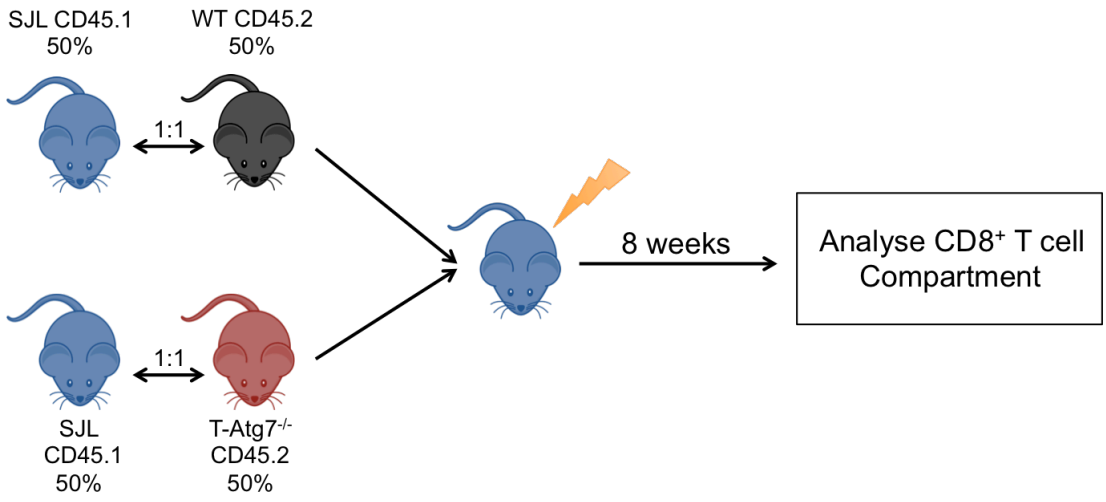


Figure 3.11 *Bone marrow chimera experimental set-up. Three million bone marrow cells from either WT or T-Atg7$^{-/-}$ mice were mixed with three million BM cells from SJL CD45.1 mice. The mixed bone marrow was transplanted by i.v injection into SJL CD45.1 hosts. BM Reconstitution took place over 8 weeks before mice were culled and the CD8⁺ T cell compartment analysed. Normal C57BL/6 WT and T-Atg7$^{-/-}$ mice acted as controls.*

Eight weeks after BM transplantation, the reconstitution of the haematopoietic system was assessed in the spleen and blood (data not shown). As expected, both CD45.1 and CD45.2 donors contributed equally to the host's haematopoietic system (Figure 3.12).

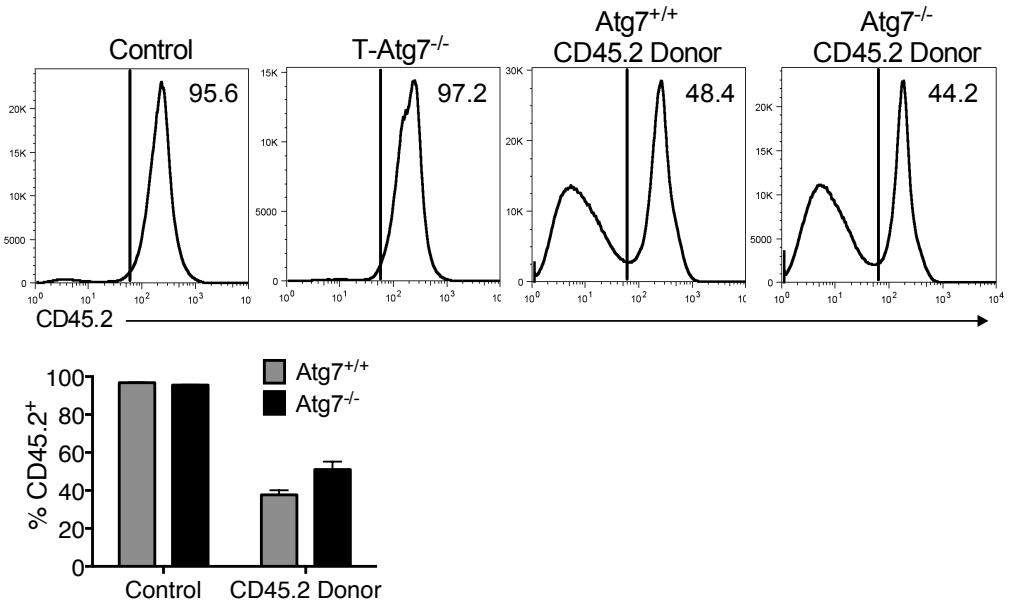


Figure 3.12 *Haematopoietic reconstitution in BM chimera mice. Eight weeks after BM transplantation, the frequency of donor CD45.2 cells was assessed in the spleen by flow cytometry. Quantified is the frequency of splenocytes expressing CD45.2 in BM chimera mice and in normal (CD45.2⁺) WT and T-Atg7⁻/⁻ mice that acted as controls, (n=4).*

Even in the presence of a full T cell niche, as is provided by a mixed BM chimera, the frequency of *Atg7⁻/⁻* CD8⁺ T cells in secondary lymphoid organs was decreased (Figure 3.13). While the wildtype CD45.2 donor contributed approximately 40% to the overall CD8⁺ T cell compartment, *Atg7⁻/⁻* donor CD8⁺ T cells made up only 20% of the total CD8⁺ T cell pool. This suggests that the survival defect previously described for autophagy-deficient CD8⁺ T cells [146] [9,53] is cell intrinsic in nature.

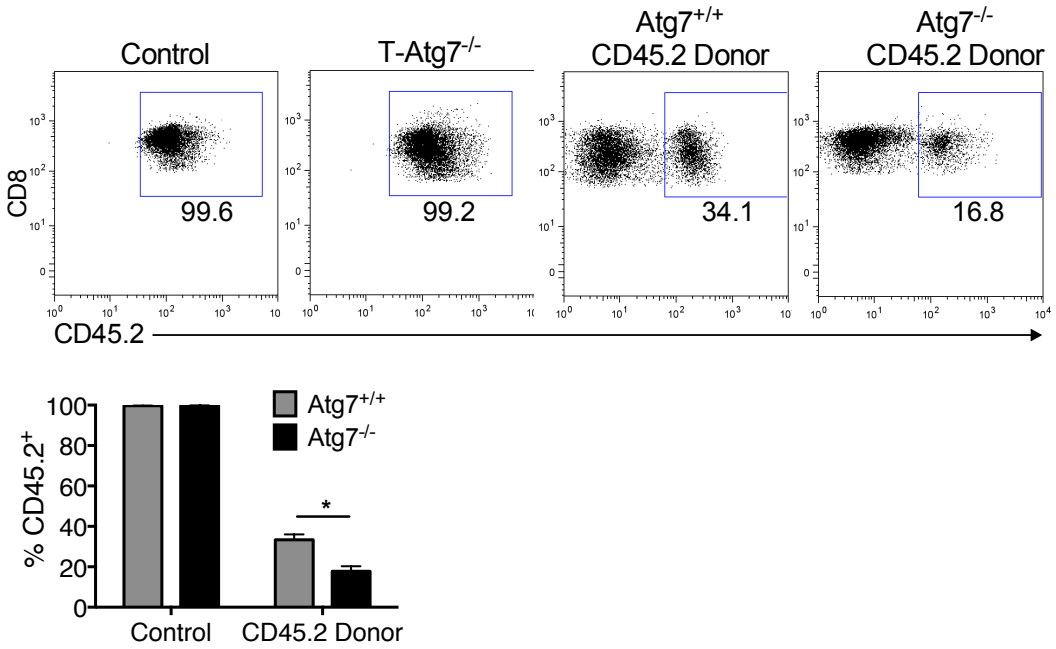


Figure 3.13 *Frequency of CD8+ T cells that are CD45.2 donor-derived in BM chimera mice. Example dot plots are shown and gated on CD8+ T cells. Quantified is the frequency of CD8+ T cells that express CD45.2 in the spleen of BM chimera and normal WT and T-Atg7-/- mice that acted as controls. p*=0.0286 by Mann-Whitney U-test (n=4).*

3.10. *Atg7-/-* T cells display an increased propensity for apoptosis

To further investigate the survival defect in the autophagy-deficient T cell pool, levels of apoptosis were examined by determining levels of active Caspase-3 in T cells from wildtype and T-Atg7-/- mice. Caspase-3 is an executioner caspase, activated in both the extrinsic (death ligand) and intrinsic (mitochondrial) apoptosis pathways, and therefore makes for a useful target in assessing apoptotic events. The percentage of cells staining positive for active Caspase-3 was two-fold higher in *Atg7-/-* CD4+ and CD8+ T cells compared to wildtype T cells, indicative of increased apoptosis (Figure 3.14). This increased susceptibility of autophagy-deficient T cells to cell death is the likely cause of the depleted T cell niche in T-Atg7-/- mice.

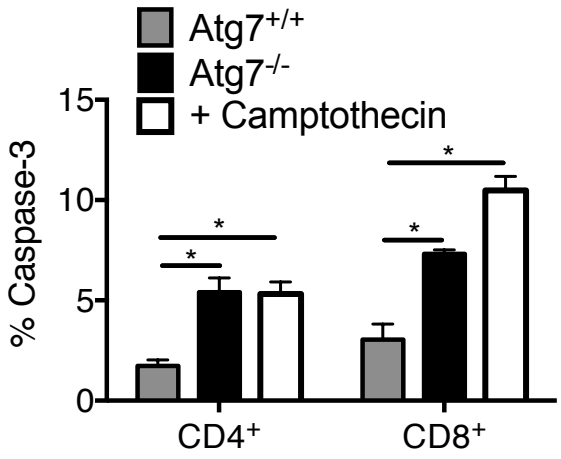


Figure 3.14 *Increased levels of apoptosis in Atg7$^{-/-}$ T cells. Splenocytes were stained ex vivo with the relevant cell surface marker and active caspase-3 and assessed by flow cytometry. As a positive control, wildtype splenocytes were incubated for 4 hours in the presence of camptothecin, which induces apoptosis through the introduction of DNA double strand breaks. Statistics – Mann Whitney U-test (n=5).*

3.11. *Atg7$^{-/-}$* CD8⁺ T cells respond normally to the homeostatic cytokine IL-7

In an attempt to explain why loss of autophagy increases susceptibility to cell death in CD8⁺ T cells, the ability of *Atg7$^{-/-}$* CD8⁺ T cells to respond to IL-7 was investigated. IL-7 is a gamma-chain cytokine required for the survival and homeostasis of peripheral T cells. It was reasoned that if autophagy-deficient T cells failed to respond efficiently to IL-7, perhaps through altered expression of the IL-7 receptor, this might explain the depleted T cell niche in T-Atg7$^{-/-}$ mice. However, the frequency of CD8⁺ T cells expressing IL-7Rα (an essential component of the IL-7R complex) was normal in T-Atg7$^{-/-}$ mice compared to wildtype controls (Figure 3.15), as was expression on Atg7$^{-/-}$ donor CD8⁺ T cells in BM chimera mice. IL-7Rα (CD127) intensity on a per

cell basis was also equivalent between *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ CD8⁺ T cells (data not shown).

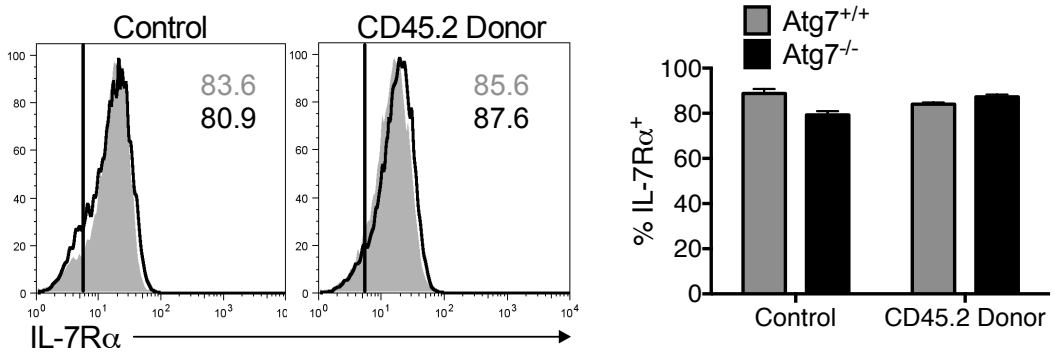


Figure 3.15 *Normal expression of IL-7Rα (CD127) on Atg7*$^{-/-}$ *CD8⁺ T cells. Splenocytes from BM chimera mice, made as described in* ***Figure 3.11****, and WT and T-Atg7*$^{-/-}$ *mice that acted as controls were analysed for IL-7R expression by flow cytometry. Example histograms are shown (n=5).*

The ability of *Atg7*$^{-/-}$ CD8⁺ T cells to respond to IL-7 was also assessed *in vitro*. Purified CD8⁺ T cells from wildtype and T-Atg7$^{-/-}$ mice were cultured with or without IL-7 for three days before survival was assessed by flow cytometry. Supplement of the culture medium with IL-7 improved the survival of wildtype CD8⁺ T cells six-fold (Figure 3.16). A similar fold-increase in survival in response to IL-7 was observed in *Atg7*$^{-/-}$ CD8⁺ T cells. These data rule out defective responses to IL-7 as a valid explanation for T cell lymphopaenia in T-Atg7$^{-/-}$ mice.

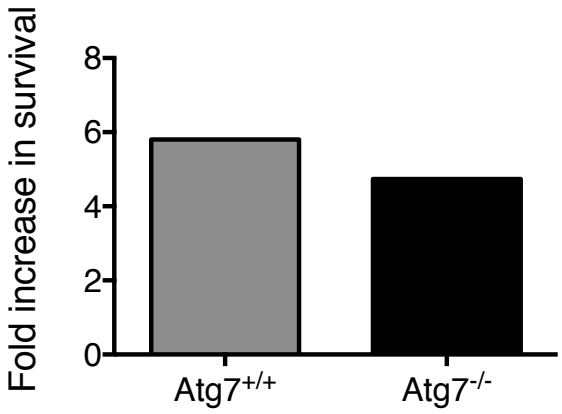


Figure 3.16 *Atg7-/- CD8+ T cells respond normally to IL-7 in vitro. MACS-purified CD8+ T cells from WT and T-Atg7-/- mice were cultured in the presence or absence of 10 ng/ml IL-7 for three days. Cells were then stained with a viability dye to assess the number of live cells remaining in culture. The fold increase in survival was calculated by dividing the mean survival of cells in the presence of IL-7 by mean survival in its absence (n=7 pooled mice).*

3.12. Atg7-/- T cells exhibit defects in mitochondrial organelle homeostasis

In previous studies, loss of autophagy in various cell types has been associated with an increased volume of malfunctional mitochondria [53 9 150]. We hypothesised that defective mitochondrial homeostasis might explain the increased levels of apoptosis observed in CD8+ T cells from T-Atg7-/- mice. Using MitoTracker Green, a dye that localises to mitochondrial membranes regardless of their membrane potential, the mitochondrial mass of *Atg7-/-* CD8+ T cells was examined in BM chimera mice as described in Figure 3.11. Within the same CD8+ T cell compartment, *Atg7-/-* donor CD8+ T cells exhibited significantly greater MitoTracker staining compared to wildtype CD45.1 donor CD8+ T cells (Figure 3.17). A similar phenotype was observed in control mice, where CD8+ T cells from T-Atg7-/- mice displayed a two-fold

increase in mitochondrial mass compared to wildtype CD8⁺ T cells, as determined by MitoTracker green staining (Figure 3.17).

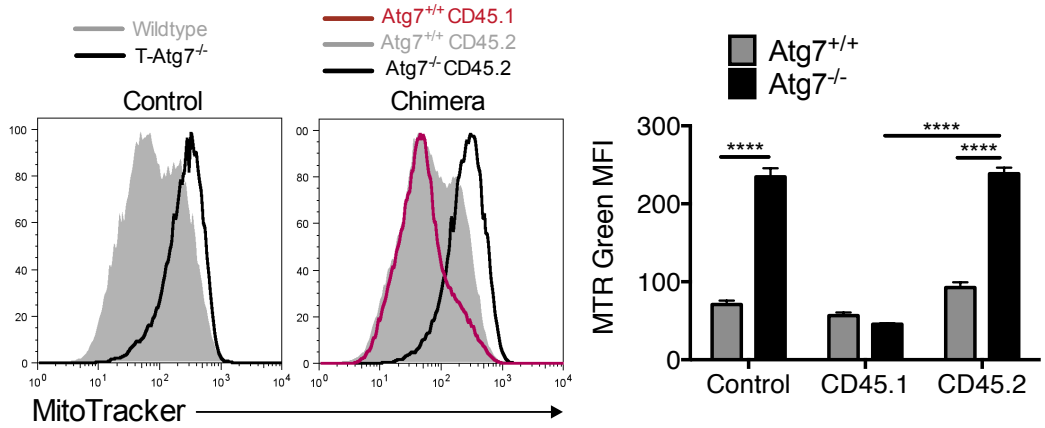


Figure 3.17 *Atg7-/- CD8⁺ T cells display increased mitochondrial mass. Bone marrow chimeras were set up as described in Figure 3.11. Splenocytes from BM chimera mice and WT and T-Atg7-/- mice that acted as controls were stained with the mitochondrial dye MitoTracker Green and assessed by flow cytometry. Data is gated on CD8⁺ T cell and is representative of three independent experiments. Representative histograms are shown. p***<0.0001 by Student's t-test (n=6).*

Similar to what has been shown elsewhere [53], mitochondrial mass appears to be developmentally regulated in CD8⁺ T cells. Mitochondrial load is halved on maturation from CD4⁺CD8⁺ DP thymocytes to mature SP CD8⁺ T cells in the thymus of wildtype mice (Figure 3.18). Once in the periphery, CD8⁺ T cells appear to undergo a further round of mitochondrial clearance, with mitochondrial volume a tenth of what it is in the thymus. *Atg7-/-* CD8⁺ T cells fail to regulate mitochondrial clearance in the same manner. Mitochondrial load is equivalent in DP thymocytes and mature thymic CD8⁺ T cells, and while mitochondrial mass does drop once cells enter the periphery, it is still ten-fold higher than what is observed in wildtype cells (Figure 3.18).

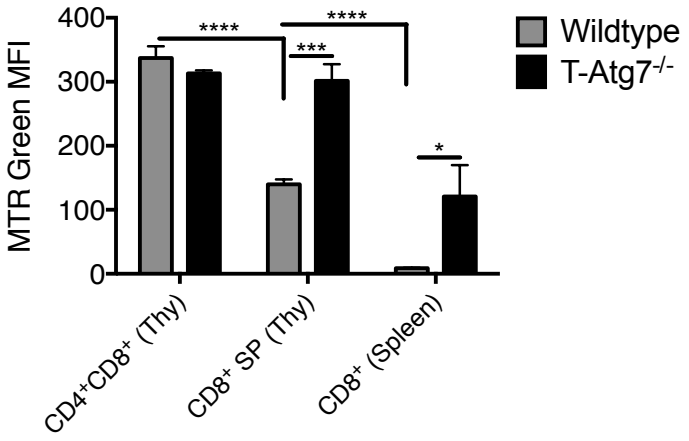


Figure 3.18 *Mitochondrial load is developmentally regulated in CD8⁺ T cells. Mean fluorescence intensity of MitoTracker Green staining in the thymus and spleen of 6-week-old WT and T-Atg7 mice. p=0.048, p***=0.003, p****<0.0001 by Student's t-test (n=6).*

Mitochondria are significant generators of reactive oxygen species (ROS), produced as a by-product of the electron transport chain. Autophagy is known to play a role in limiting ROS accumulation within cells, thereby maintaining cellular integrity [151]. We hypothesised that a failure to regulate mitochondrial volume in autophagy-deficient cells would lead to the accumulation of damaged mitochondria and with it, elevated levels of mitochondrial ROS. Generation of mitochondrial ROS was assessed using MitoSOX, a fluorogenic dye that specifically labels mitochondrial superoxide. Indeed, mitochondrial superoxide levels were significantly higher in *Atg7*$^{-/-}$ CD8^{+} T cells compared to wildtype controls (Figure 3.19). A failure to efficiently regulate mitochondria leading to increased ROS production may be the cause of intensified T cell death observed in T-Atg7$^{-/-}$ mice.

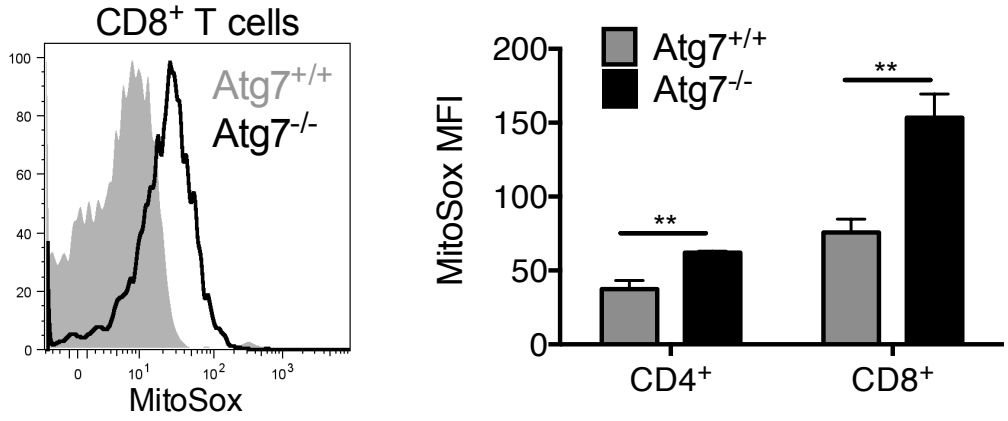


Figure 3.19 *Increased mitochondrial superoxide production in Atg7-/- CD8 T cells. Splenocytes were stained with the mitochondrial superoxide indictor MitoSOX and analysed by flow cytometry. Representative histogram is shown and is gated on CD8⁺ T cells. Data is representative of 5 independent experiments. p**<0.01 (n=5-7).*

3.13. CD8⁺ T cells from T-Atg7-/- mice display an activated, memory-like phenotype

In previous studies where T cell-specific deletions of autophagy genes have been utilised, various changes in T cell phenotype have been reported emphasising a shift towards a memory phenotype [152] [153]. Here, it is shown that this is also true for T-Atg7-/- mice. In the wildtype CD8⁺ T cell compartment, approximately 30% of cells displayed high expression for the memory/activation marker CD44, in contrast to the T-Atg7-/- CD8⁺ T cell lineage where as many as 50% of cells where found to be CD44hi (Figure 3.20a). Furthermore, when CD44 expression was tracked over time in the blood a dramatic phenotype was observed in T-Atg7-/- mice such that by one year of age nearly all CD8⁺ T cells were found to be CD44hi (Figure 3.20b). When this CD44hi population was observed in more detail, T-Atg7-/- mice exhibited a striking gain of effector memory phenotype (CD44hi CD62L-) CD8⁺ T cells, increased 75% in frequency compared to the same population in

wildtype mice (Figure 3.20c). The central memory (CD44hi CD62L+) subset was unaffected by the loss of autophagy.

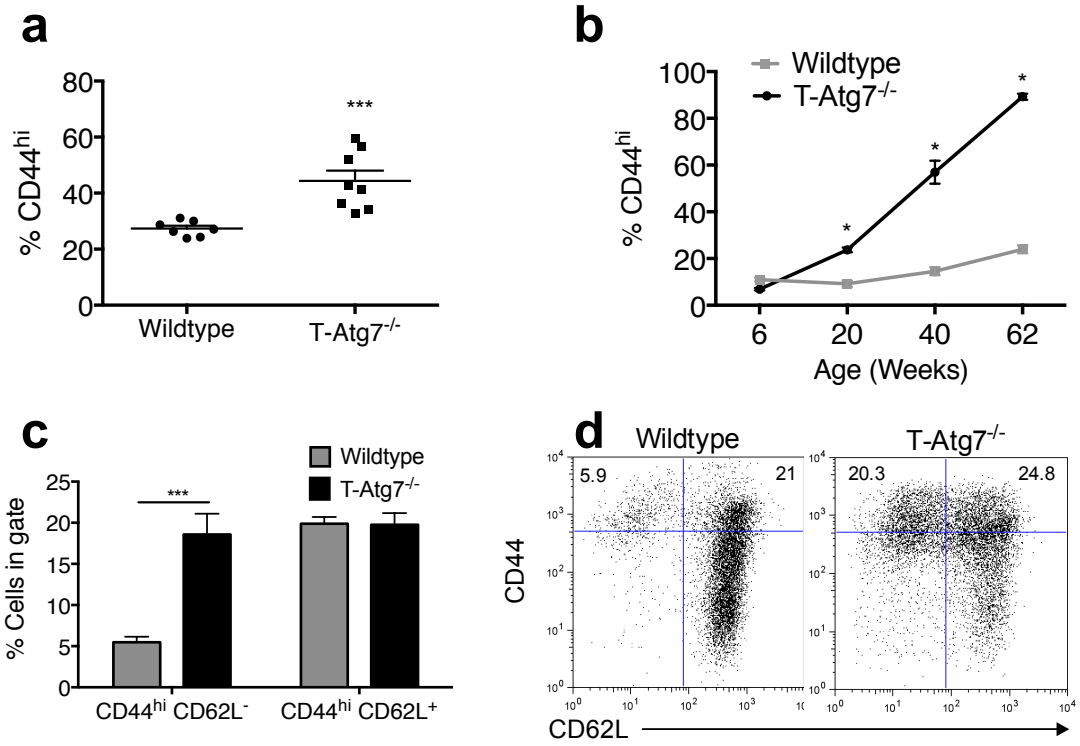


Figure 3.20 *T-Atg7-/- mice display an activated/memory-like phenotype.* ***a)*** *Splenocytes from 9-week old WT and T-Atg7-/- mice were stained for the cell surface markers CD44 and CD62L and assessed by flow cytometry. Quantification shows the frequency of CD44hi cells among gated CD8+ T cells p***=0.0003 (n=7-8).* ***b)*** *CD44 expression was tracked on CD8+ T cells in the blood through serial bleeding of the lateral tail vein at the time points indicated. p*=0.0286 (n=4).* ***c)*** *Effector (CD44hi CD62L-) vs central (CD44hi CD62L+) memory frequency in the splenic CD8+ T compartment from 9-week-old WT and T-Atg7-/- mice. p***=0.0003 (n=7-8). Representative dot plots of CD44 and CD62L staining is shown in* ***d).*** *All statistics are Mann-Whitney U-tests.*

3.14. CD8+ T cells from T-Atg7-/- mice exhibit an exhausted phenotype and signs of terminal differentiation

To confirm the increased activation status of *Atg7-/-* CD8+ T cells, the expression status of the T cell activation marker PD-1 [154] [155] was assessed in wildtype and T-Atg7-/- mice. The frequency of naïve (CD44lo) CD8+ T cells expressing PD-1 was increased by approximately 80% in T-Atg7-/- mice

compared to wildtype controls. However, this affect was most pronounced in $CD44^{hi}$ $CD8^+$ T cells, where a three fold increase in the frequency of PD-1 expression was observed in knockout mice relative to the same cells in wildtype mice (Figure 3.21a). These findings add to the evidence for an expanded activated/memory compartment in T-$Atg7^{-/-}$ mice.

Expression of PD-1 with other co-inhibitory receptors, such as TIM-3 or LAG-3, is a hallmark of T cell exhaustion [156]. Levels of exhaustion were investigated in T-$Atg7^{-/-}$ mice using the co-expression of PD-1 and TIM-3. Virtually no cells co-expressed PD-1 and TIM-3 in the $CD8^+$ compartment of wildtype mice. This is in contrast to knockout mice, where approximately 8% of $CD8^+$ T cells were found to have an exhausted phenotype (Figure 3.21b). In T-$Atg7^{-/-}$ mice, PD-1/TIM-3 co-expression was only observed in the expanded $CD44^{hi}$ subset (data not shown).

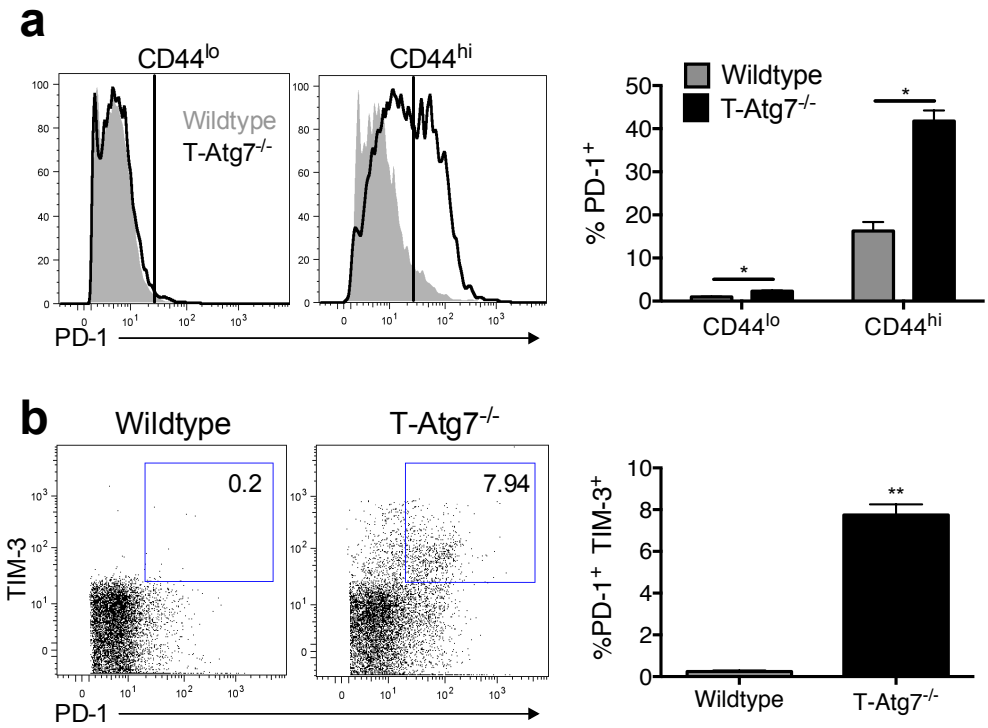


Figure 3.21 *Increased levels of activation and exhaustion in CD8+ T cells from T-Atg7-/- mice.* ***a)*** *PD-1 expression on splenic CD8+ T cells from 7 week old WT and T-Atg7-/- mice. p*=0.0286 (n=4).* ***b)*** *Analysis of PD-1 and TIM-3 co-expression on CD8+ T cells from the spleen of 7 week old WT and T-Atg7-/- mice. Representative dot plots are shown and are gated on CD8+ T cells. p**=0.0079 (n=5). All statistics are Mann-Whitney U-test.*

A signification proportion of CD8+ T cells from T-Atg7-/- mice display an activated, memory-like, and exhausted phenotype. To more closely examine CD8+ T cell differentiation status, the surface marker killer-cell lectin-like receptor G1 (KLRG1) was utilised. KLRG1 has previously been described as a marker of terminally differentiated, senescent CD8+ T cells, as well as being upregulated on antigen-specific CD8+ T cells in the course of viral infection [157]. The frequency of CD8+ T cells expressing KLRG1 was significantly higher in T-Atg7-/- mice compared to wildtype mice, suggesting a greater proportion of CD8+ T cells having reached terminal differentiation and senescence (Figure

3.22).

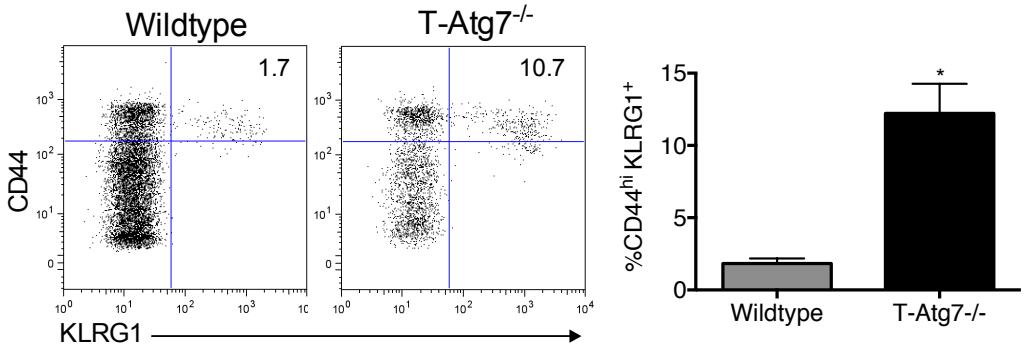


Figure 3.22 *Atg7-/- CD8+ T cells express higher levels of the senescence marker KLRG1. Splenocytes from 7-week-old WT and T-Atg7-/- mice were stained with the relevant cell surface marker and assessed by flow cytometry for the co-expression of CD44 and KLRG1. Data is gated on CD8+ T cells and is representative of three independent experiments. p*=0.0159 by Mann Whitney U-test (n=4-5).*

While KLRG1 expression can give an indication of the differentiation status of CD8+ T cells, its upregulation in other contexts makes it an unreliable readout for senescence. Therefore, we examined two markers associated with general cellular senescence. The expression of transcription factors *p16INK4A* and p21Cip1 are upregulated when cells of most type reach terminal differentiation and senescence [158]. Accordingly, we assessed *p16INK4A* and *p21Cip1* gene expression levels in CD8+ T cells from wildtype and T-Atg7-/- mice by q-PCR. CD44hi KLRG1+ CD8+ T cells from two-year-old mice were used as a positive control under the assumption that these highly differentiated cells, found at high frequency in aged mice, will exhibit high levels of senescence and thereby high levels of *p16INK4A* and *p21Cip1* expression. The CD8+ T cell compartment of T-Atg7-/- mice displayed a 10-fold and 15-fold increase in *p16INK4A* and *p21Cip1* expression, respectively, relative to young wildtype controls (Figure 3.23). The expression levels in *Atg7-/-* CD8+ T cells were

comparable to that of the enriched, highly differentiated CD44^{hi} KLRG1^{+} fraction found in aged mice, suggesting a substantially increased frequency of true end-stage, senescent CD8^{+} T cells in T-Atg7$^{-/-}$ mice.

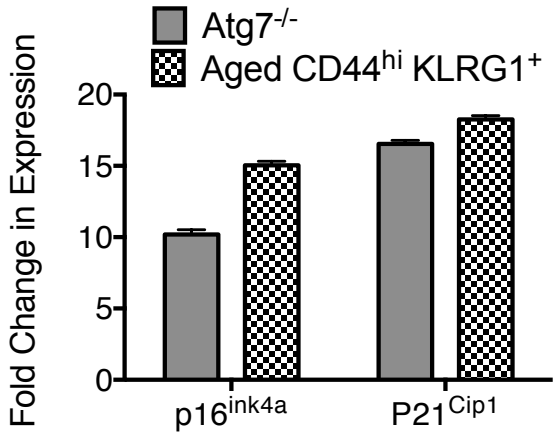


Figure 3.23 *p16^{INK4A} and p21^{Cip1} gene expression in Atg7$^{-/-}$ CD8^{+} T cells. Splenocytes from T-Atg7$^{-/-}$ mice were pooled and FACS sorted for CD8^{+} T cells, mRNA was then extracted for q-PCR analysis. Gene expression was normalised to GAPDH and relative to p16^{INK4A} and p21^{Cip1} expression in young wildtype FACS-sorted CD8^{+} T cells. As a positive control, splenocytes from four 2-year-old mice were pooled and FACS-sorted for CD8^{+} KLRG1^{+} T cells. Error bars – standard deviation on ΔC_T of triplicates (n=4-7).*

3.15. The CD8^{+} T cell compartment of Vav-Atg5$^{-/-}$ mice is similar in phenotype to that of T-Atg7$^{-/-}$ mice

Is the altered CD8^{+} T cell compartment of T-Atg7$^{-/-}$ mice specific to the loss *Atg7*, or does deletion of other autophagy genes result in a similar phenotype? To answer this question we examined the CD8^{+} T cell lineage in Vav-Atg5$^{-/-}$ mice, where the essential autophagy gene Atg5 is deleted in the haematopoietic system under the control of the *Vav* promoter. Vav-Atg5$^{-/-}$ mice exhibit severe lymphopaenia in the periphery (Figure 3.24). By six weeks of age, the frequency of CD4^{+} T cells and CD8^{+} T cells in the spleen is

decreased 4-fold and 3-fold respectively in Vav-Atg5$^{-/-}$ compared to wildtype mice.

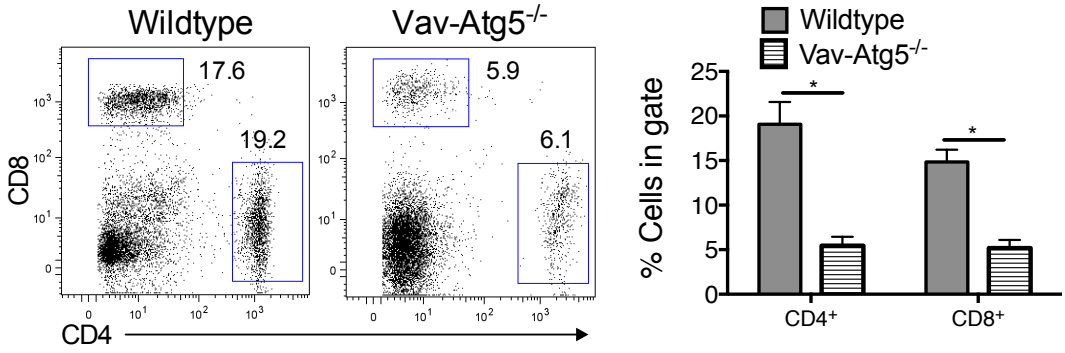


Figure 3.24 *Vav-Atg5$^{-/-}$ mice are T cell lymphopaenic. Splenocytes from 6-week-old WT and Vav-Atg5$^{-/-}$ mice were stained with the relevant cell surface markers in order to assess T cell frequency by flow cytometry. Representative dot plots are shown. p*=0.0286 (n=4) by Mann-Whitney U-test.*

Like in T-Atg7$^{-/-}$ mice, a large proportion of CD8^{+} T cells from Vav-Atg5$^{-/-}$ mice express markers associated with memory and cell activation. The frequency of CD44^{hi} CD8^{+} T cells is increased five-fold in Vav-Atg5$^{-/-}$ mice (data not shown), while the effector memory population (CD44^{hi} CD62L^{-}) is also significantly increased (Figure 3.25a), identical to the phenotype observed in T-Atg7$^{-/-}$ mice. Furthermore, Vav-Atg5$^{-/-}$ mice display a 10-fold increase in the frequency of cells expressing the activation marker PD-1 relative to wildtype controls (Figure 3.25b).

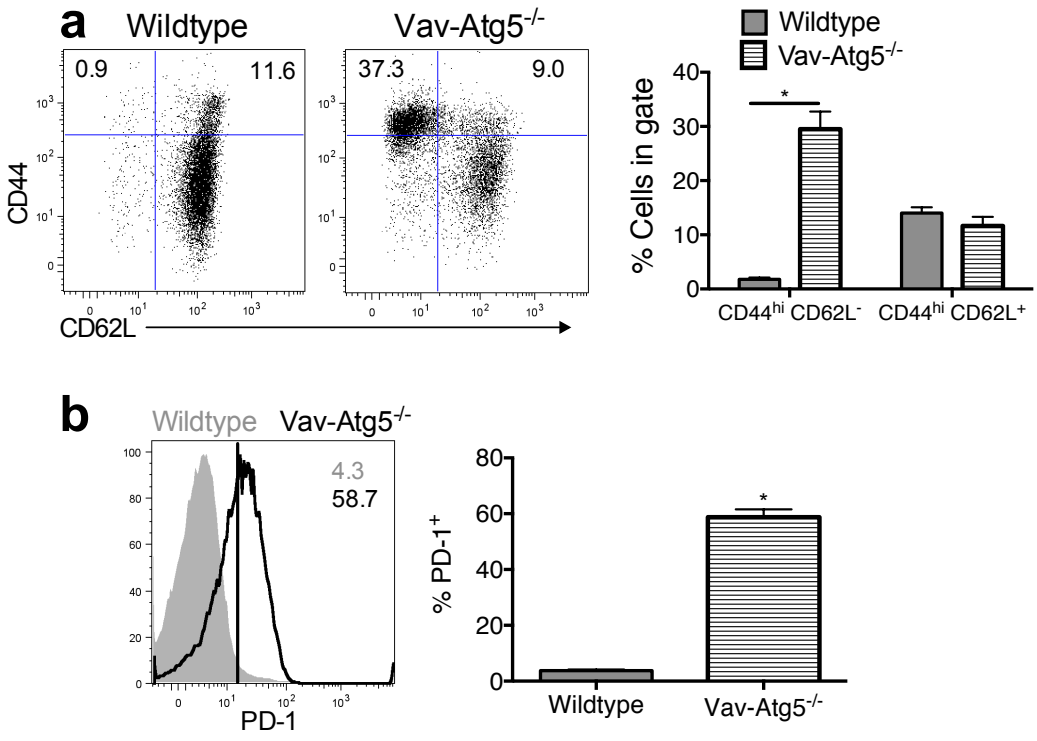


Figure 3.25 *Increased memory/activated CD8+ T cell phenotype in Vav-Atg5-/- mice.* ***a)*** *Splenocytes from 6-week-old WT and Vav-Atg5-/- were assessed for CD44 and CD62L expression by flow cytometry. Representative dot plots are shown and gated on CD8+ T cells. p*=0.0286 (n=4).* ***b)*** *Frequency of splenic CD8+ T cells from WT and Vav-Atg5-/- mice that are PD-1+. p*=0.0286 (n=4). All statistics are Mann-Whitney U-test.*

The increased frequency of CD44hi KLRG1+ CD8+ T cells observed in T-Atg7-/- mice is also observed in the Vav-Atg5-/- strain. Here, there is a seven-fold increase in the percentage of CD8+ T cells expressing the senescence marker KLRG1 (Figure 3.26). Thus, loss of *Atg5-/-* in the CD8+ T cell lineage also leads to a CD8+ T cell compartment defined by lymphopaenia, and an expanded population of cells exhibiting markers of activation and memory that is subsequently pushed towards terminal differentiation and senescence. Therefore, this phenotype is not specific to the loss of *Atg7*; rather it is driven by loss of functional autophagy caused by the absence of proteins essential for

autophagosome elongation, such as Atg5 or Atg7.

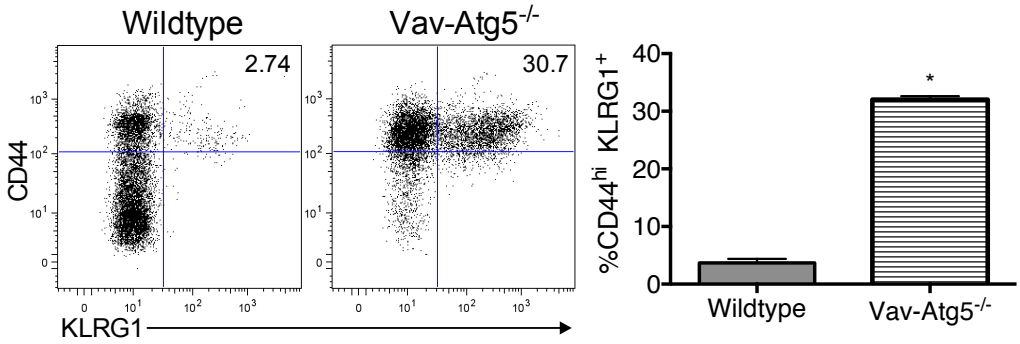


Figure 3.26 *Increased frequency of senescent-phenotype CD8 T cells in Vav-Atg5$^{-/-}$ mice. Lymph nodes from 6-week-old WT and Vav-Atg5$^{-/-}$ mice were assessed for CD44 and KLRG1 expression by flow cytometry. Representative dot plots are shown and are gated on CD8⁺ T cells. p*=0.0286 by Mann Whitney U-test (n=4).*

3.16. *Atg7$^{-/-}$* CD8⁺ T cells undergo profound homeostatic proliferation

During normal T cell homeostasis, CD8⁺ T cells undergo a basal level of cell proliferation controlled by the action of cytokines and interaction with self-peptide presenting MHC [159]. This low level turnover in the absence of antigen is termed homeostatic proliferation, levels of which were investigated in wildtype and T-Atg7$^{-/-}$ mice. To investigate CD8⁺ T cell turnover, two techniques were employed. The first utilised Ki-67, a marker of proliferation. Although its function within the cell remains unclear, the Ki-67 nuclear protein is only expressed during active stages of the cell cycle, namely G1, S, G2 and mitosis (M), yet absent from cells undergoing cell cycle arrest in G0 [160]. Naïve CD8⁺ T cells from wildtype and T-Atg7$^{-/-}$ mice displayed a similar level of homeostatic turnover according to Ki-67 staining. As expected, the rate of cell turnover was higher in the memory (CD44^{hi}) compartment compared to naïve cells [159]. However, in T-Atg7$^{-/-}$ mice 80% of CD44^{hi} CD8⁺ T cells

were found to be Ki-67-positive compared to just 40% in wildtype controls (Figure 3.27a). BrdU incorporation *in vivo* was also utilised to measure CD8⁺ T cell division. An analogue of thymidine, BrdU is incorporated into DNA during DNA synthesis and therefore is a read out for S-Phase activity and cell turnover. Mice were pulsed with one of injection of BrdU and uptake in CD8⁺ T cells was measured three days later. Similar levels of BrdU incorporation was observed in splenic CD8⁺ T cells from wildtype and T-Atg7$^{-/-}$ mice. However, in the bone marrow *Atg7*$^{-/-}$ CD8⁺ T cells displayed significantly increased BrdU uptake compared to wildtype cells (Figure 3.27b), suggesting an elevated rate of homeostatic proliferation in *Atg7*$^{-/-}$ CD8⁺ T cells.

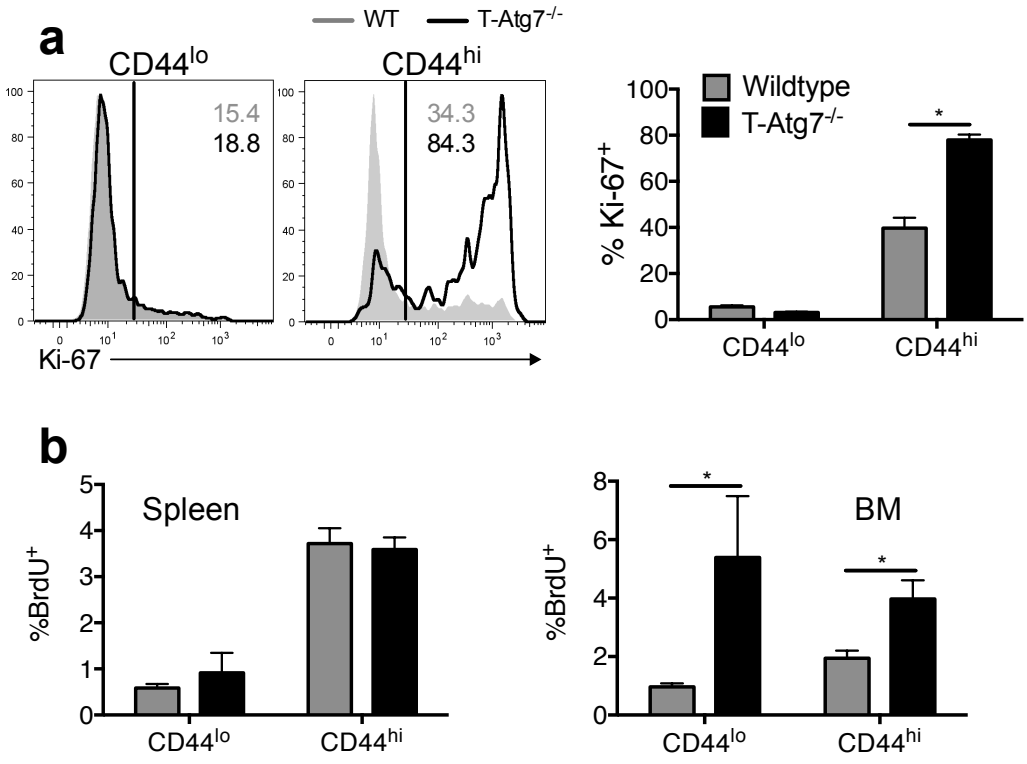


Figure 3.27 *CD8⁺ T cell homeostatic proliferation is increased in T-Atg7*$^{-/-}$ *mice.* ***a)*** *Percentage of CD44*^{lo} *and CD44*^{hi} *CD8⁺ T cells expressing Ki-67 in the spleen of 8 week old WT and T-Atg7*$^{-/-}$ *mice. Bar graphs are representative of three independent experiments, p*=0.0286 (n=4).* ***b)*** *17 week old mice were pulsed intraperitoneally with 1 mg BrdU and uptake measured in CD8⁺ T cells three days later in the spleen and bone marrow. Bar graphs*

are representative of two independent experiments, p<0.05 (n=5). Data are representative of 2 independent experiments. All statistics are Mann-Whitney U-test.*

To confirm that the increased rate of cell replication in CD8⁺ T cells was indeed due to amplified homeostatic proliferation, expression of the cell surface protein CD24 was assessed on CD8⁺ T cells. CD24, as well as being a marker of T cell maturation, is essential for T cell proliferation in a lymphopaenic environment. *CD24*$^{-/-}$ T cells adoptively transferred into irradiated hosts fail to effectively proliferate in response to lymphopaenia [161]. CD24-expressing CD8⁺ T cells were three times more frequent in the T-Atg7$^{-/-}$ mice relative to the wildtype controls, suggestive of elevated rates of homeostatic proliferation in knockout mice (Figure 3.28).

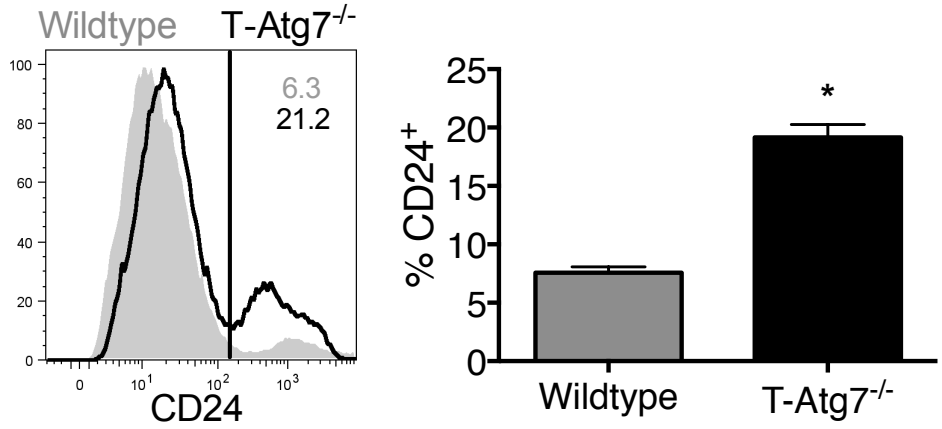


Figure 3.28 *CD24-expressing CD8⁺ T cells are increased in frequency in T-Atg7$^{-/-}$ mice. Splenocytes from 10-week-old WT and T-Atg7$^{-/-}$ mice were assessed by flow cytometry for CD24 expression. A representative histogram is shown. Data is gated on CD8⁺ T cells and representative of three experiments, p*=0.0286 (n=4).*

To confirm that lymphopaenia is the driving force behind the observed increase in homeostatic proliferation, cell turnover was analysed using BM chimeras. When *Atg7*$^{-/-}$ CD8⁺ T cells were placed in a replete T cell niche,

homeostatic proliferation was comparable with *Atg7*$^{+/+}$ donor CD8⁺ T cells (Figure 3.29). These data suggest that auto-proliferation is not an intrinsic feature of *Atg7*$^{-/-}$ CD8⁺ T cells, rather an attempt of T-Atg7$^{-/-}$ mice to refill the lymphopaenic T cell niche.

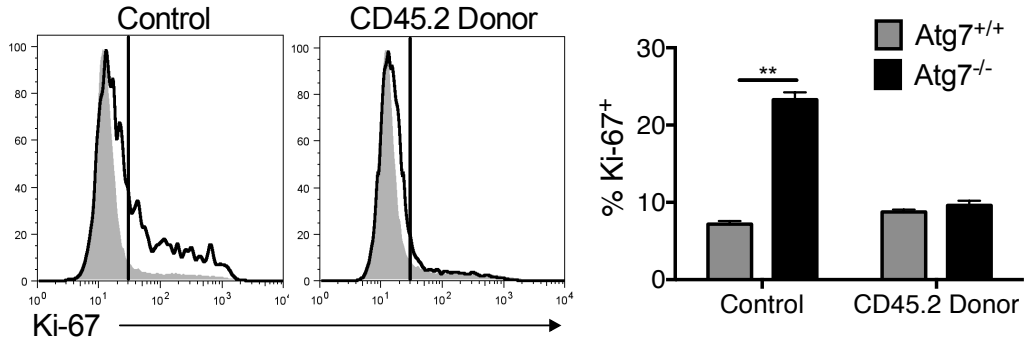


Figure 3.29 *Homeostatic proliferation of Atg7*$^{-/-}$ *CD8 T cells is normal in a replete T cell niche. Cell turnover was assessed in CD45.2⁺ donor CD8⁺ T cells by assessing Ki-67 nuclear expression. BM chimera mice were set up as described in* ***Figure 3.11****. CD8⁺ T cells from WT and T-Atg7*$^{-/-}$ *mice acted as controls. Representative histograms are shown and are gated on CD8⁺ T cells. p**=0.001 by Mann-Whitney U-test (n=5).*

3.17. The altered CD8⁺ T cell phenotype in T-Atg7$^{-/-}$ mice is driven by lymphopaenia and homeostatic proliferation

To investigate the cause behind the activated/memory phenotype CD8⁺ T cells in T-Atg7$^{-/-}$ mice we again made use of BM chimeras. It was hypothesised that the act of abnormal homeostatic cell turnover was bringing about this change in surface phenotype where enhanced proliferation would push cells towards exhaustion and terminal differentiation. When *Atg7*$^{-/-}$ CD8⁺ T cells are placed into a replete T cell compartment, normal levels of homeostatic proliferation are observed. On that basis, the *Atg7*$^{-/-}$ CD8⁺ T cell phenotype should also be normal when placed amongst a full compliment of

T cells. Indeed, in BM chimera mice the activated/memory phenotype is no longer present on *Atg7*$^{-/-}$ CD8⁺ T cells and the frequency of CD8⁺ T cells expressing the memory marker CD44 is comparable between wildtype and *Atg7*$^{-/-}$ donor CD8⁺ T cells (Figure 3.30a). In T-Atg7$^{-/-}$ mice, a large proportion of CD8⁺ T cells lack expression of CD62L, normally a sign of activation and antigen-experience. However, in BM chimera mice, the frequency of cells expressing CD62L is also comparable between wildtype and *Atg7*$^{-/-}$ CD8⁺ T cells (Figure 3.30b).

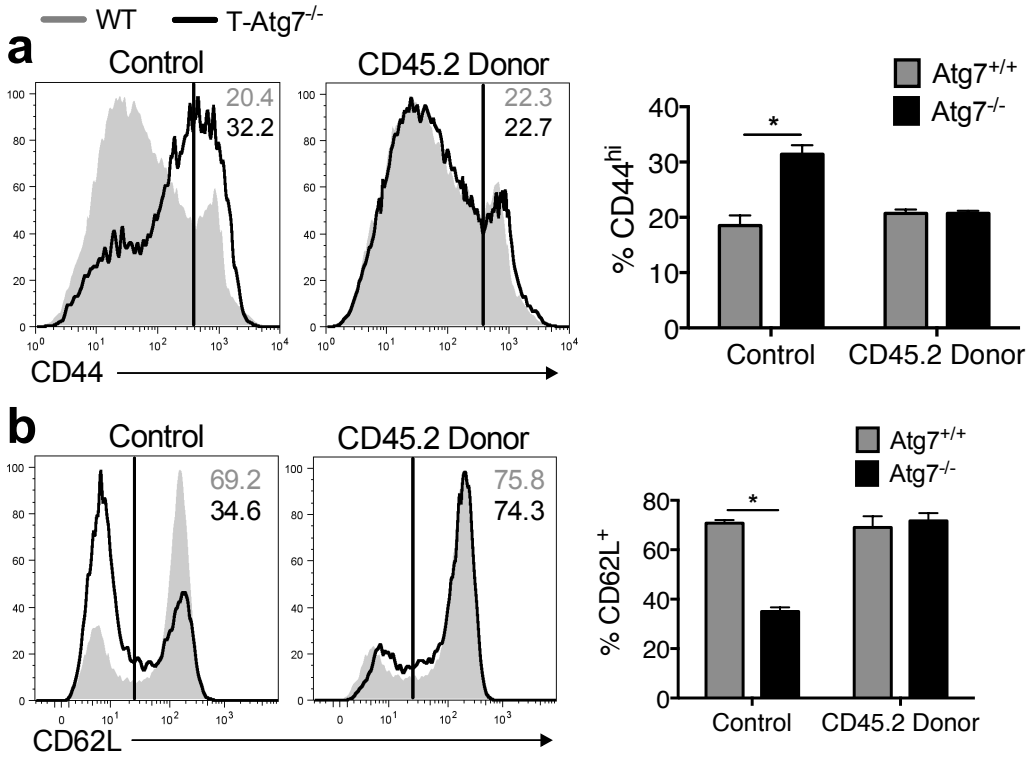


Figure 3.30 *Normal expression pattern of CD44 and CD62L on Atg7*$^{-/-}$ *CD8⁺ T cells in a replete environment. Bone marrow chimera mice were generated as in* ***Figure 3.11****. 8 weeks after transplantation the frequency of CD44*^{hi} *and CD62L⁺ cells were assessed in the donor CD8⁺ T cell compartment. Normal wildtype and T-Atg7*$^{-/-}$ *mice were used as controls. p*<0.05 by Mann-Whitney U-test.*

Chapter 3: Discussion

Autophagy is required for naïve CD8⁺ T cell homeostasis

Discussion

3.18. Autophagy is largely dispensable for T cell development

In this study, two murine models were used to assess autophagy in T cell development: one in which *Atg7* is deleted in the haematopoietic lineage (Vav-Atg7$^{-/-}$); and another in which the same gene is deleted specifically in T cells (T-Atg7$^{-/-}$). Use of T-Atg7$^{-/-}$ mice in this respect is somewhat limited as *Atg7* excision only occurs at the double positive stage of development, therefore making it impossible to investigate autophagy during the early stages of T cell maturation. In Vav-Atg7$^{-/-}$ mice, there is an altered frequency of early-stage DN1 cells. However, maturation appears to proceed in a normal fashion as the frequency of DN2-DN4 cells is comparable with wildtype mice. This altered DN1 frequency in Vav-Atg7$^{-/-}$ may originate from a smaller number of lymphoid progenitors seeding the thymus and was proposed as an explanation for low thymus cellularity in *Beclin1*$^{-/-}$ *Rag*$^{-/-}$ chimeras [162]. Previous work has shown how Vav-Atg7$^{-/-}$ mice exhibit altered haematopoietic stem cell (HSC) function and maintenance that eventually leads to bone marrow collapse [93]. This failure at the HSC level might lead to fewer lymphoid progenitors entering the thymus at the DN1 stage. These early T cell progenitors are unlikely to be functionally defective as DN1-DN4 development is normal. Although not assessed, one might expect to see increased proliferation of DN1 cells in Vav-Atg7$^{-/-}$ mice to compensate for a low number of seeding early T cell progenitors (ETPs). Changes in the local stroma could further impact upon thymus size. Although *Atg7* excision does not occur in the thymic stroma, this compartment is developmentally and

functionally symbiotic with the surrounding thymocytes [110,163] [164] [165]. While epithelial precursors arise by thymocyte-independent mechanisms, maturation of these epithelial progenitors into cortical and medullary thymic stromal cells is T cell-dependent [165]. On that basis, *Atg7*$^{-/-}$ ETPs and DN cells may be functionally aberrant in their ability to maintain the thymic stroma microenvironment resulting in loss of thymus integrity and cellularity. Low thymic cellularity has additionally been observed in *Atg5*$^{-/-}$ foetal liver chimeras suggesting this is not an *Atg7*-specific phenomenon [146]. Interestingly, T-Atg7$^{-/-}$ mice show no such fall in thymus cellularity. This is at odds with similar models in which *Atg3* or *Atg5* is deleted under the Lck promoter where thymic cellularity is decreased [145,166]. These conflicting observations may stem from differences in the timing of *Atg7* excision in *Lck-Cre* or *CD4-Cre* mice. *Lck*-mediated excision occurs at the DN stage, in contrast to *CD4*-controlled excision which takes place at the DP stage. These temporal differences might explain the disparity between thymus cellularity in *Atg5*$^{flox/flox}$ *Lck-Cre* and T-Atg7$^{-/-}$ mice. In support of this, *Vps34*$^{flox/flox}$ *CD4-Cre* mice also exhibit normal thymus cellularity [167].

Although autophagy is active during all stages of T cell maturation [166], it appears to be dispensable for T cell development. Mice with T cell-specific deletions of *Atg3*, *Atg5, Atg7,* and *Vps34* all exhibit normal frequencies of DN, DP, and mature SP CD4⁺ and CD8⁺ T cells, as do T-Atg7$^{-/-}$ mice [145] [146] [166] [167]. A previous study has suggested that the cells sensitive to negative selection are the SP thymocytes expressing high levels of the heat-stable antigen CD24 [168]. These SP cells are considered to be semi-mature as they become apoptotic

in response to antigen. This is not the case for $CD24^{low}$ SP thymocytes, and therefore lack of CD24 expression is suggested to be a marker of mature SP T cells [112]. The frequency of $CD24^{low}$ SP T cells was comparable between wildtype and T-$Atg7^{-/-}$ mice, confirming that thymocyte development and mature T cell production is normal in the absence of autophagy.

3.19. T-$Atg7^{-/-}$ mice are lymphopaenic in the periphery

While T cell development in the absence of autophagy is normal, peripheral T cell survival is significantly perturbed. In the blood and secondary lymphoid organs of T-$Atg7^{-/-}$ mice there is a precipitous drop in the frequency of both $CD4^+$ and $CD8^+$ T cells with absolute numbers down at least 50%. These data concur with similar models where loss of *Atg3* and *Atg5* in the T cell lineage also exhibit peripheral lymphopaenia [145] [166]. This adds weight to the idea that autophagy is essential for cell survival *in vivo* that has been clearly demonstrated in other systems such as the central nervous system, where conditional deletion of *Atg5* or *Atg7* leads to neurodegeneration associated with increased cell death [169,170]. In this study, we show that loss of autophagy in T cells results in high levels of apoptosis that results in depletion of the T cell compartment.

Previous studies have identified a role for autophagy in organelle regulation [9,171,172]. Here, it was shown that the mitochondrial burden of autophagy-deficient $CD8^+$ T cells was significantly higher to that of wildtype cells. Furthermore, levels of mitochondrial superoxide were also elevated *$Atg7^{-/-}$* $CD8^+$ T cells. Thus, in the absence of autophagy, T cells fail to effectively regulate mitochondrial mass resulting in the accumulation of damaged

organelles and excess ROS production (Figure 3.31). What is the importance of this? The data presented here suggests that mitochondrial volume is developmentally regulated in T cells. DP thymocytes exhibit greater mitochondrial load than SP thymocytes, which in turn show higher levels than peripheral T cells. This implies a need to remove mitochondria during T cell development, but also upon egress from the thymus into the periphery. Why the requirement to drastically remove mitochondria between the thymus and the periphery? Environmental factors could play a key role. The low oxygen tension in the thymus might require high levels of mitochondria in order to meet the metabolic and energetic demands of developing T cells. The oxygen tension in the blood is also higher than in the thymus [173], and therefore a large mitochondrial volume is no longer necessary. A failure to remove these superfluous mitochondria will lead to excess ROS formation as they become damaged and worn. In wildtype T cells, this elevated ROS level is likely to be temporary as cells efficiently remove mitochondria to appropriate levels once in the periphery. Indeed, ROS might in fact drive the observed mitochondria clearance as evidence shows that ROS can induce autophagy through Atg4 [174]. However, in autophagy-deficient T cells the failure to remove mitochondria in the periphery would lead to prolonged ROS production and exposure resulting eventually in cell death (Figure 3.31). Strict regulation of ROS production is particularly important in T cells which are demonstrably sensitive to excess ROS levels [175,176].

These observations might explain the differences in T cell survival between thymus and the periphery in T-Atg7$^{-/-}$ mice. Thymocytes appear

accustomed to high mitochondrial loads and therefore a lack of mitochondrial regulation in *Atg7*$^{-/-}$ cells is less likely to impact upon survival. In the periphery, mitochondrial-regulation becomes essential and it is here that a lack of autophagy becomes fatal. Alternatively, the excess apoptosis observed in peripheral T cells might reflect cumulative build up of damage that begins in the thymus but only becomes deleterious once ROS levels have reached a tipping point in the periphery. Developmental regulation of mitochondria appears much more dramatic in CD8⁺ T cells compared to CD4⁺ T cells (data not shown), suggesting that CD8⁺ T cells might be more sensitive to the presence of superfluous mitochondria in the cytosol. This might imply why CD8⁺ T cells are generally more prone to apoptosis in the absence of *Atg7*.

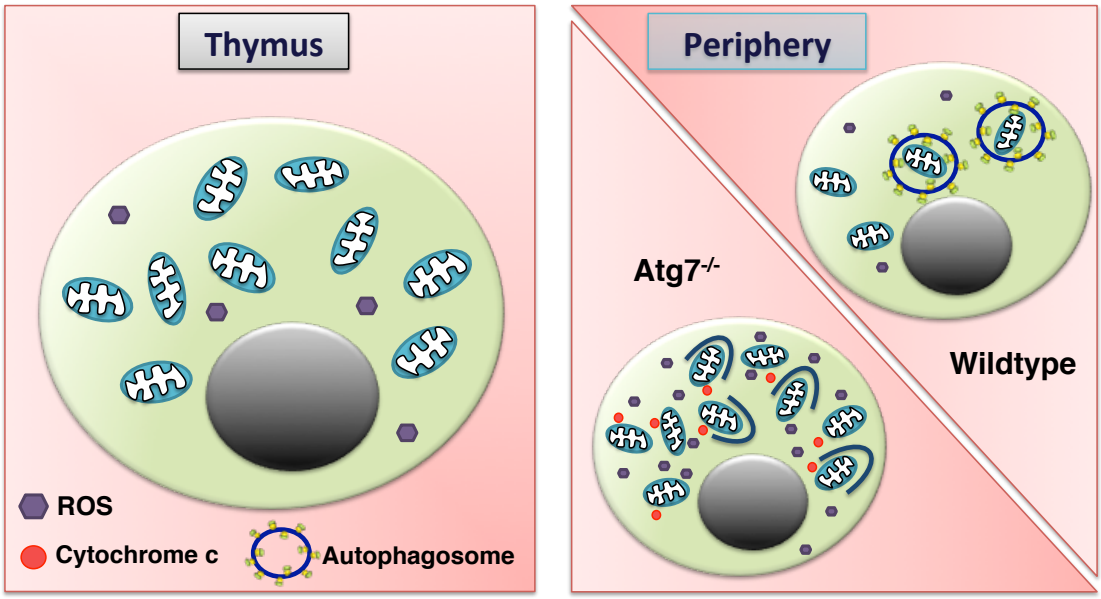


Figure 3.31 *Mitochondrial regulation by autophagy is important for peripheral T cell viability. In thymocytes, mitochondrial burden is high, probably as a consequence of the large metabolic requirements during T cell selection and development. In the periphery, the metabolic demands of quiescent naïve T cells are low and superfluous mitochondria are removed by autophagy in wildtype cells. This acts to maintain the quality of the mitochondrial pool and limit ROS production, whilst also being a possible reactionary event to high oxygen tension found in the blood. In Atg7*$^{-/-}$ *CD8⁺ T cells, autophagy cannot progress past the formation of the isolation membrane and the mitochondrial burden of*

peripheral cells remains high due to failed mitophagy. This leads to the accumulation of damaged mitochondria and ROS that deleteriously impact upon T cell survival.

3.20. *Atg7*$^{-/-}$ CD8^{+} T cells undergo increased homeostatic proliferation

Experimental evidence presented here suggests a high frequency of CD8^{+} T cells in T-Atg7$^{-/-}$ mice appear to be in cell cycle. While naïve *Atg7*$^{-/-}$ CD8^{+} T cells are comparable to wildtype cells in their rate of proliferation, this cannot be said for CD44^{hi} CD8^{+} T cells that undergo profound homeostatic cell turnover. Using BrdU in a pulse-chase experiment, no differences were observed in the rate of cell division between wildtype and knockout T cells in the spleen. However, in the bone marrow BrdU uptake was significantly higher in both naïve and memory-phenotype (MP) CD8^{+} T cells from T-Atg7$^{-/-}$ mice. The rate of basal cell turnover for wildtype T cells observed here is in agreement with previous work showing a low rate of cell proliferation in naïve T cells relative to memory-phenotype cells [177]. The difference in BrdU uptake observed between the spleen and the bone marrow was surprising, but perhaps not wholly unexpected. Previous work by Becker *et al* has shown how the bone marrow is the preferred site for homeostatic proliferation [178]. This suggests a model where *Atg7*$^{-/-}$ CD8^{+} T cells home to the bone marrow to homeostatically proliferate before egressing back to the secondary lymphoid organs. Li *et al* have previously shown how CD24 expressed on T cells is required for homeostatic proliferation [161]. We showed that the frequency of CD24-expressing CD8^{+} T cells is significantly greater in T-Atg7$^{-/-}$ mice compared to wildtype controls, confirming this increased rate of turnover is homeostatic in nature and not driven by antigen[161].

When placed in a replete T cell environment in bone marrow chimera experiments, homeostatic turnover of *Atg7*$^{-/-}$ CD8^{+} T cells returns to levels observed for wildtype cells. This observation implies that elevated cell turnover in the absence of antigen is not cell intrinsic to autophagy-deficient CD8^{+} T cells, but rather a consequence of a depleted T cell niche in T-Atg7$^{-/-}$ mice. This spontaneous proliferation in response to a severely reduced T cell compartment is a well-described phenomenon that is often termed "lymphopaenia-induced proliferation"(LIP) [179]. Both naïve and memory T cells may undergo LIP in response to T cell depletion [180,181], and this might explain the elevated levels of BrdU uptake in naïve as well as memory cells in the bone marrow observed here. LIP has been demonstrated in previous work where adoptive transfer of T cells into syngeneic irradiated hosts resulted in elevated cell turnover. This process is facilitated by IL-7 and TCR-MHC interactions [182] [183] [184] [120] [119]. The scarcity of T cells leads to enhanced availability of IL-7, which acts to amplify the normally weak TCR signalling observed following contact with self-peptide-MHC [116,185]. This confirms the ability of autophagy-deficient T cells to respond to IL-7, while also suggesting TCR signalling is unaffected by the absence of *Atg7*, at least in response to self-ligands. The overall combined strength of the signalling from the TCR and IL-7R appears to determine the propensity for T cells to undergo LIP. Accordingly, T cells with high-affinity TCR for self-peptide-MHC undergo faster rates of LIP [140,186]. Thus, a form of microevolution takes place during lymphopaenia where cells bearing high affinity TCRs for self are increasingly selected over time [187,188]. One might expect that in T-Atg7$^{-/-}$ mice the pool of T

cells should become gradually more auto-reactive over time. Consistent with this notion, aged mice with a T cell-specific deletion of *Vps34* develop an inflammatory wasting syndrome characterized by weight loss and intestinal inflammation [167]. In Vav-Atg7$^{-/-}$ mice that are also lymphopaenic, an expanded population of inducible T_{regs} (iT_{reg}) are observed (data not shown) and this might be driven from such an increase in auto-reactive T cell clones. No such iT_{reg} expansion was observed in T-Atg7$^{-/-}$ mice, however. Under common circumstances, LIP functions to restore the depleted T cell niche back to a replete state. However, LIP in T-*Atg7*$^{-/-}$ mice fails to re-establish the T cell compartment. The half-life of *Atg7*$^{-/-}$ CD8$^+$ T cells must be such that survival does not outpace homeostatic turnover. The result is that the T-Atg7$^{-/-}$ T cell niche remains in a constant state of depletion, unable to boost numbers due to the shortened lifespan of autophagy-deficient T cells.

3.21. CD8$^+$ T cells from T-Atg7$^{-/-}$ mice display an activated/memory phenotype

The data presented in this chapter illustrates how naive T-Atg7$^{-/-}$ mice display an expanded population of CD8$^+$ T cells bearing markers of memory and/or activation. By nine-weeks of age the frequency of cells expressing the memory marker CD44 is double that found in wildtype mice. This expansion of MP CD8$^+$ T cells continues with age such that a year after birth as many as 80% of *Atg7*$^{-/-}$ CD8$^+$ T cells are of a memory phenotype, a four-fold increase on what is observed in wildtype mice. While central memory CD8$^+$ T cells (CD44^{hi} CD62L$^+$) are observed at normal frequency, there is a significant increase in the relative abundance of effector memory cells (CD44^{hi} CD62L$^-$).

Why do T-Atg7$^{-/-}$ CD8^{+} T cells acquire this memory phenotype? An obvious answer is provided by the lymphopaenic state of the T cell compartment. A number of previous studies have illustrated how T cells emerging in the aftermath of lymphopaenia-induced homeostatic proliferation acquire characteristics of effector/memory T cells [189] [190] [191] [192] [193] [194]. Similar experiments where T cells are adoptively transferred into irradiated or antibody-depleted hosts show identical results [195] [196]. Loss of CD62L expression is also a feature of T cells residing in a lymphopaenic environment [118] and this may well explain the high occurrence of effector memory cells in T-Atg7$^{-/-}$ mice. In this scenario, it is probably wrong to describe this observation as an increase in effector memory cells *per se*. Whether these cells are true effector memory cells is questionable. It is more reasonable to suggest that in T-Atg7$^{-/-}$ mice these cells are merely masquerading as effector memory cells following phenotypic changes induced by lymphopaenia. Therefore, the apparent acquisition of effector memory cells is in actual fact a consequence of MP cells downregulating CD62L as part of lymphopaenia-induced activation.

The conversion to a memory phenotype seems to be inextricably linked to homeostatic proliferation. Indeed, CD8^{+} T cells bearing the TCR-specific for the H-Y male antigen do not proliferate in syngeneic lymphopaenic females [197] [182] [193] [198] and do not differentiate into memory cells [199]. The severe homeostatic proliferation detected in T-Atg7$^{-/-}$ mice is in line with this notion and is highly likely to be the cause of the expanded memory-phenotype population. The truth of this statement was confirmed using bone marrow chimeras. When *Atg7$^{-/-}$* CD8^{+} T cells were placed in a normal T cell

compartment devoid of excessive homeostatic turnover, no such expansion of MP cells was observed in the knockout donor CD8⁺ T cell pool. As such, the propensity to form MP cells in T-Atg7$^{-/-}$ mice is not an intrinsic feature and is a consequence of a lymphopaenic environment.

Although similar in phenotype to true antigen-specific CD8⁺ T cells, MP CD8⁺ T cells differ in that they differentiate directly from naïve to MP, omitting the effector phase all together [200]. Interestingly, despite the differences in their mode of differentiation, MP cells generated after LIP are remarkably similar to antigen-specific memory cells in their ability to undergo rigorous secondary responses [201]. This makes for an interesting prediction that T-*Atg7*$^{-/-}$ mice might mount potent CD8⁺ T cell responses to antigen if the antigen-specific CD8⁺ T cell is primed from the MP population. From the perspective of the *Atg7*$^{-/-}$ MP CD8⁺ T cell, this first encounter with antigen would induce a secondary effector-style response, with LIP acting as a previous pseudo primary challenge.

A key final question posed by these observations is why does LIP induce conversion to a memory phenotype? Perhaps augmented TCR signalling following self-peptide-MHC ligation in the presence of elevated concentrations of IL-7 is enough to induce activation and differentiation similar to that of foreign antigenic stimulation. Does the act of proliferation inherently lead to an activated phenotype? Work from Murali-Krishna and Ahmed goes some way to supporting this [195]. Conceivably, this change in cell surface phenotype should perform some functional relevance, perhaps in facilitating access to niches that support homeostatic proliferation. Indeed,

upregulation of CD44 is known to enable access to the bone marrow, a preferred site for homeostatic turnover [178].

3.22. CD8⁺ T cells from T-Atg7$^{-/-}$ mice display characteristics of exhaustion and terminal differentiation

The conversion of a large number of CD8⁺ T cells in T-Atg7$^{-/-}$ mice to a memory phenotype is accompanied by the acquisition of traditional signs of exhaustion in these cells. T cell exhaustion was originally described as a state of gradual non-responsiveness in antigen-specific T cells during chronic infection, such as with LCMV and HIV [156]. CD8⁺ T cell exhaustion in mice and humans is typically associated with the upregulation of inhibitory co-receptors such as PD-1 and TIM-3 with low expression of CD62L [202] and IL-7R [203] [204]. CD8⁺ T cells from T-Atg7$^{-/-}$ mice display a very similar phenotype to this, with approximately 8% of CD8⁺ T cells co-expressing PD-1 and TIM-3, while no such co-expression is observed on wildtype cells. The appearance of exhausted T cells in the absence of antigen, such as during times of lymphopaenia, has not previously been reported. However, it is important to note that the expression of inhibitory receptors does not necessarily indicate a state of exhaustion. For instance, human T cells expressing PD-1 and CTLA-4 can exhibit functional activity after activation [205]. The optimal approach to test for true exhaustion would be to assess responsiveness to antigenic stimulation in *Atg7$^{-/-}$* CD8⁺ T cells expressing PD-1 and TIM-3. However, this is difficult to achieve without use of a TCR transgenic system. PMA-induced secretion of IFN-γ and TNF-α, also affected during exhaustion, could provide some level of insight in the T-Atg7$^{-/-}$ system. A further limitation of this study is that

classical traits of exhaustion were not assessed at the transcriptional level. Formation of exhausted T cells follows a distinct transcriptional programme that includes expression of genes such as BLIMP-1 [206] [207]. BLIMP-1 acts to suppress IL-2 transcription [208] preventing further proliferation in exhausted cells. Investigating PD-1 and TIM-3-expressing knockout CD8⁺ T cells for signs of exhaustion at the transcriptional level would have provided greater insight into the functional status of these cells. Like with the conversion to a memory phenotype, it seems likely that the appearance of exhausted-phenotype cells is a corollary of a lymphopaenic environment, where profound LIP pushes cells to exhaustion. Indeed, when *Atg7*$^{-/-}$ CD8⁺ T cells were placed into a normal T cell compartment in bone marrow chimera mice, the exhausted phenotype of these cells was no longer observed. The upregulation of inhibitory receptors on the surface of knockout T cells might be also an attempt to limit the amount of homeostatic turnover in T-Atg7$^{-/-}$ mice, limiting the outgrowth of auto-reactive clones. In support of this, one might expect to observe high inhibitory receptor expression only in the cells to have underground multiple rounds of replication (and thus most reactive to self). Future experiments utilising Ki-67 or CFSE to correlate phenotype with the proliferation status might unravel the answer to this question. Under this hypothesis, a balance must be found in the depleted T cell pool: where proliferation in response to cytokine plus self is permitted in order to restore the T cell niche, but where particularly self-reactive clones are limited by the upregulation of inhibitory receptors.

$CD8^+$ T cells from T-$Atg7^{-/-}$ mice also appear to show signs of terminal differentiation. At the phenotypic level, this was based on the expression of the co-inhibitory receptor KLRG1. In mice, KLRG1 has previously been used to determine cells in a state of terminal differentiation [209] [210] [211] [212] [213]. In humans, the expression of KLRG1 rises dramatically with age, with greater than 90% expression of KLRG1 observed on $CD8^+$ T cells from individuals over 65 years of age [212] [214] [211] [213] [215]. The frequency of $CD8^+$ T cells from T-$Atg7^{-/-}$ mice expressing KLRG1 was ten times higher than that observed on wildtype cells, suggesting a significant proportion of the knockout $CD8^+$ T cell pool is terminally differentiated and/or senescent. However, unlike the human system where changes in multiple surface receptors is indicative of terminal differentiation ($CD57^+$ $CD27^-$ $CD28^-$ $CD45RA^+$), use of a solitary cell surface receptor to achieve this is in the murine model suggests caution should be exercised. Blockade of KLRG1 signalling also reverses the proliferative dysfunction of highly differentiated human $CD8^+$ T cells, suggesting that KLRG1 might regulate pathways associated with exhaustion too [211]. In murine T cells, the cross-linking of the TCR and KLRG1 by plate-bound antibodies lowers Ca^{2+} influx and IL-2 production [216] supporting this claim. Therefore, similar to the upregulation of PD-1 and TIM-3, expression of KLRG1 on *Atg7*$^{-/-}$ $CD8^+$ T cells might be an attempt to exert negative regulatory control on this homeostatically dividing population. Adding support to the idea of high levels of terminal differentiation in the knockout $CD8^+$ T cell compartment, *Atg7*$^{-/-}$ $CD8^+$ T cells displayed high mRNA expression of two transcription factors associated with senescence (*p21*^{Cip1} and

p16^{INK4A}). Like many of the other changes in CD8⁺ T cell phenotype in T-Atg7$^{-/-}$ mice, the lymphopaenic environment probably plays a large part in driving these changes with LIP pushing cells into a state of terminal differentiation. As a consequence, one would expect to observe KLRG1, *p21*^{Cip1} and *p16*^{INK4A}, expression only in cells shown to have undergone multiple rounds of proliferation (a limitation that this was not performed in this study). The excess ROS produced in autophagy-deficient CD8⁺ T cells might also be a causal factor in their senescence. Cellular senescence can occur independent of cell proliferation in response to various stress response pathways [217] [218]. Such pathways including the DNA-damage response have previously been shown to induce senescence in T cells [202] [219] [220]. Thus, DNA damage in *Atg7*$^{-/-}$ CD8⁺ T cells caused by excess ROS levels observed in these cells may also promote terminal differentiation.

3.23. Conclusions

The data presented here suggests a crucial role for autophagy in maintenance of the T cell compartment. While T cells develop normally in the thymus in the absence of autophagy, the peripheral T cell pool is markedly lymphopaenic. This lymphopaenia drives homeostatic proliferation of surviving T cells in an attempt to refill the depleted niche, inducing the acquisition of classical memory markers. In addition, *Atg7*$^{-/-}$ CD8⁺ T cells show signs of exhaustion and terminal differentiation, likely driven by prolonged homeostatic turnover. However, these affects are not intrinsic to loss of autophagy and use of bone marrow chimeras show that *Atg7*$^{-/-}$ CD8⁺ T cell phenotype and turnover is normal when in a replete T cell niche. This

loss of T cell viability in the absence of autophagy is possibly driven by defective mitophagy, preventing the effective removal of mitochondria that is normally observed in wildtype T cells between thymus and periphery. The resulting increased mitochondrial burden is associated with elevated ROS levels that compromises cell viability and T cell homeostasis.

Chapter 4: Introduction

Autophagy is a critical regulator of memory CD8⁺ T cell formation

4. Introduction

CD8⁺ T cells are key players in the adaptive immune system that mediate immunity to intracellular pathogens and tumours. During initial encounter with a foreign microbe, CD8⁺ T cells bearing a TCR specific for the infecting agent are selected to undergo clonal expansion. This process is driven by the interaction of the TCR with short pathogen-derived peptides presented by MHC class I molecules on the surface of professional APCs [221] that induces a program of activation and differentiation into effector CD8⁺ T cells (also known as cytotoxic T lymphocytes (CTLs)). Following the peak effector response and antigen clearance, the majority of these effectors die leaving behind a stable, long-lived fraction of memory CD8⁺ T cells. The longevity of these cells confers long-lasting immunity to reinfection through the rapidity of their secondary responses. Thus, in collaboration with B cells, memory CD8⁺ T cells form the basis of immunological memory – the hallmark of the acquired immune system.

4.1. CD8⁺ T cell activation and expansion

Following production, CD8⁺ T cells egress from the thymus and proceed to circulate the blood and lymphatic system. This continuous migration of T cells through the secondary lymphoid organs (SLOs) is important for allowing T cells to survey the antigenic landscape. For naïve T cells, this means scanning MHC class I molecules on the surface of dendritic cells [222]. These cells are strategically positioned as a dense network in the T cell zones of SLOs and are continuously scrutinised by recirculating CD8⁺ T cells for the expression of foreign antigen [223]. During infection, foreign antigen can gain

access to the cytosol of APCs where they will be processed by the proteasome into peptides. Hereafter, peptide fragments are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) where they are loaded onto MHC Class I molecules prior to cell surface expression via the Golgi [224]. $CD8^+$ T cells can also respond to antigens from non-cytosolic sources derived from phagocytosis of exogenous material. These antigens gain access to the cytosol and the MHC Class I pathway through a process known as antigen-cross presentation that permits APCs not directly infected to efficiently activate $CD8^+$ T cells [225]. During circulation, the vast majority of $CD8^+$ T cells do not encounter their cognate antigen and are allowed to slowly percolate through the T cell zone of SLOs before re-entering the blood stream [223]. However, DCs activated in the presence of inflammatory stimuli go through a program of differentiation that leads to upregulation of MHC class I bearing foreign antigen. Recognition of this cognate antigen by surveying $CD8^+$ T cells arrests T cell migration and facilitates a period of prolonged cell-to-cell contact between DC and T cell. The interaction of TCR with peptide-bearing MHC class I, known as signal one, is not in itself enough to promote T cell activation and differentiation. Inflammatory signals during infection promote the upregulation of the co-stimulatory molecules CD80 and CD86 [226,227] on the surface of DCs that deliver a second activatory signal to the T cell through the CD28 receptor. Signalling through CD28 greatly lowers the antigen density and TCR affinity requirements for T cell activation and differentiation while also being important for IL-2 production [228,229]. In the absence of secondary co-stimulation provided by the CD28-CD80/86 axis, T cells typically do not

generate a productive effector response. After an initial round of proliferation, such cells will either become deleted or anergic – a state of non-responsiveness and thus refractory to further stimulation [230,231]. The signals provided by the TCR and CD28 result in naïve $CD8^+$ T cell activation that occurs over a period of 2-20 hours [232,233] and probably requires constant contact with the stimulatory APC [234,235]. After this initial activation, cells undergo rapid proliferation that is associated with profound changes in gene expression which endow the cell with effector function as early as the first cell division [236-238].

A number of transcription factors have been identified that promote effector $CD8^+$ T cell ($CD8^+$ T_{eff}) differentiation. The T box transcription factor Eomesodermin (EOMES) has been shown to drive the acquisition of cytotoxicity and anti-viral cytokine production [239]. A related factor, T-bet, is also crucial for $CD8^+$ T_{eff} differentiation as T-bet deficient $CD8^+$ T cells exhibit reduced cytotoxicity and cytokine production [240]. The absence of both EOMES and T-bet both result in an aberrant effector phase characterised by altered effector function and cytokine synthesis. Cytokines themselves are also important regulators of $CD8^+$ T_{eff} differentiation. IL-12 and the type I interferons (IFNα/β) are all able to promote $CD8^+$ T_{eff} expansion and differentiation [241]. However, the cytokines required for $CD8^+$ T_{eff} differentiation are not ubiquitous for all infections. $CD8^+$ T_{eff} responses to *Listeria* infection are predominantly IL-12-dependent [242,243] while responses to LCMV rely more heavily on type I IFNs [244,245]. This implies that $CD8^+$ T_{eff} differentiation can be tailored to the pathogen at large.

The expansion phase following T cell activation is a truly remarkable feature of T cell biology that is perhaps only shared with their B cell cousins. During this time, a given clone can go from virtually undetectable levels to make up more than 10% of the entire $CD8^+$ T cell compartment [246]. Few other cell types boast such prodigious capacity for cell division. The result is a large polyclonal pool of effector $CD8^+$ T cells that can now exit the SLOs and migrate to the site of infection to combat the invading pathogen.

4.2. The $CD8^+$ T cell effector phase

During $CD8^+$ T_{eff} expansion, a single progenitor can give rise to as many as 10,000 daughter cells [236,247]. These $CD8^+$ T_{eff} cells become detectable in the peripheral circulation as early as 5-6 days in some cases and typically peak 7-10 days post infection. Leading to the climax of the effector phase, $CD8^+$ T cells accumulate at the site of infection where they exercise an array of effector functions aimed at killing infected host cells. $CD8^+$ T_{eff} cells induce lysis of infected cells by two distinct molecular pathways: the perforin/granzyme pathway or the Fas-FasL axis [248,249]. These pathways, initiated following engagement of the TCR with peptide:MHC on the host cell, promote cell death through the activation of caspases leading to apoptotic cell death [176].

The cytotoxic granules that form in $CD8^+$ T_{eff} cells contain a complex cocktail of cytotoxic and pro-apoptotic proteins such as perforin and Granzymes that function to kill target cells [250] [251]. Following engagement of an infected cell, $CD8^+$ T_{eff} cells expel their cytotoxic granules into the synaptic cleft formed between itself and the target cell. Upon encountering extracellular Ca^{2+},

perforin binds lipid moieties in the target cell membrane through its C2 domain [252] and subsequently polymerises into complement-like transmembrane pores through which granzymes can diffuse into the cell [253]. The main Granzyme involved in mediating cell death once in the cytosol, Granzyme B, can directly activate caspases in some circumstances, but more often host cells die through activation of the mitochondrial (intrinsic) pathway. Granzyme B cleaves the pro-apoptotic protein BID that in turn induces mitochondrial outer-membrane permeabilisation and subsequently the release of cytochrome *c* [254] [255]. Caspase activation then brings about the final stages of apoptosis and cell death through DNA fragmentation [256]. The observation that perforin is conserved between mice and primates highlight its importance in resistance to infection across the branches of vertebrate life. Indeed, perforin-deficient mice are susceptible to many viral pathogens and to spontaneous and carcinogen-induced neoplasms [257] [258] [253]. Similarly, deletion of Granzyme B in $CD8^+$ T cells impairs the speed at which target cells are killed [221]. Redundancy exists in the Granzyme system however, as Granzyme A and Granzyme M can both induce target cell apoptosis, [259,260] [261].

In the FasL-Fas pathway, ligand binding recruits the adaptor FADD to the receptor complex in the target cell which then associates with pro-caspase-8 [262]. At this point, the death-inducing signalling complex (DISC) is formed resulting in the autocatalytic activation of caspase-8. Casapase-8 activation leads to triggering of the execution phase of apoptosis through Caspase-3, resulting in cell death [263].

In addition to the more direct effects of cytolysis, $CD8^+$ T_{eff} cells produce an

array of cytokines that can also contribute to viral clearance. For instance, LCMV clearance is significantly delayed in *IFN-γR*-deficient mice [264] suggestive of a role for IFN-γ in viral immunity. The same cytokine is thought to have similar effects during hepatitis B virus (HBV) infection. Although all hepatocytes are infected in this system, viral clearance proceeds without large-scale liver damage. When MHC class I or perforin-deficient $CD8^+$ T cells are transferred into HBV transgenic mice, viral neutralisation is observed as normal [221] [265]. However, the ability of $CD8^+$ T cells to abrogate viral gene expression is inhibited when either IFN-γ or IFN-γR are blocked. Neutralisation of TNF-α demonstrates similar results [221] [265]. Therefore, although $CD8^+$ T cells are not demonstrably the source of IFN-γ in these instances, it is strongly suggestive of viral clearance independent of cytotoxic-mediated cell death through the action of cytokines.

The cumulative action of cytokines and cytotoxicity by $CD8^+$ T cells eventually incurs viral clearance. This leaves a large proportion of $CD8^+$ T_{eff} cells in proximity to tissues and organs armed with the most deadly weaponry the organism affords. To prevent immunopathology and maintain homeostasis, these now superfluous cells must be removed, and quickly.

4.3. $CD8^+$ T_{eff} cell contraction

Whether the T cell response is effective in eliminating the pathogen or not, the number of pathogen-specific $CD8^+$ T cells undergoes a precipitous decline shortly after the peak of the effector response, known as contraction (Figure 4.1). Typically during this time 90-95% of pathogen-specific $CD8^+$ T_{eff} cells are removed, limiting the risk of immunopathology and facilitating a balanced

TCR repertoire able to respond to a multitude of future infections [266]. The onset of the contraction phase often correlates with pathogen clearance. This led to the notion that contraction is regulated by antigen load. However, in studies where microbes were cleared using antibiotics after 2 days (as with *L. monocytogenes*), premature attenuation of the infection had no bearing on the on the onset or magnitude of $CD8^+$ T cell contraction [267]. Furthermore, the kinetics of $CD8^+$ T cell contraction is similar during acute and chronic infection [203,268]. This suggests that contraction is a programmed event initiated during activation and expansion that is independent of antigenic load [267]. The mechanisms controlling contraction appear to work independently of certain death receptors as contraction is normal in CD95- and TNFR-deficient $CD8^+$ T cells, plus humans lacking Fas do not accumulate activated $CD8^+$ T cells [269] [270] [271]. An involvement of pro- and anti-apoptotic molecules supports the idea that contraction is a cell intrinsic feature of the $CD8^+$ T cell response. Deletion of pro-apoptotic BIM (Bcl-2 interacting mediator of cell death) dramatically reduces the deletion of $CD8^+$ T cells following herpes virus infection [272]. Likewise, T cells lacking pro-apoptotic molecules Bak and Bax exhibit a survival advantage [273,274]. Conversely, overexpression of anti-apoptotic Bcl-2 enhances T cell responses *in vivo* through the associated increase in cell viability [275]. The anti-apoptotic function of Bcl-2 lies partly in its ability to bind pro-apoptotic BIM. During the onset of contraction, Bcl-2 levels fall in $CD8^+$ T_{eff} cells leading to elevated levels of free BIM in the cytosol that in turn activates the executioner Bak, leaving the cell at the precipice of apoptosis.

The action of cytokines can also influence contraction; in *IFN-γ*-deficient mice $CD8^+$ T cells fail to contract following *L. monocytogenes* and LCMV infection [276,277]. The IFN-γ-mediated signals important for contraction may come as early 12 hours post-infection, although it is not yet clear whether the influence of IFN-γ is directly on $CD8^+$ T cell or through other cells acting as intermediaries [278] [277,279]. The absence of IL-12 receptor on antigen-specific $CD8^+$ T cells also affects contraction after infection [243]. Evidence also suggests that $CD8^+$ T_{eff} cells are kept alive by IL-15. Lack of IL-15 receptor signalling leads to a particularly severe contraction phase whilst injection of exogenous IL-15 can prevent contraction in mice [280] [281] [282]. This suggests a model where as inflammation is resolved, $CD8^+$ T_{eff} cells are decreasingly exposed to inflammatory cytokines that help to maintain cell viability, resulting in cell death.

Although contraction is substantial, it is incomplete and a fraction of $CD8^+$ T_{eff} cells go onto survive and persist in the tissues and peripheral circulation (Figure 4.1). These cells are known as memory $CD8^+$ T cells ($CD8^+$ T_{mem} cells) and act to provide long-term protection perhaps throughout the entire life span of an organism. Following contraction, a given $CD8^+$ T_{mem} clone can be found at a frequency that substantially exceeds that of its naïve precursor, forming a readily available population of cells able to respond rapidly to re-infection should the need arise.

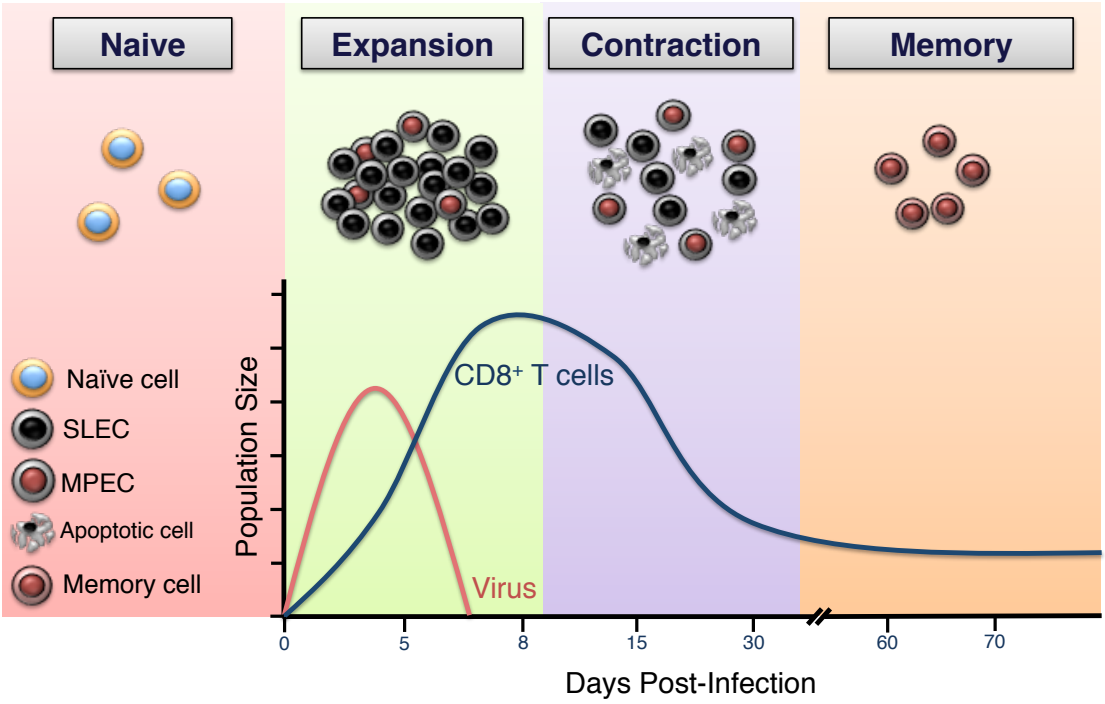


Figure 4.1 *CD8⁺ T cell kinetics following infection. Following activation, naïve CD8⁺ T cell clones undergo profound expansion significantly increasing the size of the antigen-specific CD8⁺ T cell pool. This population of effector cells compromise short-lived effector cells (SLECs), destined to die following antigen clearance, and memory precursor effector cells (MPECs) that go on to enter the memory pool. Once infection has been resolved, effector CD8⁺ T cells endure large-scale apoptosis in a process known as contraction, leaving a small population of long-lived memory CD8⁺ T cells that provide long-lasting protection.*

4.4. Memory CD8⁺ T cell differentiation

A key question in T cell biology is what dictates which cells survive the contraction phase? Conceivably, two different models are possible: First, all CD8⁺ T_{eff} cells are equipotent and chance encounters with survival factors at the peak of the effector phase determine which cells survive and turn into CD8⁺ T_{mem} cells. Alternatively, a situation where some form of imprinting is in place, such that some effectors are destined to die while others are predisposed to form the memory pool. The evidence thus far favours the latter option with modified expression of the IL-7Rα chain playing a prominent role [280]. Relatively recent experiments in mice have illustrated

how at the peak of the response two types of $CD8^+$ T_{eff} cells exist: short-lived effector cells (SLECs), which die during contraction, and memory precursor effector cells (MPECs) that go onto seed the memory compartment (Figure 4.1). These two populations can be identified through the differential expression of KLRG1 and IL-7Rα (CD127), with SLECs being $KLRG1^+$ $CD127^-$ and MPECs being $KLRG1^-$ $CD127^+$ [283] [284]. CD127 expression seems to be particularly important in determining $CD8^+$ T_{eff} cell survival. While naïve cells are $CD127^+$, cell activation induces downregulation of this receptor chain. However, progression to memory appears to be accompanied by CD127 re-expression. Furthermore, when $CD8^+$ T_{eff} cells are sub-divided into $CD127^-$ and $CD127^+$, only the latter population is able to confer protection and respond to secondary infection [283-285] [286]. Thus, the ability to survive contraction appears to rest heavily on the selective responsiveness to IL-7. However, CD127 expression is not an absolute requirement for effector cell survival. Transgenic expression of CD127 could not rescue SLECs from contraction and seemingly not all $CD127^+$ MPECs maintain viability [287]. Perhaps IL-7 responsiveness not only requires CD127 expression, but the ability to home to locations where it is produced. Fibroblastic reticular cells in the T cell zones of SLOs are an important source of IL-7. Correspondingly, MPECs are preferentially found in the T cell zones in the spleen, whereas SLECs are located in the red pulp [288]. This implies that only $CCR7^+$ MPECs might survive contraction due to their ability to home to sites of IL-7 production.

Both SLECs and MPECs are genuine effector cells able to produce cytokines

and cytolytic effector molecules such as Granzyme B and perforin [280]. Differences do exist however, with SLECs producing higher levels of cytotoxic effector molecules while MPECs synthesis more IL-2. Distinct transcriptional programs also govern SLEC and MPEC fate. T-Bet and BLIMP-1 are necessary for the development of SLECs particularly through their ability to repress the *CD127* loci [284] [289] [290]. T-bet also induces expression of CD122, the β chain of the IL-15 receptor, which helps maintain SLEC viability [291]. It has been proposed that a gradient of T-bet may be established in $CD8^+$ T_{eff} cells, with high levels of T-bet promoting terminal effector cell differentiation (SLECs) whilst low levels permit memory precursor development [292] [196]. BLIMP-1 is also posited to act in a graded fashion similar to that of T-bet [292] [293].

This all begs the question of how heterogeneity in the $CD8^+$ T_{eff} response is generated? What determines SLEC or MPEC fate? One possibility is that different naïve precursors develop into either SLECs or MPECs. However, previous work has shown how a single antigen-specific $CD8^+$ T cell can give rise to a diverse population of effector and memory cells [294,295]. When a single naïve T cell was transferred into a congenic host, its progeny after infection consisted of both $CD8^+$ T_{eff} and $CD8^+$ T_{mem} cells. Further support for this model comes from elegant experiments where naïve $CD8^+$ T cells were engineered to carry unique genetic "barcodes" which permit tracing of progeny cells. When mice bearing these $CD8^+$ T cells were immunised, the same barcodes were found in both effector and memory cells, suggesting these populations arose from the same cell [295]. If SLECs and MPECs are truly derived from different naïve precursors, a specific barcode would be

found exclusively in effector cells and not memory cells, or *vice versa*. This suggests that each $CD8^+$ T cell clone can give rise to both SLECs and MPECs and therefore both $CD8^+$ T_{eff} and $CD8^+$ T_{mem} cells.

Exactly how a single cell gives rise to both SLEC and MPEC progeny is supported by two disparate models (Figure 4.2). In one, the fate decision between SLEC and MPEC is taken during the first asymmetric cell division of the naïve $CD8^+$ T cell, where one daughter cell adopts the SLEC fate and the other the MPEC fate [296]. Evidence for this model comes from experiments showing that following interaction with cognate peptide-loaded APCs, polarised organisation occurs in the naïve $CD8^+$ T cell with molecules either moving to the site of interaction or the opposite side of the cell [296] (Figure 4.2). During the subsequent cell division, this polarisation results in an unequal distribution of molecules between the two daughter cells. The proximal daughter cell was shown to receive greater levels of molecules associated with effector fate such as Granzyme B and T-bet [296] [297], resulting in a SLEC phenotype. The distal daughter cell however received more CD127, a marker of MPEC fate. Neat experiments subsequently showed how transfer of distal daughter cells conferred greater protection than proximal ones, providing further support for this hypothesis [297]. In the second model, naïve $CD8^+$ T cells generate equipotent daughter cells (Figure 4.2). Some of these daughter cells develop into SLECs and others into MPECs depending on signals encountered during the early effector phase [280]. This idea has been supported by data showing that $CD8^+$ T_{mem} cells can derive from cells with effector characteristics. This conclusion was drawn from experiments mice were engineered to express an inducible *Granzyme-Cre* that would lead to

yellow fluorescent protein expression (YFP) following *Cre* expression. This allowed tracing of cells that had one time expressed Granzyme B and their subsequent progeny. When Granzyme B was induced during the early stages after primary infection, YFP⁺ CD8⁺ T_{mem} cells were observed during the memory phase. This suggests that a proportion of CD8⁺ T_{mem} cells had a Granzyme B-expressing ancestor and therefore was derived from a SLEC population [298]. However, the proposers of this hypothesis do not take into consideration previous findings that *bone fide* MPEC cells are able to produce Granzyme B [289] and therefore, cannot rule out that YFP⁺ CD8⁺ T_{mem} cells in their system were not derived from Granzyme B-expressing MPECs.

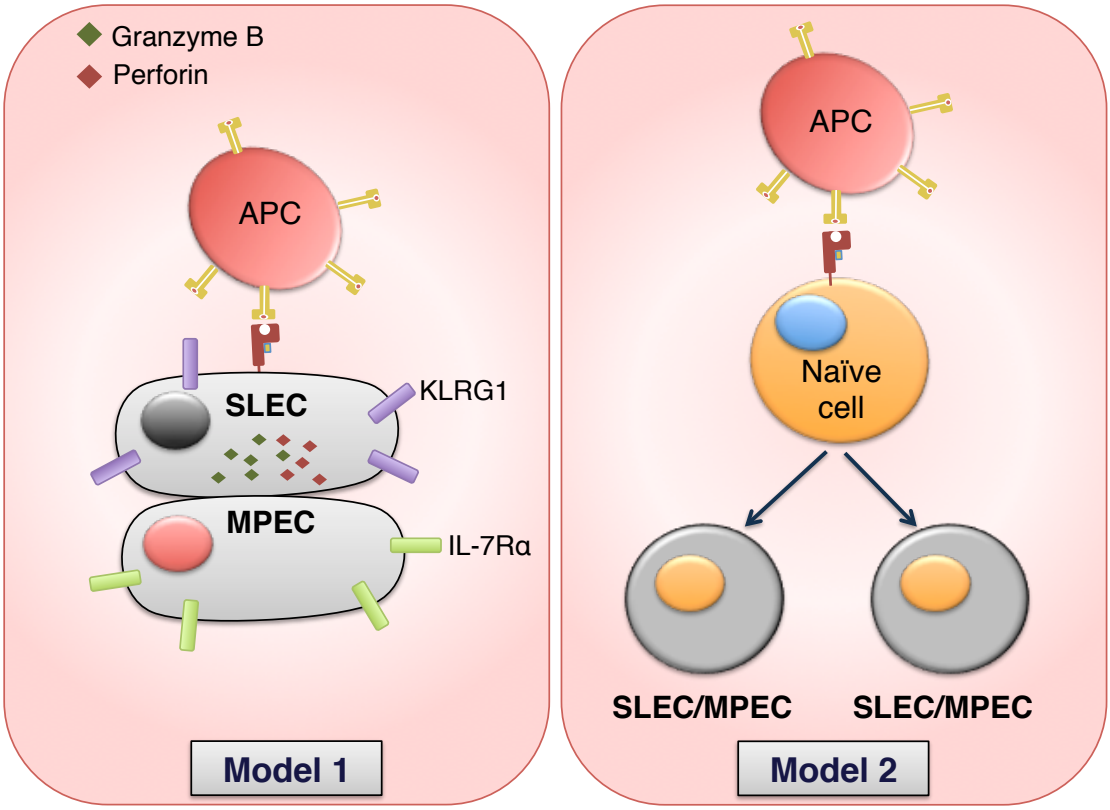


Figure 4.2 *Models of CD8⁺ T cell differentiation. After T cell expansion, the effector pool is comprised of short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). The determinants of SLEC and MPEC fate are currently best described by two disparate models. In* ***Model 1****, asymmetric T cell division leads to one cell adopting a SLEC phenotype and the other an MPEC determined by the polarised partitioning of effector molecules and transcription factors. In this model, the proximal daughter cell receives molecules such as*

T-bet, Granzyme B and perforin that drives SLEC fate. These cells are determined by the expression of KLRG1 and the absence of the IL-7Rα chain. In turn, the distal daughter cell receives IL-7Rα that is essential for MPEC persistence and entry to the memory pool. In ***Model 2****, naïve CD8⁺ T cell division generates equipotent daughter cells with both SLEC and MPEC potential. Here, the fate of these daughter cells is dependent on signals encountered during the effector phase.*

When exactly does an MPEC differentiate into a bone fide CD8⁺ T_{mem} cell? Accepted wisdom within the field is that CD8⁺ T_{mem} cells are typically formed by day 30 post-infection. Therefore, CD8⁺ T_{mem} cell differentiation is posited to occur between the peak effector response (day 7-10) and day 30. Experimental approaches have shown how CD8⁺ T cells can acquire memory properties well before the primary response is over. Male-specific CD8⁺ T cells recovered from at day 15 post-antigen stimulation have been shown to possess cytokine secretion capacities in line with that of memory cells. In contrast day 7 male-specific cells still behaved as naïve in this respect [293]. In response to LCMV, day 21, but not day 7, antigen-specific CD8⁺ T cells have acquired a gene expression profile similar to that of CD8⁺ T_{mem} cells [299]. These observations suggests after the peak of the effector phase there is a gradual differentiation of CD8⁺ T_{eff} cells toward a CD8⁺ T_{mem} phenotype with CD8⁺ T_{mem} cells beginning to appear between days 15-21 depending on the source of antigen and type of infection. This period of time after primary infection may be referred to as the memory conversion phase. During this conversion, CD8⁺ T_{mem} cells acquire the potential to rapidly produce IFN-γ and TNF-α and to quickly reacquire cytotoxic activity when re-exposed to antigen [300] [301] [302] [299]. This capacity to rapidly perform effector functions upon re-stimulation is one property that distinguishes naïve and memory CD8⁺ T cells and confers CD8⁺ T_{mem} cells with greater ability to provide protective immunity. The

acquisition of disparate homing properties is also an important distinction of $CD8^+$ T_{mem} cells. Through differential expression of the CD62L and CCR7, $CD8^+$ T_{mem} cells are able to maintain a vigil both systemically and at the site of infection, ready to respond with pace if the same pathogen should strike again.

4.5. Memory $CD8^+$ T cell subsets

The $CD8^+$ T_{mem} population that eventually forms upon pathogen clearance is heterogeneous and evolves further over time. The most widely characterised division of the $CD8^+$ T_{mem} compartment is that of central and effector memory cells. This partition of the memory compartment is based on the expression of the lymph node homing receptors CCR7 and CD62L. Both molecules interact with components displayed on the high endothelial venules of lymph nodes – CD62L interacting with carbohydrate moieties termed peripheral node addressins, while CCR7 binds the SLO-homing chemokines CCL19 and CCL21. Memory cells lacking expression of these two markers are defined as non-lymphoid tissue effector memory cells (T_{EM}), while $CCR7^+$ $CD62L^+$ cells are classed as lymphoid tissue resident central memory cells (T_{CM}) [303]. Studies in mice suggest both T_{EM} and T_{CM} stem from $CD127^+$ MPECs [283 285].

Work from Rafi Ahmed's lab has delineated the relationship between T_{CM} and T_{EM}. Using TCR transgenic $CD8^+$ T cells specific for LCMV, it was shown that following pathogen clearance there is a linear differentiation path from T_{EM} to T_{CM}. This indicates that these subsets are part of a continuum of $CD8^+$ T cell differentiation, rather than separate lineages that arise from the $CD8^+$ T_{eff} pool [304]. Once the memory compartment has formed and antigen cleared, T_{EM} cells

gradually convert to T_{CM} and only then gain the ability to homeostatically proliferate. This homeostatic growth also contributes towards the gradual skewing of the memory pool toward T_{CM} cells. During the conversion to T_{CM}, T_{EM} lose Granzyme B expression and the ability to kill *ex vivo* [304]. T_{CM} are seemingly unable to transform in the opposite direction to T_{EM} during steady state conditions. This situation changes upon secondary infection, where T_{CM} can rapidly differentiate into T_{EM} and elicit rapid effector function. Numerous transcription factors have been demonstrated to control the balance of T_{EM}/T_{CM} differentiation. Activation of the PI3K/mTOR pathway downregulates both CCR7 and CD62L expression via the transcription factor Klf2, inducing T_{EM} formation [305]. In addition, the transcription factors ID2, T-bet and BLIMP-1 all favour T_{EM} differentiation, while Bcl-6 favours TCM development [306]. The linear differentiation pathway that these factors regulate culminates in the generation of a cell type, T_{CM}, which boasts the two hallmark characteristics of $CD8^+$ T_{mem} cells: rapid recall ability and the stem-like quality of self-renewal.

While previous work has suggested that T_{CM} is the superior subset for long-term protection [304], the picture is somewhat complicated in the case of vaccination. Secondary challenge with the same antigen changes the representation of the memory pool, with conversion of T_{CM} back to a T_{EM} phenotype, decreasing T_{CM} frequency even after antigen has been cleared for a second time [303]. Indeed, during prime/boost vaccine strategies, the frequency of T_{CM} can become low within the antigen-specific $CD8^+$ T_{mem} pool which becomes dominated by T_{EM} cells [307] [308] [309]. Therefore, while prime/boosting clearly has benefit for high affinity B cell responses, it

might degrade the protective capacity of the CD8⁺ T_{mem} compartment by increasing the relative frequency of less protective T_{EM} cells. On the other hand, T_{EM} cells induced by heterologous prime/boosting were associated with improved protection against simian immunodeficiency virus (SIV) challenge [310] [311]. Thus, the relative importance of T_{EM} and T_{CM} for long-term protection may be specific to the invading pathogen or perhaps even the route of infection.

Interestingly, a further CD8⁺ T_{mem} subset has been described that populates a diverse array of non-lymphoid tissues [312] [237]. While these cells were originally though to be part of the T_{EM} pool, studies showed they were in fact a distinct population of non-recirculating cells termed tissue resident CD8⁺ T_{mem} cells (T_{RM}) and have been observed in the skin, brain, salivary glands and amongst the intraepithelial lymphocyte population in mice [313] [312] [314] [315] [316]. Recently, similar populations have been observed in the human skin, where infection at a single skin site results in T_{RM} cells located throughout the entire skin surface forming a protecting first line of defence to secondary infection [317]. T_{RM} cells in other tissues might provide a similar function, providing a rapid first wave of CD8⁺ T_{eff} cells that is then reinforced by T_{EM} cells produced in lymphoid tissues from T_{CM} precursors.

Recent evidence has suggested the existence of a specialised self-renewing population of CD8⁺ T_{mem} cells that shares signalling pathways with HSCs, known as memory stem cells [318] [319]. Wnt signalling was shown to suppress EOMES and generate CD8⁺ T_{mem} cells with features similar to that of stem cells. These cells can go through many more rounds of division than normal

$CD8^+$ T cells and can proliferate and differentiate in response to antigen. Importantly, transfer of these cells into T cell depleted hosts led to reconstitution of the T cell compartment [319] [320]. However, a number of conflicting studies offer opposing evidence that Wnt signalling does not regulate production of the memory stem cell pool. Thus, controversy remains as to whether we can add these cells to the slowly growing list of $CD8^+$ T_{mem} subsets.

4.6. Memory $CD8^+$ T cell maintenance

To maintain immunity, it is important that the organism responds quickly and efficiently to a second encounter with the same pathogen. This principle is the basis of successful vaccination and one that is afforded by the action of lymphocytes. Thus, while clearance of $CD8^+$ T cells following resolution of an infection is necessary to maintain homeostasis, mechanisms must exist that allow a small pool of antigen experienced cells to persist so that they may act as sentinels for secondary infection [321]. These cells are the memory $CD8^+$ T cells and they may provide life-long protection. But what permits their long-term survival? What special features do they possess that endow these cells with exceptional longevity?

The pool sizes of memory and naïve $CD8^+$ T cells are independently regulated [322] [323] which safeguards the preservation of a diverse repertoire of TCR specificities to cope with novel infections. Physical space is a crucial factor in this regard; with finite space available, the number of $CD8^+$ T_{mem} cells has to be tightly regulated such that pre-existing memory cells are often sacrificed to make way for new ones - a process known as attrition [324] [325]. Independent

regulation of the naïve and memory compartments helps to limit this erosion of memory towards previously encountered pathogens. Thus, naïve and memory subsets occupy unique environmental niches requiring distinct, albeit similar mechanisms for their maintenance. Naïve $CD8^+$ T cells require the continuous presence of MHC for long-term survival with complimentary help from certain cytokines. However, evidence suggests that neither foreign antigen nor MHC molecules are required for $CD8^+$ T_{mem} persistence [326] [327]. This implies a process of TCR desensitisation during memory conversion that results in ignorance to self-MHC ligands. Instead, $CD8^+$ T_{mem} relies heavily on the action of soluble mediators to maintain viability.

Evidence that cytokines affect $CD8^+$ T_{mem} homeostasis came from experiments where injection of Polyinosinic:polycytidylic acid (Poly:IC) or LPS in the absence of antigen caused a transient surge in the turnover rate of antigen-specific and bystander $CD8^+$ T_{mem} cells [328] [133]. These adjuvants were subsequently found to elicit the production of type I IFN and IFN-γ, which in turn induce IL-15 synthesis by an array of supporting cells. IL-15 then acts directly on $CD8^+$ T_{mem} cells inducing their homeostatic turnover [328] [133]. The role of IL-15 in $CD8^+$ T_{mem} maintenance was confirmed in *il-15*-deficient mice which lacked IL-15 responsive $CD122^{hi}$ $CD8^+$ T_{mem} cells, but maintained a population of $CD122^{lo}$ $CD8^+$ T_{mem} cells. Further support was provided by observations in which $CD8^+$ T_{mem} cells were transferred into *il-15*$^{-/-}$ hosts and were rapidly shown to die [329], and where *il-15* transgenic mice exhibit an expanded $CD8^+$ T_{mem} pool [330].

Like naïve $CD8^+$ T cells, $CD8^+$ T_{mem} cells are also dependent on IL-7 as well as

IL-15. While basal levels of IL-7 are not sufficient to support CD8⁺ T_{mem} maintenance in *il-15*-deficient hosts, overexpression of *il-7* can rescue this phenotype when *il-15*$^{-/-}$ mice are crossed onto an *il-7* transgenic background [124]. Such findings have led to the paradigm that CD8⁺ T_{mem} requires contact with both IL-7 and IL-15, where IL-15 controls homeostatic proliferation and IL-7 maintains cell viability. Indeed, in the absence of IL-7R signalling, naïve CD8⁺ T cells can differentiate into CD8⁺ T_{mem}, but these cells gradually disappear from the periphery over time [331] [332].

4.7. Secondary effector CD8⁺ T cell responses

The purpose of maintaining CD8⁺ T_{mem} cells is to provide a more efficient immune response to secondary infection resulting in rapid elimination of the pathogen. After re-challenge, CD8⁺ T_{mem} cells undergo a second clonal expansion phase which is typically quicker and of greater intensity than the primary response [333]. This speed of secondary CD8⁺ T_{eff} cells in infiltrating the site of infection is due to a number of contributing factors, such as increased expression of adhesion molecules [321] and a lower dependency on co-stimulatory signals that are required for priming naïve T cells [233]. Furthermore, CD8⁺ T_{mem} cells downregulate the expression of classical cell cycle inhibitors such as $p27^{kip}$ and maintain high levels of pre-activated cyclin D/CDK6 complexes. Therefore, CD8⁺ T_{mem} cells are found in a continued state of readiness with many of the factors required for cell division pre-synthesised and assembled [334] [335]. The result is efficient activation and rapid entry to cell cycle, coupled to steadfast recruitment to peripheral tissues which ensures the precocity of the recall response. In general, the secondary

response undergoes a lengthened contraction phase, culminating in a greater number of $CD8^+$ T_{mem} cells than existed previously [333].

$CD4^+$ T cells are also important for efficient secondary $CD8^+$ T cell responses. Whereas the frequency and effector function of $CD8^+$ T cells generated in the absence of $CD4^+$ T cells are not altered during the primary response, such cells are demonstrably impaired in proliferative and effector capacity upon antigen re-encounter [336]. The most interesting aspect of $CD4^+$ T cell help is that it occurs during initial $CD8^+$ T cell priming upon first exposure and seems dispensable during secondary expansion. $CD8^+$ T cells primed in $CD4^+$ T cell-deficient mice mount a defective recall response even when transferred into normal hosts [337] [338]. By contrast, $CD8^+$ T cells primed in normal mice exhibit effective recall responses when transferred into $CD4^+$ T cell-deficient mice. The $CD4^+$ T cell helper effect is known to involve CD40-CD40L interactions. Deficiencies in either CD40 or CD40L leads to impaired $CD8^+$ T cells responses [339] [340] and anti-CD40 antibodies can substitute for $CD4^+$ T cell help [341] [342]. Initial observations suggested that such interactions were between CD40L-expressing $CD4^+$ T cells that would activate CD40-expressing APCs which may then efficiently prime $CD8^+$ T cells. However, experiments showing that efficient recall responses can occur in the presence of CD40-deficient APCs spoke against that hypothesis [343] [344]. Instead, $CD4^+$ T cell help is thought to be derived following direct contact between CD40-expressing $CD4^+$ T cells and CD40L-expressing $CD8^+$ T cells [343].

4.8. Metabolism and $CD8^+$ T cell differentiation

It was recognised over 30 years ago that it was important for $CD8^+$ T cells to

control cellular metabolism in order to maintain efficient effector function [345] [346] [347]. In recent years, the study of T cell metabolism has gone through somewhat of a renaissance, driven in part by advances in technology permitting simpler yet more sensitive ways to measure energy usage. Much more is known about the metabolic pathways that support T cell activation and proliferation than support naïve, quiescent T cells. The conventional view is that naïve CD8⁺ T cells interchangeably break down glucose, amino acids, and fatty acids in order to fuel the tricarboxylic acid (TCA) cycle and generate ATP in their mitochondria via oxidative phosphorylation (OXPHOS) (Figure 4.3) [348]. Thus, naïve CD8⁺ T cell metabolism is catabolic in nature and this metabolic signature is actively maintained in order to ensure quiescence [349] [350] [351]. TSC1, a regulator of the mTOR pathway, is an active player in this process and establishes a quiescent gene expression program that links metabolism to cell proliferation [350]. Likewsie, FOXO (Forkhead box class O) and KLF (Kruppel-like factor) also maintain naïve T cell quiescence through metabolic regulation [352].

During antigenic stimulation, CD8⁺ T cells undergo a switch to anabolic metabolism in the form of glycolysis to fuel growth, proliferation and effector function (Figure 4.3) [353] [354] [355]. Following TCR stimulation, signals from growth factors such as IL-2, in addition to CD28 ligation, lead to an increase in glycolysis through Akt activation [356] [357] [358]. Akt acts through the mTOR pathway to increase glycolytic enzyme activity while enhancing the expression of nutrient transporters, enabling increased glucose utilisation [359] [360] [361]. An unusual metabolic aspect of proliferating CD8⁺ T cells is their preference for glycolysis even in the presence of sufficient oxygen to

support mitochondrial OXPHOS [362] [345] [363]. This phenomenon is known as the Warburg effect [364] and is also a feature of cancer cell metabolism. A likely explanation for this discrepancy is that glycolysis provides building blocks that can be incorporated into new biomass, facilitating rapid proliferation and ensuring a speedy response to infection. This is often achieved by routing glycolytic intermediates into the pentose phosphate pathway that provides precursors for nucleotide and amino acid synthesis while indirectly supporting lipid and cholesterol production needed for membrane synthesis [365].

Although glycolysis is important for mediating $CD8^+$ T cell proliferation during the initial effector phase, it eventually comes at a price to the cell during contraction. Prolonged glycolysis render $CD8^+$ T cells bioenergetically unstable due to a failing ability to generate energy from diverse substrates via OXPHOS. This lack of metabolic flexibility compromises cell viability when growth factor signalling (such as IL-2) that supports glycolysis dissipates after infection [365]. Thus, $CD8^+$ T cell metabolism is a defining factor that determines survival and death during $CD8^+$ T cell contraction. What are the metabolic determinants for $CD8^+$ T_{eff} survival and $CD8^+$ T_{mem} differentiation? Work from Erika Pearce's lab has illustrated the importance of mitochondria and IL-15 in this process. $CD8^+$ T_{mem} cells maintain significantly higher spare respiratory capacity (SRC) in comparison to naïve and effector cells. SRC is the extra mitochondrial capacity available in a cell to produce energy under increased work or stress, such as during recall responses to secondary infection [366] [367]. SRC is underscored by enhanced mitochondrial biogenesis during memory formation, a process that is modulated by IL-15 [368].

Following these observations, a model was suggested where energetic instability during the effector phase is also driven by a failure to maintain and/or induce mitochondrial biogenesis resulting in a SRC below the threshold required for $CD8^+$ T_{mem} formation. The cells that go onto to form the memory pool are effectors that have maintained mitochondrial function and volume through exposure to IL-15 or other growth factor cytokines. High mitochondrial mass permits greater use of OXPHOS, facilitating cell survival in the absence of pro-glycolysis signals [368] (Figure 4.3). More recent studies have added weight to this argument, showing that memory precursor cells exhibit low levels of glycolysis relative other $CD8^+$ T_{eff} cells, and that blocking glycolysis results in significantly greater number of cells entering the $CD8^+$ T_{mem} compartment [369]. Pearce *et al* has also described an essential role for fatty acid oxidation (β-oxidation) in $CD8^+$ T_{mem} formation [370]. Here, it was shown that $CD8^+$ T cells unable to perform β-oxidation due to a deletion of *TRAF6* mounted normal effector responses, but failed to differentiate into $CD8^+$ T_{mem} cells. Indeed, inducing β-oxidation through the drug metformin led to enhanced $CD8^+$ T_{mem} formation to *L. monocytogenes* infection [370]. Surprisingly, this requirement for fatty acids to support memory formation rests on the intrinsic breakdown of lipids within the cell driven by glucose uptake, rather than uptake of exogenous fatty acids from the environment [371].

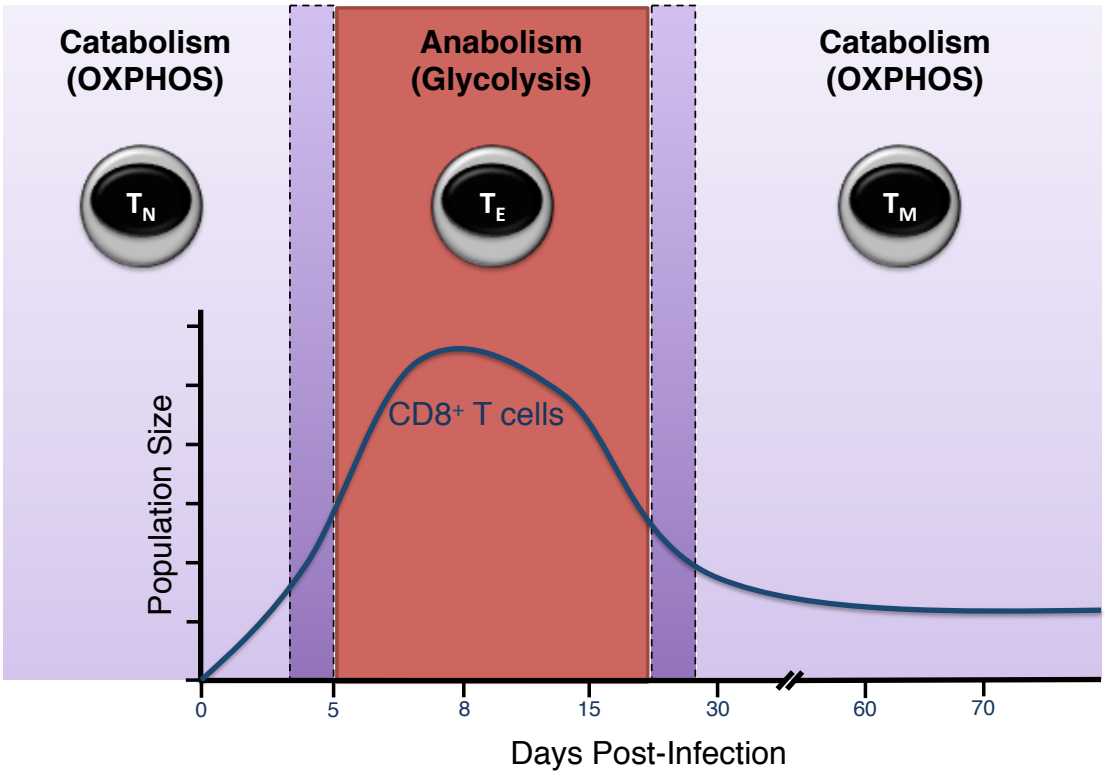


Figure 4.3 *Metabolism during CD8⁺ T cell differentiation. Quiescent naïve CD8⁺ T cells interchangeably use glucose, amino acids, and fatty acids to catabolically fuel ATP generation. Following activation, T cell expansion is driven by glycolysis that provides the anabolic building blocks to support cell growth. After contraction, memory CD8⁺ T cells reacquire OXPHOS metabolism. The oxidation of fatty acids in the mitochondria is particularly important for entry into the memory pool. Adapted from* [352].

4.9. Chapter summary: Autophagy is a critical regulator of memory CD8⁺ T cell formation

Up to now, studies on autophagy in T cells have been restricted to observations in naïve T cells, with its importance in antigen-specific CD8⁺ T cells unknown. In this chapter, an essential requirement for autophagy in memory CD8⁺ T formation will be presented. In response to viral infection, autophagy-deficient CD8⁺ T cells proceed normally through the effector phase, but an absence of antigen-specific CD8⁺ T cells is observed at later time points. Experiments will show how this defect is cell intrinsic and stems, in part, from dysregulated mitochondrial homeostasis. *Atg7*$^{-/-}$ antigen-

specific CD8⁺ T cells display increased mitochondrial mass and elevated ROS production and the failure to form a memory compartment can be rescued through antioxidant treatment. Furthermore, *Atg7*$^{-/-}$ CD8⁺ T cells exhibit an altered metabolic profile that is suggestive of a failure to switch from glycolysis to fatty acid oxidation, an essential requirement for CD8⁺ T_{mem} differentiation.

Chapter 4: Results

Autophagy is a critical regulator of memory CD8⁺ T cell formation

Results

4.10. Autophagy is dispensable for effector CD8⁺ T cell responses to influenza

In order to assess the role of autophagy during the effector phase of the CD8⁺ T cell response, wildtype and T-Atg7$^{-/-}$ mice were immunised with PR8 influenza (H1N1). On day 10 of infection, the antigen-specific CD8⁺ T cell response to the influenza nucleoprotein (NP) was analysed in the lungs by tetramer staining. The frequency of CD8⁺ T cells specific for NP in the lungs was similar between wildtype and T-Atg7$^{-/-}$ mice (Figure 4.4). However, the absolute number of NP-specific CD8⁺ T cells was significantly lower in knockout mice (Figure 4.4), likely due to the lymphopaenic nature of the T-Atg7$^{-/-}$ T cell compartment.

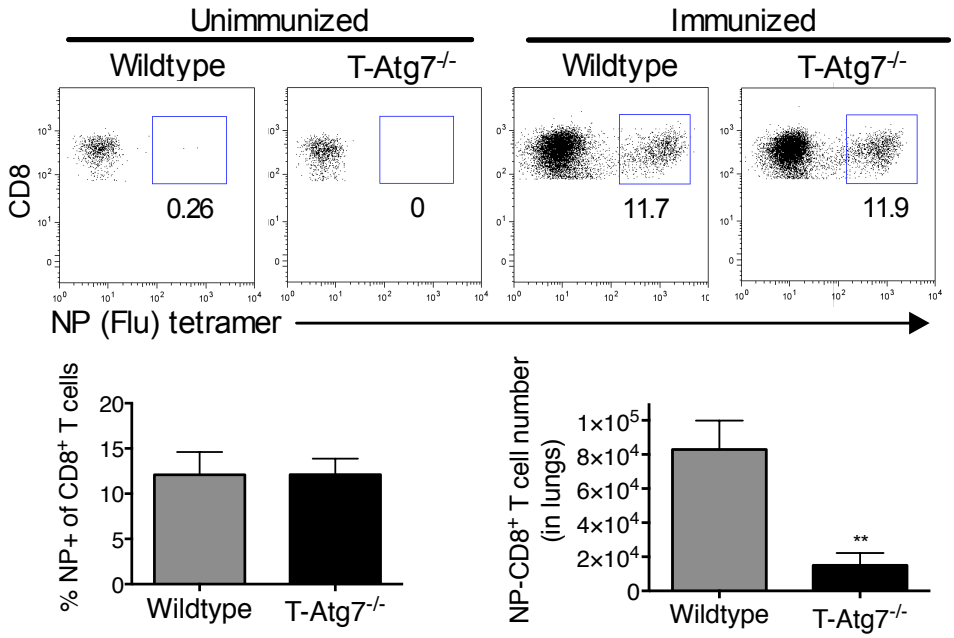


Figure 4.4 *The effector CD8⁺ T cell response to influenza is normal in T-Atg7$^{-/-}$ mice. Wildtype and T-Atg7$^{-/-}$ mice were immunised with 0.00032 HAU PR8 influenza and the response was assessed in the lungs on day 10 of infection by tetramer. Unimmunized mice were used as controls. Example dot plots are shown in the upper panel, data is representative of 5 independent experiments. p**=0.0089 by Student's t-test (n=6-9).*

4.11. Autophagy is not required for effector CD8⁺ T cell responses to MCMV

To confirm that autophagy is not needed for efficient effector $CD8^+$ T cell ($CD8^+$ T_{eff}) responses to viral infection, we immunized T-$Atg7^{-/-}$ mice with murine cytomegalovirus (MCMV). During the acute response to MCMV, the $CD8^+$ T cell response is directed against epitopes that drive classic $CD8^+$ T cell kinetics, with a large expansion phase followed by contraction. However, during chronic infection dominant responses switch to so called 'inflationary' epitopes that do not exhibit contraction. The $CD8^+$ T_{eff} response to the classical, non-inflating epitope m45 was analysed in the blood at the peak of infection on day 7. In wildtype mice, as many as 10% of circulating $CD8^+$ T cells were specific for m45 and this frequency was similar in T-$Atg7^{-/-}$ mice (Figure 4.5a). The $CD8^+$ T cell response to m45 was also assessed in multiple peripheral organs at day 8 of infection. In the spleen, lungs and salivary glands (SGs) the frequency of $CD8^+$ T_{eff} cells specific for m45 was equivalent between wildtype and knockout mice (Figure 4.5b), supporting the notion that autophagy is not required for normal $CD8^+$ T_{eff} expansion.

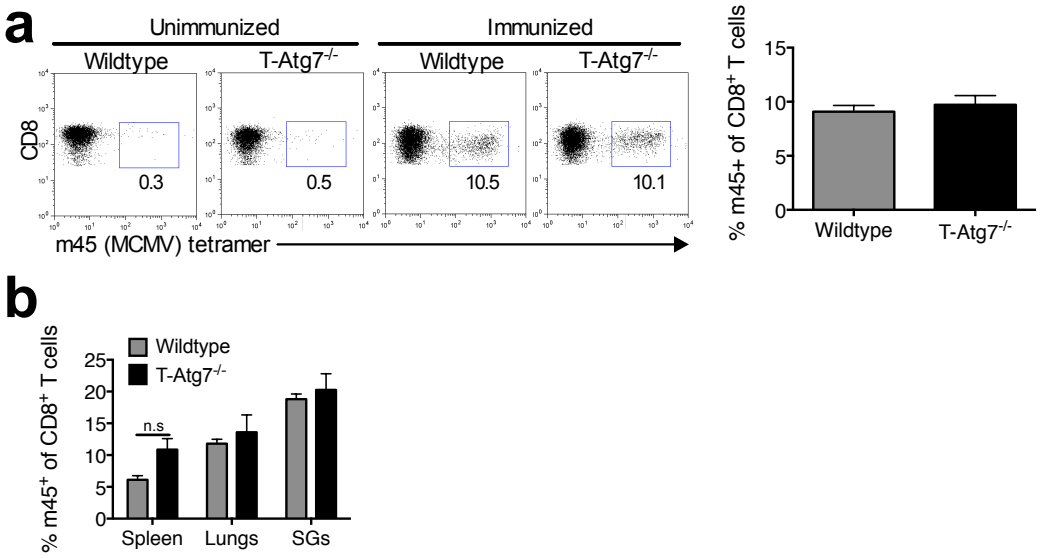


Figure 4.5 *The CD8+ T_{eff} response to MCMV is normal in T-Atg7-/- mice.* ***a)*** *Wildtype and T-Atg7-/- mice were immunized with MCMV and CD8+ T cells from blood were stained with m45 tetramer on day 7 post-infection. Dot plots show the frequency of m45-tetramer+ cells gated on CD8+ T cells. Bar graph indicates % CD8+ T cells specific for m45 (n=4–5) and is representative of three independent experiments.* ***b)*** *MCMV-immunized mice were assessed for their CD8+ T_{eff} response to m45 in the organs indicated at day 8 of infection.*

4.12. Autophagy is required for memory CD8+ T cell formation

While autophagy is dispensable during the acute phase of viral infection, we next assessed its role during memory CD8+ T cell formation. Wildtype and T-Atg7-/- mice were immunized with influenza and the CD8+ T cell response during the memory phase was analysed on day 50. While a small, long-lived population of NP-specific CD8+ T cells remained in the lungs in wildtype mice, no such population was observed in T-Atg7-/- mice where there was a profound absence of antigen-specific CD8+ T cells (Figure 4.6).

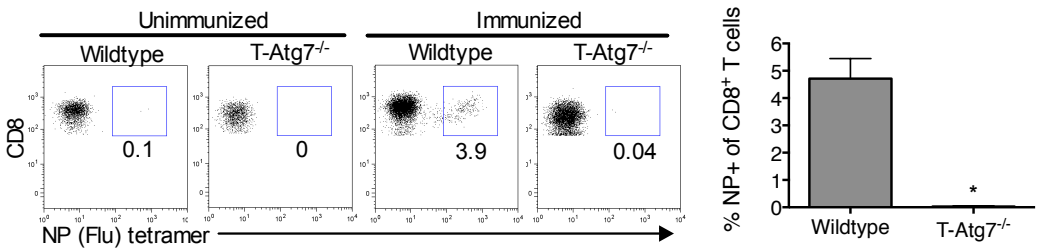


Figure 4.6 *Autophagy is required for memory CD8+ T cell formation to influenza. Wildtype and T-Atg7-/- mice were immunised with 0.00032 HAU PR8 influenza and the frequency of antigen-specific CD8+ T cells assessed by tetramer in the lungs on day 50. Example dot plots are shown and are gated on CD8+ T cells. Bar graph is representative of three independent experiments. p*=<0.05 by Mann-Whitney U-test.*

Next, wildtype and T-Atg7$^{-/-}$ mice were immunized with MCMV and the CD8$^+$ T cell response was investigated on day 100 of infection in the liver. In wildtype mice, a long-lived population of m45-specific cells was found comprising approximately 3% of liver CD8$^+$ T cells. This was in contrast to T-Atg7$^{-/-}$ mice, where the frequency CD8$^+$ T cells specific for this epitope was similar to levels seen in naïve mice (Figure 4.7a). To investigate whether the failure to form memory CD8$^+$ T cells (CD8$^+$ T_{mem}) in T-Atg7$^{-/-}$ mice was an epitope-specific phenomenon, the CD8$^+$ T cell response to a second MCMV epitope was assessed. On day 65 post-immunization in the lungs, approximately 6% of CD8$^+$ T cells in wildtype mice were specific for the inflationary epitope IE3. Once again, this was in contrast to T-Atg7$^{-/-}$ mice where a complete absence of IE3-specific CD8$^+$ T cells was observed (Figure 4.7b). These data suggest that autophagy is essential for the formation of CD8$^+$ T_{mem} cells in response to viral infection.

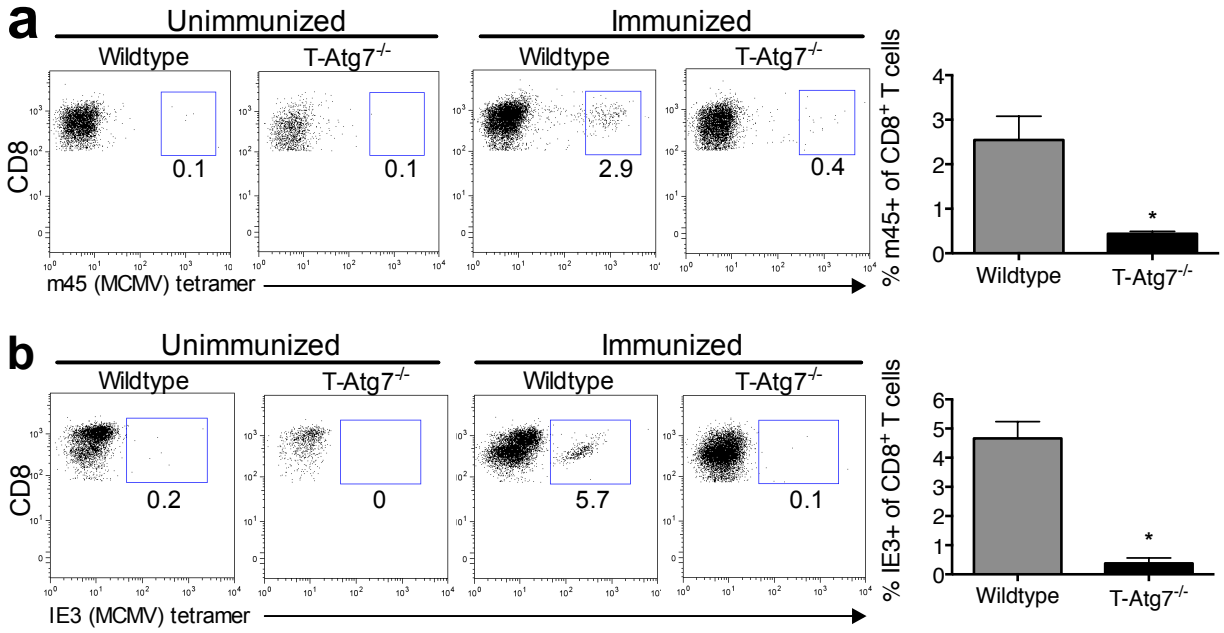


Figure 4.7 *Autophagy is required for memory CD8+ T cell formation during MCMV infection.* ***a)*** *Frequency of CD8+ T cells specific for m45 in the liver of MCMV-immunized WT and T-Atg7-/- mice on day 100 post-infection. Dot plots are gated on CD8+ T cells; bar graphs are representative of two independent experiments, p*<0.05 (n=4).* ***b)*** *Lung CD8+ T cells on day 65 post-infection were stained with IE3-tetramer. Dot plots are gated on CD8+ T cells. Quantitation depicts frequency of IE3 specific CD8+ T cells, p*<0.05. All statistics are Mann-Whitney U-test.*

4.13. T-Atg7-/- mice display significantly altered CD8+ T cell kinetics to viral infection

In order to more closely investigate the CD8+ T cell response to infection in the absence of autophagy, the kinetics to influenza and MCMV infection was tracked in the blood over time. During influenza infection in wildtype mice, the NP-specific CD8+ T cell response peaks at day 14 and then contracts to form a stable CD8+ T_{mem} population, in line with classical CD8+ T cell kinetics (Figure 4.8). However, in T-Atg7-/- mice the circulating antigen-specific CD8+ T cell pool undergoes a catastrophic collapse by day 21 resulting in a failure to form a CD8+ T_{mem} population. A similar phenomenon was observed when serial bleeding was performed on MCMV-infected mice in response to the

inflationary epitopes m38 and IE3. In response to these epitopes, wildtype CD8⁺ T cells continue to expand throughout MCMV chronic infection. However, *Atg7*$^{-/-}$ CD8⁺ T cells fail to inflate and instead undergo a dramatic contraction after the effector phase leading to a failure to form a MCMV-specific CD8⁺ T cell memory pool (Figure 4.8). These data support previous observations that autophagy is required to form the long-lived CD8⁺ T_{mem} pool following viral infection.

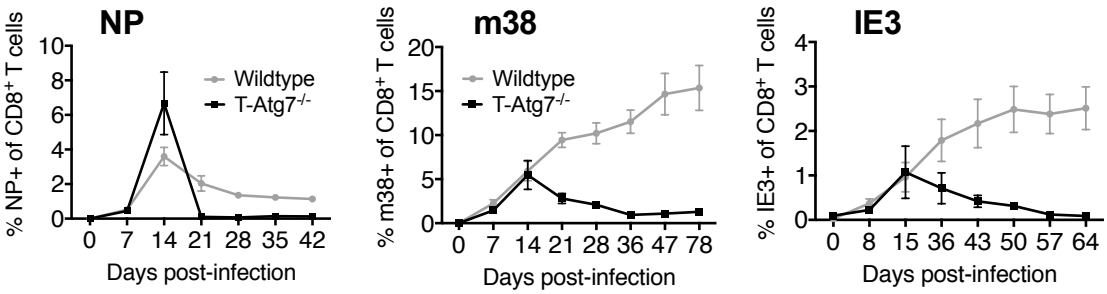


Figure 4.8 *Altered kinetics of Atg7-/- CD8⁺ T cell cells to viral infection.* ***(Left panel)*** *WT and T-Atg7⁻/⁻ mice were immunized with 0.00032 HAU PR8 influenza and the CD8⁺ T cell response tracked over time in blood by tetramer.* ***(Middle & right panel)*** *CD8⁺ T cell response to epitopes m38 and IE3 were tracked over time in blood by tetramer in MCMV-immunized WT and T-Atg7⁻/⁻ mice.*

4.14. The clinical response of T-Atg7$^{-/-}$ mice to influenza infection is normal

In light of the lymphopaenic nature of the T cell compartment, it was unclear how T-Atg7$^{-/-}$ mice would respond to infection. While the expansion of CD8⁺ T_{eff} cells is normal, their absolute number is significantly decreased compared to wildtype mice. Following infection with a sub-lethal dose of PR8 influenza, wildtype mice exhibit weight loss between days 4-7 before recovering by day 14. T-Atg7$^{-/-}$ mice also survive this infection, but unlike wildtype mice do not show signs of weight loss in the days after challenge (Figure 4.9a). When a

lethal dose ten-times greater was administered, wildtype and T-Atg7$^{-/-}$ mice lost weight in a similar fashion from day 5 post-infection and both arrived at the 20% weight loss limit by day 8 at which point mice were culled (Figure 4.9b). These observations suggest that the clinical response to influenza infection is not severely affected by the loss of *Atg7* in the T cell lineage.

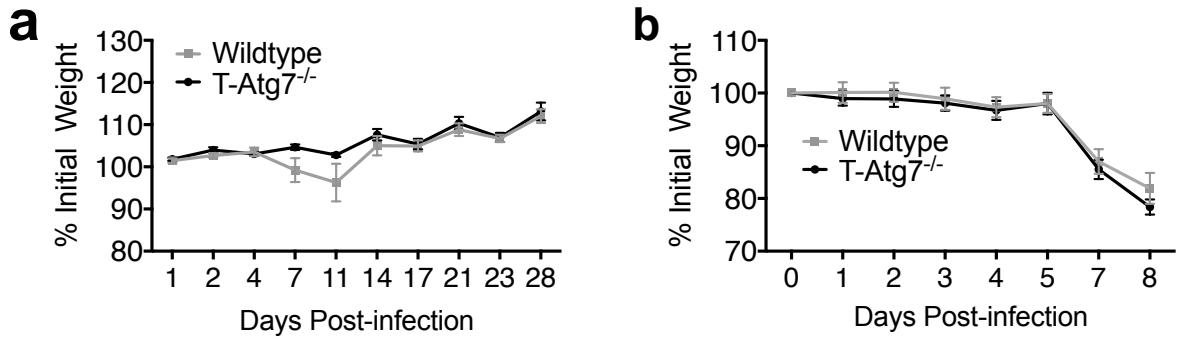


Figure 4.9 *Weight loss following influenza infection in T-Atg7$^{-/-}$ mice.* ***a)*** *Mice were immunized with 0.00032 HAU PR8 influenza and weight tracked daily following infection.* ***b)*** *Mice were immunized with 0.0032 HAU PR8 influenza and weight tracked daily following infection. Data is representative of two independent experiments (n=5).*

4.15. T-Atg7$^{-/-}$ mice clear influenza virus less efficiently than wildtype mice

Next, the ability of *Atg7$^{-/-}$* CD8⁺ T cells to clear virus from the lungs of influenza-infected mice was tested. While viral titers of wildtype and T-Atg7$^{-/-}$ mice were comparable at day 3 of infection, they were significantly higher on day 6 in T-Atg7$^{-/-}$ mice suggestive of lower efficiency in viral clearance (Figure 4.10). This difference is probably due to the lower number of antigen-specific CD8⁺ T_{eff} cells in the lungs of knockout mice. As T-Atg7$^{-/-}$ mice survived influenza challenge, one would expect that virus is eventually cleared as in wildtype mice.

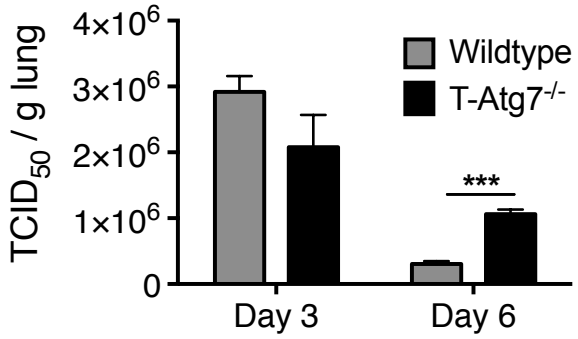


Figure 4.10 *Lung viral titres following influenza infection. WT and T-Atg7$^{-/-}$ mice were culled at days 3 and 6 post-immunization with PR8, and lungs were collected and snap frozen in liquid nitrogen. Virus titres were determined using MDCK-SIAT1 cells. p***=0.0002 as determined by Student t-test (n = 4).*

4.16. Autophagy is induced in antigen-specific CD8+ T cells

To confirm that antigen-specific CD8+ T cells utilise autophagy during differentiation, NP-specific CD8+ T cells on day 9 of influenza infection were stained with CytoID, a dye that specifically labels autophagosomes. Splenic and lung NP-specific CD8+ T cells displayed a four-fold greater staining intensity with CytoID compared to naive CD8+ T cells, suggestive of significantly more autophagosomes in the antigen-specific population (Figure 4.11).

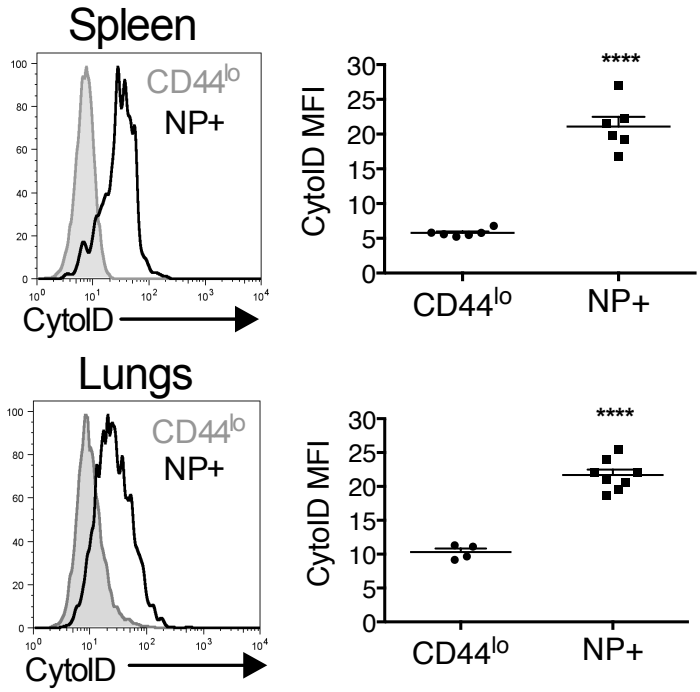


Figure 4.11 *Higher CytoID levels in antigen-specific CD8+ T cells relative to naïve cells. WT mice were immunized with 0.00032 HAU PR8 influenza. Splenic and lung CD8+ T cells were stained with CytoID at day 9 post-infection and assessed by flow cytometry. Histograms show examples of CD44lo CD8+ T cells from unimmunized mice (filled grey line) and in NP-specific CD8+ T cells from immunized mice (open black line). Quantification is by mean fluorescence intensity (MFI) of CytoID on indicated cell population and representative of two independent experiments. ****p < 0.0001 by Student t test (n = 6).*

4.17. T-Atg7-/- mice exhibit defective CD8+ T cell responses to secondary infection

Considering that T-Atg7-/- mice fail to maintain a population of CD8+ T_{mem} cells following primary infection, an obvious point to address is how these mice would respond to secondary infection. Would the *Atg7-/-* CD8+ T cell compartment be able to mount a robust recall response to a second immunization? To answer this question, heterologous influenza strains were utilised to minimise the complicating issue of antibody during clearance of a secondary challenge. This form of immunity, termed heterotypic, relies heavily on cross-reactive CD8+ T cell responses [372] [373], as opposed to

homotypic immunity to which influenza-specific antibodies contribute [373] [374]. In the influenza strains used here, PR8 (H1N1) and X31 (H3N2) differ in the their external proteins but share the core components of PR8. This strategy avoids the complication of cross-neutralising antibodies and allows T cell immunity to be examined directly since the anti-H1 and anti-N1 antibodies generated during PR8 infection cannot neutralise X31. To test recall responses in wildtype and T-Atg7$^{-/-}$ strains, mice were immunized with PR8 followed by a second challenge with X31 30 days later. While wildtype mice mounted a fast and strong recall response upon X31 challenge (PR8 + X31), this was drastically diminished in T-Atg7$^{-/-}$ mice in the lungs on day 5 in both frequency and number (Figure 4.12). Even when three live virus immunizations were administered (PR8 primed, challenged with X31 then with PR8), T-Atg7$^{-/-}$ mice were unable to mount a robust recall response, while the wildtype tertiary response was of greater intensity than the primary and secondary challenge (Figure 4.12).

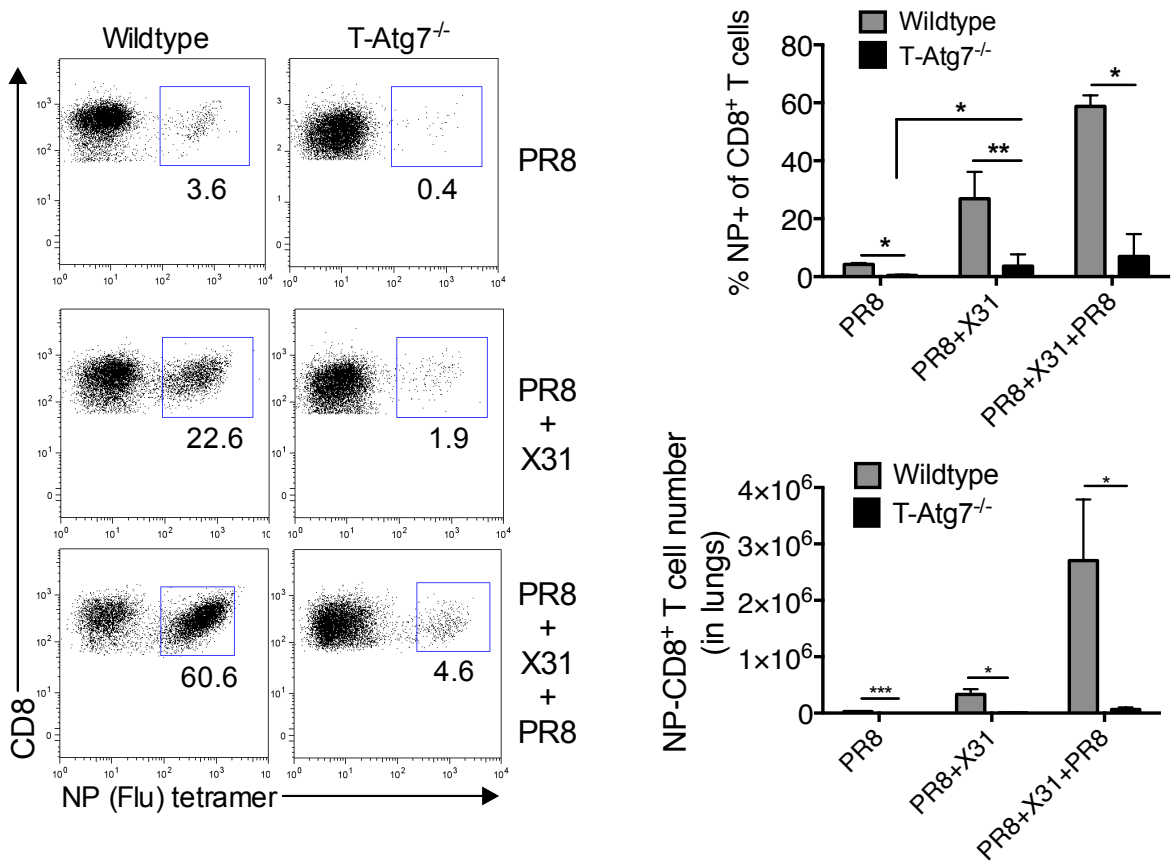


Figure 4.12 *Atg7⁻/⁻ CD8⁺ T_{mem} mount a significantly reduced recall response to secondary influenza infection. WT and T-Atg7⁻/⁻ mice were split into three groups. In one, mice were immunized with 0.00032 HAU PR8 influenza and the NP-specific CD8⁺ T cell response was measured in lungs on day 24 (PR8, n=4). In another group, mice were immunized with 0.00032 HAU PR8 influenza followed by 0.32 HAU X31 influenza challenge on day 24. The recall response to this challenge was measured on day 5 post–challenge using NP-specific tetramers (PR8+X31, n=6). In the third group, mice were immunized with 0.00032 HAU PR8 influenza, followed by 0.32 HAU X31 on day 24, and 30 days later immunized for a third time with 32 HAU PR8. The recall response to this third infection was measured in the lungs on day 5 post–challenge by tetramer (PR8+X31+PR8, n=4). Upper bar graph shows frequency of CD8⁺ T cells that are NP-specific and are representative of two independent experiments. *p < 0.05, **p < 0.01, by Mann–Whitney U-test. The lower bar graph depicts the absolute number of NP-specific CD8⁺ T cells in the lung following each challenge. p*<0.05, p***=0.0002 by Student t-test. Data is representative of two independent experiments.*

Due to the significantly diminished recall response of T-Atg7$^{-/-}$ mice to a heterologous secondary infection one might predict a poor clinical outcome in these mice. Surprisingly however, PR8-primed T-Atg7$^{-/-}$ mice survived the

heterotypic challenge with X31 influenza despite the vastly diminished number of secondary CD8+ $T_{eff\ cells}$. Unexpectedly, T-Atg7$^{-/-}$ mice lost less weight in the days after secondary infection compared to wildtype mice (Figure 4.13a). Similarly, T-Atg7$^{-/-}$ mice survived a lethal third immunization with PR8 and again exhibited less weight loss than wildtype mice (Figure 4.13b). Although in this case, antibodies would provide a contributing factor to viral clearance and clinical outcome.

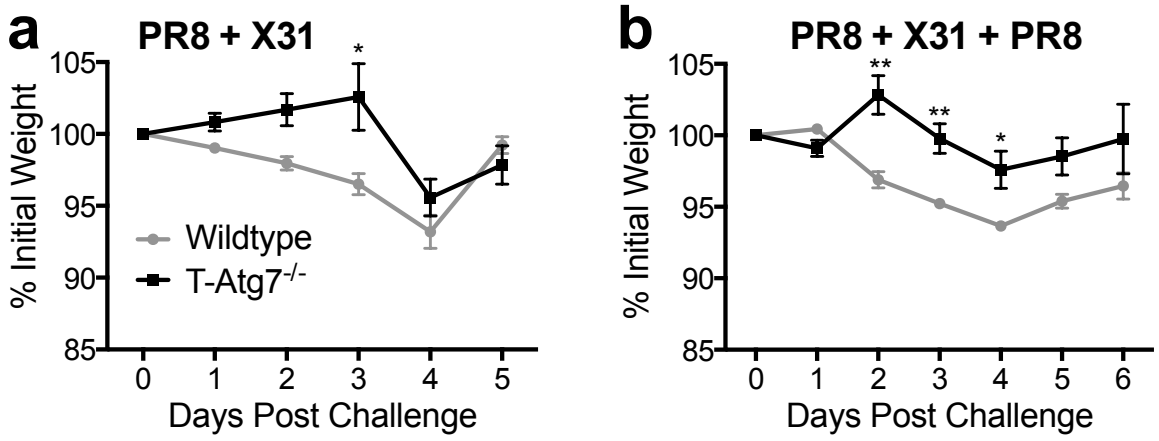


Figure 4.13 *T-Atg7$^{-/-}$ mice survive heterologous secondary infection with influenza.* ***a)*** *WT and T-Atg7$^{-/-}$ mice were primed with 0.00032 HAU PR8 influenza followed by 0.32 HAU X31 influenza challenge 24 days later and mice weight tracked. p*<0.05 (n=4-6).* ***b)*** *WT and T-Atg7$^{-/-}$ mice were immunized with 0.00032 HAU PR8 influenza, followed by 0.32 HAU X31 on day 24, and 30 days later immunized for a third time with 32 HAU PR8. p*<0.05, p**<0.01 (n=4-6). All statistics are Student's t-test.*

To confirm that T-Atg7$^{-/-}$ mice fail to mount robust recall responses to secondary influenza infection, a vaccine-based model was implemented. Using an established non-replicating pseudotyped influenza vaccination protocol [97], we next tested whether H1N1 vaccinated T-Atg7$^{-/-}$ mice could mount secondary CD8+ T responses to heterologous viral challenge. Mice were vaccinated twice, 22 days apart then challenged with a lethal dose of

X31 influenza. Large numbers of influenza NP-specific $CD8^{+}$ T cells were detected in the lungs of vaccinated wild-type mice 5 days post-challenge, but not in T-Atg7$^{-/-}$ mice (Figure 4.14a). When the $CD8^{+}$ T cell kinetics to the vaccine regimen were followed over time in the blood, influenza-specific $CD8^{+}$ T cells were detected at normal frequencies to primary immunization in T-Atg7$^{-/-}$ mice but the secondary 'booster' vaccine was unable to induce an increase in NP-specific $CD8^{+}$ T cell frequency like it could in wild-type mice (Figure 4.14b). Similarly, vaccinated T-Atg7$^{-/-}$ mice failed to generate significant $CD8^{+}$ T cell responses to viral challenge in the blood (Figure 4.14c). It was expected that a failure to form $CD8^{+}$ T_{mem} cells to vaccination would result in a lack of protection to lethal heterologous live viral challenge. However, vaccinated T-Atg7$^{-/-}$ mice survived, losing a similar amount of weight to wildtype mice in the process (Figure 4.14d). In summary, these data indicate that autophagy is required for the $CD8^{+}$ T cell recall response to either repeated immunizations with live virus, or vaccination followed by live viral challenge.

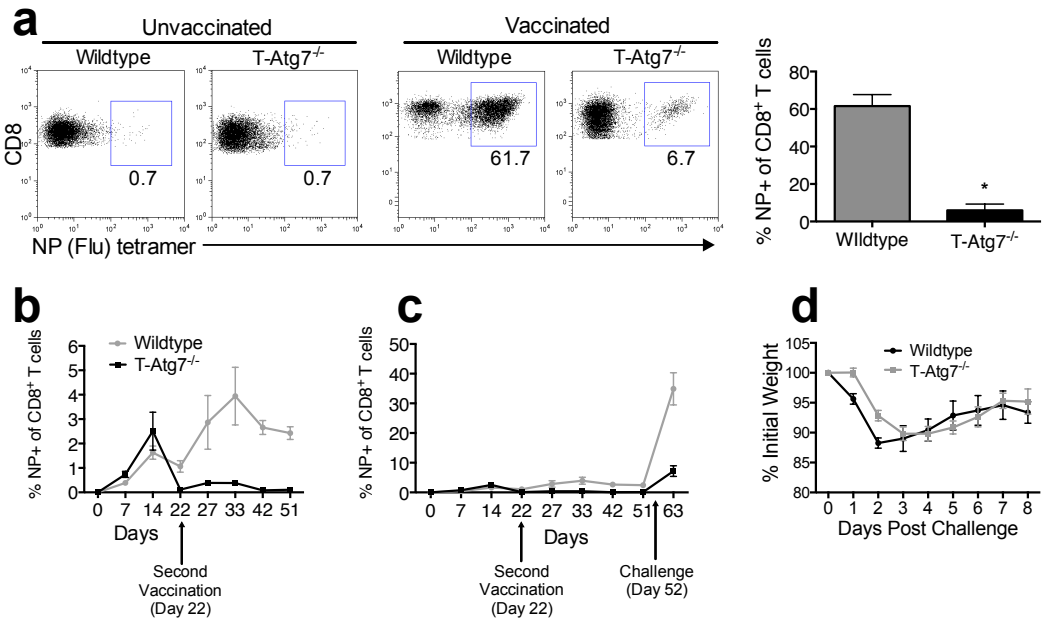


Figure 4.14 *Vaccinated T-Atg7-/- mice fail to mount a normal CD8⁺ T cell recall response to viral challenge.* ***a)*** *WT and T-Atg7-/- mice were vaccinated twice, 22 days apart with 32 HAU of the live attenuated H1N1 vaccine, S-Flu. 30 days after the last vaccination, mice were challenged with 32 HAU X31 influenza. The CD8⁺ T cell response to NP was measured in the lungs on day 23 post–challenge by tetramer. As a control, unvaccinated mice were challenged with 32 HAU X31 and culled on day 4 due to weight loss and morbidity. Quantification indicates the percentage of CD8⁺ T cells in the lung that are specific for NP on day 23 post–challenge. Example dot plots are shown. Data are representative of two independent experiments. *p<0.05 by Mann–Whitney U-test (n=4–5).* ***b)*** *WT and T-Atg7-/- mice were vaccinated with S-Flu twice, 22 days apart, and the CD8⁺ T cell response to NP was tracked over time in blood by tetramer (n=4–6).* ***c)*** *WT and T-Atg7-/- mice were vaccinated as described in (b). 30 days after the last vaccination, mice were challenged with 32 HAU X31 influenza. Using NP-specific tetramers, the CD8⁺ T cell response to the vaccine regime and the challenge was tracked in the blood over time (n=4–6).* ***d)*** *Mice were immunized as described in (a) and weight was tracked after X31 challenge.*

4.18. T-Atg7-/- mice mount a normal secondary CD8⁺ T_{eff} response to an inflationary epitope of MCMV

To investigate further the role of autophagy in secondary CD8⁺ T_{eff} responses, MCMV-primed T-Atg7-/- mice were administered a second immunization with the same virus and the CD8⁺ T cell response to two epitopes was assessed. Interestingly, a second immunization with MCMV did not induce a secondary CD8⁺ T_{eff} response to a classical expanding/contracting epitope in

wildtype mice, nor did it in T-Atg7$^{-/-}$ mice (Figure 4.15a). Similarly, this second immunization also failed to provoke a CD8$^+$ T cell recall response to the inflationary m38 epitope in wildtype mice (Figure 4.15b), suggesting that MCMV challenge does not incur CD8$^+$ T cell expansion in primed mice. However, secondary MCMV infection in T-Atg7$^{-/-}$ mice produced a potent recall response to the same inflationary epitope that could be measured in the circulation 5 days post-challenge, reminiscent of true memory kinetics. This secondary CD8$^+$ T_{eff} population was transient in nature and had disappeared from the peripheral blood by day 10 post-challenge (Figure 4.15b). These data suggest that while autophagy-deficient CD8$^+$ T cells fail to mount recall responses to classical epitopes, they may do so to the distinct inflationary epitopes found during chronic MCMV infection.

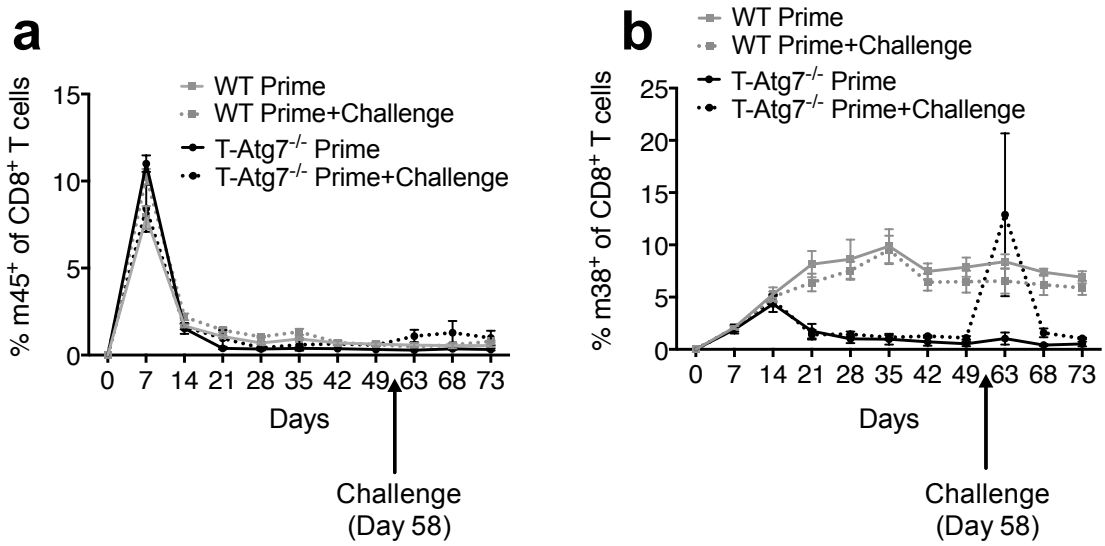


Figure 4.15 *Secondary responses of T-Atg7-/- mice to MCMV infection.* ***a)*** *WT and T-Atg7-/- mice were immunized intravenously with MCMV twice, 58 days apart. The CD8⁺ T cell response to m45 was tracked in the blood through serial bleeding at the time points indicated using m45-specific tetramers (n=4-5).* ***b)*** *WT and T-Atg7-/- mice were immunized as in (a). The CD8⁺ T cell response to m38 was tracked in the blood through serial bleeding at the time points indicated using m38-specific tetramers (n=4-5).*

4.19. *Atg7*$^{-/-}$ CD8⁺ T cells mount normal recall responses in bone marrow chimera mice

To investigate why autophagy-deficient CD8⁺ T cells fail to mount secondary CD8⁺ T_{eff} responses, bone marrow chimeras were utilised. This addresses two key points that might contribute to these observations: the lymphopaenic T cell compartment of T-Atg7$^{-/-}$ mice and defective CD4⁺ T cell help that is a requirement for secondary CD8⁺ T_{eff} responses [375,376] [336]. Like before, heterologous influenza strains were used and bone marrow chimeras were set up as in Figure 3.11. Eight weeks after transplantation, mice were immunised with PR8 followed by a second infection with X31, 30 days later. When *Atg7*$^{-/-}$ CD8⁺ T cells were placed into the replete T cell environment of bone marrow chimera mice, a normal expansion of secondary CD8⁺ T_{eff} cells was observed in the lungs 5 days post-X31 challenge (Figure 4.16). This result suggests defective recall responses is not cell intrinsic to *Atg7*$^{-/-}$ CD8⁺ T cells and that a feature of the lymphopaenic T cell pool in T-Atg7$^{-/-}$ mice has deleterious consequences for secondary responses; or alternatively, that bone marrow chimera mice provide adequate CD4⁺ T cell help to support secondary effector expansion of *Atg7*$^{-/-}$ CD8⁺ T cells that is missing in T-Atg7$^{-/-}$ mice.

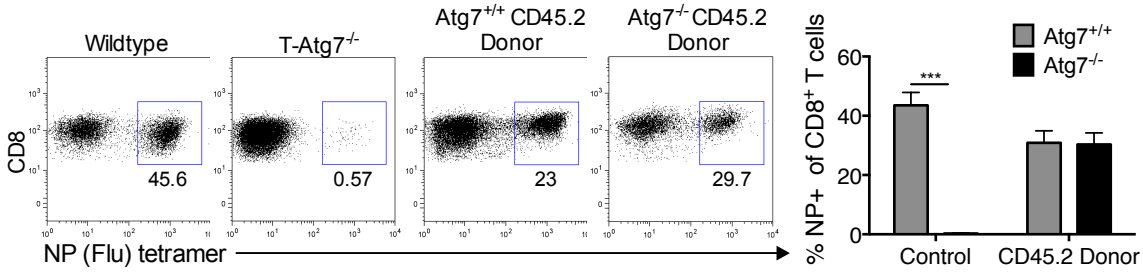


Figure 4.16 *Normal recall response of Atg7*$^{-/-}$ *CD8⁺ T cells in bone marrow chimera mice. Bone marrow chimera mice were set up as described in Figure 3.11. 8 weeks after reconstitution mice were immunized with 0.0005 HAU PR8 influenza. 30 days later, mice were challenged with 0.5 HAU X31 influenza and the recall response assessed in the lungs 5 days post-challenge. Normal WT and T-Atg7*$^{-/-}$ *mice were used as controls. p***=0.0006 by*

Mann-Whitney U-test (n=4-5).

4.20. The failure of *Atg7*$^{-/-}$ CD8^{+} T cells to form a memory pool is cell intrinsic

Is the inability of *Atg7*$^{-/-}$ CD8^{+} T cells to differentiate into CD8^{+} T_{mem} cells an intrinsic feature driven by the loss of autophagy in these cells? As *CD4*-expressing APCs will also be autophagy-deficient in T-Atg7$^{-/-}$ mice, perhaps altered CD8^{+} T cell priming by defective APCs is a contributing factor. Similarly, is CD8^{+} T_{mem} formation affected by a lymphopaenic environment? To address these points, memory formation of *Atg7*$^{-/-}$ CD8^{+} T cells was assessed using 1:1 mixed bone marrow chimeras as described in Figure 3.11. Bone marrow chimera mice were immunised with PR8 influenza and the CD8^{+} T_{mem} response was analysed in the lungs on day 40. In mice where both donors were of wildtype origin, both CD45.1 and CD45.2 donors contributed equally to the NP-specific CD8^{+} T_{mem} pool. However, in hosts containing T-Atg7$^{-/-}$ donor bone marrow, the NP-specific CD8^{+} T_{mem} pool was formed exclusively from (CD45.1) wildtype cells (Figure 4.17). Thus, even in the context of a mixed bone marrow chimera, *Atg7*$^{-/-}$ CD8^{+} T cells fail to differentiate into CD8^{+} T_{mem} cells, suggesting this defect is cell intrinsic. These data rule out lymphopaenia; defective CD8^{+} T cell priming by *Atg7*$^{-/-}$ APCs; or altered CD4^{+} T cell help as a potential mechanism.

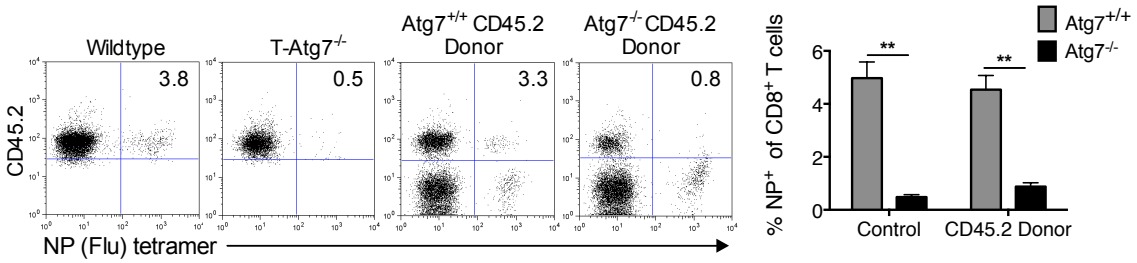


Figure 4.17 *Defective memory formation of Atg7-/- CD8+ T cells is cell intrinsic. Bone marrow chimera mice were generated as in Figure 3.11. 8 weeks after reconstitution, mice were immunized with 0.00032 HAU PR8 influenza, and the CD8+ T_{mem} response of the CD45.2 donor to NP was assessed in lungs by tetramer on day 40. Normal wildtype and T-Atg7-/- mice were used as controls. Quantitation shows frequency of (donor) CD45.2+ CD8+ T cells that are NP-specific. **p < 0.01, by Mann–Whitney U-test (n=4–7).*

4.21. Normal memory precursor frequency in the *Atg7-/-* antigen-specific CD8+ T cell pool.

It is conceivable that memory precursor cells are affected by the loss of autophagy and this impacts upon CD8+ T_{mem} formation. To investigate this, the frequency of short-lived effector (SLEC, $CD127^+KLRG1^-$) and memory precursor (MPEC, $CD127^-KLRG1^+$) CD8 T cells was analysed in MCMV-infected 1:1 mixed bone marrow chimeras on day 10 of infection. While the effector pool appeared to be skewed towards SLECs in T-Atg7-/- mice with a profound loss of MPECs, use of bone marrow chimeras showed in fact that SLEC and MPEC distribution were normal during the *Atg7-/-* CD8+ T_{eff} phase (Figure 4.18). The discrepancy between T-Atg7-/- and bone marrow chimera mice is likely derived from changes in cell surface phenotype driven by lymphopaenia in T-Atg7-/- mice, making the use of bone marrow chimeras important for analysing *Atg7-/-* CD8+ T cell phenotype. Altered memory precursor frequency is not an explanation for the observed CD8+ T_{mem} defect in the absence of autophagy.

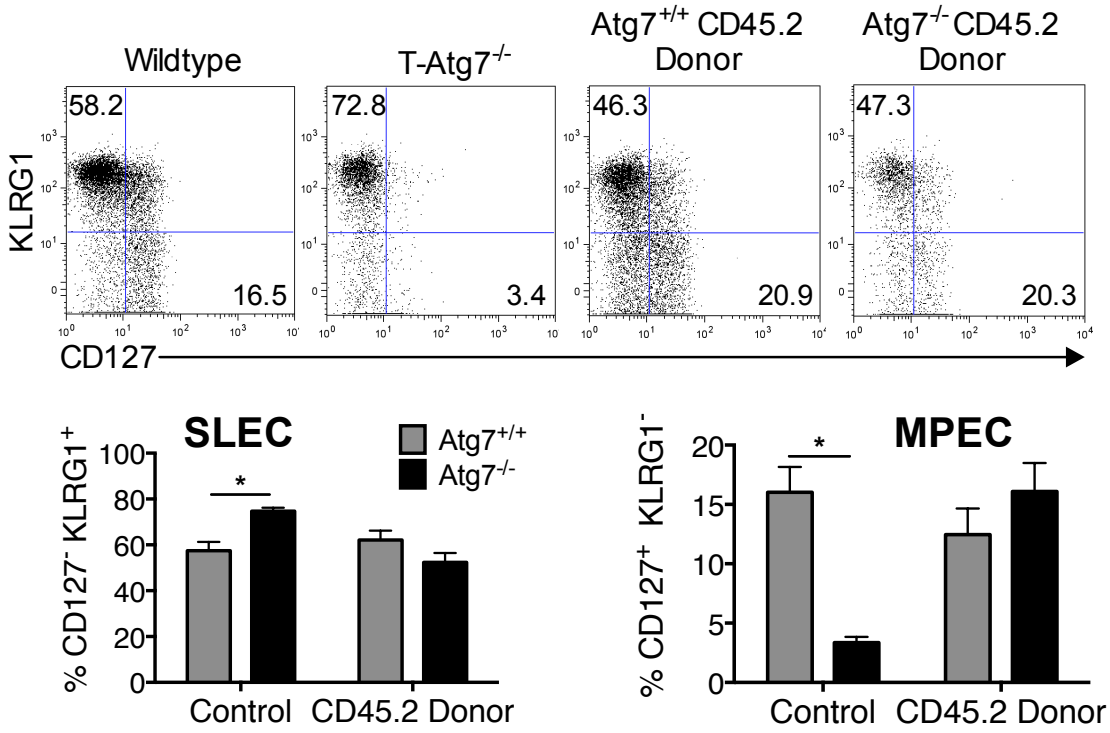


Figure 4.18 *Normal memory precursor frequency in the Atg7-/- CD8⁺ T_{eff} pool. Mixed BM chimera (generated as in Fig 3.11) were immunized with MCMV 8 weeks after transplantation. Dot plots show example of KLRG1 and CD127 expression on gated CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells on day 10 post-infection. Left bar graph depicts the % of CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells that are CD127⁻ KLRG1⁺ (SLECs). Right bar graph shows the % of CD127⁺ KLRG1⁻ (MPECs) in the same population. Example dot plots are depicted in the upper panel. Normal WT and T-Atg7-/- mice were used as controls. *p < 0.05, by Mann–Whitney U-test (n=4–7).*

4.22. *Atg7-/-* antigen-specific CD8⁺ T cells exhibit increased levels of apoptosis during memory transition

To further understand the mechanism that results in memory formation failure in the absence of autophagy, we next checked if *Atg7⁻/⁻* antigen-specific CD8 T cells were undergoing more cell death than wildtype cells. To explore this, wildtype and T-Atg7-/- mice were immunized with MCMV and levels of apoptosis were analysed at varying stages of CD8⁺ T cell differentiation. As expected, in wildtype mice the highest levels of cell death was found among antigen-specific CD8⁺ T cells just after the peak of the

effector phase on day 9 and steadily decreased as cells progressed towards CD8⁺ T_{mem} formation. In contrast, in T-Atg7$^{-/-}$ mice cell death was comparable with wildtype mice during the effector phase, but instead was found to increase over time, peaking during the memory transition phase on day 22 (Figure 4.19a). In an attempt to explain these observations, levels of the anti-apoptotic protein Bcl-2 were measured during antigen-specific CD8⁺ T cell differentiation. However, levels were comparable between *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ antigen-specific CD8⁺ T cells (Figure 4.19b).

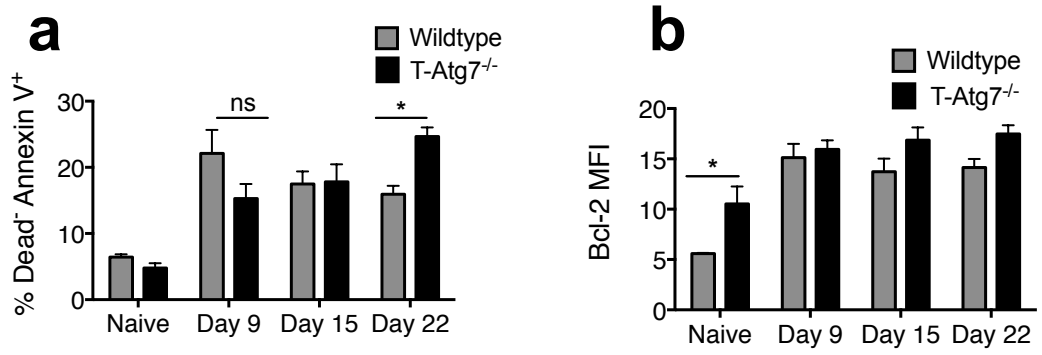


Figure 4.19 *Increased cell death but normal Bcl-2 levels in Atg7$^{-/-}$ antigen-specific CD8⁺ T cells.* ***a)*** *The spleens of unimmunized and MCMV-infected mice were stained with the apoptotic marker Annexin V and a dead cell dye that stains cells with disrupted membranes on the time points indicated. Apoptotic cells were defined as dead cell dye-negative Annexin V⁺. As a control, CD44^{lo} CD8⁺ T cells from unimmunized mice were used (naïve). Quantification is gated on m45-tetramer⁺ CD8⁺ T cells, *p < 0.05, by Mann–Whitney U-test (n=4–5).* ***b)*** *MCMV-immunized WT and T-Atg7$^{-/-}$ mice were assessed for Bcl-2 expression in splenic m45-specific CD8⁺ T cells on day 9, 15 and 22 post-infection. As a control, Bcl-2 was measured in CD44^{lo} CD8⁺ T cells from unimmunized mice (naïve). Quantification shows Bcl-2 mean fluorescence intensity. Statistics – Student's t-test (n=4–5).*

4.23. The expression of receptors important for memory maintenance are normal on *Atg7*$^{-/-}$ antigen-specific CD8⁺ T cells

To probe the underlying cause of elevated cell death in *Atg7*$^{-/-}$ antigen-specific CD8⁺ T cells, the expression of IL-7Rα (CD127) and IL-15Rα, important for CD8⁺ T_{mem} homeostasis and survival, was measured. Once again, the

presence of lymphopaenia in T-Atg7$^{-/-}$ mice prevents coherent analysis of cell surface receptor expression; therefore expression of these markers was assessed using mixed bone marrow chimeras (as described in Figure 3.11). Following bone marrow transplant and reconstitution, mice were immunized with MCMV and the expression of CD127 and IL-15Rα was assessed on MCMV-specific CD8⁺ T cells on day 10 post-infection. The frequency of antigen-specific CD8⁺ T cells expressing CD127 was comparable between wildtype and T-Atg7$^{-/-}$ donor CD8⁺ T cells (Figure 4.20a). IL-15Rα expression on *Atg7*$^{-/-}$ MCMV-specific donor CD8⁺ T cells was also equivalent to that of wildtype donor cells (Figure 4.20b). Similarly, the expression of CD122 (a subunit shared between the IL-2R and IL-15R) was normal on these cells (data not shown). Accordingly, defective cytokine receptor expression in the absence of autophagy does not explain the observed defective CD8⁺ T_{mem} formation.

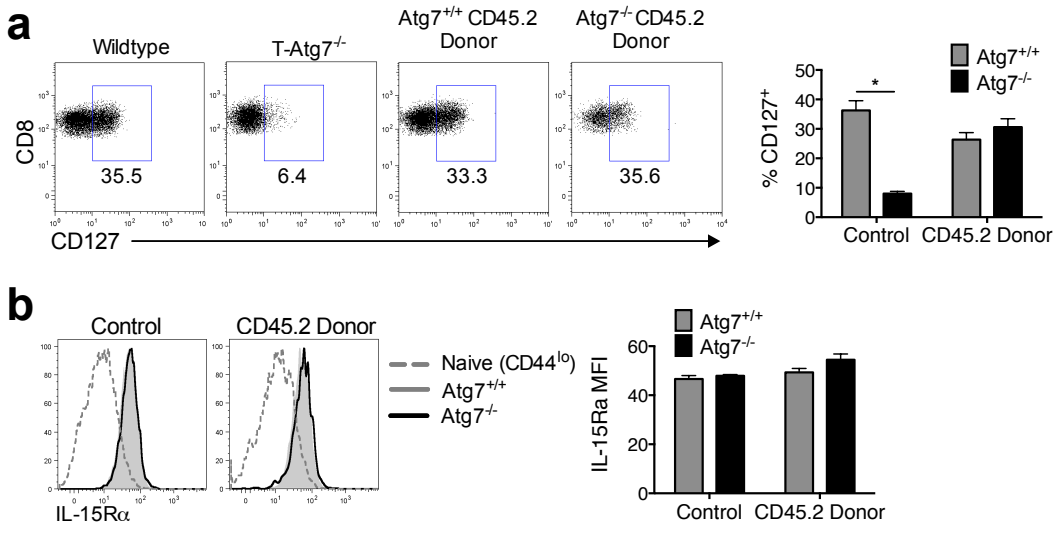


Figure 4.20 *Normal expression of IL-7Rα and IL-15Rα on Atg7*$^{-/-}$ *antigen-specific CD8⁺ T cells.* ***a)*** *CD127 expression on Atg7*$^{-/-}$ *m45-specific CD8⁺ T cells from the spleens of MCMV challenged BM chimeras on day 10 of infection. Example dot plots of CD127 staining on gated CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells are shown. Normal wildtype and T-Atg7*$^{-/-}$ *mice are used as controls. *p < 0.05, by Mann–Whitney U-test (n=4–7).* ***b)*** *IL-15Rα*

expression on day 10 splenic Atg7⁻/⁻ MCMV-specific CD8⁺ T cells in MCMV challenged BM chimeras. Histograms depict IL-15Rα expression in CD44^{lo} CD8⁺ T cells from unimmunized mice (grey dotted line), Atg7⁺/⁺ CD45.2⁺ m45-tetramer CD8⁺ T cells (grey filled line), and Atg7⁻/⁻ CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells (black line). The left histogram shows expression in control normal WT and T-Atg7⁻/⁻ mice, the right histogram indicates staining in donor CD45.2⁺ cells from BM chimera mice. Quantified is IL-15Rα mean fluorescence intensity on gated CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells (n=4–7).

4.24. Lack of CD8⁺ T_{mem} formation in T-Atg7$^{-/-}$ mice is not due to exhaustion

Next, it was tested if the lack of memory formation was caused by exhaustion in autophagy-deficient antigen-specific CD8⁺ T cells. During the terminal stages of exhaustion, antigen-specific CD8⁺ T cells are deleted from the T cell pool [156] and this phenomenon may explain the lack of long-lived *Atg7$^{-/-}$* antigen-specific CD8⁺ T cells. T-Atg7$^{-/-}$ and mixed bone marrow chimera mice were immunized with MCMV and day 10 antigen-specific CD8⁺ T cells were assessed for exhaustion by the co-expression of PD-1 and TIM-3 [156]. In wildtype mice, the antigen-specific CD8⁺ T cell pool was virtually devoid of exhausted-phenotype cells. This is on contrast to T-Atg7$^{-/-}$ mice where approximately a third of all MCMV-specific CD8⁺ T_{eff} cells at day 10 co-expressed PD-1 and TIM-3. However, these observations were not matched in bone marrow chimera mice, where comparable levels of PD-1 and TIM-3 co-expression was observed between wildtype and *Atg7$^{-/-}$* donor MCMV-specific CD8⁺ T cells (Figure 4.21). This indicates that without lymphopenia, exhaustion is not a hallmark of *Atg7$^{-/-}$* CD8⁺ T cell responses and cannot explain the observed diminished CD8⁺ T_{mem} compartment.

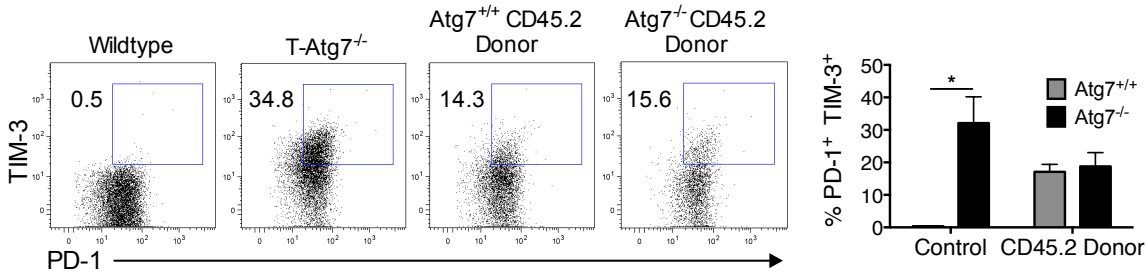


Figure 4.21 *Normal levels of exhaustion on Atg7-/- antigen-specific CD8+ T cells. Bone marrow chimera mice (generated as in Fig 3.11) were immunized with MCMV 8 weeks after transplantation. The frequency of m45-specific CD8+ T cells expressing PD-1 and TIM-3 was assessed by flow cytometry on day 10 post-infection (in spleens). Dot plots depict example of PD-1 and TIM-3 staining on gated CD45.2+ m45-tetramer+ CD8+ T cells. Bar graph quantifies the percentage of (donor) CD45.2+ m45-tetramer+ CD8+ T cells that are PD-1+ TIM-3+ at day 10 post-infection. Normal wildtype and T-Atg7-/- mice were used as controls. *p<0.05, by Mann–Whitney U-test (n=4–7).*

4.25. Transcription factors important for CD8+ T cell differentiation are expressed normally in *Atg7-/-* CD8+ T cells

To investigate if autophagy compromises effector/memory differentiation at the transcriptional level, the expression of two transcription factors important during the antigen-specific CD8+ T cell response were measured. Using flow cytometry, it was found that the expression of IRF4, responsible for sustaining the expansion and differentiation of CD8+ T_{eff} cells [377], and EOMES, a factor that promotes CD8+ T_{mem} formation [291], behaved normally during the effector and memory transition phase in *Atg7-/-* CD8+ T cells (Figure 4.22). These data indicate that effector to memory differentiation functions normally at the transcriptional level in the absence of autophagy.

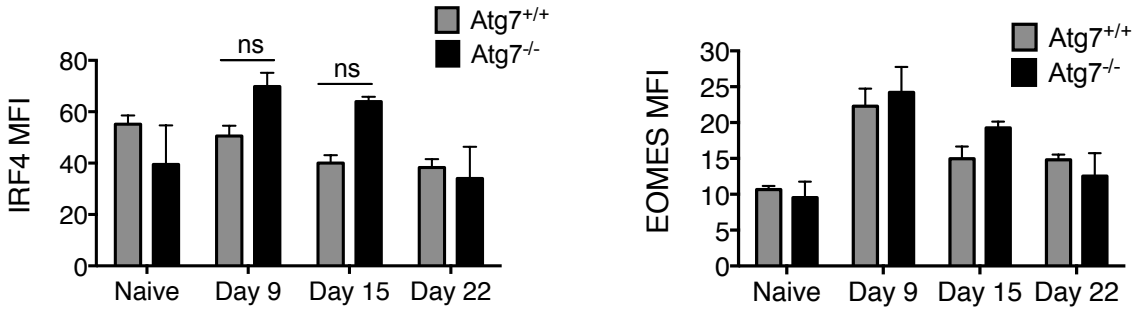


Figure 4.22 *Normal expression of IRF4 and EOMES during CD8+ T cell differentiation to MCMV. WT and T-Atg7-/- mice were immunized with MCMV and IRF4 & EOMES expression was measured in m45-specific CD8+ T cells from the spleen on day 9, 15, and 22 of infection. As a control, IRF4 & EOMES was also measured in CD44lo CD8+ T cells from unimmunized mice (naïve). Quantification shows IRF4/EOMES mean fluorescence intensity on gated m45-tetramer+ CD8+ T cells and CD44lo CD8+ T cells (naïve) (n=4–5).*

4.26. Autophagy is required to maintain mitochondrial homeostasis in antigen-specific CD8+ T cells

Previous work presented earlier in this thesis and elsewhere [53] has detailed a role for autophagy in naïve T cell mitochondrial homeostasis. Conceivably, autophagy might also be a mechanism for mitochondrial quality control in antigen-specific CD8+ T cells. When mitochondrial content was measured using MitoTracker green on MCMV-specific CD8+ T cells 15 days post-infection, autophagy-deficient cells had significantly increased mitochondrial volume compared to wildtype controls (Figure 4.23a). Furthermore, levels of mitochondrial ROS were also significantly elevated in *Atg7-/-* MCMV-specific CD8+ T cells relative to wildtype cells (Figure 4.23b).

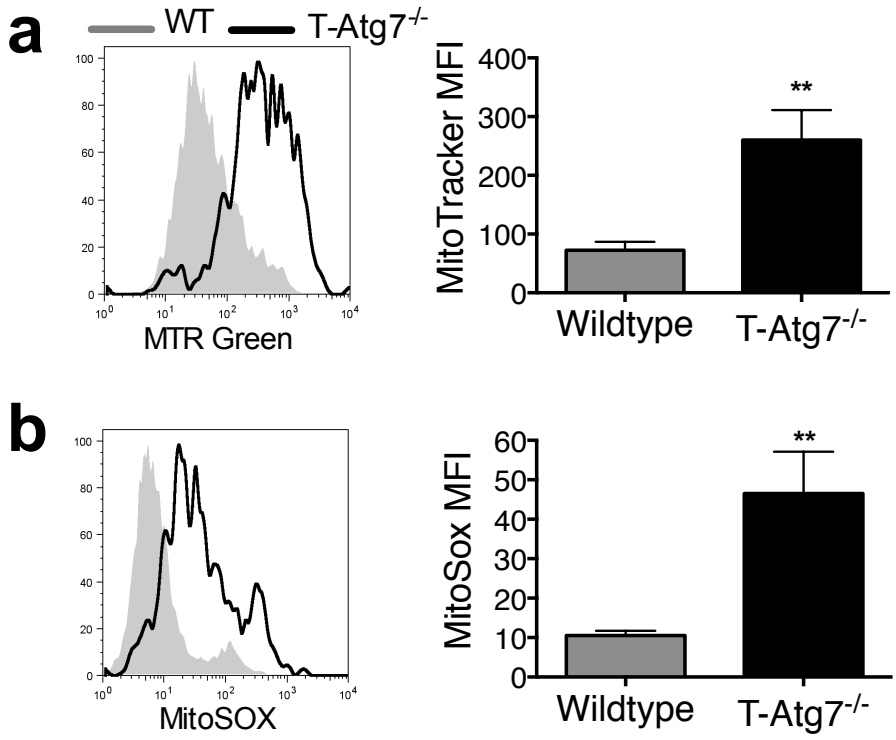
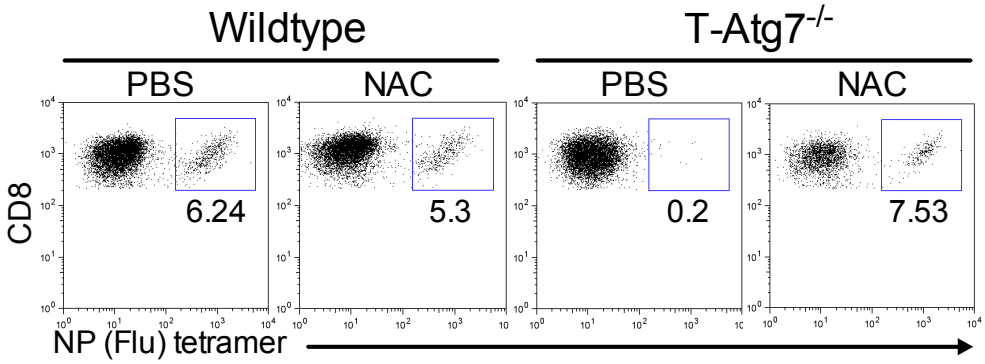


Figure 4.23 *Defective mitochondrial homeostasis in Atg7-/- antigen-specific CD8+ T cells.* ***a)*** *Spleens from MCMV-immunized WT and T-Atg7-/- mice were stained with MitoTracker Green on day 15 post-infection. Quantification depicts mean fluorescence intensity on m45-tetramer+ CD8+ T cells and is representative of three independent experiments. **p < 0.01, Student t-test (n=4–5).* ***b)*** *Spleens from MCMV-immunized WT and T-Atg7-/- mice were stained with MitoSox Red on day 15 post-infection and analysed by flow cytometry. Bar graph depicts mean fluorescence intensity on m45-tetramer+ CD8+ T cells and is representative of three independent experiments. **p < 0.01, by Student t-test (n=4–5).*

4.27. The CD8+ T_{mem} defect in T-Atg7-/- mice can be rescued through antioxidant treatment *in vivo*

Atg7-/- CD8+ T cells display signs of altered mitochondrial homeostasis. The lack of autophagy in these cells likely results in the accumulation of mitochondria that would normally be degraded. Furthermore, the increased levels of mitochondrial ROS indicate a proportion of these organelles are damaged and dysfunctional. Could deleterious ROS levels be the underlying cause of the dramatic collapse of the CD8+ T_{eff} pool in T-Atg7-/- mice? As a

corollary to this, might scavenging these excess ROS restore CD8⁺ T_{mem} differentiation in autophagy-deficient cells? To explore this hypothesis, T-Atg7$^{-/-}$ mice were treated with the antioxidant N-acetyl cysteine (NAC) following influenza infection and the CD8⁺ T_{mem} response was assessed on day 28 in the lungs. NAC treatment had no effect on the frequency of NP-specific CD8⁺ T_{mem} cells in wildtype mice, but could rescue the NP-specific CD8⁺ T_{mem} pool in T-Atg7$^{-/-}$ mice that was absent in untreated controls (Figure 4.24). Although not statistically significant due to relatively small sample size and variation in the degree of rescue, flow cytometry revealed *bona fide* CD8⁺ T_{mem} populations in the lungs of all NAC-treated mice by tetramer staining. This suggests that autophagy is required to regulate ROS levels in antigen-specific CD8⁺ T cells that if left unchecked, leads to collapse of the CD8⁺ T_{eff} pool and a failure to form a CD8⁺ T_{mem} compartment.



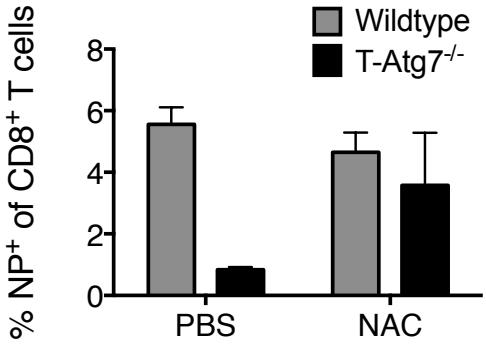


Figure 4.24 *Administration of NAC restores the CD8+ T_{mem} compartment in T-Atg7-/- mice. Wildtype and T-Atg7-/- mice were immunized with 0.0005 HAU PR8 and injected intraperitoneally with 10 mg/kg of NAC or PBS every other day following infection. On day 28, the frequency of NP-specific CD8+ T cells was assessed in the lungs by flow cytometry, (n=4-5).*

4.28. *Atg7-/-* CD8+ T cells display an altered metabolic profile

In recent years, a gluttony of papers have come to the fore detailing a crucial role of metabolism in the differentiation of CD8+ T cells following activation [378]. There now exists a clearly defined paradigm as to how energy is utilised by T cells response to infection. During the effector phase, CD8+ T cells utilise glycolysis to support cell proliferation [365] [353,356], while CD8+ T_{mem} cells switch to oxidative phosphorylation, particular turning to the oxidation of fatty acids to aid their survival [370] [368]. Mitochondria are also important in this process, providing key energy reserves, termed mitochondrial spare respiratory capacity, allowing CD8+ T_{mem} cells to respond rapidly to secondary infection [368]. Previous work from our lab has shown how loss of autophagy in other immune cells results in a shift toward a glycolytic phenotype (Stranks *et al*, In Press). Moreover, autophagy has been described to be an important regulator of lipid metabolism in murine hepatocytes, especially in the breakdown of intracellular lipid stores [43] - a process also important for CD8+ T_{mem} formation [371]. Putting these observations together, it was hypothesised that autophagy was important for mediating the switch to fatty acid (FA) metabolism during CD8+ T_{mem} differentiation through the release of FAs from intracellular lipid stores. Therefore, a lack of autophagy would result in sustained glycolysis during the effector phase and a failure to shift to FA metabolism, inhibiting the formation of the CD8+ T_{mem} pool.

The current gold standard to measure the metabolic profile of a cell population is Seahorse, where the rate of glycolysis and oxidative phosphorylation can be measured in real time in a sensitive manner. However, the absence of CD8⁺ T_{mem} cells in T-Atg7$^{-/-}$ mice and the relative scarcity of antigen-specific CD8⁺ T cells in the physiological infection models used here meant it was not possible to obtain the quantity of cells required to perform Seahorse analysis. To first test if *Atg7*$^{-/-}$ CD8⁺ T cells exhibit prolonged and elevate glycolysis during the effector phase, measurement of a surrogate marker of glycolysis was resorted to during various stages of the CD8⁺ T cell response to MCMV - the glucose transporter GLUT-1. This was detected using two different techniques: (a) the fluorescently labelled GLUT-1 binding domain of HTLV (human T cell leukaemia virus), which specifically detects GLUT-1 [379] [380], and (b) GLUT-1 antibody staining.

As expected, GLUT-1 is upregulated on CD8⁺ T_{eff} cells (day 9) and then downregulated on late CD8⁺ T_{eff}/early CD8⁺ T_{mem} cells (day 22) in wildtype mice as CD8⁺ T cells switch to mitochondrial respiration. However, m45-specific *Atg7*$^{-/-}$ CD8⁺ T_{eff} cells express more GLUT-1 throughout all stages of differentiation and downregulation does not occur to the same extent at later time points compared to wildtype cells (day 22) (Figure 4.25a). Similar results were obtained using the GLUT-1 antibody, where a significantly increased frequency of m45-specific *Atg7*$^{-/-}$ CD8⁺ T cells were found expressing GLUT-1 during the early (day 9) and late (day 22) effector phase relative to wildtype controls (Figure 4.25b).

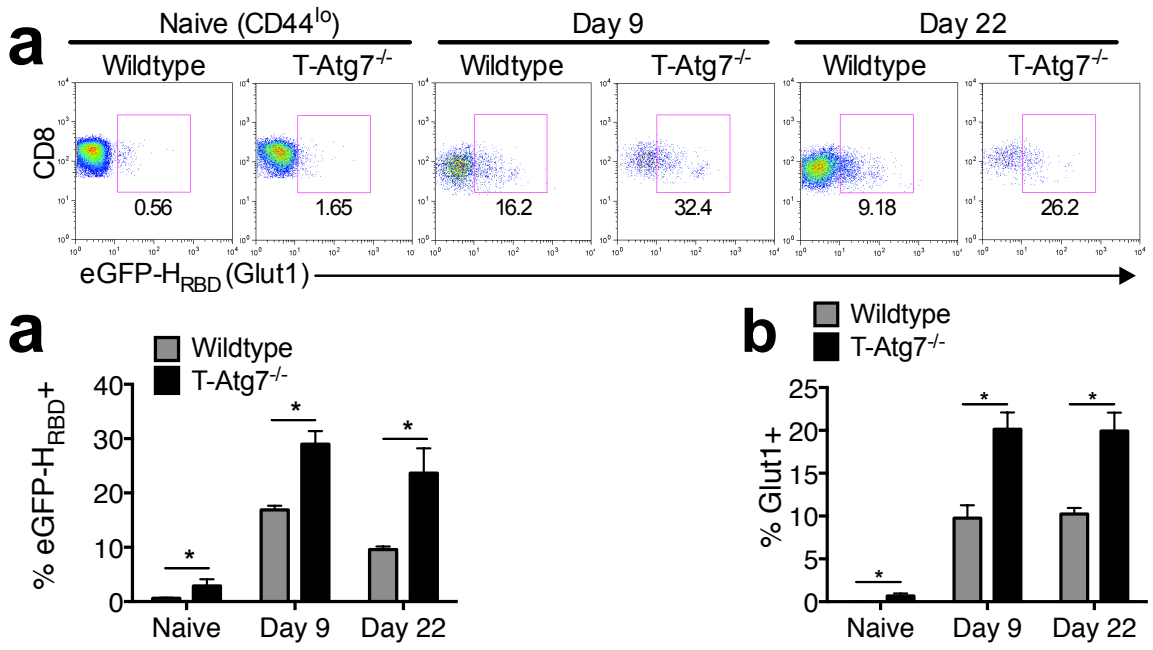


Figure 4.25 *Increased frequency of GLUT-1 expressing CD8⁺ T cells during the Atg7-/- effector phase.* ***a)*** *m45-tetramer⁺ CD8⁺ T cells from the spleens of MCMV-immunized WT and T-Atg7-/- mice were stained with the GFP-tagged HTLV receptor binding domain (eGFP-H_{RBD}) that binds GLUT-1 at the time points indicated. As a control, GLUT-1 was also measured on CD44lo CD8⁺ T cells from unimmunized mice (naïve). Example dot plots are shown and are gated on m45-tetramer⁺ CD8⁺ T cells. Bar graph shows the percentage of cells expressing GLUT-1. $p < 0.05$ (n=4–5).* ***b)*** *GLUT-1 antibody staining on day 9 and day 22 post-infection of MCMV-immunized WT and T-Atg7⁻/⁻ mice (spleens). As a control, GLUT-1 was assessed in CD44lo CD8⁺ T cells from unimmunized mice (naïve). Bar graphs indicate the frequency of m45-tetramer⁺ CD8⁺ T cells and CD44lo CD8⁺ T cells (naïve) that express GLUT-1. p*<0.05 (n=4–5). All statistics are Mann-Whitney U-test.*

Another method to investigate the rate of glycolysis is the measurement of glucose uptake using the fluorescent glucose analogue 2-NBDG. 2-NBDG is taken up normally by the cell and is retained in the cytoplasm following its phosphorylation by hexokinase, permitting the analysis of glucose uptake on a per cell basis. NP-specific CD8⁺ T cells from influenza-immunised T-Atg7-/- mice exhibited modest but significantly increased glucose uptake during the early (day 10) and late (day 18) effector phase compared to wildtype cells (Figure 4.26). Put together with earlier GLUT-1 observations, these results

suggests a sustained and increased glycolytic profile of *Atg7*$^{-/-}$ CD8^{+} T cells during the effector and memory transition phase.

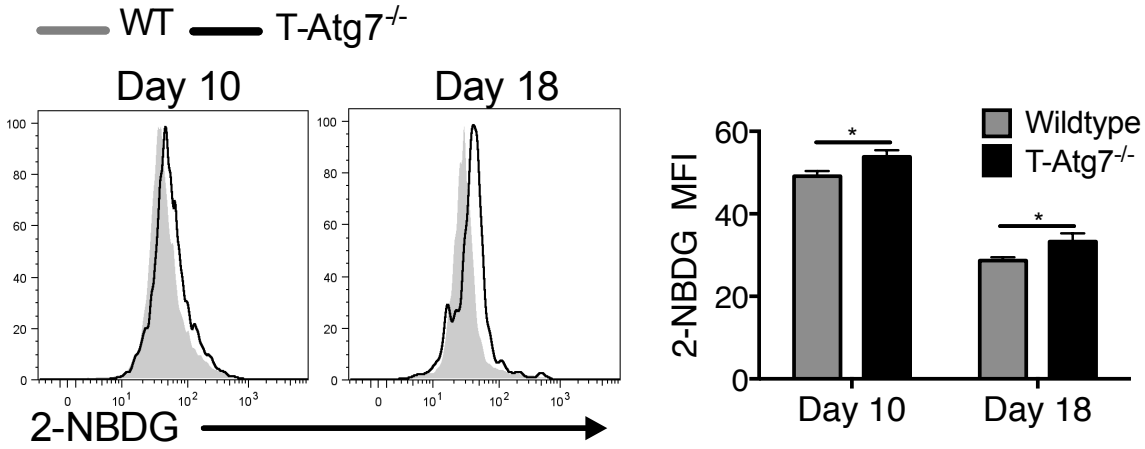


Figure 4.26 *Increased glucose uptake of Atg7*$^{-/-}$ *CD8*$^{+}$ *T*$_{eff}$ *cells. NP-specific CD8*$^{+}$ *T cells from the lungs of PR8 influenza-immunized WT and T-Atg7*$^{-/-}$ *mice were incubated for 1 hour at 37°C with 50 μg/ml 2-NBDG and uptake quantified using flow cytometry. Example histograms show 2-NBDG uptake in NP-specific cells from day 10 and day 18 post-infection. Bar chart depicts 2-NBDG MFI of NP-specific CD8*$^{+}$ *T cells. p*<0.05 by Student's t-test (n=5-6).*

A recent study by O'Sullivan *et al* demonstrated how CD8^{+} T cells utilise lipolysis to provide a source of FAs for fatty acid oxidation (FAO) [371]. To some surprise, the source of these FAs did not come from extracellular uptake during the memory phase, rather CD8^{+} T$_{mem}$ cells used extracellular glucose to synthesise lipids which were then broken down to free FAs through lysosomal lipolysis [371]. Here, it is hypothesised that autophagy provides a source of free FAs for FAO by facilitating lipolysis through the delivery of lipid stores to the lysosome. When the total neutral lipid content of NP-specific CD8^{+} T cells was assessed using BODIPY, wildtype cells entering the memory phase (day 18) had a lower lipid content than cells at the peak of the effector response (day 10) (Figure 4.27), in line with the work of O'Sullivan *et*

al which suggest that CD8⁺ T_{mem} cells break down lipid to supply FAs for FAO. *Atg7*$^{-/-}$ NP-specific CD8⁺ T cells had significantly higher lipid content than wildtype cells at day 10 and day 18 post-influenza infection (Figure 4.27), supporting the notion that autophagy might be required to degrade lipids to support the switch to FAO needed for CD8⁺ T_{mem} differentiation.

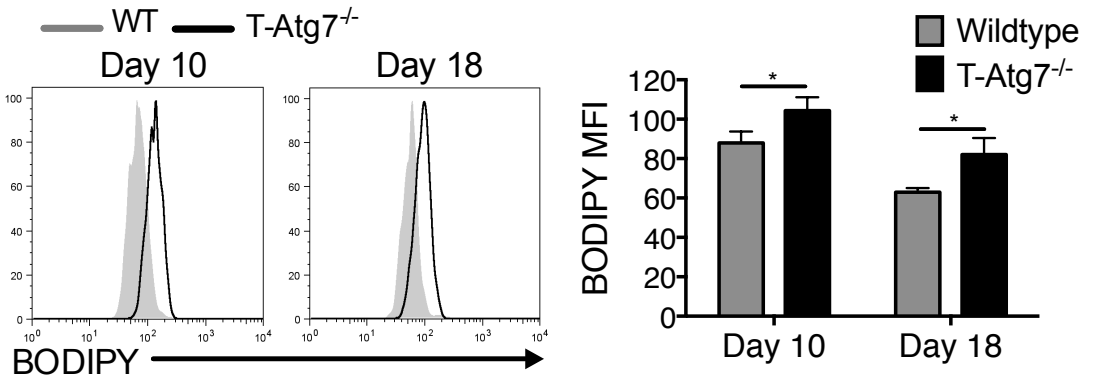


Figure 4.27 *Increased intracellular neutral lipid in Autophagy-deficient CD8⁺ T_{eff} cells. Splenocytes from influenza-immunized wildtype and T-Atg7*$^{-/-}$ *mice from day 10 and day 18 post-infection were cultured with BODIPY to assess neutral lipid content. Example histograms and bar graph is gated on NP-specific CD8⁺ T cells. p*<0.05 by Student t-test (n=5).*

Free FAs are taken up predominantly during the effector phase and are thought to be used to support cell growth during proliferation [371]. Given the change in lipid content observed in *Atg7*$^{-/-}$ CD8⁺ T_{eff} cells, the uptake of FAs was investigated by assessing intake of fluorescently conjugated palmitate (BODIPY FL C_{16}) *ex vivo*. As expected, in wildtype NP-specific CD8⁺ T_{eff} cells palmitate uptake was highest during the effector phase (day 10) and had decreased upon reaching the memory transition stage (day 18)(Figure 4.28). However, in *Atg7*$^{-/-}$ CD8⁺ T_{eff} cells, the profile of palmitate uptake was reversed compared to wildtype cells. Instead of decreasing, palmitate uptake was increased during the memory transition phase and was significantly

greater than that of wildtype cells during this time (day 18) (Figure 4.28). In fact, palmitate uptake at day 18 in *Atg7*$^{-/-}$ cells was at levels similar to that of day 10 wildtype CD8$^+$ T_{eff} cells, suggesting that FA uptake is prolonged into CD8$^+$ T_{mem} differentiation in the absence of autophagy. This may be an attempt to compensate for a decrease in lipolysis in *Atg7*$^{-/-}$ CD8$^+$ T_{mem} cells, in an effort to provide extracellular FAs to circumvent the lack of intrinsic FA production.

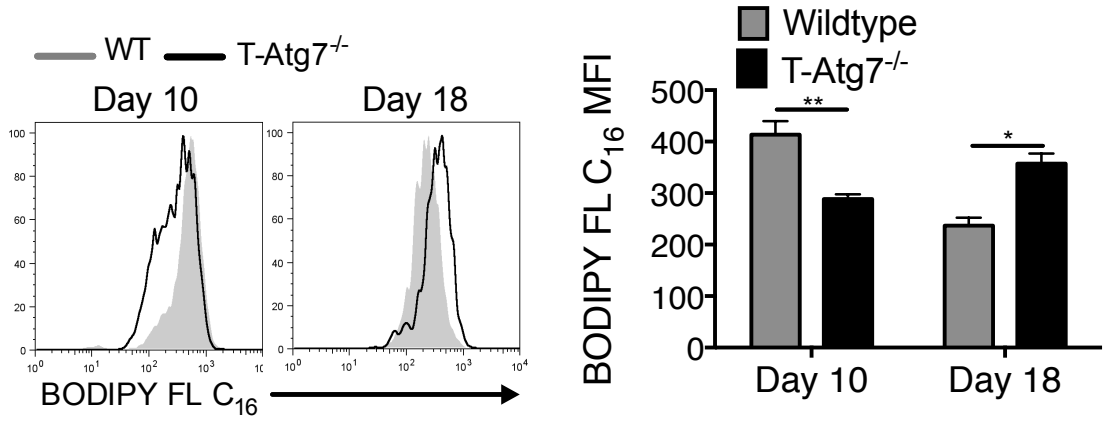


Figure 4.28 *Atg7*$^{-/-}$ *CD8*$^+$ T_{eff} *cells display altered FA uptake kinetics during differentiation. Splenocytes from influenza-immunized wildtype and T-Atg7*$^{-/-}$ *mice from day 10 and day 18 post-infection were cultured with BODIPY FL* C_{16} *to assess palmitate uptake. Example histograms and bar graph is gated on NP-specific CD8*$^+$ *T cells. p*=0.0269, p**=0.0025 by Student t-test (n=5-6).*

4.29. Autophagy is induced during the effector phase

If autophagy is required to regulate the switch to FAO that occurs during CD8$^+$ T_{eff} to CD8$^+$ T_{mem} differentiation, it might be expected that autophagy is induced prior to the formation of the CD8$^+$ T_{mem} pool, while the effector phase is on-going. To investigate this, autophagy levels were tested using CytoID at varying stages of the antigen-specific CD8$^+$ T cell response to influenza. NP-specific CytoID levels were highest during the peak of the effector

response on days 9 and 13 post-infection. CytoID staining at these time points was significantly higher than in CD8⁺ T_{mem} cells on day 30, which exhibited CytoID levels similar to that of naïve cells (Figure 4.29). Thus, autophagy levels appear to be highest during the effector phase with low levels observed in CD8⁺ T_{mem} cells.

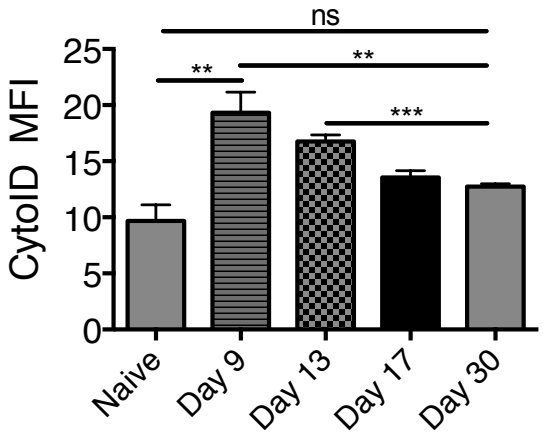


Figure 4.29 *High CytoID levels are seen in CD8⁺ T_{eff} but not CD8⁺ T_{mem} cells. NP-specific CD8⁺ T cells from the lungs were stained with CytoID at varying days of infection from PR8 influenza infected WT and T-Atg7⁻/⁻ mice. Quantification shows CytoID mean fluorescent intensity as a readout for autophagy analysed by flow cytometry. CytoID was also measured in CD44^{lo} CD8⁺ T cells from the lungs of unimmunized mice (Naïve). p**<0.01, p***=0.0002 by Student's t-test (n=4-5).*

4.30. The memory compartment remains stable when *Atg5* is excised in CD8⁺ T_{mem} cells

To further explore exactly when autophagy is required during CD8⁺ T cell differentiation, *Atg5* was excised in CD8⁺ T_{mem} cells using an inducible Cre-recombinase system. In this setting, *Cre-recombinase* is placed under the control of *Mx-1*, ubiquitously expressed in the presence of interferon that can be induced by the addition of the dsRNA analogue Poly:IC. Thus, *in vitro* or *in vivo* administration of Poly:IC induces IFN production leading to

expression of *Mx-1/Cre-recombinase* resulting in *Atg5* excision. In this model, *Atg5* is excised in all cell types.

To knockout *Atg5* in CD8⁺ T_{mem} cells, bone marrow from *Atg5*$^{Flox/Flox}$ *Mx1-Cre*⁺ or *Atg5*$^{Flox/Flox}$ *Mx1-Cre*⁻ mice (CD45.2) was transplanted 1:1 with wildtype (CD45.1) bone marrow into lethally irradiated CD45.1 hosts to create a mixed bone marrow chimera. After reconstitution, bone marrow chimera mice were then immunised with influenza and left for 30 days for a CD8⁺ T_{mem} population to form. To excise *Atg5*, Poly:IC was administered between days 30-34 post-infection and the CD8⁺ T_{mem} compartment was analysed in the lungs 3 weeks later. A schematic of the experimental design can be seen inFigure 4.30.

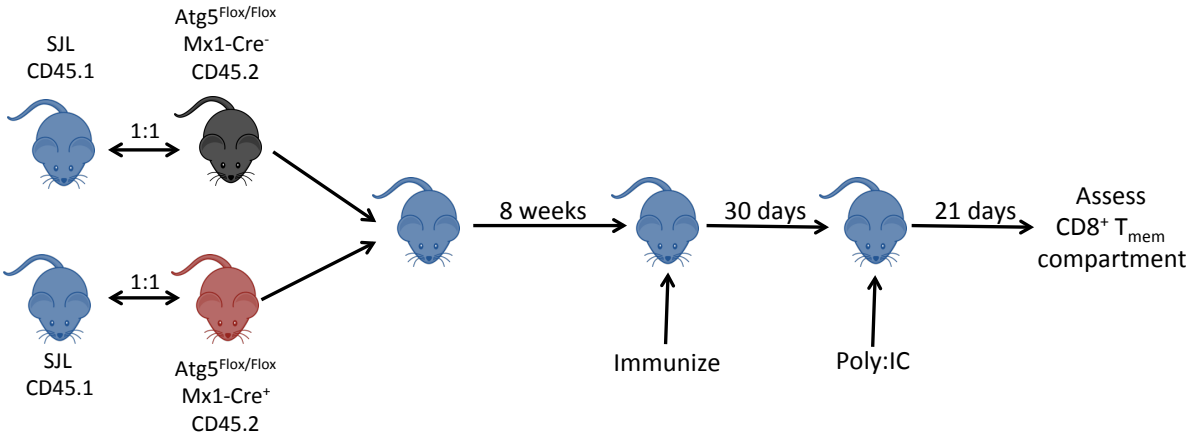


Figure 4.30 *Atg5*$^{Flox/Flox}$ *Mx1-Cre bone marrow chimera set up. Bone marrow from either Atg5*$^{Flox/Flox}$ *Mx1-Cre- or Mx1-Cre⁺ was transplanted 1:1 with wildtype SJL CD45.1 mice into SJL CD45.1 hosts. 8 weeks after transplantation mice were immunized with 0.0005 PR8 influenza. At day 30, 32, and 34 post-infection Poly:IC was administered i.p. 21 days after the last Poly:IC injection, the frequency of NP-specific CD8⁺ T cells was measured in the lungs (day 55 post-infection). PBS was administered as a control.*

In naïve bone marrow chimera mice, Poly:IC treatment resulted in a significant decrease in the frequency of *Atg5*$^{Flox/Flox}$ *Mx1-Cre*⁺ donor-

derived CD4⁺ and CD8⁺ T cells found in the spleen. No such decrease was observed in Mx1-Cre⁻ donor cells or *Mx1-Cre*⁺ mice treated with PBS (Figure 4.31a). This phenotype is similar to that of Vav-Atg5$^{-/-}$ and T-Atg7$^{-/-}$ mice, suggesting *Atg5* is efficiently excised following Poly:IC treatment. Poly:IC administration did not affect the frequency of Mx1-Cre⁺ CD19⁺ cells (Figure 4.31a). One major limitation of a Poly:IC-inducible system to study CD8⁺ T_{mem} is the risk of premature excision caused by the natural IFN response to virus infection. In T-Atg7$^{-/-}$ mice, where *Atg7* is excised prior to T cell activation, a significant decrease of CD8⁺ T cells is observed in the lungs of both naïve and influenza infected mice. In PBS-treated *Mx-1 Cre*⁺ chimera mice, influenza infection did not affect the frequency of donor derived polyclonal CD8⁺ T cells in the lungs. This is in contrast to Poly:IC treated mice, where a 30-40% decrease in donor-derived Mx1-Cre⁺ polyclonal CD8⁺ T cells was observed (Figure 4.31b). This suggests that influenza infection alone does not result in Atg5-excision in the lungs. Identical results were obtained in the spleen of influenza-infected *Mx1-Cre*⁺ chimera mice treated with PBS and Poly:IC (data not shown).

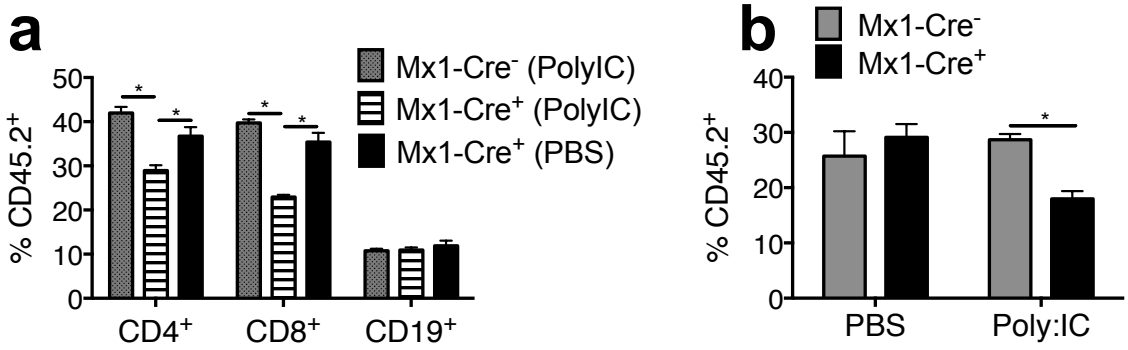


Figure 4.31 *Atg5 excision by Poly:IC leads to a decrease in the frequency of Mx1-Cre⁺ donor T cells.* ***a)*** *Bone marrow chimera mice were set up as described in* ***Figure 4.30****. 111 days after reconstitution and 21 days after Poly:IC treatment, the frequency of certain donor*

populations was assessed in the spleen by flow cytometry. p<0.05 (n=4-6). Quantified is the frequency of CD4⁺, CD8⁺, CD19⁺ cells that are donor derived (CD45.2⁺)* ***b)*** *Bone marrow chimera mice were set up as described in Figure 4.30. 3 weeks after Poly:IC treatment in influenza-immunized mice, the frequency of donor CD8⁺ T cells was measured in the lungs by flow cytometry. Quantified is the frequency of CD8⁺ T cells that are donor-derived (CD45.2⁺), p*=0.0286 (n=4-6).*

When the CD8⁺ T_{mem} compartment was measured on day 55 post-infection in the lungs, *Atg5*-excision by Poly:IC treatment during the memory phase had no effect on the frequency of NP-specific CD8⁺ T_{mem} cells (Figure 4.32). This suggests that autophagy may be dispensable for maintenance of the CD8⁺ T_{mem} pool once formed, instead being an essential requirement during initial CD8⁺ T_{mem} differentiation functioning to regulate ROS and the switch to oxidative phosphorylation and FAO.

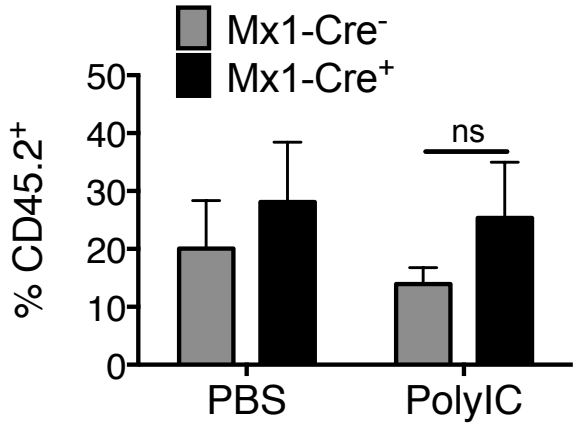


Figure 4.32 *Atg5-excision during the memory phase does not affect the frequency of CD8⁺ T_{mem} cells. Bone marrow chimera mice were set up as described in* ***Figure 4.30****. Mice were immunised with 0.0005 HAU PR8 influenza and on days 30, 32, 34 post-infection mice were administered Poly:IC by i.p injection. 21 days after Poly:IC treatment (day 55 post-infection), the frequency of NP-specific CD8⁺T_{mem} cells that were donor derived was quantified by flow cytometry using tetramers.*

Chapter 4: Discussion

Autophagy is a critical regulator of memory CD8⁺ T cell formation

Discussion

A role for autophagy in naïve CD8⁺ T cell homeostasis has been clearly demonstrated in numerous previous studies [9] [145,146]. Here, for the first time a requirement for autophagy in antigen-specific CD8⁺ T cells is shown. Whilst the CD8⁺ T cell effector phase proceeds in typical fashion with clonal expansion observed at similar levels to that of wildtype cells, *Atg7*$^{-/-}$ CD8⁺ T cells fail to establish a population of memory CD8⁺ T cells proceeding viral infection. When CD8⁺ T cell kinetics were tracked to influenza and MCMV infections in T-Atg7$^{-/-}$ mice, the CD8⁺ T_{eff} population underwent a catastrophic collapse during memory transition, prohibiting CD8⁺ T_{mem} formation. These observations were corroborated by work from Rafi Ahmed's lab, published simultaneously with this author, that also show a lack of CD8⁺ T_{mem} cells in the absence of *Atg7* and also *Atg5* [381].

4.31. Autophagy is dispensable for CD8⁺ T_{eff} responses

The finding that CD8⁺ T cell expansion was normal in T-Atg7$^{-/-}$ mice was initially unexpected as previous *in vitro* work had shown that deletion of autophagy-related genes resulted in compromised T cell proliferation following stimulation through the TCR [146]. The discrepancy between these two observations almost certainly lies in the vastly different condition between *in vitro* stimulation and *in vivo* priming. Whilst autophagy-deficient T cell proliferation is affected in the relatively simplistic scenario of stimulation via anti-TCR and anti-CD28 antibodies, when cells have access to the full compliment of accessory molecules and growth factors *in vivo*, not to mention the presence of antigen, clonal expansion may proceed normally

without autophagy. Signalling strength may thus be an important determinant in the differences between *in vitro* and *in vivo* studies. However, in experiments on the detailed kinetics of autophagy induction on TCR transgenic T cells performed by Rafi Ahmed's group, autophagy was inversely correlated with cell proliferation and levels reached their maximum at the peak of the effector phase [381]. Similar results were obtained in this study where CytoID levels (a marker of autophagosomes) were also highest at the peak of the effector response following influenza infection. Due to the physiological infection models utilised here, it was not possible to perform this analysis on early effector cells undergoing proliferation as typically they are not detectable by tetramer staining. Both studies support the idea that autophagy is not required for initial $CD8^+$ T cell activation and expansion.

In around 50% of the experimental repeats analysing the influenza and MCMV effector phase, the peak of the T-$Atg7^{-/-}$ response was actually of greater intensity than that of wildtype mice. In young wildtype mice, infection will typically lead to the priming of $CD44^{lo}$ naïve $CD8^+$ T cells. However, immunization of T-$Atg7^{-/-}$ mice presents an interesting proposition in that the high frequency of $CD44^{hi}$ memory phenotype (MP) cells means that $CD8^+$ T cell priming may occur from the $CD44^{hi}$ pool. What effects might this have on the *Atg7*$^{-/-}$ $CD8^+$ T_{eff} phase? In previous studies, when MP $CD8^+$ T cells were taken from naive lymphopaenic mice and stimulated, these cells had many of the hallmarks of true $CD8^+$ T_{mem} cells [191] [195]. These cells are not true antigen-experienced cells, rather naïve cells masquerading as memory cells. MP $CD8^+$ T cells could respond to lower doses of antigen, lyse target cells directly *in vitro* and *in vivo* and produce IFN-γ faster than naïve cells

[191]. This phenomenon might explain the instances of heightened effector phase in T-Atg7$^{-/-}$ mice. In those cases, if priming occurred on clones within the MP pool, these cells would react to this primary response in a manner reminiscent of a recall response, leading to a greater CD8$^+$ T_{eff} response in terms of frequency. A greater concentration of growth factors, such as IL-2 and IL-7 which support T cell proliferation, is also likely to be apparent in lymphopaenic mice and this might facilitate increased clonal expansion.

A limitation of this study was that CD8$^+$ T_{eff} function was not assessed in detail. T-Atg7$^{-/-}$ mice clear influenza virus less efficiently than wildtype controls, perhaps suggesting a lower killing capacity. However, the significantly lower number of antigen-specific CD8$^+$ T cells in these mice is the likely reason for this. While MCMV is a chronic infection, all T-Atg7$^{-/-}$ mice survive influenza infection making it probable that virus is eventually completely cleared from the lungs. *Atg7$^{-/-}$* CD8$^+$ T cells synthesise normal quantities of IFN-γ and TNF-α *in vitro* when stimulated with PMA and ionomycin (data not shown), but whether this is true for *Atg7$^{-/-}$* antigen-specific CD8$^+$ T cells stimulated with cognate peptide is unknown. It is also not known if autophagy has any role in CD8$^+$ T_{eff} degranulation. However, published studies have identified a role for autophagy in effective mast cell degranulation [382] [383], making this a potentially interesting area for future investigation.

4.32. Autophagy regulates memory CD8$^+$ T cell formation

While autophagy was not important for the CD8$^+$ T cell response during the effector phase, without it CD8$^+$ T cells failed to differentiate into long-lived

$CD8^+$ T_{mem} cells. When the kinetics to both influenza and MCMV were tracked by tetramer, the population of antigen-specific $CD8^+$ T cells collapsed around the time $CD8^+$ T_{eff} cells are normally undergoing conversion to $CD8^+$ T_{mem} cells. A number of complicating issues arise when using T-$Atg7^{-/-}$ mice to investigate $CD8^+$ T cell responses, including the lymphopaenic T cell compartment and the fact that *Atg7* is also excised in *CD4*-expressing APCs. Using immunized bone marrow chimeras, it was demonstrated that the failure to form the $CD8^+$ T_{mem} pool is cell intrinsic and not due to defective priming by $Atg7^{-/-}$ APCs, nor due to altered $CD8^+$ T cell differentiation in a lymphopaenic environment. This also rules out aberrant interactions with $Atg7^{-/-}$ $CD4^+$ T cells, which are typically essential for the maintenance of functional memory and recall responses, rather than a determinant of entry into the memory pool [384] [338] [336].

Bone marrow chimera experiments were also utilised to show a normal distribution of short-lived effectors (SLECs) and memory precursors (MPECs) amongst $Atg7^{-/-}$ $CD8^+$ T_{eff} cells, ruling out defective MPEC differentiation in explaining altered memory formation. The paradigm of MPECs providing the basis for the future memory pool is heavily centred on re-expression of the IL-7Rα subunit after it is initially downregulated following activation [286] [283] [285]. Levels of IL-7Rα are found normally expressed on $Atg7^{-/-}$ $CD8^+$ T_{eff} cells suggesting they are responsive to IL-7 – an important factor in memory development. While in terms of phenotype and frequency MPECs appear normal, it is not possible to rule out that they are somehow flawed functionally in the absence of autophagy. One paradigm of MPEC formation

suggests a model where activated CD8⁺ T cells undergo asymmetric cell division to produce daughter cells that are either SLEC or MPEC in nature [296]. Autophagy might be important for the partition of molecules between daughter cells, ensuring MPEC fate through the delivery of factors required to survive contraction and drive memory development.

The expression of receptors like IL-7R, important for memory formation and maintenance, are ultimately under the control of transcription factors that act as master regulators of CD8⁺ T cell differentiation. In this study, the expression of two such transcription factors was assessed over time during the CD8⁺ T cell response to MCMV. *Atg7*$^{-/-}$ CD8⁺ T cells exhibited normal expression of IRF4, responsible for sustaining the expansion and differentiation of CD8⁺ T_{eff} cells [377], and EOMES, a factor that promotes CD8⁺ T_{mem} formation [291]. One function of EOMES is to regulate IL-15R expression, also important for memory differentiation and maintenance [291,330] [329]. Indeed, *Atg7*$^{-/-}$ CD8⁺ T cells express levels of IL-15Rα and IL-15Rβ comparable to that of wildtype cells; supporting the concept that autophagy is not required to respond effectively to extrinsic signals important for memory formation. At the level of the gene, loss of autophagy does not seem to lead to defective transcriptional regulation of CD8⁺ T_{mem} differentiation, although this study was limited to observations on two genes only. Other factors such as T-bet and FOXO1 are also important for CD8⁺ T_{mem} formation and it is a drawback of this study that their expression was not analysed [385] [284].

In naïve T-Atg7$^{-/-}$ mice, the CD8⁺ T cell compartment shows signs of exhaustion that is driven by lymphopaenia-induced proliferation. In CD8⁺ T

cells responding to chronic virus infection, cells that become exhausted are eventually deleted from the T cell pool. It was hypothesised that exhaustion of *Atg7*$^{-/-}$ CD8^{+} T_{eff} cells might result in failure to differentiate into CD8^{+} T_{mem} cells. MCMV-specific CD8^{+} T cells from T-Atg7$^{-/-}$ mice did express markers of exhaustion as early as day 10 of infection, but when these cells were analysed in a mixed bone marrow chimera setting, markers of exhaustion were similar to that of wildtype cells ruling this out as a plausible explanation.

4.33. Dysregulated mitochondrial homeostasis in *Atg7*$^{-/-}$ CD8^{+} T_{eff} cells

Previous studies on autophagy and T cells, including data presented in this thesis, show that autophagy is important in naïve CD8^{+} T cell mitochondrial homeostasis [53]. Up to now, it was not known how autophagy regulated mitochondria in antigen-specific CD8^{+} T cells. Like in naïve cells, absence of autophagy resulted in significantly elevated mitochondrial mass in antigen-specific CD8^{+} T cells. This was observed with a concomitant increase in mitochondria-derived superoxide production. Mitochondria are known to be dynamically regulated during CD8^{+} T cell differentiation that begins early after activation when cells up-regulate mitochondrial mass [386] [387] [388]. This increase in mitochondrial volume is required to provide mitochondria for daughter cells and an initial surge of energy in preparation for a strong burst of proliferation [386]. During the effector phase, a further round of mitochondrial biogenesis takes place in memory precursor cells, this time driven by IL-15 as these cells prepare to switch from glycolysis to mitochondrial-driven OXPHOS [368,369]. Thus, memory precursor cells boast a relatively high mitochondrial load in contrast to short-lived effectors and

naïve cells. One might expect that in *Atg7*$^{-/-}$ CD8$^+$ T cells the mitochondrial pool is of lower quality due to a failure to remove damaged mitochondria through mitophagy. When the effector phase peaks and cells relax the use of glycolysis and begin to utilise OXPHOS once more, an increased burden is now placed on these organelles. The kick-starting of OXPHOS would lead to a flood of electrons being shed from the electron transport chain that can interact with molecular oxygen, resulting in superoxide production [389]. In autophagy-deficient CD8$^+$ T_{eff} cells, the increased volume of mitochondrial membranes would naturally equate to increased superoxide production, which is exactly what is observed in *Atg7*$^{-/-}$ antigen-specific CD8$^+$ T cells. While ROS production is a normal occurrence within T cells and are indeed required for efficient activation [390] [176,391,392], T cells are abnormally sensitive to aberrant ROS production due their inability to synthesise and take up glutathione precursors that can scavenge these deleterious intermediates [393]. Therefore, it is highly possible that the increased superoxide production during the *Atg7*$^{-/-}$ CD8$^+$ T_{eff} phase drives the observed increase in apoptosis and collapse of the CD8$^+$ T_{eff} population. This notion is partly supported by published results that report T cell apoptosis in the presence of excess ROS [176,392] [350] [351]. Crucially, when treated with the antioxidant N-acetyl cysteine (NAC), the capacity of T-Atg7$^{-/-}$ mice to form a CD8$^+$ T_{mem} compartment was restored, strongly supporting a role for autophagy in the prevention of superfluous ROS levels through regulation of mitochondria. Indeed, a role for autophagy as an antioxidant system in human T cells has been described [394]. These results also depict a role for NAC in preventing ROS-induced T cell death. A similar phenomenon has been observed in eosinophils where

NAC treatment was able to protect against apoptosis following sodium arsenite-induced ROS production [395]. Similarly, treatment of *in vivo* primed T cells with a superoxide dismutase analogue, able to degrade ROS, was able to prolong T cell viability in culture [176]. This implies that ROS levels are exquisitely fine-tuned in T cells and mechanisms must be in place to prevent excess production by mitochondria during the turbulent time of effector expansion and memory formation when large energetic demands are prevalent.

4.34. *Atg7*$^{-/-}$ CD8^{+} T_{eff} cells exhibit an altered metabolic phenotype

Recently published work from our lab has shown how glycolysis is upregulated in myeloid cells in the absence of autophagy (Stranks *et al*, In Press). This appears to be a general trend as similar unpublished observations also show this to be true in HSCs and various downstream progenitors. While glycolysis is required during the CD8^{+} T cell effector phase, entry to the memory pool requires a switch to OXPHOS, particularly FAO [370,371]. Autophagy has been demonstrated to regulate lipid metabolism in other cell types [43] and therefore it was hypothesised that autophagy is required to mediate the switch to FAO during memory differentiation. Backing these claims, GLUT-1, required for glucose uptake and a surrogate marker of glycolysis, was expressed on a significantly higher proportion of *Atg7*$^{-/-}$ CD8^{+} T_{eff} cells at the peak of the effector phase compared to wildtype controls. While wildtype CD8^{+} T_{eff} cells downregulated GLUT-1 toward the end of the effector phase as cells switched to OXPHOS, GLUT-1 remained stably expressed on autophagy-deficient cells. In addition, glucose uptake was also

significantly greater in *Atg7*-deficient cells during the early and late effector phase. Thus, like in other haematopoietic cell subsets, absence of autophagy in T cells results in an enhanced glycolytic profile. This observation may also explain why autophagy is dispensable for effector expansion, which relies on glycolysis to support cell proliferation. The intracellular lipid content of early and late *Atg7*$^{-/-}$ CD8^{+} T_{eff} cells was also different to that of wildtype cells, with elevated levels observed at day 10 and day 18. This adds weight to the idea that autophagy is required to break down lipids to provide fatty acids (FAs) for FAO. Experiments from Xu *et al* add support to this, using a liquid chromatography-coupled mass spectrometry metabolics platform; they observed alterations in metabolites important for FAO in *Atg7*$^{-/-}$ CD8^{+} T_{eff} cells. Interestingly, initial observations that FAO was required for CD8^{+} T_{mem} formation came in mice with *TRAF6*-deficient T cells that were unable to perform FAO and form CD8^{+} T_{mem} cells [370]. In subsequent publications, TRAF6 was found to regulate autophagy initiation in a variety of cells including immune subsets [396] [60], further linking autophagy to FA metabolism. Incorporating these observations, a model can be envisioned where *Atg7*$^{-/-}$ CD8^{+} T cells undergoing differentiation to CD8^{+} T_{mem} cells are unable to utilise the oxidation of FAs for energy due to a failure to mobilise FA metabolites from larger lipid stores (Figure 4.33). Even in the presence of FAs, the presence of damaged mitochondrial with impaired inner membranes cannot support efficient OXPHOS to the same degree as wildtype cells. This leaves glycolysis as the only effective way to generate energy in the cell. While this is fine during the effector phase, during the transition to memory

the lack of metabolic flexibility causes bioenergetic instability resulting in cell crisis, death, and a collapse of the late CD8⁺ T_{eff} pool.

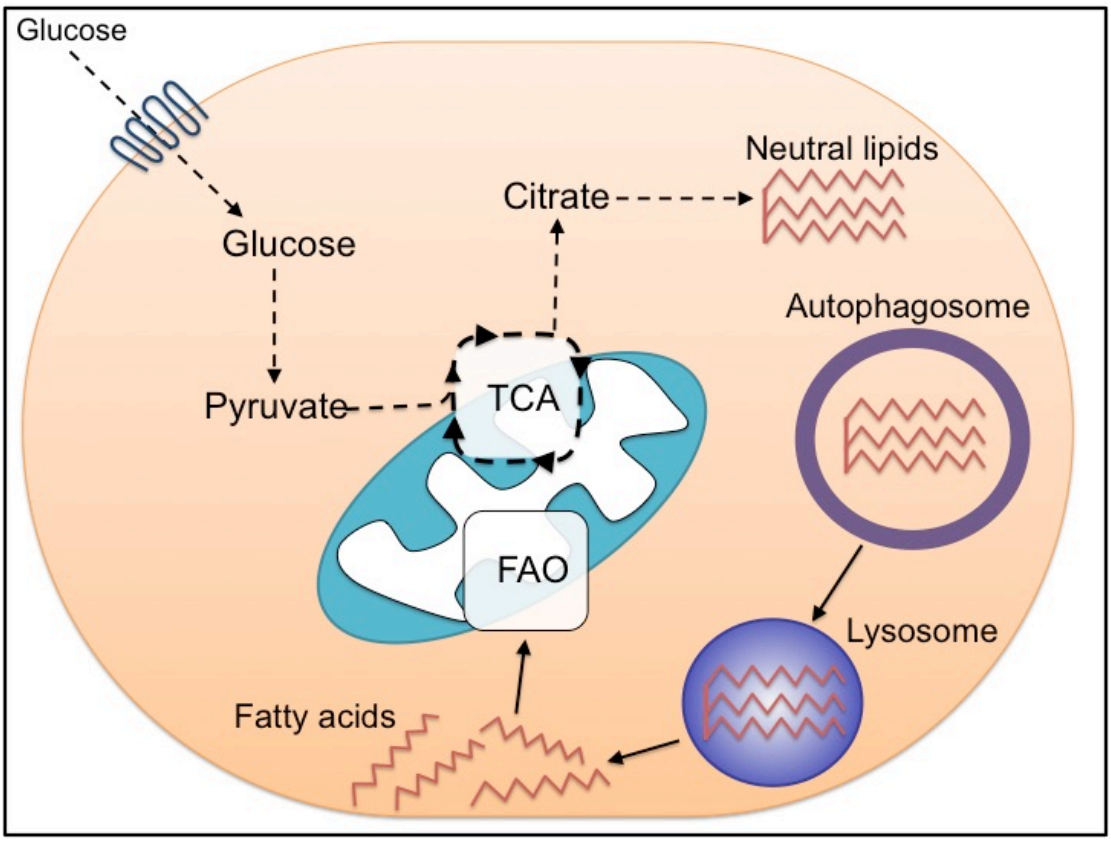


Figure 4.33 *Autophagy and fatty acid metabolism are important for CD8⁺ T_{mem} formation. During contraction, memory precursor cells must switch to the oxidation of fats to maintain viability. Extracellular glucose is used to fuel lipid synthesis from tricarboxylic acid cycle (TCA) precursors. In this model, autophagy is then required to sequester neutral lipid stores in autophagosomes in order to deliver them to the lysosome for degradation. The resulting free fatty acids can then enter the mitochondria for fatty acid oxidation (FAO) and energy production.*

This also leads to the important of question of precisely when autophagy is active and required during CD8⁺ T cell differentiation. Is autophagy required for entry into the memory pool or is it required for the maintenance of the memory compartment once formed? Evidence put forward here provides support for the former scenario. During the CD8⁺ T cell response to influenza, autophagy was highest at the peak of the effector phase, just as cells begin to die during contraction. This was confirmed by the work Xu *et al* that

similarly showed maximum autophagic activity during the peak effector phase [381]. Interestingly, when *Atg5* was knocked out in the already-formed memory compartment to influenza, the frequency of $CD8^+$ T_{mem} cells remained stable. However, there were limitations to the approach used here as Poly:IC treatment may not effectively excise NP-specific $CD8^+$ T_{mem} cells resident in the lung. However, other T cells were significantly reduced in the lung following PolyI:C administration, but it cannot be certain that these cells were not excised in the periphery before migrating into the lung. It is important to note that it is not the case that loss of *Atg5* does not affect $CD8^+$ T_{mem} formation as the phenotype of $Atg5^{-/-}$ and $Atg7^{-/-}$ T cells are largely interchangeable [146], and the recent work from Rafi Ahmed's group have shown an essential requirement for *Atg5* in memory $CD8^+$ T cell formation [381]. Thus, it appears that autophagy is required for the entry of cells into the memory compartment, rather than regulation of the memory pool once formed. This is in line with the paradigm where autophagy is required to provide substrates for FAO during the effector phase in preparation for $CD8^+$ T_{mem} formation, a time when autophagy is most active. Interestingly, inhibition of mTOR with the autophagy-inducing drug rapamycin during the effector phase has been show to improve memory responses in both mice and primates [397]. With mTOR acting as a major negative regulator of autophagy, in hindsight this effect might have been mediated through autophagy induction. Repeating these experiments in T-$Atg7^{-/-}$ mice would go some way to proving this link.

4.35. Secondary CD8⁺ T_{eff} responses in the absence of *Atg7*

Initially, it was unclear whether T-Atg7$^{-/-}$ mice would survive a second immunization or be able to mount a recall response given the lack of antigen-specific CD8⁺ T cells to the first infection. To investigate this, T-Atg7$^{-/-}$ mice were primed and then challenged with heterologous influenza strains, a type of immunity that relies heavily on T cells as antibodies are not cross-reactive between strains [398]. However, T-Atg7$^{-/-}$ mice could mount a secondary CD8⁺ T_{eff} response, although this was severely reduced in intensity compared to wildtype mice. Surprisingly, T-Atg7$^{-/-}$ mice survived this second challenge, suggesting the small number of number of recalled T cells are enough to provide protection. This raises interesting questions as to why secondary infections induce such vigorous T cell responses when protection can be achieved with a fraction of what is observed. It is unlikely that this small recall response in knockout mice is a new primary response, brought about through the priming of fresh clones released from the thymus between infections, as presence of antigen-specific cells a mere 5 days after challenge is typical of memory kinetics. Therefore, a small population of CD8⁺ T_{mem} cells must persist in T-Atg7$^{-/-}$ mice that are below the detection limit of the tetramer, and these cells must exhibit normal memory function and proliferative capacity. Why these cells survive is not known although they might be cells that have escaped *Atg7* excision or have managed to adjust to prolonged glycolytic metabolism.

A shift in epitope dominance away from the usually dominant NP epitope ($NP_{366-374}$) detected by the tetramer might also explain the blunted recall

responses of *Atg7*$^{-/-}$ cells. This again is unlikely however, as during secondary influenza infections, $NP_{366-374}$-specific $CD8^+$ T cells in fact become 'over-dominant', accounting for up to 80% of all influenza-specific $CD8^+$ T cells [399] [400].

$CD4^+$ T cell help is essential for proper recall responses to secondary infection [336]. To investigate if aberrant $CD4^+$ T cell help was at play in the poor recall responses observed in T-Atg7$^{-/-}$ mice, secondary responses were assessed in bone marrow chimera mice, placing *Atg7*$^{-/-}$ antigen-specific cells alongside wildtype $CD4^+$ T cells. In bone marrow chimera mice, *Atg7*$^{-/-}$ $CD8^+$ T cells could mount a robust response to a second influenza challenge and exhibited secondary $CD8^+$ T_{eff} expansion similar to that of wildtype donor cells. These data imply that sub-standard recall responses in T-Atg7$^{-/-}$ mice might be due to defective $CD4^+$ T cell help, implying a problem for *Atg7*$^{-/-}$ $CD4^+$ T cells in providing the necessary signals during initial $CD8^+$ T cell priming.

This view is somewhat complicated by the observation that in T-Atg7$^{-/-}$ mice $CD8^+$ T cells can mount a recall response to inflationary epitopes (but not classical) during secondary MCMV infection. Also of interest is the finding that in wildtype mice, secondary MCMV infection does not induce a $CD8^+$ T cell recall response. With one inflationary epitope alone making up as much as 15% of the whole $CD8^+$ T cell pool at the time of re-infection, it is likely that wildtype mice already contain enough T cells to clear virus efficiently without the need for renewed expansion. To this author's knowledge, this is the first report of the analysis of secondary $CD8^+$ T cell responses to MCMV. In T-Atg7$^{-/-}$ mice, these large MCMV-specific populations do not exist, hence the

requirement to undergo a second effector response. It is possible that secondary responses to MCMV are $CD4^+$ T cell-independent, especially to non-typical inflationary epitopes. This might explain why the secondary *Atg7*$^{-/-}$ $CD8^+$ T_{eff} response is directed at an inflationary epitope and not a classical one, as might be expected.

As previously mentioned, T-Atg7$^{-/-}$ mice unexpectedly survived a second influenza infection. Not only that, but outward clinical signs in the form of reduced weight loss suggests that the effects of infection were less severe in these mice. In this instance, the low-level recall responses might have the beneficial advantage of reducing disease severity by lowering T cell-mediated immunopathology. The decreased number of infiltrating secondary $CD8^+$ T_{eff} cells into the lungs would likely lead to reduced collateral tissue damage and ultimately less weight loss. Indeed, we have shown (presented in the next chapter) that weight loss following influenza infection in vaccinated mice is correlated to the strength of the NP-specific T cell response. In this study, lung sections were not taken for pathology needed to confirm the link between weight loss and T cell-driven immunopathology. However, in a TCR transgenic mouse model, devoid of antibodies, influenza-specific $CD8^+$ T cells have been shown to exacerbate lethal influenza pneumonia [401]. $CD8^+$ T cell-mediated immunopathology has also been highlighted in other settings. With LCMV, intracranial infection of B6 mice results in lethal CNS disease mediated by $CD8^+$ T cells [402]. Similarly, intracranial infection of perforin-deficient mice with Theiler's virus results in death, where wildtype mice survive, implicating $CD8^+$ T cells in disease severity [403].

4.36. Conclusions

In this chapter, a novel role for autophagy in antigen-specific CD8⁺ T cells has been described. While dispensable for efficient expansion of CD8⁺ T_{eff} cells, mice bearing autophagy-deficient T cells (T-Atg7$^{-/-}$) display a distinct absence of CD8⁺ T_{mem} cells in the latter stages following infection and vaccination. This defect was found to be cell intrinsic in nature and closer analysis revealed dysregulated mitochondrial homeostasis in *Atg7*$^{-/-}$ antigen-specific CD8⁺ T cells that was accompanied by increased levels of mitochondrial ROS. When these ROS were scavenged following *in vivo* administration of the antioxidant NAC, the ability of T-Atg7$^{-/-}$ mice to form CD8⁺ T_{mem} cells was restored. This implies autophagy acts as a key antioxidant pathway in antigen-specific CD8⁺ T cells through regulation of mitochondrial number and integrity, preventing excessive ROS accumulation. Autophagy-deficient CD8⁺ T_{eff} cells also display an altered metabolic profile that shows signs of prolonged glycolysis when CD8⁺ T_{eff} cells are normally switching to OXPHOS metabolism in order to enter the memory pool. Moreover, the intracellular lipid content of *Atg7*$^{-/-}$ CD8⁺ T_{eff} cells is significantly increased while fatty acid uptake is also altered, providing support for the hypothesis that autophagy is important to mobilise fatty acids from intracellular lipid stores, an important step for memory differentiation. These findings highlight autophagy as a novel pathway important for CD8⁺ T_{mem} formation and offer the prospect of CD8⁺ T cell modulation through autophagy.

Chapter 5: Introduction

Rejuvenation of aged CD8⁺ T cell responses through autophagy

5. Introduction

5.1. Autophagy and ageing

The ubiquitous nature of autophagy in eukaryotic cells has led to a vast array of evolved functions, from tissue remodelling [404] during morphogenesis to the removal of intracellular bacteria [72] [405]. However, autophagy is perhaps best known for its ability to clear unwanted cytosolic components. The characteristic accumulation of unwanted or damaged proteins and organelles during ageing have led many suggest autophagy as a key anti-ageing mechanism. These deposits of altered components are particularly detrimental in post-mitotic cells such as neurons and cardiomyocytes where cytoplasmic debris cannot be diluted to daughter cells [406]. In this context, the observed decrease in autophagy levels is thought to contribute to the ageing process through failure to maintain a healthy cytoplasm [407] [408]. While natural autophagy levels have been shown to fall in aged lower organisms [409] [408] [410] [411], their ability to induce this pathway in response to stress also becomes defective [412]. It is unclear what drives this decline, although evidence exists that altered insulin growth factor (IGF) receptor signalling and the accumulation of undigested material in lysosomes (lipofuscin) that prevent fusion with autophagosomes lies at the heart of these observations [406]. Autophagy is strongly induced in the absence of IGF signalling and a loss of function mutation in the downstream insulin-like tyrosine kinase *daf*-2 extends life-span in *C. elegans* two-fold [413]. Other mutations in the insulin signalling pathway have been shown to extend lifespan in flies and mice [414] [415], hinting at an anti-ageing role for autophagy across taxa. Along these lines,

elimination or depletion of the negative autophagy regulator Tor also extends longevity [416]. The first direct evidence linking autophagy to ageing came from the observation that RNAi inactivation of *bec-1* (nematode ortholog of mammalian *beclin-1*) cancelled the life-span extending phenotype of *daf-2* mutants [413]. Subsequent studies in *Drosophila* confirmed a critical role for autophagy in longevity, based on the observation that *Atg7*-deficient flies are short-lived [417], whereas promoting autophagy levels enhanced longevity [418]. Similarly, spermidine, a naturally occurring polyamine, also extends life-span in mice by inducing autophagy [419]. Furthermore, calorie restriction, the most steadfast way of improving life-span across species [420] [409] [421] [422] [423] [424] is also thought to work by evoking autophagy through lowered IGFR signalling or preventing the age-related decline in autophagic activity [425] [426]. Tellingly, the pro-longevity effects of calorie restriction were abolished when autophagy genes were knocked out or knocked down [427]. Therefore, boosting autophagy appears to be strongly beneficial for extending life-span. How autophagy achieves this is uncertain, but it may well be brought about by increased protection against oxidative damage, perhaps through the removal of damaged mitochondria [428], as well as mechanisms involved in the repair and replacement of damaged DNA, proteins, and lipids [429]. However, whilst autophagy is strongly linked to aged related phenomena in tissues such as the liver [430] and the brain [431], what involvement it has during the large scale changes that occur during immune ageing, termed immunosenescence, is largely unknown.

5.2. Innate Immunosenescence

Virtually all tissues are affected by the passage of time and the immune system is no exception. Wholesale changes occur throughout the innate and specific arms of immunity culminating in a system that can no longer respond optimally to infection. This unfortunate decline comes at a significant detriment to the organism at large, leaving the individual at increased risk of both infection and malignancy. In humans, the incidence and severity of infectious diseases such as pneumonia [432], meningitis [433], sepsis [434] or influenza [435] all increase with age. Aged animals, like aged humans, are also more susceptible to infection [436] [437] [438] [439]. Significantly, this waning of immune function eventually limits preventative prophylaxis and vaccine immune responses are reduced in elderly adults [440]. In recent years, it has been possible to construct a clear picture of the changes that occur during immunosenescence. Typically, these changes are most substantial in the adaptive arm; however, the innate immune system does not escape unscathed.

Neutrophils are key players in early innate immune responses and are activated by compounds that bind to PRRs. However, in elderly humans neutrophils show impaired activation to PRR ligands [441] and altered signal transduction [442] [443]. This is in contrast to neutrophils isolated from aged mice, which have been found to be unchanged in respiratory burst, exocytosis, chemotaxis and phagocytic activity compared with young mice [444]. Similar to neutrophils, macrophages also have defects in immune signalling cascades with the TLR pathway in particular showing reduced efficiency in in old age [445] together with lower TLR expression (TLR1-9) [446] [447]. Perhaps as a

consequence of this, LPS-induced production of TNF-α, IL-1β, and IL-6 by macrophages isolated from both the spleen and peritoneum of aged mice is suppressed compared to macrophages from young mice [445] [448] [446] [449]. However, production of these same mediators after *in vivo* exposure to various bacterial products is elevated in aged versus young mice [450] [451]. While these factors can be produced by an array of other host cells, it hints at an important role in the local milieu and the connected cast of surrounding cells in influencing macrophage immunosenescence.

In dendritic cells, reports suggest that DCs derived from elderly mice have an intact ability to prime adaptive responses [452], whereas others show a decreased capacity in antigen capture and presentation [453] [454] and T cell priming [455] [456]. Co-receptor expression is also decreased on aged DCs [454], as is reduced surface HLA-DR expression [457]. Impaired type I IFN responses have been observed following TLR7 and TLR9 activation by CpG-A or herpes simplex virus-2 (HSV-2) infection [458,459]. Like with macrophages, aged DCs demonstrate lower production of TNF-α, IL-6 and IL-12 upon stimulation, suggesting an altered cytokine profile. This common *in vitro* observation of decreased cytokine production in innate immune subsets is difficult to couple with extensive *in vivo* studies in mice and humans that show elevated serum levels of inflammatory cytokines such as IL-6 and TNF-α in old age [460] [461] [462] that has been coined 'inflam-ageing'. Levels of these cytokines in the elderly are predictive of mortality risk and are thought to mediate chronic low-level inflammation that is a contributing factor to various inflammatory conditions and morbidity [463] [462].

5.3. Adaptive immunosenescence

Innate immunity functions immediately after birth and manifests relatively little change through life. In contrast, adaptive immunity is immature at birth, peaks during the transition to early adulthood, and progressively declines thereafter. It is the substantial changes in the adaptive arm that most significantly contribute to waning immunity during ageing.

B cells

In terms of subsets, a gradual decrease in naïve B cells coupled to an increase in effector B cells is observed with ageing, which is thought to result in reduced diversity of antibody responses [464]. In addition, generation of high affinity antibodies in the elderly is impaired due to defects in somatic hypermutation and isotype switching [465]. One dramatic change is the oligoclonal expansion of $CD5^+$ B cells producing low affinity auto-antibodies, albeit with no known pathophysiological consequences [466]. However, these cells can go onto occupy niches that can then no longer be filled by more 'useful' B cell subsets with the ability to produce high affinity antibody [467] [468]. Furthermore, it has been demonstrated that the bone marrow of aged mice has diminished ability to support B cell lymphopoiesis and survival of antibody-producing plasma cells [469] [470]. Perhaps as a result of this altered bone marrow environment, pre-B cells are markedly decreased with age due to a block between pro- and pre-B cell developmental stages. In this respect, reduced responsiveness of developing B cells to IL-7 [471] [472] and decreased V-DJ recombination of immunoglobulin genes [473] are also thought to deleteriously affect B cell development during ageing. Together, these

changes profoundly affect B cell function and can lead to significantly impaired humoral immunity. Indeed, antibody responses decline to both infection and vaccination in elderly adults [474] [475,476] [477]. In the case of influenza, generally elderly antibody responses are reduced for both IgG and IgA with a delayed peak in antibody titres [476] [478] [479]. Antibody titres against seasonal H1N1 influenza have also been reported to be significantly lower in old age [480], with a published meta-analysis suggesting antibody vaccine responses reduced 25% and 50% to H1 and H3 antigens, respectively [475]. In randomised, placebo-controlled clinical trial of healthy young adults, the influenza vaccine was 70-90% effective in preventing serologically confirmed influenza illness [481] [482]. In the elderly, this figure was as low as 17% [475].

T cells

Of all age-associated changes in the immune system, regression of the thymus is arguably the most dramatic, ubiquitous and recognisable. With increasing age, there is a decrease in thymic epithelial space and thymic cellularity, collectively called thymic involution. In mice, loss of epithelial space is caused by a gross reduction in thymus size [483] [484], whereas in the human thymus there is an increase in perivascular space which is progressively replaced with fat during ageing [485] [486]. In the mouse, a decline in thymus cellularity is observed at 6 weeks of age [487]. In humans, thymic regression begins as early as 9 months of age [488] and then undergoes gradual involution throughout life [485]. By the time of middle age, most parenchymal tissue is replaced by fat, although islets of intact thymic cortex and medulla persist in humans at least until the sixth decade of life and are the site of on-going TCR

rearrangement [489] [490] [491] [492] [490]. Thymic involution is likely to be a multifactorial event, but evidence exists that changes in the aged bone marrow niche contribute. One report has proposed a decline in the ability of aged bone marrow to reconstitute T cell populations in lethally irradiated hosts [493]. Furthermore, purified HSCs from old mice exhibit decreased differentiation potential toward lymphoid lineages *in vitro* and *in vivo* [494]. These observations might explain the reported decline in early T cell progenitors in aged mice that are also inefficient at seeding foetal thymic lobes and generating DP and SP thymocytes [495]. IL-7 has also been held up as a possible culprit driving thymic involution. Produced by thymic epithelial cells (TEC), IL-7 is a vital cytokine for thymopoiesis and levels have been shown to decline with age [496]. To support this, treatment of mice with blocking antibodies against IL-7 resulted in a phenotype similar to that of thymic involution [497]. Similarly, administration of aged mice with exogenous IL-7 increased thymus weight and cellularity. However, when thymic stromal cells engineered to constitutively express IL-7 were transplanted into aged mice, no change in the rate or degree of thymic involution was found even in the presence of increased numbers of DN progenitors [498]. Thus, while IL-7 may rescue early defects in thymopoiesis, it is unable to successfully prevent involution. Other cytokines may also play role however, with overproduction of IL-6, leukaemia inhibitory factor, oncostatin M, and stem cell factor all thought to actively contribute toward loss of thymic cellularity in old age [491]. The major consequence of involution is reduced thymic export of T cells into the periphery. Eventually, this reduced rate of T cell production can no longer adequately replenish the peripheral T cell pool and maintenance of pool

size becomes almost completely dependent on homeostatic proliferation [499]. The lack of new T cell specificities emanating from the thymus, coupled to increased homeostatic proliferation, results in a dramatic contraction in the peripheral TCR repertoire [500], reducing the number of clones available to combat new infections.

Clonal expansions in the elderly predominantly involve CD8⁺ T cells; outgrowth of CD4⁺ T cell clones is much less common and preferentially occurs in certain autoimmune diseases [501] [142]. Studies in NZW and C57L mice show that a 50% reduction in TCR diversity leads to holes in the repertoire to a variety of antigens [502] [503]. Similar observations were made by Yager *et al*, where low naïve precursor frequency for given epitopes was observed during old age in mice that effectively led to an immunological blind spot to certain antigens that impaired immunity to influenza. Thymectomised mice exhibited a comparable phenotype, confirming the role of the thymus in maintaining a diverse TCR repertoire [504]. Clonal outgrowth of CD8⁺ T cells in clinically healthy humans is often a consequence of chronic viral stimulation [505], particularly with cytomegalovirus (CMV) [506] [507], but can also arise from uneven homeostatic proliferation [508] [509] [510] [511]. Outgrowths of CMV-specific CD8⁺ T cells can come to dominate the peripheral repertoire, with up to 23% of all CD8⁺ T cells specific for a single CMV epitope in some cases [507].

The outgrowth of clones specific for chronic viruses inevitably leads to a preponderance of memory CD8⁺ T cells over naïve cells during the latter stages of life. This shift in T cell subset and phenotype also reflects the influence of cumulative exposure to foreign pathogens throughout life.

Elevated levels of homeostatic proliferation in old age will also result in the conversion of naïve cells to a memory phenotype, exacerbating the predilection towards memory cells in the aged T cell compartment [512]. Both $CD4^+$ and $CD8^+$ T cells undergo the same principle phenotypic shifts; however, the rate at which these occur and accumulate is vastly different [513]. In humans, the relative proportions of memory subsets remain reasonably stable in $CD4^+$ T cells, while for $CD8^+$ T cells, the ageing central memory compartment contracts by as much as 50% with a corresponding increase in terminally differentiated effector cells. These end-stage cells are defined by the loss of CD28 and CD27 and gain of CD45RA, CD57 and CD85j [513] and are typically specific for CMV or other chronic viruses [513]. The increased frequency of $CD8^+$ $CD28^-$ T cells have been correlated with poor humoral responses to influenza vaccination and reduced cytotoxic activity to influenza stimulation *in vitro* [514] [515] [516], suggesting changes in the frequency and type of memory subsets has clinical relevance. In mice, there is a gradual accumulation of cells bearing the $CD44^{hi}$ $CD62L^{lo}$ memory phenotype [517] [518] [512] that may also express the terminal differentiation marker KLRG1, somewhat mimicking the human system [157].

Crucially, what effects do these changes in old age have on the ability to respond to infections, such as with influenza? Studies in aged mice indicate a delay in influenza clearance, which is accompanied by delayed peak in T cell expansion and cytotoxic activity [519] [438] [520] [521] [522] [523]. These observation have been linked to poor proliferation and IL-2 production in T cell responding to influenza in aged mice [439]. Similar results have been obtained following *in*

vitro studies in human showing a reduced frequency of IFN-γ-secreting cells in the elderly following flu peptide stimulation [524].

The decline in T cell mediated immunity in old age has a significant impact on clinical outcomes to infections like influenza. For instance, influenza-related complications disproportionately affect the elderly. In the USA, more than 90% of the 36,000 influenza related annual deaths occur in those aged 65 or over [525] [526] [527]. The mean influenza-associated mortality rate for this age group is 22.1 deaths per 100,000 people, compared to 0.2 deaths among those 5-49 years of age [527]. Poor vaccine protection levels in this demographic only act to compound the problem. Influenza infection in the elderly places a huge economic burden on nation's health services, and with the ageing population in western countries growing this issue is only likely to magnify. This makes finding ways to restore elderly immunity or improve vaccination in the elderly a major priority in the coming decades if we are going to achieve the real feat of extending healthy life-span rather than chronological longevity.

Chapter Summary: Rejuvenation of aged CD8⁺ T cell responses through autophagy

In this chapter, experiments will show how the CD8⁺ T cell kinetics to viral infection and vaccination are altered in aged mice. These findings support previous work that demonstrate delayed T cell expansion and diminished memory formation in elderly mice and humans. Autophagy has been linked to the ageing process in a multitude of organs and species, here autophagy levels are shown to fall in antigen-specific CD8⁺ T cells, linking autophagy to T cell ageing. When aged mice were treated with the autophagy-inducing compound spermidine, the CD8⁺ T cell response to vaccination was

dramatically improved, back to similar levels observed in young mice. These results imply that falling autophagy levels act as a cell intrinsic driving force behind the age-related decline in T cell function. Similar experiments will show how spermidine can also augment the $CD8^+$ T cell response to vaccination in young mice, suggesting spermidine might function as a novel immunomodulatory agent.

Chapter 5: Results

Rejuvenation of aged CD8⁺ T cell responses through autophagy

Results

5.4. Autophagy levels fall in CD8⁺ T cells from aged mice

Autophagy has been linked to the ageing process at the whole organism level and falling levels are thought to explain the appearance of age related phenomena in various tissues [430] [410]. However, its involvement in immune ageing, if any, remains unclear. Given the importance for autophagy in T cell memory formation described in this thesis, it was hypothesised that falling autophagy levels in T cells might contribute to poor CD8⁺ T_{mem} formation and inefficient vaccine responses observed in the elderly. Previous published results from our lab have shown that autophagy levels are significantly reduced in CD8⁺ T cells of aged healthy human donors (>65 years) as compared to young (<30 years) [13]. We next sought to determine if this was also true for murine cells. Indeed, mRNA levels of essential autophagy genes were decreased in sorted $CD44^{lo}$ and $CD44^{hi}$ CD8⁺ T cells from naïve old mice (>2 years) relative to the same cells in young mice (8 weeks) (Figure 5.1). The CD8⁺ $CD44^{hi}$ memory compartment is particularly affected, as is *Atg7* expression, which is down 60-fold in $CD44^{hi}$ cells from aged mice (Figure 5.1).

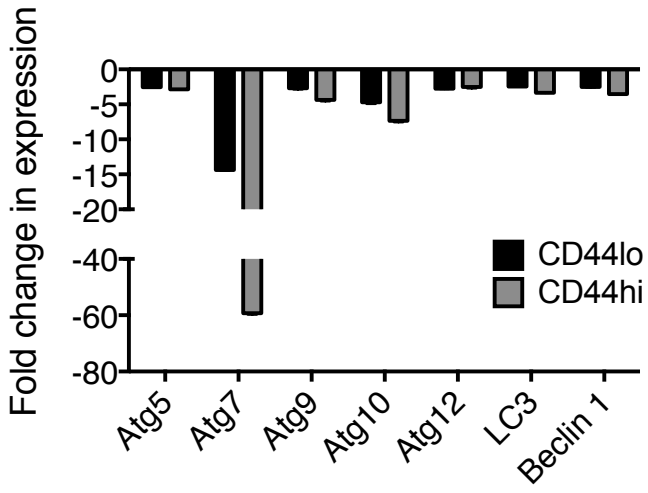


Figure 5.1 *Autophagy gene expression in CD8+ T cells from young and elderly mice. CD44lo and CD44hi CD8+ T cells were purified from the spleens of 6 week old and 2 year old mice using fluorescent activated cell sorting. mRNA was extracted and the expression of essential autophagy genes was measured by q-PCR. Shown is the fold change in expression in CD8+ T cells from old mice relative to expression in young mice (normalized to gapdh and hprt)(n=4, pooled).*

CD8+ T cells from naive old mice also showed significantly decreased autophagic flux, detected by counting LC3 spots in CD44hi CD8+ T cells from young and old mice, both in the presence and absence of an autophagy flux inhibitor (Figure 5.2a,b).

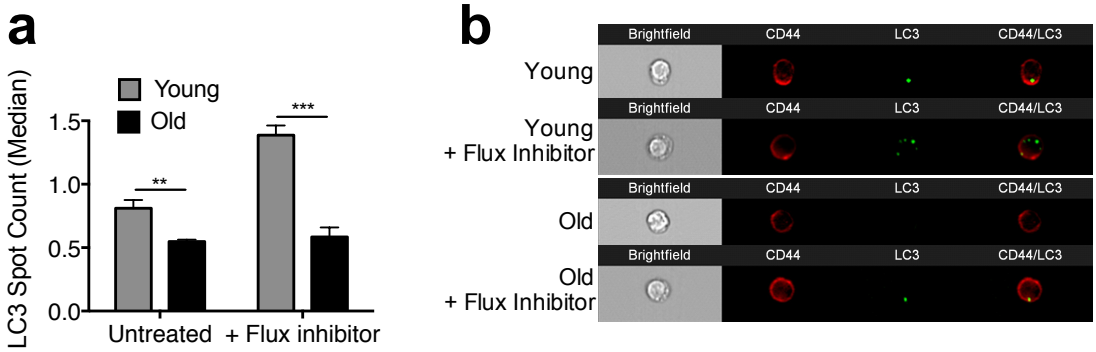


Figure 5.2 *LC3 Spot count in CD8+ T cells from young and old mice.* ***a)*** *Splenic CD8+ T cells from 8 week old and 2 year old mice were treated with an autophagy flux inhibitor for 2 hrs, as a control cells were left untreated. LC3 spot count was determined on CD8+ CD44hi T cells using ImageStream.* ***b)*** *Representative images are shown (x60 magnification).*

***p=0.0082, ***p=0.0004 as determined by Student t-test (n=4–5). With thanks to Isabel Panse for help with analysis.*

Furthermore, using two flow cytometry-based autophagy detection techniques, autophagy levels were found to be significantly reduced in influenza-specific CD8⁺ T cells from aged mice compared to the same cells in young (Figure 5.3). On day 10 post-infection, NP-specific CD8⁺ T cells from old mice exhibited reduced CytoID levels (Figure 5.3a) and lower LC3-II staining intensity (Figure 5.3b) relative to young controls. These are indicative of falling autophagy levels in antigen-specific CD8⁺ T cells in old age.

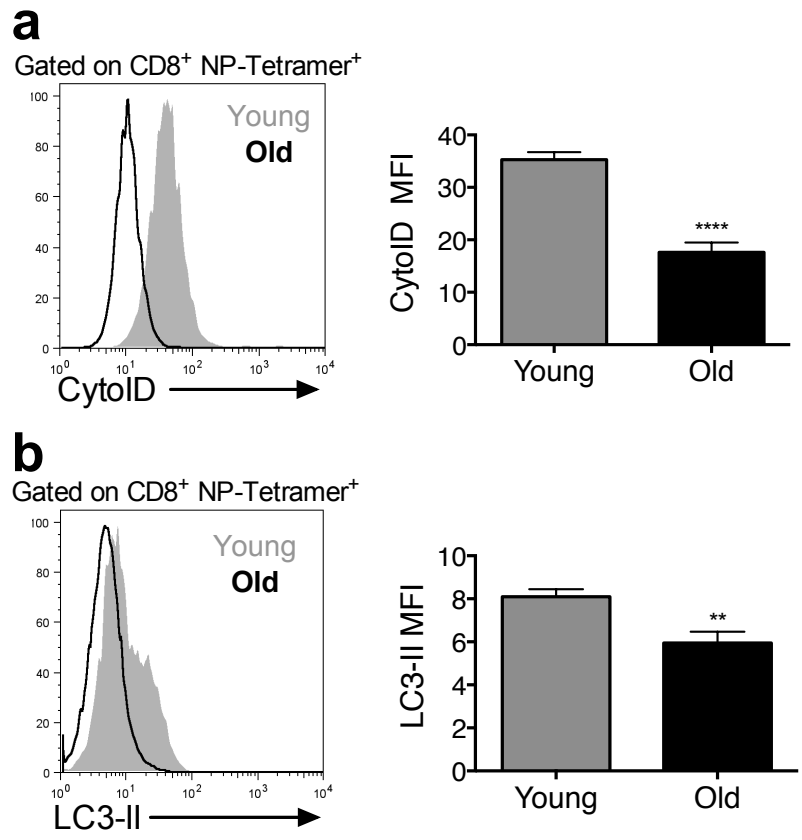


Figure 5.3 *Autophagy levels are significantly reduced in antigen-specific CD8⁺ T cells from aged mice. Autophagy levels by CytoID* ***(a)*** *and LC3-II staining* ***(b)*** *in antigen-specific CD8⁺ T cells from young and old mice. 8-week-old and 2 year old mice were immunized with PR8 influenza. On day 10 post-infection lungs were harvested and stained with CytoID or for LC3-II to assess autophagy levels in NP-Tetramer⁺ CD8⁺ T cells by flow cytometry.* ***(a)*** *****p< 0.0001 (n = 7) by Student's t-test.* ***(b)*** ***p=0.0056, (n=7).*

5.5. Altered CD8⁺ T cell responses to virus infection in aged mice

To confirm that CD8⁺ T cell responses are affected during old age, two-year-old mice were immunised with MCMV and the kinetics of the CD8⁺ T cell response tracked in the blood. To the conventional, dominant m45 epitope elderly mice exhibited significantly altered kinetics. While in young mice the peak T cell response was observed at day 7 and represented approximately 8% of all circulating CD8⁺ T cells, the peak response in aged mice was not seen until day 14, and was 50% reduced in intensity making up only 4% of circulating CD8⁺ T cells (Figure 5.4a). To the inflationary epitope that is dominant during the chronic response, m38, the aged CD8⁺ T cell response also exhibits delayed kinetics and reduced intensity with the frequency of circulating m38-specific CD8⁺ T cells never reaching the same levels as observed in young mice (Figure 5.4b).

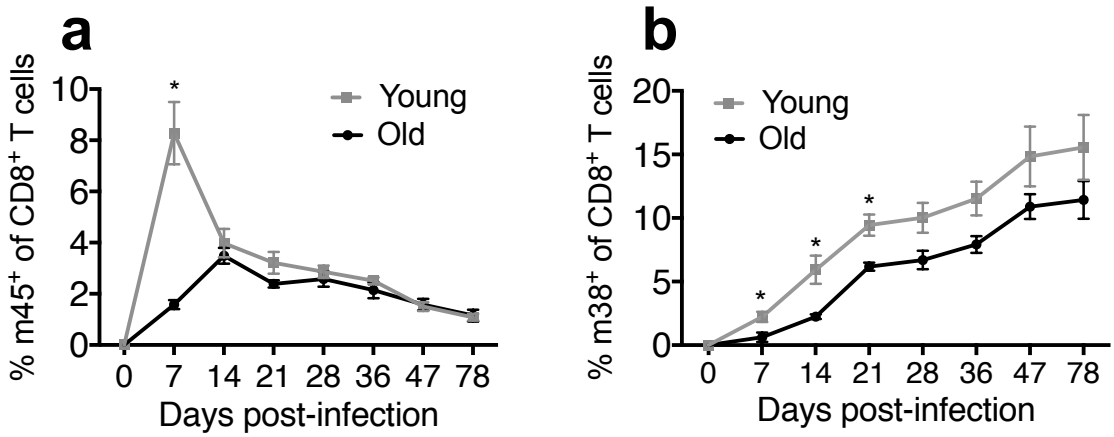


Figure 5.4 *Altered CD8⁺ T cell kinetics to MCMV infection in aged mice. Young (6-week-old) and old (2 years old) were immunised with MCMV and the m45-specific* ***(a)*** *and m38-specific* ***(b)*** *CD8⁺ T cell response tracked over time in the blood by tetramer through serial bleeding. $p<0.05$ by Mann-Whitney U-test (n=4).*

To confirm the altered CD8⁺ T cell response of aged mice to viral infection seen with MCMV, young (8 weeks) and old mice (>2 years) were immunized with influenza and the NP-specific CD8⁺ T response assessed on day 8. While young mice had already formed a significant population of NP-specific CD8⁺ T_{eff} cells in the lungs at this time, in old mice no such population was observed (Figure 5.5). This again implies delayed kinetics in elderly mice, or a failure to respond to this particular dominant epitope altogether.

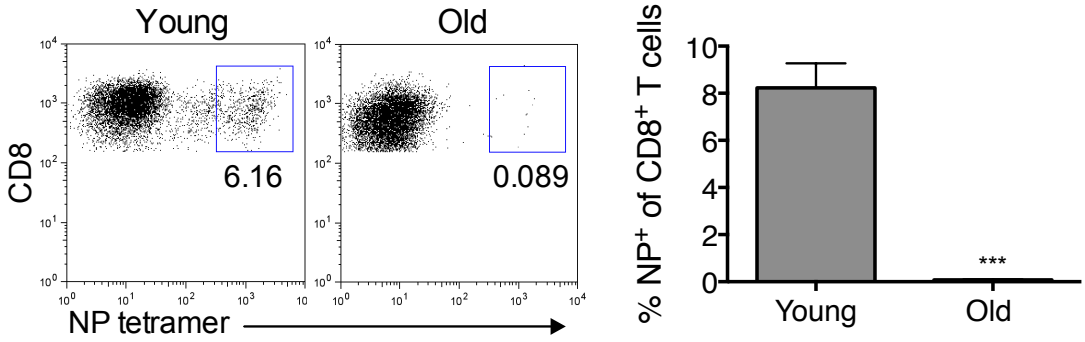


Figure 5.5 *Altered CD8⁺ T cell response to NP in aged mice. 8 week old and 2 year old mice were immunised with PR8 influenza and the NP-specific response was analysed in the lungs using tetramers by flow cytometry. p***=0.0003 by Mann-Whitney U-test (n=7-8).*

5.6. Reduced CD8⁺ T cell responses to influenza challenge in vaccinated aged mice

Next, the ability of vaccinated aged mice to respond to influenza challenge was tested. Using the pseudotyped vaccine S-Flu [97] utilised in previous experiments, we vaccinated mice twice, 3 weeks apart, with H1N1 S-Flu before providing a lethal homologous challenge with H1N1 PR8. The CD8⁺ T cell response to the dominant NP epitope was then assessed in the lungs on day 30 post-challenge. Both young and old vaccinated mice survived lethal PR8 challenge (Figure 5.6a), whereas unvaccinated mice had to be sacrificed 4

days post challenge due to excessive weight loss and morbidity (Figure 5.6a). In young mice, a large frequency of CD8⁺ T cells were specific for NP in the lungs after challenge even at day 30. While a significant NP-specific pool remained in the lungs in old mice too, it was nearly 50% reduced in frequency compared to young (Figure 5.6b,c).

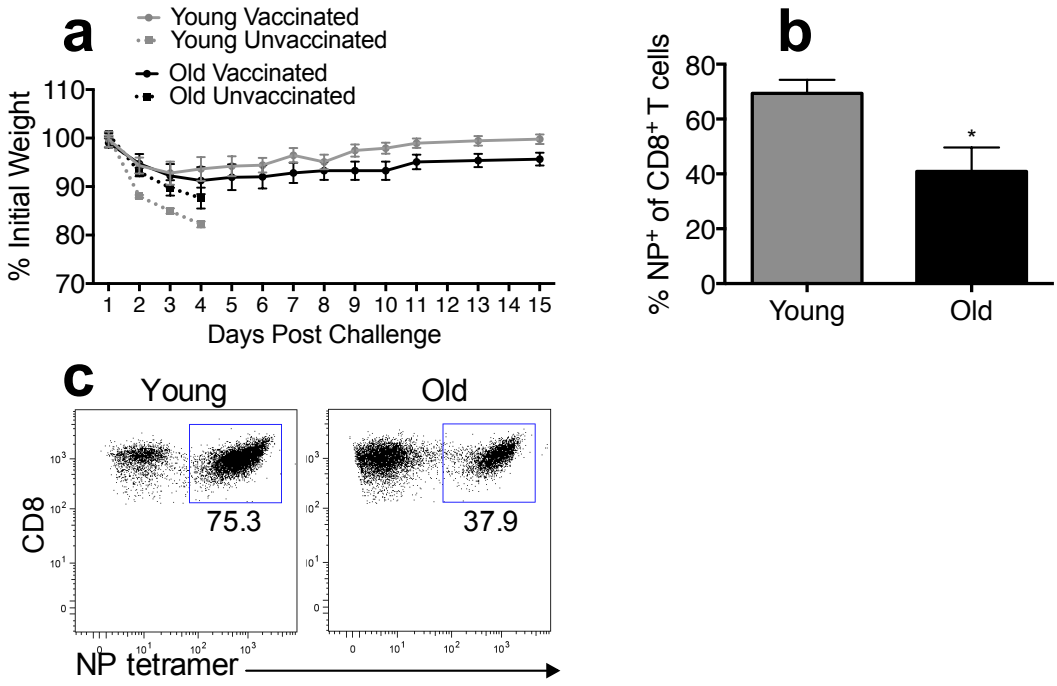


Figure 5.6 *Reduced CD8⁺ T cell responses to influenza challenge in vaccinated aged mice. 7-week-old and 22 month old mice were vaccinated with H1N1 S-Flu twice, 21 days apart, and then challenged with 32 HAU PR8 influenza. As a control, unvaccinated young and old mice were also challenged.* ***a)*** *Following challenge, weight loss was tracked daily in vaccinated and unvaccinated mice.* ***b)*** *On day 30 post-challenge, the frequency of CD8⁺ T cells specific for NP was analysed in lungs using tetramers by flow cytometry. p*=0.0152 (n=6) by Mann-Whiney U-test.* ***c)*** *Representative dot plots are shown.*

5.7. Rejuvenation of aged CD8⁺ T cell responses through spermidine and autophagy

Given the low autophagy levels observed in antigen-specific CD8⁺ T cells from elderly mice, coupled to the newly described role of autophagy in CD8⁺ T cell responses to infection and vaccination, it was reasoned that boosting

autophagy levels in old mice might rejuvenate the aged T cell response. Many positive regulators of autophagy target PI3Ks that may also affect immune signalling pathways [528]; therefore, it was important to identify a compound that could successfully induce autophagy and leave immune signalling pathways untouched. The polyamine spermidine presented as a good candidate, it was able to boost autophagy *in vitro* and *in vivo*, could be administered with no known toxicity, and was not known to interact extensively with immune pathways [419]. Thus, spermidine was administered to old and young mice via the drinking water at concentrations known to induce autophagy [419] during the influenza vaccination protocols used previously. While being continuously supplied with spermidine, mice were immunized with influenza vaccine S-Flu twice, 22 days apart. Thirty days after the last vaccination mice were given a lethal heterologous challenge with X31 influenza. While tracking the $CD8^+$ T cell kinetics to the vaccine regimen, aged mice respond similarly to young controls to primary vaccination (Figure 5.7a). However, old mice do not mount a robust $CD8^+$ T cell response to the second "booster" vaccination like young mice are able to. Twenty-nine days after the last vaccination, the frequency of circulating vaccine-specific $CD8^+$ T_{mem} cells (i.e NP-specific) is 3-4-fold lower in elderly mice relative to young. However, spermidine treatment dramatically enhanced the elderly response to both primary and secondary vaccination, and 29 days post-immunization the frequency of circulating $CD8^+$ T_{mem} cells was 5-fold higher than in untreated aged mice and was 50% increased even on young mice (Figure 5.7a). Crucially, this affect was lost in aged T-$Atg7^{-/-}$ mice, where the T cells are autophagy-deficient (Figure 5.7a). Similarly, when the response to live

virus challenge was assessed in the blood, the CD8⁺ T cell response on day 5 post-challenge was 50% diminished in frequency in aged mice compared to young (Figure 5.7b). However, spermidine administration of aged mice restored the frequency of CD8⁺ T cells specific for NP back to the level seen in young mice following X31 challenge (Figure 5.7b). In the lungs, the number of NP-specific CD8⁺ T cells responding to influenza challenge in vaccinated old mice was down three fold compared to young, but this was improved 5-6-fold in the presence of spermidine, although drug treatment had little effect on the response of aged T-Atg7$^{-/-}$ mice (Figure 5.7c).

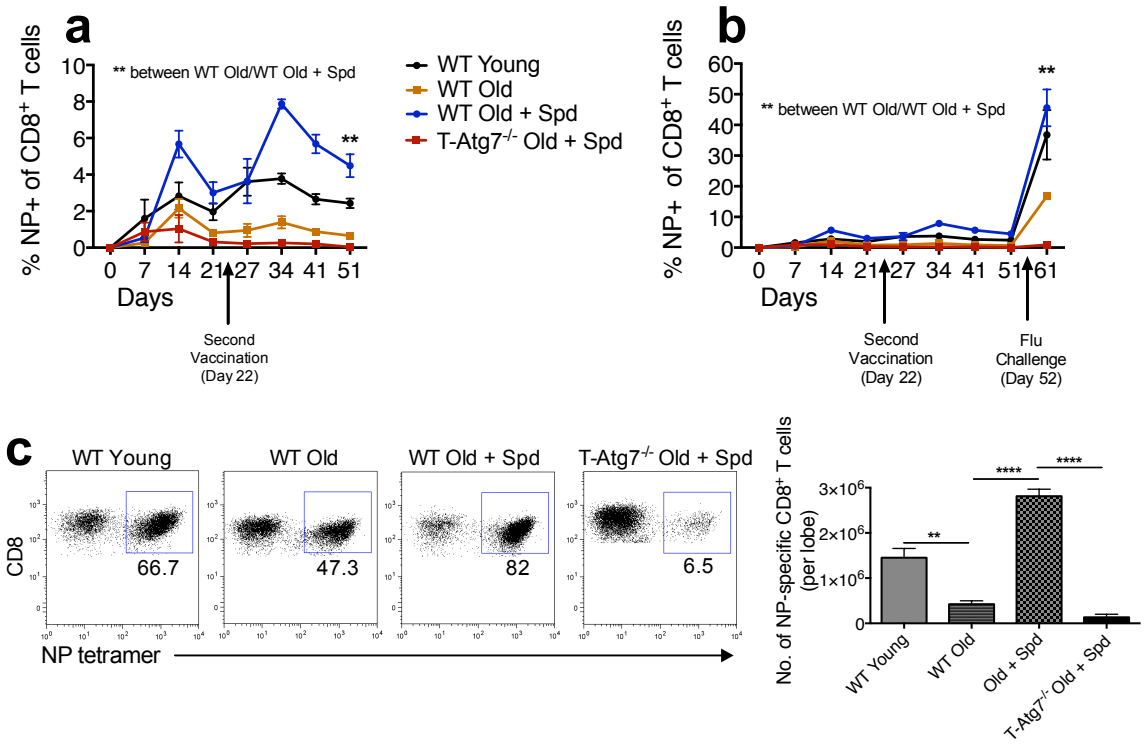


Figure 5.7 *Boosting autophagy restores CD8⁺ T cell responses to vaccination in elderly mice. 8-week-old young WT and 23 month old WT and T-Atg7⁻/⁻ mice were vaccinated 22 days apart with S-Flu and then challenged 30 days later with 32 HAU X31 influenza. 21 days prior to the first vaccination, aged WT and T-Atg7⁻/⁻ mice were administered spermidine in the drinking water at a concentration of 5 mM through to the experimental endpoint. As a control, 23 month old WT mice were administered water alone.* ***a)*** *The CD8⁺ T cell response to NP was tracked over time in the blood by tetramer to both vaccine immunizations (n=4–5). Y-axis depicts the frequency of CD8⁺ T cells that are specific for NP. **p < 0.01 by Mann–Whitney U-test.* ***(b)*** *8-week-old young WT and T-Atg7⁻/⁻ and 23 month old WT and T-*

*Atg7⁻/⁻ mice were vaccinated as described above. 30 days after the last vaccination, mice were challenged with X31 and the CD8⁺ T cell kinetics to vaccination and challenge was measured in the blood by tetramer. Y-axis indicated the percentage of CD8⁺ T cells that are specific for NP, p**=<0.01 (n=4–5) by Mann-Whitney U-test.* ***(c)*** *9 days post–challenge, lungs were harvested and the NP-specific CD8⁺ T cell response to influenza challenge was measured by tetramer. Example dot plots are gated on CD8⁺ T cells. Bar chart shows absolute counts of NP-specific CD8⁺ T cells in the lung per lobe. **p< 0.01, ****p<0.0001 by Student t test (n=4–5).*

Similar to observations with T-Atg7$^{-/-}$ mice in the previous chapter, all mice survived lethal heterologous challenge with X31, including old T-Atg7$^{-/-}$ mice that had 10-fold fewer NP-specific CD8$^+$ T cell in the lung. As all old mice survived, it was therefore not possible to demonstrate improved vaccine protection in the presence of spermidine using this model. Interestingly, although spermidine-treated mice showed vastly improved CD8$^+$ T cell responses to both vaccine and challenge, they lost significantly more weight following challenge than non-treated controls (Figure 5.8a), even though observationally they appeared clinically healthier in terms of mobility, fur condition and general well being (data not shown). This led to the idea that T cell status following challenge has an influence on the clinical phenotype following live virus challenge. Indeed, for this experiment, weight loss could be significantly correlated to the intensity of CD8$^+$ T cell response to influenza challenge in the lung (Figure 5.8b), linking the T cell response to disease severity.

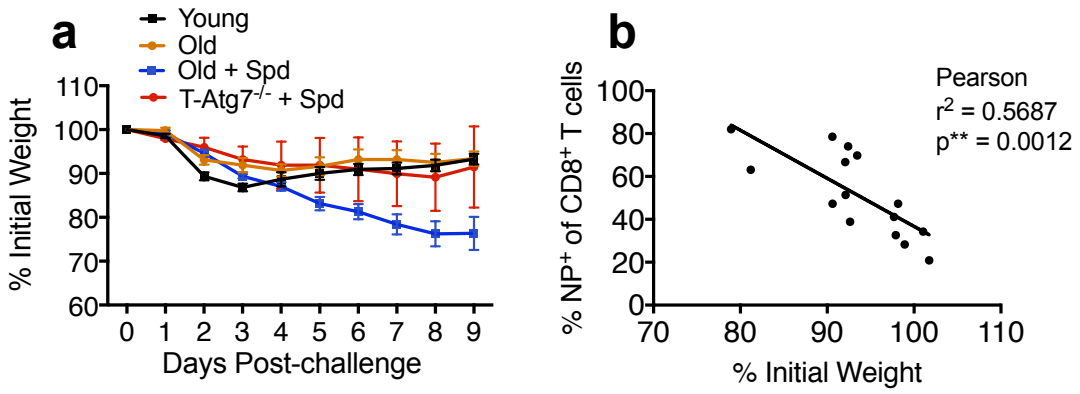


Figure 5.8 *Weight loss following influenza infection is linked to the intensity of the T cell response.* ***a)*** *Weight loss was tracked following X31 challenge in S-Flu vaccinated mice as described in* ***Figure 5.7****.* ***b)*** *Correlating weight loss to the NP-specific CD8⁺ T cell response following X31 challenge in vaccinated mice. Each data point represents an individual mouse from the experiment described in* ***Figure 5.7****.*

5.8. Spermidine improves the young CD8⁺ T cell response to vaccination through different routes of administration

Considering the profound affect spermidine has on the aged CD8⁺ T cell response to vaccination in aged mice, it was hypothesised that spermidine might function as a novel immunomodulatory compound across all ages. To test this, spermidine was administered to 6-week-old mice in the drinking water following influenza vaccination. On day 7 post-vaccination, spermidine treated mice exhibited a 5-fold increase in the frequency of lung CD8⁺ T cells specific for NP compared to untreated controls (Figure 5.9a). To investigate if spermidine could function via multiple routes, spermidine was also administered to 6-week-old mice intraperitoneally following influenza vaccination. On day 13 post-vaccination, treated mice displayed more than a 2-fold improvement in the frequency of NP-specific CD8⁺ T cells relative to untreated controls (Figure 5.9b).

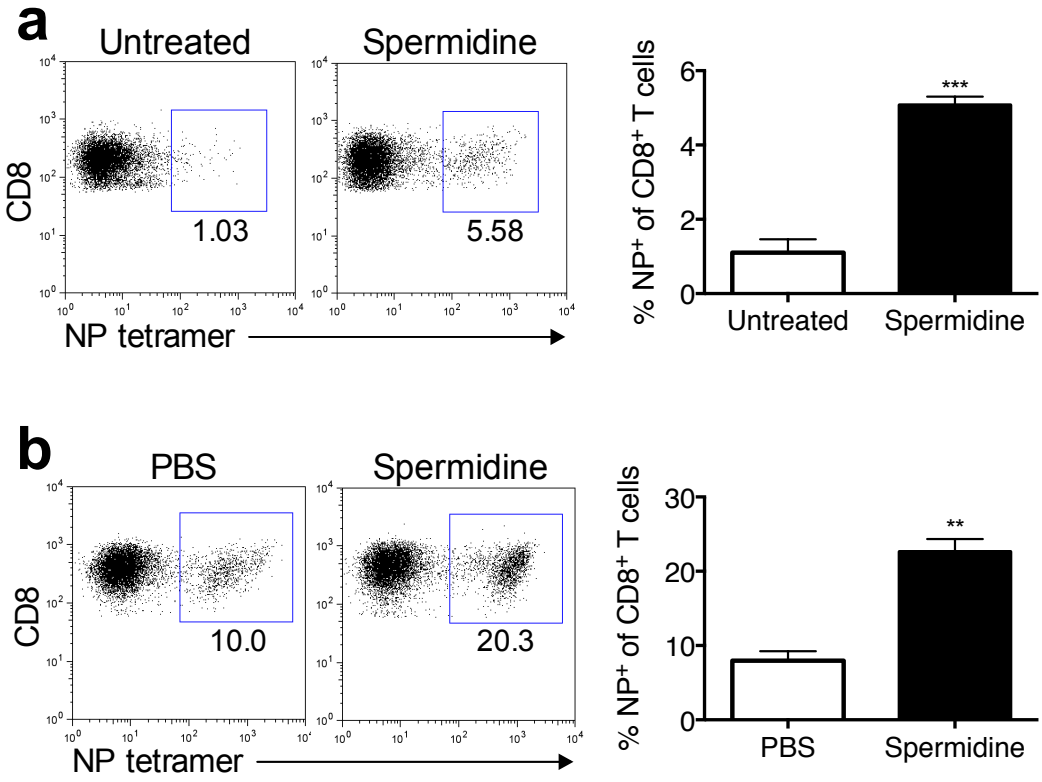


Figure 5.9 *Autophagy improves the young CD8+ T cell response to vaccination* ***a)*** *Mice were immunised with the influenza vaccine S-Flu and on day 7 the CD8+ T cell response to NP analysed in the lungs by flow cytometry using tetramers. From day 0 to day 7 mice were treated with 5 mM spermidine in the drinking water, as a control mice were administered just water. p***=0.0007 (n=8-9).* ***b)*** *Mice were immunised with the influenza vaccine S-Flu and on day 13 the CD8+ T cell response to NP analysed in the lungs by flow cytometry using tetramers. From day 0, mice were administered with 100 µg spermidine intraperitoneally every other day. As a control, mice were injected with PBS. p**=0.0043 (n=5-6). All statistics are Mann-Whitney U-test.*

5.9. Spermidine as a classical vaccine adjuvant

Whilst spermidine can modulate CD8+ T cell responses when administered for prolonged periods, it was next tested if it could function as a classical adjuvant, boosting T cell responses following a single dose. When spermidine was administered once, simultaneously with influenza vaccination, a 40% increase in NP-specific CD8+ T cells was observed on day 14 in the lungs compared to PBS treated mice (Figure 5.10). This proposes spermidine as a

potential novel vaccine adjuvant or immunomodulatory compound with significant T cell enhancing capabilities.

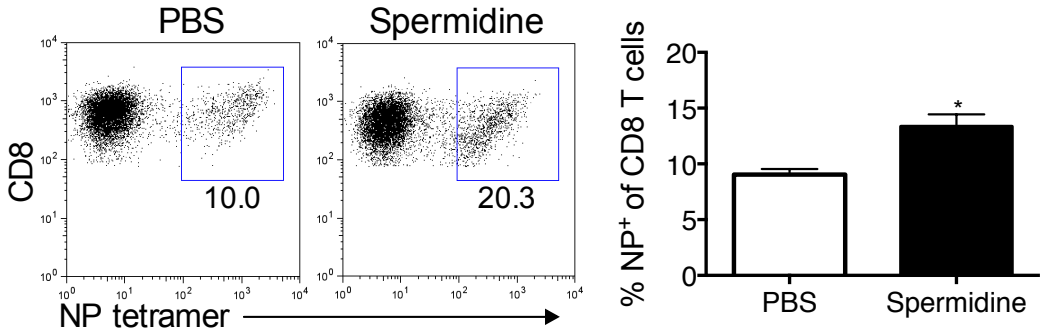


Figure 5.10 *Spermidine as a novel adjuvant to improve T cell vaccine responses. 8-week-old mice were immunized with S-Flu and simultaneously administered 100 µg spermidine or PBS by i.p injection. 14 days post vaccination the CD8⁺ T cell response to NP was assessed in the lungs by tetramer using flow cytometry. p*=0.0131 (n=4-5) by Mann-Whitney U-test.*

Chapter 5: Discussion

Rejuvenation of aged CD8⁺ T cell responses through autophagy

Discussion

5.10. Diminished CD8⁺ T cell responses to viral infection and vaccination in the aged mice

Experiments in this chapter suggest that aged mice exhibit altered CD8⁺ T cell kinetics to MCMV infection. To this author's knowledge, this is the first such report into aged MCMV T cell responses and these observations are closely aligned with published data showing diminished CD8⁺ T cell responses to a multitude of other viruses in old age [520] [438] [436]. The CD8⁺ T cell response to a classical MCMV epitope (m45) took 7 days longer to peak in old mice and was diminished in intensity relative to the maximal response in young mice. In conflict with previous studies, these altered kinetics during the acute phase did not translate to diminished memory formation and 78 days after infection young and old mice showed nearly identical levels of circulating CD8⁺ T_{mem} cells [438] [436]. This was not the case for the CD8⁺ T cell response to the inflationary m38 epitope, where the kinetics of aged mice were also delayed, but the frequency of circulating m38-specific cells never reached the same level seen in young mice. When the CD8⁺ T cell kinetics were tracked to influenza vaccination, the recall response to secondary immunization was significantly reduced in aged mice and 30 days after this vaccination the frequency of circulating CD8⁺ T_{mem} cells was reduced by 50% compared to young. Similar results were obtained in elderly vaccinated mice where the frequency of NP-specific CD8⁺ T cells in the lung following homologous and heterologous challenge was diminished to a similar degree. However, aged mice survived both lethal types of challenge, suggesting that in even this lower frequency of vaccine/influenza-specific CD8⁺ T cells is sufficient to

mediate protection. The reduced CD8⁺ T cell vaccine responses observed here supports previous reports that also show diminished CD8⁺ T cell responses to influenza vaccination and poor memory formation to infection [436 520 524 504 529].

When the response of aged mice to influenza infection was analysed, 2-year-old mice displayed a virtual absence of NP-specific CD8⁺ T cells at day 8, whereas a significant population of NP-specific cells were present in young mice at this time. These results confirm the work of previous studies that have also detailed diminished T cell responses to viral infection [530 438 436 439 524 531 520] that often correlate with delayed viral clearance [438]. Effros *et al* have also shown a complete absence of influenza-specific CD8⁺ T cell activity in old mice at a time when the response in young was peaking [439]. Similar experiments performed by Po *et al* demonstrate how the peak response to the same NP epitope in C57BL/6 mice peaks at day 14 in the lungs of aged mice and day 10 in young mice [438]. However, it cannot be ruled out that these mice might have completely failed to form a response to this particular epitope. Additional analysis of the NP-specific response at a later time point of infection would have gone some way to answering this question. However, if the latter case is true, it would add weight to the idea that repertoire contraction in aged mice can lead to 'holes' in the TCR repertoire rendering mice unable to respond to certain epitopes [504]. In this setting, 'hole formation' in the repertoire might be a stochastic event or alternatively, certain specificities may be more liable to attrition.

What factors drive these diminished CD8⁺ T cell responses in old age? Low or absent precursor frequency as a result of repertoire contraction during old age

is likely to have a significant bearing on the results presented here and elsewhere [500] [502] [503] [504]. The loss of clones representing any given specificity would inevitably lead to a smaller burst size following activation and could explain the delayed and diminished acute T cell response to influenza and MCMV infection observed here in the aged. The smaller peak during the effector phase might also result in a reduction in cells able to enter the memory pool, explaining the diminished frequency of $CD8^+$ T_{mem} cells in old age to both MCMV and influenza vaccination. IL-2 might be an important factor in these observations. Effros and Walford demonstrated diminished IL-2 production from aged $CD8^+$ T cells *in vitro* following stimulation in with influenza. Exogenous IL-2 supplementation could augment influenza-specific CTL activity [530], suggesting diminished IL-2 production could hinder efficient T cell expansion in old mice. T cell migration is also thought to be impaired during ageing and a delay in T cell infiltration to the lungs following influenza infection has been observed in old mice [532]. This might also explain the discrepancy between the normal primary $CD8^+$ T cell response to vaccination in the blood, but drastically reduced primary response in the lungs to influenza infection that is presented here. These observations are likely to be part of a host of extrinsic factors that contribute to defective $CD8^+$ T cell expansion and memory generation in aged mice that might also include altered antigen-presentation [453] [454] [455] [456], defective $CD4^+$ T cell help [533] [529], and diminished IL-7 and IL-15 production [534] [496]. However, intrinsic factors might also be at play; *in vitro* work suggests that a large proportion of $CD8^+$ T cells from aged mice do not proliferate at all following mitogenic stimulation, and of the ones that do, the burst size is smaller and a significant percentage

start proliferating later and stop sooner [519]. $CD8^+$ T_{mem} cells from aged mice also function poorly when adoptively transferred into young animals [535], supporting a cell intrinsic influence to poor T cell function in old age.

5.11. Decreased autophagy levels in $CD8^+$ T cells from aged mice

Falling autophagy levels have been implicated as a cell intrinsic-driving force behind the aging process in a multitude of cells, systems, and organisms [94]. We hypothesised that falling autophagy levels in T cells might explain altered T cell responses in old age, particularly defective memory formation. Indeed, autophagy levels were shown to fall in polyclonal $CD8^+$ T cells in old age as well as in antigen-specific $CD8^+$ T cells in response to influenza infection. This confirms a previous finding that autophagy levels fall in $CD8^+$ T cells from elderly humans [13]. How might falling autophagy levels impact upon T cell function in old age? It is possible that falling autophagy levels in APCs could deleteriously affect antigen-presentation and as a corollary T cell priming. In old mice and humans, the $CD8^+$ T cell kinetics following activation is drastically affected; yet in the previous chapter, autophagy was shown to be dispensable during the T cell effector phase. However, it is still feasible that low autophagy levels might influence T cell priming and expansion. Autophagy is generally an essential requirement for T cell homeostasis and survival [536]. Low autophagy levels in old age might therefore destabilise T cell viability, contributing to the decreased T cell frequency observed in aged mice that is predominantly driven by thymic involution [537]. As a consequence, diminished precursor frequency would in turn impact upon the magnitude of the effector phase. Low autophagy levels might also influence cell migration,

previously suggested to be a factor in delayed T cell kinetics in old age [532]. Unpublished data from our lab shows that cell size in a number of haematopoietic cells such as T cells, macrophages, and neutrophils increases in the absence of autophagy. This might result from dysregulated organelle homeostasis that drives an increase in cytoplasmic volume to accommodate larger and more abundant organelles. In turn, this increase in cell size could impair the ability of T cells to extravasate from the peripheral circulation to the site of infection, delaying the appearance of antigen-specific T cells. To support this, pharmacological inhibition of autophagy by 3-Methyladenine has been shown to inhibit cancer cell migration, whilst the autophagy associated factors DRAM1 [538] and p62 have also been linked to cell migration [539].

Low autophagy levels in T cells from aged organisms is also likely to influence T cell memory formation in much the same way as it does in T-Atg7$^{-/-}$ mice. Low autophagy during ageing would lead to the accumulation dysfunctional mitochondrial and consequently an increase in ROS that might promote cell death in memory precursor cells (*Figure 5.11*). While data is limited on T cell mitochondrial function in old age, decreased function in other cell types has been observed during ageing [540] [541] [428] [542]. Furthermore, the inability to breakdown lipid stores to provide FAs for β-oxidation would also reduce memory formation by preventing efficient switching to OXPHOS metabolism, ultimately resulting in energetic instability and cell death [370] [371] (Figure 5.11). A decrease in functional mitochondria in the presence of low autophagy levels would exacerbate this problem through damaged

mitochondrial membranes with low potential to perform OXPHOS.

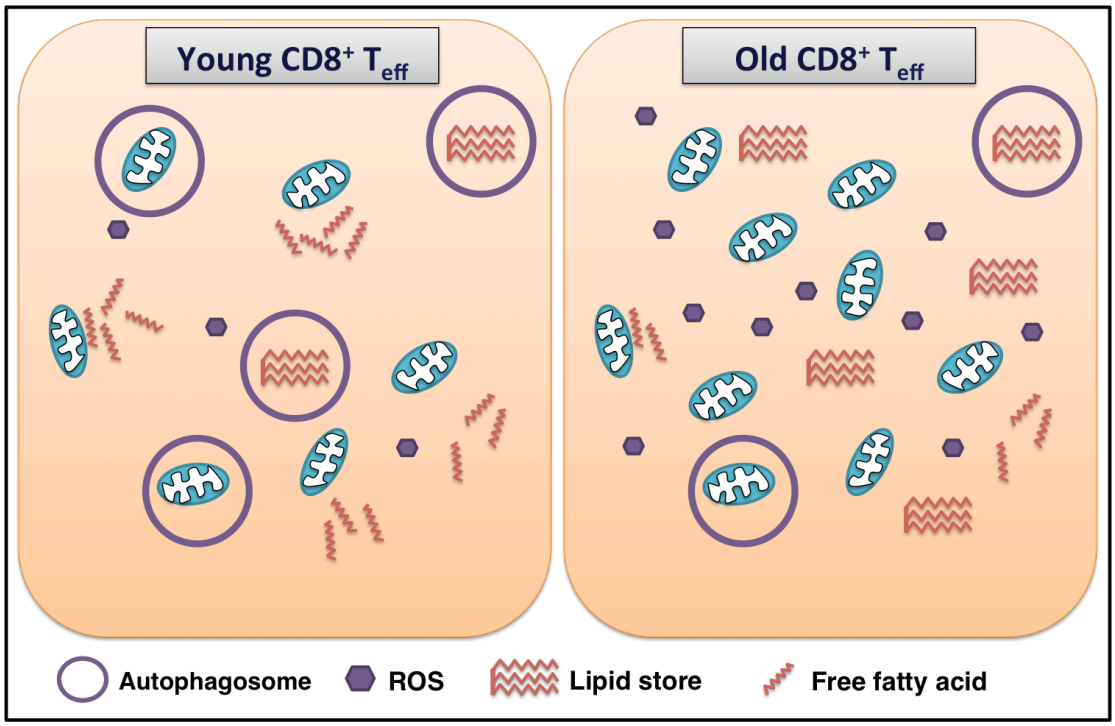


Figure 5.11 *The effect of low autophagy in CD8⁺ T cells during memory conversion in old age. During contraction, young CD8⁺ T_{eff} cells have robust autophagy levels and so can effectively maintain mitochondrial mass and quality, limiting the production of ROS. Furthermore, autophagy is able to degrade lipid stores leading to the release of free fatty acids that are subsequently utilised for fatty acid oxidation, a requirement to enter the long-lived memory pool. Conversely, CD8⁺ T_{eff} cells from aged mice exhibit low autophagy. Correspondingly, this results in low mitophagy and the accumulation of mitochondria, many of which will be dysfunctional leading to excess ROS production. Low autophagy also results in the failure to effectively mobilise fatty acids for FAO. Together, these factors reduce viability of CD8⁺ T_{eff} cells, lowering the number of cells entering the memory compartment.*

These effects would cumulatively result in enhanced cell death of memory precursors restricting the number of cells entering the memory pool during old age following infection and vaccination. Supporting circumstantial evidence comes from Kapasi *et al* that show it is defective memory generation that is prevalent in old age, not defective memory maintenance [436], mirroring the phenotype of T-Atg7$^{-/-}$ mice where lack of autophagy inhibits entry to the

memory pool.

A fundamental question is why do autophagy levels fall with age? Data presented here suggests that autophagy gene transcription is decreased in CD8⁺ T cells from aged mice, but it is unclear what might drive changes at the genetic level. Epigenetic changes or altered signalling events that result in reduced induction of autophagy gene transcription might provide some insight into this. However, a major factor is likely to originate from lysosomal dysfunction driven by the accumulation of the ageing-associated pigment lipofuscin. Lipofuscin is an intralysosomal, polymeric substance primarily composed of cross-linked protein residues formed due to iron-catalyzed oxidative processes. Moreover, it is undegradable and cannot be removed via exocytosis [543]. The presence of lipofuscin within the lysosome renders it functionally redundant. Where lipofuscin accumulation is high, the availability of lysosomes free to fuse with autophagosomes is low, reducing autophagic flux [544]. The lower autophagic flux is likely to result in increased oxidative stress, a factor also shown to promote lipofuscin accumulation [544], suggesting the presence of a deleterious positive feedback loop that eventually renders the cell non-functional or apoptotic [545]. It is not entirely clear why lipofuscin accumulation occurs with time, but attenuated proteolytic capacity in old age is thought to be an important underlying cause. Nevertheless, these observations suggest that lipofuscin clearance might be a route to normalise autophagy levels in old age, resulting in improved CD8⁺ T cell memory formation.

5.12. Rejuvenation of aged CD8⁺ T cell responses through autophagy

With a link between autophagy, CD8⁺ T cell memory, and ageing established, it was postulated that targeting the autophagy pathway might be desirable in order to improve T cell immunity in the aged. To achieve this, aged mice were treated with polyamine spermidine, shown previously to induce autophagy both *in vitro* and *in vivo* [419]. Polyamines are low molecular weight, water-soluble, aliphatic polycations found both free in the cytoplasm and bound to DNA and RNA [546] [547]. First identified in 1678 [548], the polyamine family is comprised of the compounds spermine, spermidine and putrescine that have wide-ranging cell and tissue distribution [549] [550]. Considering their ubiquitous nature, the known biological functions of polyamines are surprisingly small, although their importance in cell proliferation is well established [551] as is their ability to stabilise DNA:chromatin complexes [552] [553]. Polyamines are synthesised from amino acid precursors where ornithine is converted into putrescine by the action of ornithine decarboxylase (ODC). Putrescine may then be converted to spermine, which in turn can be converted to spermidine in a set of reversible reactions [548]. The polyamine spermidine was a good candidate to target autophagy *in vivo* as it had previously been administered over long time periods with no reported toxicity and was not known to influence many immune signalling pathways [419]. When the CD8⁺ T cell kinetics to vaccination was tracked in the blood, aged mice exhibited a significantly reduced response to both first and second vaccination and the frequency of CD8⁺ T_{mem} cells formed after immunisation was also significantly lower. However, spermidine treatment dramatically improved

the aged response to vaccination in the blood to both primary and secondary vaccinations. Spermidine administration also enhanced the frequency of antigen-specific $CD8^+$ T_{mem} cells 5-6-fold 30 days after vaccination. In fact, $CD8^+$ T_{mem} cell levels were brought to a level above what was observed even in young mice. Importantly, this effect was lost in spermidine-treated aged T-Atg7$^{-/-}$ mice, suggesting spermidine is reliant on autophagy in T cells. A similar situation was observed following heterologous challenge with live virus, non-treated aged mice exhibited a 3-fold lower number of NP-specific $CD8^+$ T cells in the lung and this was improved dramatically when old mice were treated with spermidine. Again, this improvement was lost in T-Atg7$^{-/-}$ mice. These data provide first proof of concept that utilising autophagy-boosting compounds such as spermidine can improve T cell immunity to vaccination and infection. However, these results are similar to those reported by Hacker-Shahin and Droge, where injections of putrescine strongly augmented *in vivo* priming for secondary *in vitro* responses to syngeneic T cell lymphoma cells and minor histocompatible allogeneic spleen cells [554]. Although autophagy levels were not analysed in $CD8^+$ T cells from spermidine treated aged mice, a limitation of this study, the observation that spermidine-mediated effects are lost in T-Atg7$^{-/-}$ cells suggests that this function is heavily reliant on autophagy within the T cell lineage. The effect of boosting autophagy in T cells from aged mice is likely to be centred on the newly described role for autophagy in $CD8^+$ T cell memory formation presented in this thesis and simultaneously elsewhere [555] [381]. Primarily, increased autophagy levels would serve to improve mitochondrial health and fitness, supporting elevated OXPHOS, whilst also improving metabolic

flexibility by enhancing fatty acid availability for fatty acid oxidation that would act to improve CD8⁺ T_{mem} generation. Increased autophagy levels would also improve T cell viability by reducing oxidative stress. Indeed, spermidine administration has previously been shown to limit oxidative stress in ageing mice through autophagy [419]. Also, it cannot be ruled out the spermidine-mediated effect is solely down to enhanced autophagy just in CD8⁺ T cells. Spermidine might also function on APCs to improve antigen presentation and T cell priming whilst also influencing CD4⁺ T cells, perhaps by enhancing CD4⁺ T cell-help to CD8⁺ T cells. *Ex vivo* treatment of TCR transgenic T cells before adoptive transfer into untreated syngeneic hosts would go some way to answering whether the effects of spermidine are specific to CD8⁺ T cells. One interesting observation was that spermidine-treated aged mice displayed significant weight loss following influenza challenge, relative to non-treated aged mice. It cannot be completely ruled out that this effect is due to spermidine-associated toxicity, but this is unlikely as aged T-Atg7$^{-/-}$ mice given spermidine did not show similar weight loss and spermidine has been administered for prolonger periods previously with no reported toxicity [419]. Interestingly, weight loss following challenge could be correlated to the intensity of the NP-specific CD8⁺ T cell response, implying that CD8⁺ T cell mediated immunopathology in the lung could be an important factor in weight loss disease severity following influenza infection. A similar role for CD8⁺ T cells in immunopathology has been described before in mice [401 402 403].

A key question is how does spermidine induce autophagy? The previous study to utilise spermidine *in vivo* suggested that it triggers epigenetic

deacetylation of histone H3 through inhibition of histone acetyltransferases (HAT) resulting in altered autophagy gene expression. However, these results could not be repeated in our lab and moreover, it is unlikely that upregulation of a select few downstream genes is solely enough to potently boost a pathway that requires that action of over 50 genes. Unpublished data from our lab suggests that spermidine induces autophagy by facilitating a unique modification in the elongation factor eIF5A. eIF5A contains a unique spermidine-dependent hypusine modification on a specific lysine residue [556] which is essential for eIF5A function [557]. We have recently shown that hypusinated-eIF5A promotes ER stress (Zhang *et al.,* unpublished observations), a potent inducer of autophagy [558] [559]. Therefore, eIF5A-mediated ER stress may contribute to autophagy induction by spermidine. On the other hand, inhibitors of the eIF5A hypusination pathway inhibit autophagy in an ER stress-independent manner. So further experiments are needed to determine the precise molecular pathway of spermidine's role in autophagy.

The loss of improved $CD8^+$ T cell response in treated T-$Atg7^{-/-}$ mice provides evidence that spermidine is functioning through the autophagy pathway. However, it is not possible to eliminate non-autophagic functions for spermidine in enhancing $CD8^+$ T cell responses. A notable function of polyamines is in promoting cell proliferation. Depletion of spermidine prevents cell cycle progression with cells arrested at the G_1/S boundary. This is thought to be due to a slowed rate of DNA synthesis and increased levels of $p27^{Kip1}$ and $p21^{Cip1/WAF1}$, inhibitors of cyclin-dependent protein kinases [560] [561] [562] [563] [564]. Direct evidence of this has been observed in peripheral blood

lymphocytes *in vitro* where polyamine depletion with the polyamine synthesis inhibitors α-difluoromethylornithine (DFMO) and MGBG (methylglyoxal-bis(guanylhydrazone)) inhibited T cell proliferation and CTL induction following activation [565] [566] [567] [568] [569] [570]. These studies are however contrasted with the observation that addition of exogenous polyamines impairs the mitogen-stimulated transmembrane Ca^{2+} mobilization in murine T cells, suggesting a potential immunosuppressive role for polyamines [571]. Similar to this, Brune *et al* demonstrated that polyamines prevented Ca^{2+}-induced endonuclease activation, DNA fragmentation and ultimately apoptosis in thymocytes [572]. Thus, spermidine treatment might enhance $CD8^+$ T cell responses by increasing $CD8^+$ T_{eff} and $CD8^+$ T_{mem} viability, increasing the frequency of antigen-specific T cells after expansion and contraction. Thus, while spermidine may enhance $CD8^+$ T_{mem} formation by increasing autophagy levels, spermidine might boast a pleiotropic role in enhancing immunity, particularly through the regulation of T cell proliferation. The observation that ODC, the enzyme required for polyamine synthesis, is induced following T cell activation [573] [574] [575] [576] suggests polyamines might play an important intrinsic role in T cell activation and expansion and warrants further investigation.

Interestingly, decreased polyamine levels and diminished ODC activity with old age has been described in multiple species including humans [577] [578] [579] [580] [581]. This effect was most pronounced in the spleen and thymus in rodents, suggesting that falling polyamine levels might be a contributing factor to T cell immunosenescence [580] [579]. In this case, exogenous addition of spermidine,

as was done here, would also restore the non-autophagy-related functions of spermidine that are lost in old age due to low polyamine levels. Furthermore, when this system is modelled *in vitro* during DFMO-mediated polyamine depletion of Jurkat T cells and primary CD8⁺ T cells, autophagy levels are severely compromised [555]. Therefore, low polyamine levels in old age might also explain the decreased autophagy levels in T cells from elderly mice.

The results presented in this chapter suggest that targeting the polyamine or autophagy pathways are desirable to improve aged immunity. Other autophagy boosting compounds would be expected to mediate a similar effect such as resveratrol and rapamycin. Indeed, calorie restriction, shown to work primarily through autophagy [427], has been demonstrated to enhance immunity to influenza in aged mice [421]. Shown here to enhance the CD8⁺ T cell responses to vaccination in both young and old mice, spermidine offers the prospect of a novel immunomodulatory drug able to operate through multiple administration routes and as a classical vaccine adjuvant. While also being naturally occurring, spermidine is significantly more attractive than previously published T_{mem}-boosting compounds such as metformin [370] and rapamycin [397], that come with unwelcome side effects and toxicity in humans. The elucidation of the precise role of spermidine will be the subject of future work and will enable its development as a safe, readily administrable immune-modulating drug.

5.13. Conclusions

In this chapter, the CD8⁺ T cell response to viral infection and vaccination of aged mice was assessed. In the case of influenza, experiments confirmed

previous studies showing that T cell expansion is delayed and diminished in magnitude in old mice. $CD8^+$ T_{mem} formation following influenza vaccination was also significantly impaired in the elderly. Confirming this, novel data showed that altered kinetics in old age is also true during infection with MCMV. Autophagy is heavily linked to the aging process and here, for the first time, it is linked to immunesenescence in T cells. Autophagy levels were found to fall in antigen-specific $CD8^+$ T cells in old age. Improving autophagy levels with the compound spermidine could dramatically improve aged $CD8^+$ T cell responses to both vaccination and infection. This is an important finding giving the low rates of vaccine protection in elderly populations driven by poor T and B cell responses. Given the poor antibody correlates of protection in the elderly [537] [582], modulation of T cell responses might be especially beneficial, particularly in the setting of any future pan-influenza vaccine where cross-reactive T cells would be of great importance. Importantly, spermidine was able to augment $CD8^+$ T cell responses in both young and old mice whilst also functioning as a classical vaccine adjuvant, opening the door to it's use a novel immunomodulatory drug or a first in class directly T cell boosting vaccine adjuvant. However, mechanisms for the enhancement of T cell responses by spermidine are at present speculative and further work is required to delineate this novel immunomodulatory compound's role in T cell biology.

Chapter 6: General Conclusions

6. General Conclusions

6.1. Autophagy in T cell development

The role of autophagy in T cell development was analysed using Vav-Atg7$^{-/-}$ mice where *Atg7* is excised in all haematopoietic cells as early as the HSC. In this model, a decreased frequency of early T cell progenitors was observed (DN1 cells), although after this point T cell development progressed unperturbed. The reduced numbers of DN1 cells might originate from defects at the HSC and lymphoid progenitor stage as *Atg7* has previously been shown to regulate the HSC niche [93]. The effects of *Atg7* on HSC and progenitor viability could result in a lower number of early T cell progenitors seeding the thymus, which might also account for the reduced thymic cellularity observed in Vav-Atg7$^{-/-}$ mice. However, *Atg7* does not appear to impact functionally on T cell development as normal frequencies of mature T cells are observed in the thymus. This is also true in T-Atg7$^{-/-}$ mice where T cell production is unaffected by the loss of autophagy at the double positive stage, confirming previously published observations [145] [166] [146].

6.2. Autophagy regulates naïve T cell homeostasis

To study the role of autophagy in T cells, the essential autophagy gene *Atg7* was excised in the T cell lineage under the control of the *CD4* promoter to create T-Atg7$^{-/-}$ mice. While T cell development proceeds normally in the thymus, in the periphery the T cell compartment is severely affected by the loss of *Atg7* resulting in significant T cell lymphopaenia. These findings concur with similar models where loss of *Atg3* and *Atg5* in the T cell lineage also exhibit peripheral lymphopaenia [145] [166] and adds weight to the idea

that autophagy is essential for cell survival *in vivo*. The loss of T cell viability in the absence of autophagy did not originate from a failure to respond to essential extrinsic homeostatic signals provided by gamma chain cytokines as *Atg7*$^{-/-}$ T cell survival was increased in the presence of IL-7 while IL-7R expression was found to be normal. However, when autophagy-deficient T cells were placed into the replete T cell environment provided by a bone marrow chimera, the survival defect remained suggesting its cell intrinsic nature.

Previous work has shown that mitochondrial mass is developmentally regulated in T cells [53]. Here, those observations were confirmed when peripheral CD8^{+} T cells were found to display a significantly reduced mitochondrial load than mature thymocytes. *Atg7*$^{-/-}$ CD8^{+} T cells exhibited a greater mitochondrial burden than wildtype cells and this was associated with elevated levels of mitochondrial superoxide. As was also posited by Pua *et al.*, these results suggest that autophagy is required to remove mitochondria between thymus and periphery [53]. The failure to do so results in the accumulation of mitochondria and ROS that has deleterious consequences for cell survival. Reasons behind the disparity in mitochondrial volume between thymus and periphery is not immediately clear, although T cell development might impose a high metabolic demand that requires large amounts of mitochondria to sustain energy production. Furthermore, the frequent proliferation required during T cell development might make a high mitochondrial load necessary so cells are prepared to pass organelles onto daughter cells. Whatever the reason, this mitochondrial load is no longer

required in the periphery and failure to remove them through autophagy puts the cell at risk from mitochondrial-mediated apoptosis.

The lymphopaenia in T-Atg7$^{-/-}$ mice also drives changes in surface phenotype with a large frequency of CD8^{+} T cells expressing markers associated with memory even in the absence of antigen. This change in phenotype is driven by profound homeostatic proliferation as *Atg7*$^{-/-}$ CD8^{+} T cells turnover in an attempt to refill the depleted T cell niche. This phenomenon of lymphopaenia-induced proliferation and the subsequent acquisition of memory characteristics is a well described occurrence during times of T cell scarcity, [189] [190] [191] [192] [193] [194] [179] [180] [181]. However, this is the first time it has been described in an autophagy-deficient T cell compartment. Excessive cell turnover of CD8^{+} T cells from T-Atg7$^{-/-}$ mice also resulted in signs of exhaustion and terminal differentiation. A fraction of autophagy-deficient CD8^{+} T cells exhibit co-expression of PD-1 and TIM-3 that is normally a feature of exhausted antigen-specific CD8^{+} T cells during chronic infection [156]. The upregulation of these markers might be an attempt to limit auto-proliferation and the outgrowth of more auto-reactive clones that have a survival advantage during times of lymphopaenia. The upregulation of the inhibitory receptor KLRG1 on *Atg7*$^{-/-}$ CD8^{+} T cells, normally a marker of terminal differentiation, might also serve to exert negative regulatory control to limit the amount of LIP.

6.3. Autophagy is an essential requirement for memory CD8^{+} T cell formation

Whilst a role for autophagy in naïve T cells had been previously described, how autophagy might be important in antigen-specific T cells was unknown.

Although, dispensable for $CD8^+$ T_{eff} expansion, evidence presented here suggests that autophagy is crucial for $CD8^+$ T_{mem} formation. When the $CD8^+$ T cell kinetics to influenza and MCMV infection were assessed, the effector phase of T-Atg7$^{-/-}$ mice proceeded in normal fashion but the antigen-specific pool suffered catastrophic collapse during contraction, resulting in the failure to form a memory compartment. Use of bone marrow chimeras showed that this failure was cell intrinsic to the loss of *Atg7* and could not be traced to defective $CD4^+$ T cell help nor altered T cell priming by *Atg7*$^{-/-}$ CD4-expressing APCs. Autophagy had been shown here and previously [53] to regulate mitochondrial removal in T cells upon egress from the thymus. Mitochondria are also dynamically regulated during $CD8^+$ T cell differentiation following antigen stimulation. After activation, cells initially up-regulate mitochondrial mass to provide mitochondria for daughter cells and an initial surge of energy in preparation for the following strong burst of proliferation [386] [386] [387] [388]. During the effector phase, a further round of mitochondrial biogenesis takes place in memory precursor cells, this time driven by IL-15 as these cells prepare to switch from glycolysis to mitochondrial-driven OXPHOS [368,369]. Thus, memory precursors boast a high mitochondrial burden and quality control must be maintained to limit the toxic potential of these organelles. In autophagy-deficient antigen-specific T cells, this mitochondrial burden was shown to be even greater and was accompanied by an increase in mitochondrial ROS relative to wildtype cells. Importantly, autophagy not only regulates the number of mitochondria within the cell, but it regulates quality also, preferentially targeting damaged organelles for deletion [44]. It was thus hypothesised that autophagy acts to limit the flow of ROS and other

toxic mediators emanating from mitochondria, maintaining cell viability but also the functionality of mitochondrial membranes so that they can support the metabolism required for $CD8^+$ T_{mem} differentiation. Indeed, when T-$Atg7^{-/-}$ mice were treated with the ROS-scavenging compound NAC, the $CD8^+$ T_{mem} compartment could be rescued, suggesting that ROS levels are exquisitely fine tuned in T cells and are controlled through efficient mitophagy.

Metabolic regulation is also a key determinant of $CD8^+$ T_{mem} differentiation [365] and cells seemingly require the oxidation of fatty acids in particular to enter the long-lived memory pool [370] [371]. *Atg7*$^{-/-}$ $CD8^+$ T_{eff} cells display an altered metabolic profile that is typified by increased intracellular lipid stores, altered fatty acid uptake, and prolonged glycolysis. Autophagy might therefore be a key factor in the switch from glycolysis to FAO during $CD8^+$ T_{mem} formation. In this model, autophagy, shown in other cell types to be important for lipid metabolism [43], is required to deliver lipid stores to the lysosome where they are broken down to free fatty acids. These fatty acids may then enter the mitochondria to provide the fuel for OXPHOS required for memory differentiation. In the absence of autophagy, this metabolic switch cannot occur due to a failure to sequester and degrade lipid stores and cells are forced to continue using glycolysis as a source of energy. While this is fine during the expansion phase when glycolysis provides essential building blocks for growth, when memory precursors return to a more quiescent state during contraction glycolysis is no longer sufficient and a failure to change to OXPHOS metabolism results in energetic instability and cell crisis,

contributing to the collapse of *Atg7*$^{-/-}$ CD8^{+} T_{eff} pool.

Put together, these results suggest that autophagy regulates entry into the memory pool, rather than maintenance of the memory compartment itself. Experiments showing that autophagy levels are at their highest during the effector phase and lowest in CD8^{+} T_{mem} cells provide support for this idea. Adding further weight is the observation that when the essential autophagy gene *Atg5* was excised during the memory phase, the frequency of CD8^{+} T_{mem} cells remained unperturbed.

6.4. Secondary CD8^{+} T cell responses in the absence of autophagy

An unexpected finding was that T-Atg7$^{-/-}$ mice could survive and mount a recall response, albeit severely reduced, to heterologous influenza challenge. This suggests that some CD8^{+} T_{mem} cells must survive at very low frequency in T-Atg7$^{-/-}$ mice, below the detection limit of the tetramer, and retain normal proliferative potential. That being said, the secondary CD8^{+} T_{eff} response was vastly diminished in intensity on what was observed in wildtype mice. CD4^{+} T cells are important for the efficient recall response of CD8^{+} T cells [336] [338] and it was hypothesised that dysfunctional CD4^{+} T cell help might explain the diminished recall response to infection in T-Atg7$^{-/-}$ mice. In bone marrow chimera mice, where *Atg7*$^{-/-}$ CD8^{+} T cells were in the presence of wildtype CD4^{+} T cells, the ability of CD8^{+} T cells to expand to secondary infection was similar to that of wildtype cells, verifying the notion of defective CD4^{+} T cell help in T-Atg7$^{-/-}$ mice. These results are confused somewhat by the finding that T-Atg7$^{-/-}$ mice could mount recall responses to an inflationary epitope during secondary MCMV infection. This is the first time secondary CD8^{+} T

cell responses to MCMV infection have been reported and therefore pre-existing knowledge is scarce. However, recall responses to atypical inflationary epitopes might be CD4-independent, explaining why T-Atg7$^{-/-}$ mice could mount secondary responses in this instance but not to epitopes that drive classical T cell responses.

6.5. Falling autophagy levels are linked to poor CD8$^+$ T cell responses in old age

In recent years, autophagy has become heavily implicated in the ageing process in lower organisms [409] [408] [410] [411]. More recently however, targeting of the autophagy pathway in mice was found to have a significant impact on longevity. With a link to CD8$^+$ T_{mem} formation now also established, it was posited that falling autophagy levels in T cells during ageing might account for the poor T cell responses often observed in the elderly [438] [520] [521] [522] [523]. Diminished CD8$^+$ T cell responses in old age was confirmed here using infection and vaccination models, where the CD8$^+$ T cell kinetics of aged mice were shown to be delayed and decreased in intensity relative to young mice. Following MCMV infection and influenza vaccination, the frequency of CD8$^+$ T_{mem} cells was also significantly reduced in aged mice compared to young controls. Both polyclonal and antigen-specific CD8$^+$ T cells from aged mice displayed significantly reduced autophagy levels compared to the same cells in young controls, supporting similar observations in T cells from aged humans [13]. It is not clear what drives this decline in autophagic activity with age, be it epigenetic changes at autophagy gene loci, or changes in extrinsic factors that regulate autophagy levels such as decreased growth factor concentration and signalling. One contributing factor could be the

accumulation of lipofuscin in lysosomes that might prevent their fusion with autophagosomes [543] [544]. Low autophagy levels are likely to impact on $CD8^+$ T_{mem} formation through inefficient mitophagy, resulting in an increase in damaged mitochondria and ROS production, and perhaps also by reducing metabolic flexibility. Diminished autophagy levels might lower the amount of free fatty acids available to fuel FAO during contraction, leading to greater energetic instability and consequently fewer cells entering the memory compartment. Importantly, when aged mice were treated with the autophagy-inducing compound spermidine, the $CD8^+$ T cell response to both vaccination and infection was dramatically improved. Spermidine could also enhance the $CD8^+$ T cell response to vaccination of young mice suggesting its potential as a novel immunomodulatory compound. These results link autophagy to the age-related decline in T cell function for the first time and demonstrate that targeting of this pathway is a way to restore immune responses to vaccination in the elderly.

6.6. In summation

It was only around the turn of the century that work unlocked a role for autophagy in the immune system. Since then, autophagy has become a ubiquitous feature in innate immunity, regulating intracellular bacteria removal, cytokine production, and neutrophil extracellular trap formation to name but a few. Until last year, its scope in adaptive immunity had remained limited to a handful of studies on naïve T cell homeostasis and B cell development. However, the work presented here means autophagy can now be recognised as a crucial cog in the formation of long-lasting immunity.

Lymphocytes appear to be exquisitely sensitive to the loss of autophagy, especially in the case of T cells where both naïve and activated antigen-specific T cells are lost in large number when autophagy is absent. Why autophagy is so important in T cells is still not clear, although its role in regulating mitochondrial homeostasis is sure to play a large part. Compared to other cell types, T cells boast a relatively small cytoplasm. Therefore, the need for strict regulation of cytosolic components is likely to be more apparent in these cells. Similarly, how autophagy is linked to immune metabolism is also currently beyond our grasp, but surely for not much longer. Will the findings in this thesis be extended to show a role for autophagy in regulating metabolic switches in other cells, such as during cancer?

The results presented here open the door to the prospect of T cell modulation through autophagy. In hindsight, the observation that the autophagy inducer rapamycin can improve memory T cell responses might have provided first proof of this, but nevertheless that principle has been confirmed here where spermidine was shown to improve vaccine T cell responses in an autophagy-dependent manner. Immuno-modulators with the ability to improve T cell responses are limited, but their need are becoming ever more important as we realise the power of T cells in fighting cancer and prominent infections such as malaria, HIV and pandemic influenza. Compounds like spermidine might therefore have important uses in immunotherapy as well as vaccination.

Polyamine biology to date has been vastly over-looked in the context of T cells. How T cells utilise polyamines following activation will be the source of

future investigation. The finding that the polyamine synthesiser ornithine decarboxylase is dynamically regulated following T cell activation suggests intrinsic polyamine production might be used to drive clonal expansion during infection. A more interesting idea is that polyamines are secreted by APCs during T cell contact, thus providing a crucial extra signal to T cells during activation that supports proliferation. The role of eIF5a is also intriguing in this respect. Do polyamines regulate T cell responses by mediating the hypusination and subsequent activation of eIF5a? As a translation initiation factor, might eIF5A then drive the translation of transcripts important for T cell activation, proliferation and differentiation?

Finally, the finding that autophagy levels diminish over time in antigen-specific $CD8^+$ T cells provides a cell-intrinsic explanation for reduced T cell memory in old age, something that the field of immunosenescence had been lacking. However, why the immune system, and indeed the organism as a whole, ages at all remains a mystery. Is ageing merely the by-product of damage accumulation in the genome and proteome, like a mechanical machine suffering from overuse and lack of repair? Or is there a selective pressure driving the ageing process? This is somewhat paradoxical, as many would agree that most age-related phenomena are deleterious to the organism. A selective pressure driving the organism to a less fit state is counter-intuitive to the principles of evolution. Or is it? Death may be an important trait in limiting the generation span. This is key, as species with short generation spans are more likely to adapt to changes in the environment. Therefore, ageing ensures death at the appropriate time, which ironically maintains fitness at the level of species. How does this bear out in the

immune system? What parts of this system are programmed to degrade, if any? TCR repertoire restriction has a heavy impact in old age in terms of lessoning the ability of the individual to respond to new infections, and this is certainly shaped by the consequence of responding to many scores of infections over the course of a lifetime. But repertoire contraction is also heavily influenced by thymic involution, which is possibly a genetically controlled event. The cumulative affect of infections over a lifetime also does not explain why macrophages and DCs age, which bear no memory of previous battles. This is where autophagy might come in to play. Our lab has shown that autophagy levels fall in T cells, macrophages and neutrophils during ageing. Thus, if the hypothesis presented here is correct, that the journey towards death is an active event under selective pressure and not a passive by-product of life, the immune system might utilise falling autophagy levels to drive the onset of immunosenescence. The falling autophagy levels observed in other tissues therefore, might also be the act of switching on the ageing process. This leads to the problematic issue of how falling autophagy levels are programmed. The answer to this question probably lies in the activity of mTOR and insulin-like growth factor signalling, both of which become dysfunctional in old age, but here the danger is falling into a reductionist loophole.

This study shows how it might be possible to limit the onset and effects of immune ageing by increasing autophagy levels pharmacologically. With efficacy of some vaccines as low as 10% in the over 65 population, coupled to the rising prevalence of elderly demographics, it is essential that we find ways to improve immunity in older adults. Similarly, targeting of

autophagy in other tissues (perhaps through spermidine) might also alleviate age-related diseases such as Alzheimer's and Parkinson's and extend healthy life-span as well as chronological longevity.

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