

Definition of a Murine CD8<sup>+</sup> MHCII-Recognizing Effector T Cell Population

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Thesis submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the Department of  
Immunology in the Graduate School  
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2019

ABSTRACT

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## Abstract

CD4<sup>+</sup> and CD8<sup>+</sup> T cells are dichotomous lineages in adaptive immunity. While conventionally viewed as distinct fates that are fixed post-thymic development, accumulating evidence indicates that these two populations can exhibit significant lineage plasticity, particularly upon TCR-mediated activation. In this work, I define a novel murine CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing population generated from effector CD4<sup>+</sup> T cells. Effector CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells downregulate expression of T helper cell-associated costimulatory molecules and increase expression of cytotoxic T lymphocyte-associated cytotoxic molecules. TCR $\beta$  repertoire sequencing and *in vivo* genetic lineage tracing in acutely-infected wild-type mice demonstrate fundamental lineage reprogramming resulting in an “ex-CD4” T cell phenotype, rather than expansion of an aberrantly-developed CD8<sup>+</sup> MHCII-restricted population. Impairing autophagy by functional deletion of the initiating kinase Vps34 or a downstream enzyme Atg7 dramatically enhances the generation of effector CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells. These findings suggest that effector CD4<sup>+</sup> T cells can exhibit a previously unreported degree of skewing towards the CD8<sup>+</sup> T cell lineage, which may point towards a novel direction for vaccine design, particularly against HIV.

*To the One who created everything I have discovered*

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## Acknowledgements

The following trainees/investigators made significant contributions to this work: Ming Zheng<sup>1</sup>, Qingshan Ni<sup>2</sup>, Siqi Liu<sup>3</sup>, Liang Chen<sup>3</sup>, Baojun Zhang<sup>3</sup>, Jian Guo<sup>3</sup>, Yuan Zhuang<sup>3</sup>, You-Wen He<sup>3</sup>, Ping Zhu<sup>1</sup>, Ying Wan<sup>2</sup>, Qi-Jing Li<sup>3</sup>. (Affiliations: <sup>1</sup> National Translational Science Center for Molecular Medicine, Xi'An, China; <sup>2</sup> Biomedical Analysis Center, Third Military Medical University, Chongqing, China; <sup>3</sup> Department of Immunology, Duke University Medical Center, Durham, NC, USA.)

M.Z. and Q.N. analyzed TCR repertoire data. S.L. performed murine experiments and prepared heatmap figures. L.C. generated murine strains. B.Z. provided expertise on *in vivo* murine experiments and generated murine strains. J.G. generated and maintained murine strains. Y.Z. provided murine strains and expertise on murine experiments. Y.-W.H. provided expertise on Vps34/Atg7 murine studies and murine strains. P.Z. and Y.W. provided TCR sequencing equipment and administrative oversight. Q.-J.L. provided experiment guidance, provided experiment reagents/equipment, provided administrative oversight and revised manuscript.

I thank the NIH Tetramer Core Facility for providing tetramer reagents. I thank Dr. Regina Lin for assistance with qPCR experiments, Jose Sevilla for assistance with mouse husbandry and Dr. Marthony Robins for helpful comments on my manuscript. I thank Dr. Fan Wang for mice and expertise on Vps34 studies.

I also thank those who have supported me personally during this thesis work. I thank my family, who has always encouraged me to pursue excellence. I thank my friends and mentors—near and far, old and young—who have supported me wholeheartedly and have kept me focused on the things that really matter. Finally, I thank my husband, Dr. Marthony Robins, for love and devotion that continually amaze me, and for making me a better woman simply by being himself.

Funding for this investigation was provided by a Duke University Center for AIDS Research (CFAR) Small Grant (Q.-J. L.), National Institutes of Health (NIH) Training Grants (2 T32 AI 052077-11 and 5 T32 AI 052077-09) (E.W.) and an American Association of Immunologists (AAI) Careers in Immunology Fellowship (Q.-J. L. and E.W.).

I have no competing financial or personal interests to disclose.

# 1. Introduction

## 1.1 *The immune system: The military of mice, monkeys and men*

The immune system is an essential factor in the battle for survival among the living organisms in any given ecosystem. It is a coordinated network of physical barriers (e.g., murine fur, plant cell wall), chemicals (e.g., lysozyme in human tears, penicillin secreted by *Penicillium* mold) and specialized cells (e.g., mammalian neutrophils and T cells), which protect the host organism from invasion and infection by foreign bodies, pathogenic organisms and aberrant host cells <sup>1</sup>. Immune mechanisms exist in organisms ranging from fungi to plants to mice to humans, with increasing complexity. Yet, there are remarkable commonalities in immune systems across species, largely due to similar genetic coding of system components <sup>2</sup>. Therefore, in seeking to uncover the molecular and cellular underpinnings of the human immune system, current experimental approaches in Immunology center around murine as well as primate animal models <sup>3</sup>, in addition to *in vitro* analyses of human cells <sup>3</sup> and patient clinical trials <sup>4</sup>.

## 1.2 *Innate vs. adaptive immunity*

A main point of divergence in immune systems is the presence of innate immunity versus adaptive immunity. Innate immunity is present in all organisms, and consists of mechanisms with either continuous activity or rapid response times (i.e., seconds to minutes), which defend against a broad spectrum of pathogens <sup>5</sup>. Meanwhile, adaptive immunity is present in jawed fish and more complex vertebrates. Its mechanisms have

slower response times than innate mechanisms (i.e., days to weeks), but they provide protection that is highly pathogen-specific and exhibit a capacity for memory (i.e., mechanisms act more robustly upon subsequent encounters with the same pathogen) <sup>1, 6</sup>. Therefore, for the purposes of elucidating how to promote long-lasting protection against specific pathogens in humans, adaptive immunity is often the focus of study.

### **1.3 CD4 and CD8 T cells**

The success of any military organization is highly dependent on the precise coordination of multiple units with distinct functions. This is certainly true of the immune system. T cells are a key unit of the adaptive immune system; in fact, different T cell types (i.e., compartments) correspondingly execute different defense functions. Adaptive immune responses are predominantly carried out by  $\alpha\beta$  T cells, so-called due to the expression of a T cell receptor (TCR) on their surface that contains  $\alpha$  and  $\beta$  peptide chains within a hexameric receptor complex (Fig. 1) <sup>8</sup>. Each individual T cell (i.e., clone) bears an identifiable TCR (i.e., clonotype), generated during thymic development by multiple genetic/epigenetic events <sup>9</sup> and selected for appropriate recognition of host molecules <sup>10</sup>. These clonotypes can be defined and enumerated using TCR sequencing technology, allowing us track and count individual T cells during the course of an immune response <sup>11</sup>.

In turn,  $\alpha\beta$  T cells are canonically separated into 2 compartments—CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. CD4<sup>+</sup> and CD8<sup>+</sup> T cells are so named due to their expression of the CD4 or CD8 $\alpha/\beta$  molecules, respectively, on their cell surface <sup>1</sup>. These co-receptors mediate

recognition of 2 different classes of molecules known as Major Histocompatibility Complex (MHC): MHC Class I (MHCI), which is recognized by CD8<sup>+</sup> T cells, and MHC Class II (MHCII), which is recognized by CD4<sup>+</sup> T cells (Fig. 1) <sup>12</sup>. These components work in concert to allow a T cell to detect and respond to pathogen. Specifically, the TCR recognizes a pathogen-derived peptide fragment (i.e., antigen), bound to either MHCI or MHCII, and presented on the surface of an antigen-presenting cell (APC), such as a dendritic cell <sup>13</sup>. CD4 or CD8 binds to this peptide-MHC complex (pMHC) to increase the avidity of the TCR-pMHC interaction, which in turn promotes intracellular signaling that activates the T cell <sup>12</sup>.

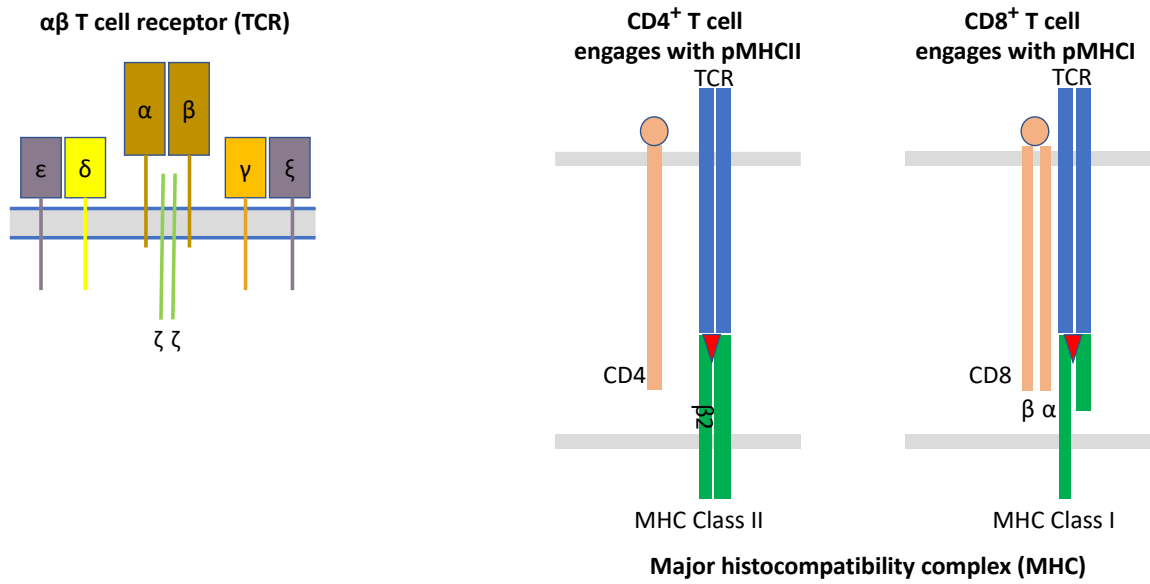


Figure 1.  $\alpha\beta$  T cell receptor and CD4/CD8 engagement with pMHC.

## 1.4 Thymic development of $\alpha\beta$ T cells

The development of  $\alpha\beta$  T cells into functional adaptive immune cells <sup>14</sup>, as well as the divergence of the CD4<sup>+</sup> and CD8<sup>+</sup> lineages <sup>15, 16, 17</sup>, occurs in the thymus.  $\alpha\beta$  T cells begin in the thymic cortex as CD4<sup>-</sup>CD8<sup>-</sup> (“double negative”, DN) thymic precursors (Fig. 2). DN thymocytes undergo 4 sub-stages of development (DN1-DN4), characterized by a series of recombination events at the locus encoding TCR $\beta$  <sup>18</sup>. These recombination events lead to the production of a pre-TCR that is composed of a unique TCR $\beta$  chain paired with a precursor TCR $\alpha$  (pre-T $\alpha$ ) chain <sup>19</sup>. T cells that successfully form a pre-TCR transition to the CD4<sup>+</sup>CD8<sup>+</sup> (“double-positive”, DP) stage. During this transition, thymocytes migrate towards the thymic medulla, proliferate, begin to express CD4 and CD8, and recombine the TCR $\alpha$  locus <sup>18</sup> to express unique TCR $\alpha$  chains that replace pre-T $\alpha$ . DP thymocytes then undergo a process of selection based on their interaction with thymic cortical epithelial cells that express MHCI and MHCII molecules presenting a variety of self-peptides <sup>10</sup>. Thymocytes with TCRs that trigger an appropriate level of TCR signaling upon engagement of self peptide-MHC are selected to proceed to the next stage of development. DP thymocytes that appropriately signal upon interaction with MHCI become committed to the CD8<sup>+</sup> MHCI-restricted T cell lineage, while DP thymocytes that appropriately signal upon interaction with MHCII become committed to the CD4<sup>+</sup> MHCII-restricted T cell lineage <sup>15, 16, 17</sup>. CD8<sup>+</sup>-committed thymocytes then downregulate expression of CD4 to become CD4<sup>-</sup>CD8<sup>+</sup>, and CD4<sup>+</sup>-committed thymocytes downregulate expression of CD8 to become CD4<sup>+</sup>CD8<sup>-</sup>. Finally, CD4<sup>+</sup> and CD8<sup>+</sup> single-positive (CD4 SP/CD8 SP) thymocytes

exit the thymus as mature T cells, which migrate to secondary lymphoid organs (e.g., lymph nodes and spleen).

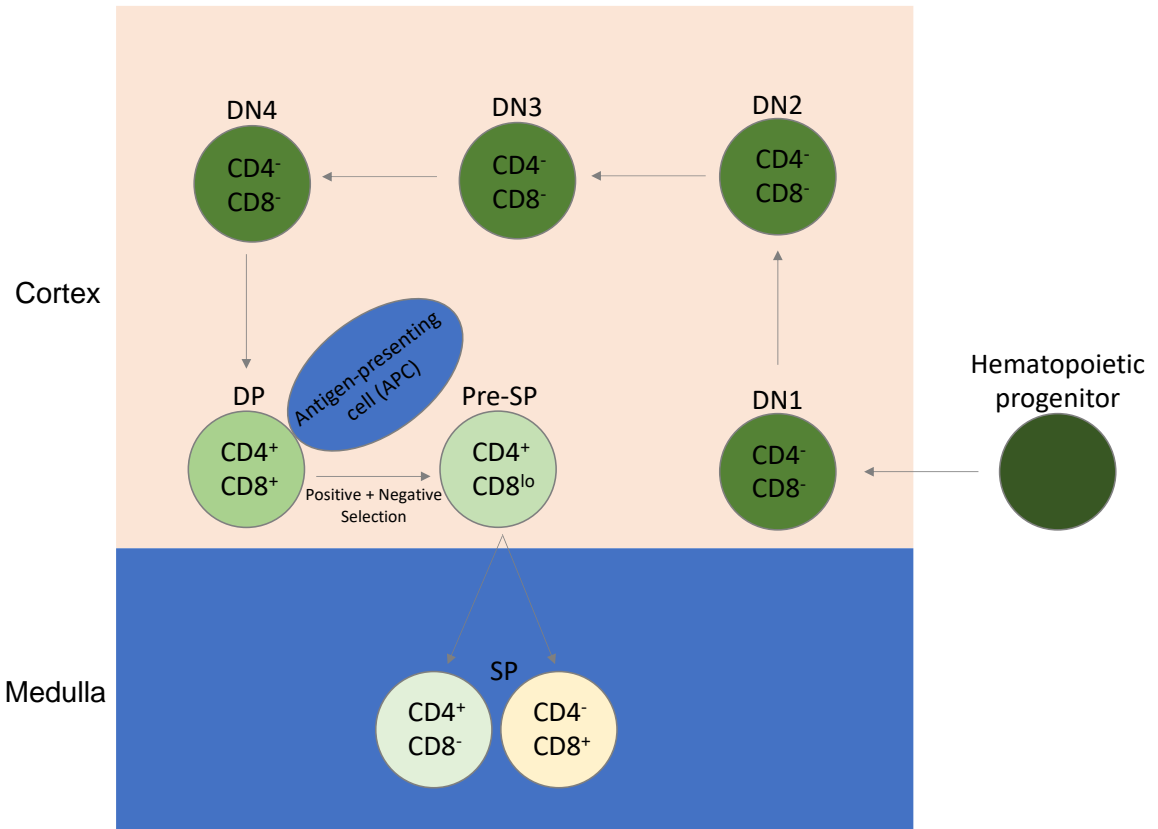


Figure 2.  $\alpha\beta$  T cell development in the thymus.

## **1.5 Multifactor “control” of CD4<sup>+</sup>/CD8<sup>+</sup> T cell lineage commitment**

As our understanding of  $\alpha\beta$  T cell development has progressed, it has become clear that the tandem process of CD4<sup>+</sup>/CD8<sup>+</sup> T cell lineage commitment is nuanced. Rather than converting directly from the DP to SP stage, it appears that  $\alpha\beta$  T cells pass through an intermediate CD4<sup>+</sup>CD8<sup>lo</sup> stage, during which DP thymocytes undergo a progressive decrease in CD8 transcription, and in turn, CD8 surface expression (Fig. 2) <sup>15</sup>. CD4<sup>+</sup>CD8<sup>lo</sup> thymocytes must then (1) either completely shut down CD8 expression and become CD4 SP, or (2) reopen the CD8 locus and shut down the CD4 locus to become CD8 SP. In essence, thymocytes in this stage are “undecided” in their lineage commitment, and must receive crucial cues for correct lineage divergence.

One of these cues is TCR signaling itself. To date, the role of TCR signaling in  $\alpha\beta$  T cell lineage commitment is most accurately described by the kinetic signaling model <sup>22</sup>. According to this model, a sustained TCR signal leads to CD4<sup>+</sup> lineage commitment, while a truncated TCR signal leads to CD8<sup>+</sup> commitment (Fig. 3). On the other hand, the expression level of CD8 itself during the DP to SP transition can determine the persistence of TCR signaling, depending on how much a given TCR depends on CD8 for binding to pMHC. As result, thymocytes may express a range of CD4/CD8 surface phenotypes immediately before commitment, yet simultaneously retain lineage bipotential.

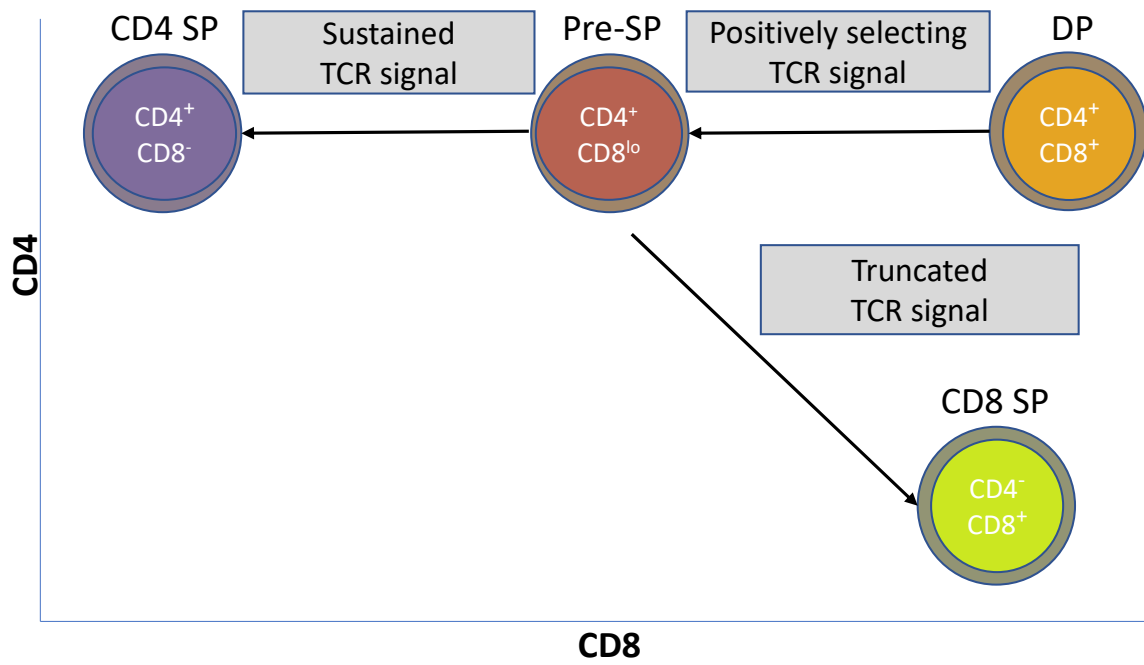
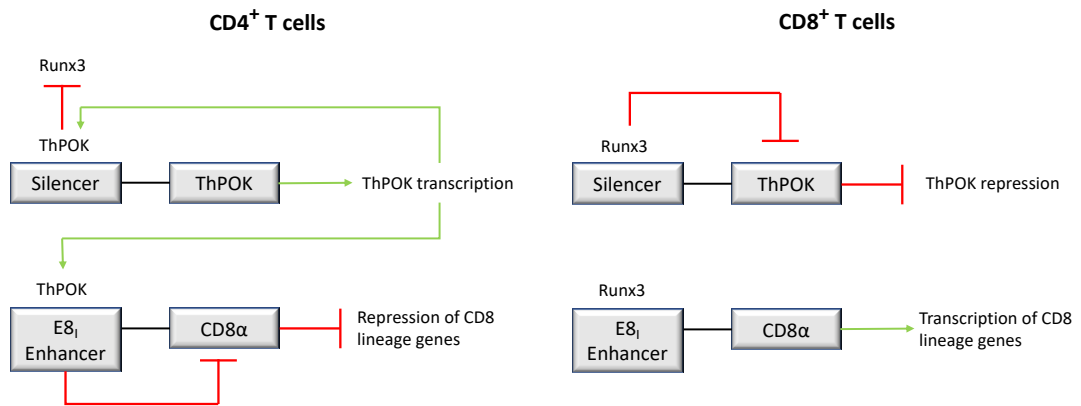
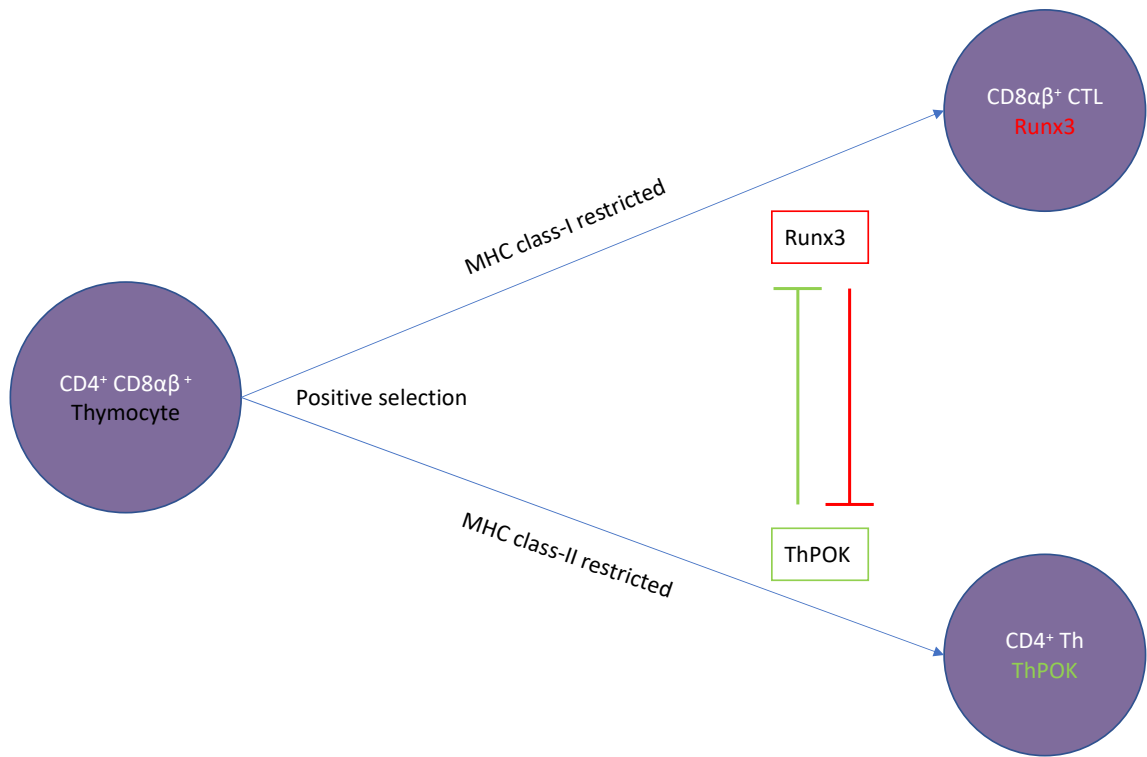


Figure 3. Kinetic signaling model of CD4<sup>+</sup>/CD8<sup>+</sup> T cell lineage commitment.

TCR signaling works in tandem with another cue—transcriptional programming—to determine T cell lineage commitment. Transcriptional programming is coordinated by a number of transcription factors and epigenetic modifiers. However, it is centrally controlled by the master transcription factors ThPOK and Runx3. ThPOK and Runx3 act in an opposing fashion: ThPOK<sup>hi</sup>Runx3<sup>lo</sup> expression sets the CD4<sup>+</sup> transcriptional program, while ThPOK<sup>lo</sup>Runx3<sup>hi</sup> expression sets the CD8<sup>+</sup> transcriptional program (Fig. 4) <sup>23</sup>. Specifically, ThPOK binds to and suppresses the silencer element contained by the ThPOK locus (Figs. 4-5) <sup>24</sup>. As a result, ThPOK sustains its own expression, which allows ThPOK to facilitate the expression of CD4<sup>+</sup> lineage genes (e.g., S4 silencer of CD4 locus (Fig. 5) <sup>25</sup>) and to suppress the expression of CD8 lineage genes (e.g., E8<sub>i</sub> enhancer of CD8 $\alpha$  locus (Fig. 5) <sup>26</sup>). In contrast, Runx3 indirectly suppresses the expression of CD4<sup>+</sup> lineage genes by binding to the ThPOK silencer element, suppressing the expression of ThPOK (Figs. 4-5) <sup>26</sup>. It also directly binds to and suppresses CD4<sup>+</sup> lineage loci (e.g., S4 silencer of CD4 locus (Fig. 5) <sup>25</sup>). On the other hand, Runx3 upregulates the expression of CD8<sup>+</sup> lineage genes by directly binding to their loci (e.g., E8<sub>i</sub> enhancer of CD8 $\alpha$  locus (Fig. 5) <sup>24</sup>). As a result, ThPOK and Runx3 coordinate two distinct programs of lineage-specific transcripts. For example, ThPOK promotes the expression of CD4 and costimulatory molecules (e.g., CD40 ligand (CD40L) and O<sub>x</sub>40), while Runx3 drives expression of CD8 and cytotoxic effector molecules (e.g., perforin, granzymes and interferon- $\gamma$  (IFN- $\gamma$ ) <sup>27</sup>). In addition, auxiliary regulation of lineage programming is provided by other transcription factors, including MAZR, TOX and GATA-3.<sup>25</sup>

Interestingly, transcriptional programming can override cues given by TCR signaling during T cell lineage commitment. This has been demonstrated by numerous genetic studies of ThPOK/Runx3. For example, enforced expression of ThPOK leads to the generation of both CD4<sup>+</sup> MHCI-restricted and CD4<sup>+</sup> MHCII-restricted CD4<sup>+</sup> T cells <sup>28, 29</sup>, and Runx3 deficiency produces a similar phenotype due to constitutive expression of ThPOK <sup>30,31</sup>. On the other hand, mice that lack functional ThPOK <sup>30,32</sup> or overexpress Runx3 <sup>31</sup> generate CD8<sup>+</sup> MHCII-recognizing T cells. Such evidence indicates that, while MHC restriction guides T cell lineage commitment, transcriptional control of lineage-specific gene expression plays the dominant role in deciding lineage fate. It also suggests that abnormal lineage commitment can occur under certain TCR signaling conditions and/or with certain relative expression levels of ThPOK/Runx3. However, the precise relationship between MHC restriction and transcriptional programming during thymocyte commitment is not yet fully understood. Furthermore, the probability that aberrant CD4<sup>+</sup>/MHCI and CD8<sup>+</sup>/MHCII T cell lineages can be produced under normal physiological conditions remains to be determined.



**Figure 4. Regulation of  $CD4^+/CD8^+$  lineage commitment by ThPOK and Runx3.**

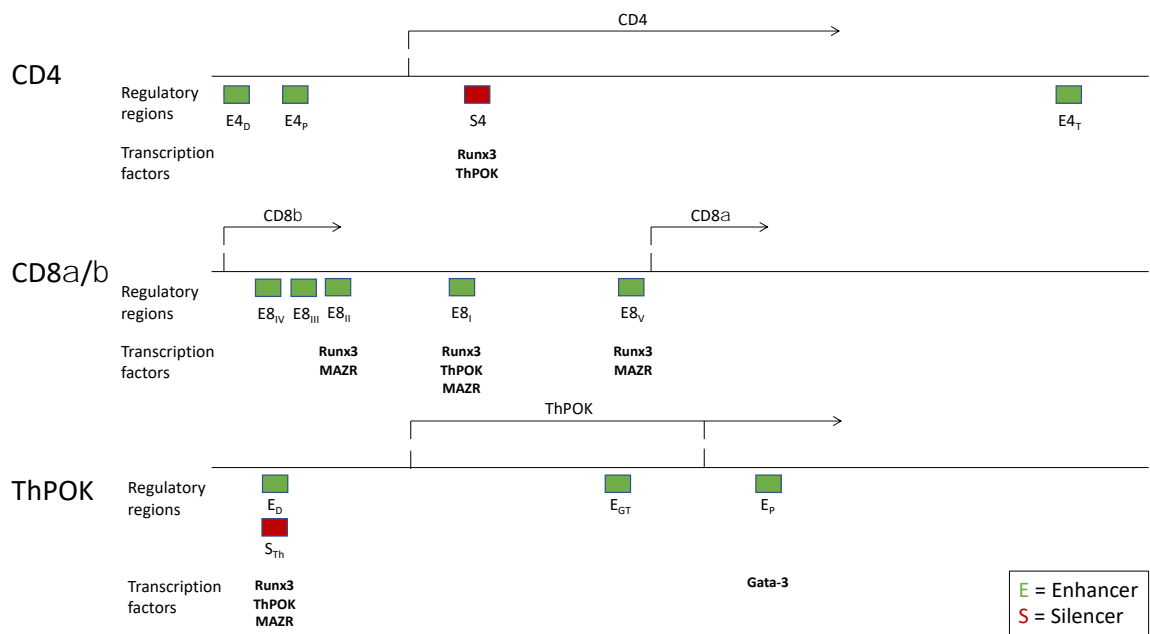


Figure 5. ThPOK/Runx3 binding sites at the CD4, CD8 $\alpha/\beta$  and ThPOK loci.

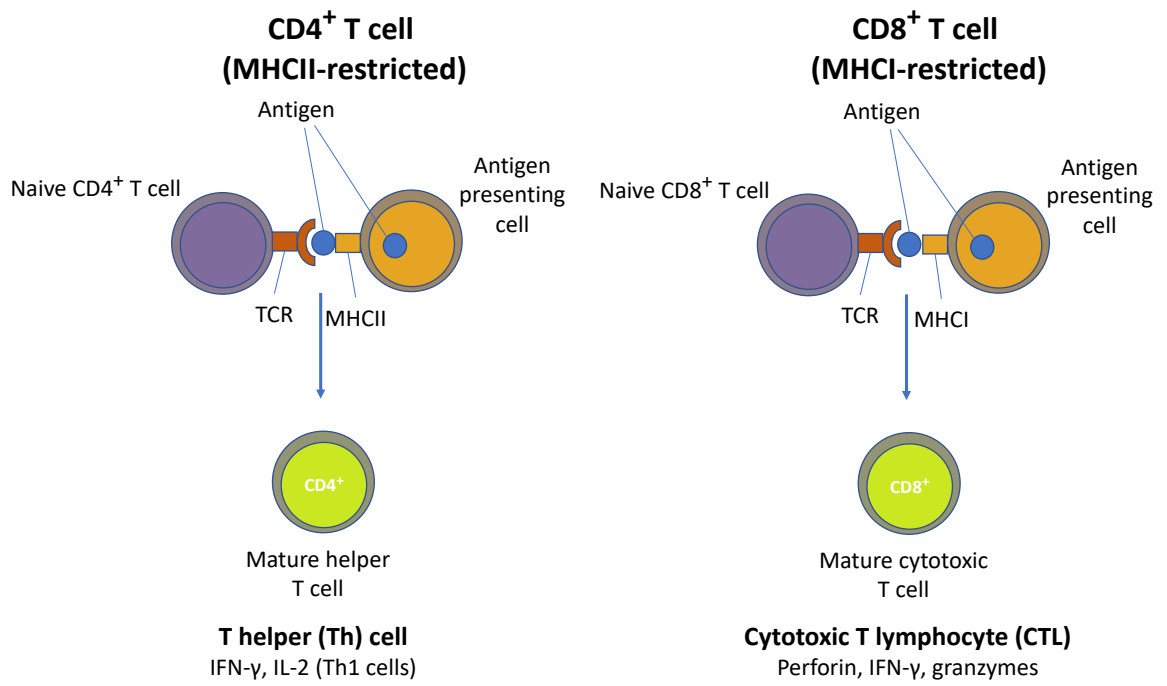
## **1.6 Antigen-induced T cell activation triggers effector differentiation**

Once mature, naïve T cells enter the peripheral immune system, the CD4<sup>+</sup> vs. CD8<sup>+</sup> distinctions in recognition capacity and molecular expression are maintained<sup>33</sup>, and they in turn catalyze the distinct functions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells upon effector differentiation<sup>34</sup>. MHC I vs. MHC II molecules display distinct classes of pathogen-derived antigen<sup>35,36</sup>; once the pMHC unit is engaged by the TCR of a naïve or memory T cell, the T cell recognizes the presence of pathogen and is activated to differentiate into an effector cell with appropriate immune function<sup>37,38</sup>. CD4<sup>+</sup> T cells facilitate the orchestration of comprehensive immune responses that are tailored to the pathogen at hand—therefore, once activated, they are known as T helper (Th) cells, of which there are distinct subtypes with distinct molecular messengers (i.e., cytokines) (Fig. 6)<sup>39</sup>. In contrast, CD8<sup>+</sup> T cells mediate cytotoxic function against host cells that are infected or cancerous, and are therefore often referred to as cytotoxic T lymphocytes (CTLs) (Fig. 6)<sup>40</sup>. Nevertheless, these lineages do not act independently of each other. Th function is required for CTL function, during both primary and memory immune responses<sup>41,42</sup>. Conversely, Th1-mediated responses require CTL activity for effective pathogen eradication<sup>40</sup>.

During effector T cell differentiation, the phenotype and function of naïve T cells undergo rapid and dramatic transformation. Upon pMHC engagement, T cells enlarge by several fold and begin to proliferate rapidly, as a result of autocrine production of the cytokine IL-2<sup>38</sup>. In addition, CD4/CD8 surface expression is upregulated—and in the case

of Th cells—other co-stimulatory molecules including CD40L and OX40<sup>39</sup>. Furthermore, TCR activation precipitates the production and release of effector molecules that correspond to each effector T cell subtype. In Th1 cells, for example, which combat intracellular pathogens and malignant host cells, IFN- $\gamma$  production increases, which facilitates CTL function (Fig. 6)<sup>39</sup>. On the other hand, CTLs express hallmark cytotoxic molecules, including perforin, IFN- $\gamma$  and granzyme B, which allow for penetration and termination of infected cells (Fig. 6)<sup>40</sup>.

In order for T cells to acquire full effector function, complete transcriptional reprogramming must occur. In naïve T cells, most of the genes that encode effector molecules are epigenetically closed, preventing access by the transcriptional machinery<sup>37, 43, 44</sup>. Upon transition to effector status, however, these effector loci must open, allowing effector gene expression to be upregulated. The expression and function of ThPOK and Runx3 play a crucial role in coordinating this transition in gene expression programming<sup>45</sup>. As a result, multiple populations of antigen-specific effector T cells arise, each with different clonotypes and similar effector function.



**Figure 6. Effector CD4<sup>+</sup> and CD8<sup>+</sup> T cell differentiation.**

## **1.7 CD4<sup>+</sup> T cell activation can give rise to lineage-intermediate T cells**

It is generally believed that mature, peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells are stable lineages, but in fact, effector differentiation provides another, post-thymic opportunity for lineage indecision in the lifespan of an  $\alpha\beta$  T cell. The gene expression upheaval that is necessary for conventional Th differentiation can produce unexpected lineage outcomes. More specifically, gene expression and function in effector CD4<sup>+</sup> T cells can shift remarkably towards a CD8<sup>+</sup>-like phenotype, resulting in observations of various CD4/CD8 lineage-intermediate phenotypes.

As an example, ThPOK expression in Th1 cells generally maintains the CD4<sup>+</sup> lineage program during the course of Th1 differentiation <sup>45</sup>. Runx3 expression, however, could be upregulated in order to promote the production of Th1 effector molecules (e.g., IFN- $\gamma$ ) <sup>47</sup>. In this context, one could imagine that the homeostatic concentration equilibrium between ThPOK and Runx3 could be disrupted by a rise in the concentration of Runx3. In turn, this could allow Runx3 to displace ThPOK at the ThPOK silencer and inhibit expression of ThPOK and the CD4<sup>+</sup> lineage program. Thus, it would be possible for a Th cell that began as a bona fide naïve CD4<sup>+</sup> T cell to shift away from the CD4<sup>+</sup> lineage program and acquire a CD8<sup>+</sup>-like lineage program.

In fact, this hypothetical scenario is actually an experimental observation <sup>47</sup>. *In vitro*-generated T helper cell type 1 (Th1) cells have been noted to upregulate Runx3 expression to become ThPOK<sup>hi</sup>Runx3<sup>hi</sup> (Fig. 7) <sup>47</sup>. Still further, it has been observed that a

sub-population of *in vitro* Th1 cells can express IFN- $\gamma$  and granzyme B, which are known as CD4<sup>+</sup> cytotoxic T lymphocytes (CD4<sup>+</sup> CTLs) (Fig. 7)<sup>48, 49, 50, 51, 52</sup>. For some time, CD4<sup>+</sup> CTLs were considered an artifact of *in vitro* Th1 differentiation. While numerous *in vivo* observations of CD4<sup>+</sup> T cells with MHCII-restricted cytotoxic activity<sup>53, 54, 55</sup> eventually debunked this assumption, this population has generally continued to be viewed as a minor variant of the Th1 lineage, with sparse study or appreciation given to its physiological relevance. However, new interest was sparked by two studies conducted by the Mucida group, which evidenced unique lineage re-programming in a subset of CD4<sup>+</sup> CTLs. In Rag 1-deficient, lymphodepleted host mice, adoptively-transferred CD4<sup>+</sup>ThPOK<sup>hi</sup> T cells gave rise to a population in the intestinal mucosa that was CD4<sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup>, downregulated ThPOK expression and upregulated expression of granzyme B, IFN- $\gamma$  and other cytotoxic molecules (Fig. 7).<sup>56</sup> Unlike Th1 cells, this population exhibited a ThPOK<sup>lo</sup>Runx3<sup>hi</sup> signature. On the other hand, unlike in CD8<sup>+</sup> CTLs, downregulated ThPOK expression in CD4<sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> T cells resulted from antigen engagement of mature CD4<sup>+</sup> T cells rather than thymic development of CD8<sup>+</sup> T cell precursors. In a subsequent study, the Mucida group found that a ThPOK<sup>lo</sup>Runx3<sup>hi</sup> signature in these intestinal CD4<sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> T cells inhibited their potential to become colitis-inducing Th17 cells<sup>57</sup>. Therefore, these two studies combined indicated that activation of conventional CD4<sup>+</sup> T cells could produce a hybrid lineage of CD4<sup>+</sup> CTLs defined by a unique combination of surface marker and functional identity, as well as transcriptional programming, with the potential to mitigate systemic disease. Another group

subsequently reported that loss of functional histone deacetylase (HDAC) 1 or 2 promotes the generation of CD4<sup>+</sup> T cell-derived CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> effector T cells, demonstrating that conventional CD4<sup>+</sup> T cells could become still more like conventional CD8<sup>+</sup> CTLs than previously thought, in a manner dependent on epigenetic regulation of lineage-specific gene expression (Fig. 7).<sup>58</sup> Paralleling these findings, lineage-intermediate populations originating from effector CD4<sup>+</sup> T cells and exhibiting various CD4/CD8 and functional gene expression profiles have been reported in other animal models,<sup>52, 53, 59, 60</sup> as well as human disease contexts that include hepatocellular carcinoma, multiple sclerosis, rheumatoid arthritis, cytomegalovirus (CMV) infection and human immunodeficiency virus-1 (HIV-1, hereafter HIV) infection <sup>48, 61, 62, 63, 64, 65</sup>.

Therefore, while the physiological importance of these unconventional populations remains ambiguous, we have some clues based on the range of disease contexts in which they have been observed. Collectively, the previous studies referenced above suggest that the presence of CTL-like effector CD4<sup>+</sup> T cells corresponds with a variety of inflammatory and infectious diseases, particularly chronic viral infection. HIV infection is a particularly interesting example. A variety of CD4/CD8 phenotypes have been observed among the peripheral blood mononuclear cells (PBMCs) of HIV patients, including CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, and most recently, MHCII-recognizing CD4<sup>-</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> <sup>66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86</sup> (Fig. 7). These various, sometimes conflicting reports, indicate that correlations exist between the presence of these populations and HIV disease status. Meanwhile, rhesus macaques vaccinated against simian immunodeficiency virus

(SIV), an ortholog of HIV, have been shown to harbor a population of CD8<sup>+</sup> MHCII-recognizing T cells, which corresponds with the maintenance of robust protection against SIV infection<sup>59, 87, 88</sup>. However, the direct role of these lineage-intermediate populations in either combating or facilitating HIV infection remains unclear.

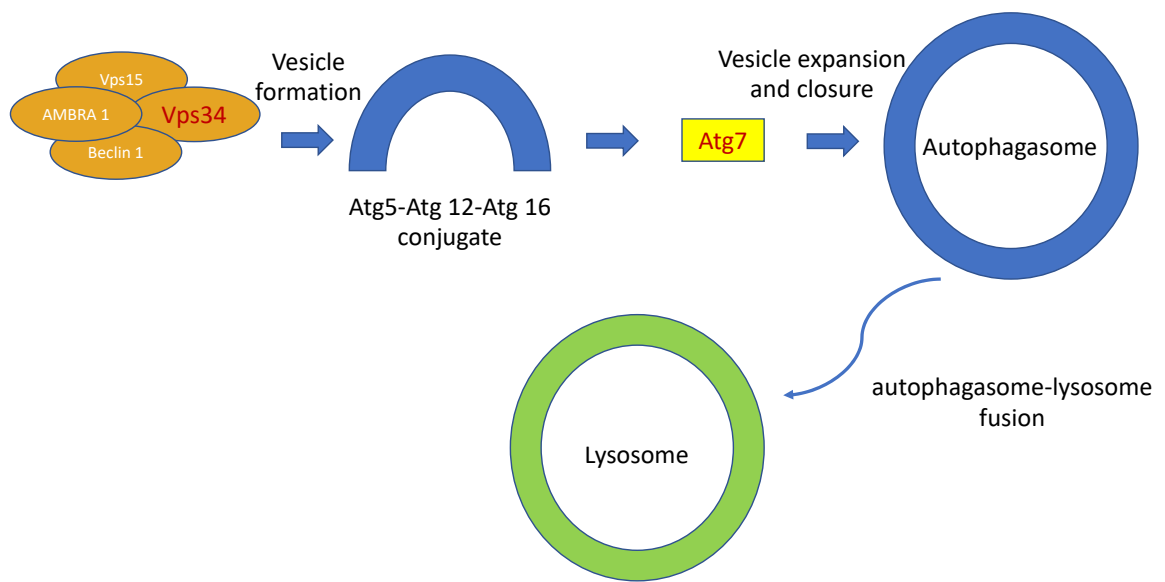
CD4 <sup>+</sup> Th1	ThPOK <sup>hi</sup> Runx3 <sup>hi</sup>
CD4 <sup>+</sup> CTL	ThPOK <sup>hi</sup> Runx3 <sup>hi</sup> IFN- $\gamma$ <sup>+</sup> granzyme B <sup>+</sup>
CD4 <sup>+</sup> IEL	CD8aa <sup>+</sup> ThPOK <sup>lo</sup> Runx3 <sup>hi</sup> IFN- $\gamma$ <sup>+</sup> granzyme B <sup>+</sup>
CD4 <sup>+</sup> CD8 <sup>+</sup>	CD8ab <sup>+</sup> ThPOK <sup>lo</sup> Runx3 <sup>hi</sup> IFN- $\gamma$ <sup>+</sup> granzyme B <sup>+</sup>
CD4 <sup>-</sup> CD8ab <sup>+</sup> MHCII-restricted	CD8ab <sup>+</sup> CD4 <sup>-</sup> IFN- $\gamma$ <sup>+</sup> granzyme B <sup>+</sup>

**Figure 7. A brief review of effector CD4<sup>+</sup> T cell-derived lineage-intermediate populations.**

Furthermore, our insight into the molecular mechanisms that facilitate or suppress the generation of lineage-intermediate effector T cells remains incomplete. Loss of ThPOK expression appears to be a lynchpin factor; reduction of ThPOK expression results in de-repression of the ThPOK silencer, in turn allowing Runx3 to bind to the silencer and further suppress expression of ThPOK.<sup>56</sup> MAZR concomitantly facilitates ThPOK suppression by activating the ThPOK silencer.<sup>89, 90</sup> Meanwhile, the ThPOK-redundant activity of the transcription factor LRF may be suppressed, leading to downregulation of CD4 expression.<sup>91</sup> On the other hand, HDAC1/2 may also be suppressed, leading to upregulation of CD8 $\alpha$ / $\beta$  expression.<sup>58</sup> As a result, the CTL transcriptional program is activated, including Runx3 and corresponding functional molecules IFN- $\gamma$ , granzymes and perforin. In fact, upregulation of Runx3 expression, and not merely downregulation of ThPOK expression, is required for the conversion of conventional effector CD4<sup>+</sup> T cell lineages to a CTL-like phenotype.<sup>57</sup> In this context, Runx3 expression is bolstered by the activity of Tbet<sup>92</sup>, and potentially, SOCS proteins<sup>93</sup> and Eomes.<sup>27</sup> The overall outcome is a shift from the CD4<sup>+</sup> lineage transcriptional program towards a CD8<sup>+</sup> CTL-like program, leading to a corresponding shift in effector lineage identity and function.

## **1.8 Autophagy regulates activated T cells**

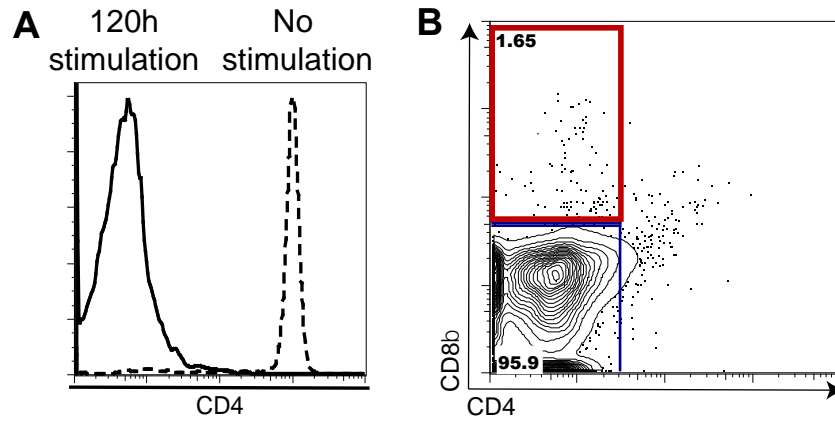
In tandem with the transcriptional reprogramming mechanisms at work during effector CD4<sup>+</sup> T cell differentiation, various homeostatic cellular processes contribute auxiliary regulation. One such process is autophagy, a catabolic survival mechanism that recycles cellular organelles and macromolecules. The major form of autophagy, known as macroautophagy, occurs by the formation of double-membrane autophagosomes, which is initiated by an enzymatic complex consisting of Beclin1, Vps15, Atg14 and Vps34 (Fig. 8)<sup>94, 95</sup>. The subsequent elongation and closure of the autophagosomal membrane to encapsulate cytosolic material is catalyzed by a series of reactions requiring the Atg proteins, including Atg7, which is the lynchpin of the membrane elongation mechanisms (Fig. 8)<sup>96, 97</sup>. Molecular cargo captured by autophagosomes are eventually recycled to the cell surface or an appropriate organelle, or sent to the lysosome for degradation (Fig. 8)<sup>98</sup>. Autophagy controls T cell effector status at multiple levels, including TCR signaling<sup>99</sup>, metabolism<sup>100, 101</sup> and memory formation<sup>102, 103</sup>. In particular, Vps34 and Atg7 have demonstrated roles in T cell organelle homeostasis<sup>104, 105</sup>, Treg function and stability<sup>101, 104</sup>, the homeostatic maintenance of naïve T cells<sup>100, 106, 107, 108</sup> and the accumulation of effector cells<sup>100, 104, 106</sup>.



**Figure 8. Molecular mechanisms of autophagy.**

## 1.9 Preliminary Data

Amidst the observations and questions I have reviewed above, my thesis project was born from an interesting, unexpected observation in 2012. I found that CD4<sup>+</sup> T cells from mice with a specific null mutation and transgenic expression of an MHCII-restricted TCR dramatically lost CD4 expression upon stimulation *in vitro* (Fig. 9A). In parallel, a small proportion of these CD4<sup>-</sup> MHCII-recognizing T cells acquired CD8 $\alpha\beta$  expression (Fig. 9B). Given that I observed this population near the time when the first report emerged of CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells in monkeys<sup>59</sup>, and a few years before a similar population was observed in humans<sup>110</sup>, I considered that the murine CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells I had observed could be a novel population of effector-derived, lineage-intermediate T cells. My investigation of this population became the basis for my thesis work.



**Figure 9. Initial observation of a murine CD4<sup>-</sup>CD8αβ<sup>+</sup> MHCII-recognizing population.** Total lymph node cells or FACS-purified CD4<sup>+</sup> T cells from mice with a null mutation and transgenic expression of an MHCII-restricted TCR were stimulated *in vitro* with cognate peptide for 120 days. (A) CD4 surface expression was almost completely abrogated, and (B) CD8αβ surface expression was upregulated in a small proportion of CD4<sup>+</sup> cells.

## **1.10 Specific Aims**

The purpose of this study was to investigate lineage plasticity in antigen-activated effector CD4<sup>+</sup> T cells, in the context of transcriptional re-programming and autophagic regulation. In this pursuit, I have discovered and defined a murine population of CD4<sup>+</sup> CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells that originates from effector CD4<sup>+</sup> T cells. The 4 specific aims of this study are outlined below, and correspond to the Results subsections in Chapter 2 as indicated:

### Specific Aim 1:

(2.3.1, 2.3.2, 2.3.5)

To define a novel population of effector CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells, based on surface molecule expression and transcriptional signature.

### Specific Aim 2:

(2.3.3)

To determine the effector CD4<sup>+</sup> T cell origin of CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells, using TCR $\beta$  sequencing and genetic lineage tracing techniques.

### Specific Aim 3:

(2.3.4)

To determine the effector function of CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells, by analyzing cytokine and co-receptor expression.

Specific Aim 4:

(2.3.6)

To determine the molecular mechanisms that underpin lineage destabilization of effector CD4<sup>+</sup> T cells towards the CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing phenotype, by investigating the role of the autophagic molecules Vps34 and Atg7 in the generation of effector CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells.

## 2. Conversion of effector CD4<sup>+</sup> T cells to a CD8<sup>+</sup> MHCII-recognizing lineage

This chapter is adapted from the following manuscript in submission:

Robins, E., Zheng, M., Ni, Q., Liu, S., Chen, L., et al. Conversion of effector CD4<sup>+</sup> T cells to a CD8<sup>+</sup> MHCII-recognizing T cell lineage. *Frontiers in Immunology*.

### 2.1 Introduction

Via the investigation described in this chapter, I have discovered and defined a murine CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing lineage that can be derived from effector CD4<sup>+</sup> T cells. Using multiple *in vitro* differentiation and *in vivo* tracing strategies, I have demonstrated that this population exhibits a CD4/CD8 lineage-intermediate phenotype, is generated from bona fide effector CD4<sup>+</sup> T cells by transcriptional reprogramming and is regulated by the key autophagy molecules Vps34 and Atg7.

### 2.2 Materials and Methods

#### 2.2.1 Mice

LLO118 $\alpha\beta$  Vps34<sup>f/f</sup> CD4-Cre mice were generated by crossing LLO118 CD4-Cre mice <sup>111, 112</sup> with Vps34<sup>f/f</sup> Lck-Cre <sup>106, 113, 114</sup> mice kindly provided by Dr. You-Wen He. LLO118 Vps34<sup>f/f</sup> or LLO118 mice were used as WT controls. Rosa26<sup>tdTomato</sup> Ox40-Cre mice were generated by crossing Rosa26<sup>tdTomato</sup> <sup>85</sup> with Ox40-Cre mice <sup>115, 116</sup> mice kindly provided by Dr. Yuan Zhuang. Thy1.1<sup>+</sup> C57BL/6 <sup>117</sup>, C57BL/6 <sup>118</sup>, OT-1 <sup>119</sup>, Pmel-1 <sup>120</sup> and Rosa26<sup>mT/mG</sup> <sup>121</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). 4-20 week-

old male and female mice were used for *in vitro* experiments, and 6-16 week-old male and female mice were used for *in vivo* experiments.

### **2.2.2 *In vitro* T cell stimulation**

For peptide-mediated stimulation, (1)  $5 \times 10^6$ - $1 \times 10^7$  total lymph node cells or (2)  $1$ - $3 \times 10^6$  FACS-sorted CD4<sup>+</sup> T cells and  $1$ - $3 \times 10^6$  CD19/B220<sup>+</sup> B cells (T:B cell ratio = 1:1) were cultured for 5 days in complete RPMI (cRPMI; RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 50  $\mu$ M beta-mercaptoethanol) in the presence of 10  $\mu$ M LLO190-205 peptide (LLO118 cells) or 1  $\mu$ g/ml SIINFEKL peptide (OT-1 cells) in a humidified incubator at 37°C/7% CO<sub>2</sub>. For antibody-mediated stimulation,  $1 \times 10^6$  FACS-sorted CD4<sup>+</sup> T cells were cultured for 5 days in cRPMI in the presence of plate-bound anti-CD3/CD28 antibody (0.1-1 $\mu$ g/ml) under Th1-skewing conditions (mouse/human recombinant IL-2 (10 ng/ml), mouse recombinant IL-12 (50 ng/ml) and anti-mouse IL-4 (11B11, 10  $\mu$ g/ml)) in a humidified incubator at 37°C/7% CO<sub>2</sub>. For cell proliferation analysis, cells were stained with Cell Trace™ Violet (0.5-5  $\mu$ M) or CFSE (0.5-10  $\mu$ M) per the manufacturer's protocol immediately prior to *in vitro* stimulation.

### **2.2.3 *In vivo* infection**

For *Listeria* studies, mice were infected with  $1 \times 10^7$  cfu *Listeria monocytogenes* by intravenous (i.v.) injection. Four days post-infection, spleen was harvested and prepared for flow cytometry analysis (tissue homogenization by mechanical disruption followed by red blood cell lysis using ACK lysis buffer). For lymphocytic choriomeningitis (LCMV) studies, mice were infected with  $1 \times 10^5$  cfu LCMV Armstrong by intraperitoneal (i.p.) injection. Seven-12 days post-infection, spleen was harvested and prepared for flow cytometry analysis. For adoptive transfer studies, naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells from total lymph nodes were FACS-sorted and transferred to recipient mice by i.v. injection ( $1 \times 10^6$  cells/mouse). Zero or 24 hours post-transfer, recipient mice were infected with  $1 \times 10^5$  cfu LCMV Armstrong by i.p. injection. Seven-8 days post-infection, spleen was harvested and prepared for flow cytometry analysis.

### **2.2.4 Flow cytometry**

For mouse cells, lymphocyte samples were suspended in FACS buffer (DPBS supplemented with 2% FBS and 2 mM EDTA). For surface marker analysis, samples were treated with Fc receptor blocking antibody (2.4G2, 0.0078 µg/µl) for 10 mins at 4°C followed by surface antibodies (1:100-500 dilution) for 15-30 mins at 4°C. For cell survival analysis, after surface staining, cells were resuspended in Annexin V binding buffer (10 mM HEPES, 140 mM sodium chloride and 2.5 mM calcium chloride in diH<sub>2</sub>O) supplemented with 2% FBS, then treated with 7-AAD Viability Staining Solution (1:40

dilution) for 15 mins at room temperature (rm temp). For dead-cell exclusion, cells were stained with Live/Dead Fixable Dead Cell Stain per the manufacturer's protocol (either before or after surface staining). For intracellular cytokine analysis, lymphocytes were stimulated for 4 hours at 37°C/7% CO<sub>2</sub> with phorbol 12, 13-dibutyrate (PDBu, 0.9 μM) and ionomycin (1 ng/μl) in the presence of brefeldin and monensin (1000X solutions, eBioscience, Inc., San Diego, CA, USA). Intracellular staining was then performed by (1) fixation (paraformaldehyde, 2%) for 20 mins at rm temp, (2) cell permeabilization (saponin, 0.1%) and Fc receptor blocking (2.4G2, 0.0078 μg/μl) for 10 mins at rm temp and (3) antibody treatment (1:100-500 dilution) for 15 mins at 4°C. All flow cytometry data was acquired using a FACSCanto II (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

## **2.2.5 Antibodies/Staining Solutions**

### **Biolegend (San Diego, CA, USA):**

#### **7AAD Viability Staining Solution**

**Annexin V:** APC

**Anti-mouse B220 (RA3-6B2):** FITC

**Anti-mouse CD4 (GK1.5):** FITC, PE, PE/Cy7, APC, Pacific Blue

**Anti-mouse CD8α (53-6.7):** FITC, PE/Cy7, APC

**Anti-mouse CD8β (YTS156.7.7):** FITC, PE, PerCP/Cy5.5

**Anti-mouse/human CD44 (IM7):** PE/Cy5, APC/Cy7

**Anti-mouse/human granzyme B (GB11):** Alexa Fluor 647®

**Anti-mouse IFN- $\gamma$  XMG1.2):** Pacific Blue

**Anti-mouse TCR $\beta$  (H57-597):** PE, PerCP/Cy5.5, APC

**Anti-mouse TCRV $\alpha$ 2 (B20.1):** APC, APC-Cy7

**Bio X Cell (West Lebanon, NH, USA):**

Anti-mouse CD16/32 (2.4G2)

**Life Technologies (Grand Island, NY, USA):**

Cell Trace™ CFSE Cell Proliferation Kit

Cell Trace™ Violet Cell Proliferation Kit

**Thermo Fisher Scientific (Waltham, MA, USA):**

**Live/Dead Fixable Dead Cell Stain:** Green, Red, Far Red, Aqua, Violet

## **2.2.6 Tetramer binding analysis**

For mouse tetramer binding analysis, cells were pre-treated with Fc receptor blocking antibody (Flow cytometry method). Cells were then stained with an LCMV-MHCII (I-A<sup>b</sup>) tetramer (LCMVgp6-20-I-A<sup>b</sup>, LCMVgp31-45-I-A<sup>b</sup>, LCMVgp66-77-I-A<sup>b</sup>, LCMVgp126-140-I-A<sup>b</sup> or LCMVnp309-328-I-A<sup>b</sup>; 6  $\mu$ g/ml; NIH Tetramer Core Facility, Atlanta, GA, USA) and control tetramer (huCLIP87-101-I-A<sup>b</sup> PE; 6  $\mu$ g/ml; NIH Tetramer Core Facility) for 1h at room temperature followed by surface staining and analyzed by flow cytometry (Flow cytometry method).

## 2.2.7 TCR $\beta$ sequencing

Splenic lymphocytes were harvested from Thy1.1<sup>+</sup> C57Bl/6 mice 7 days post-LCMV Armstrong infection (*In vivo* infection method). Cells were prepared for FACS sorting (Flow Cytometry and Tetramer binding analysis methods), and Live-Dead<sup>+</sup> huCLIP87-101- I-A<sup>b-</sup> LCMVgp66-77- I-A<sup>b+</sup> CD44<sup>hi</sup> CD4<sup>-</sup> and CD4<sup>+</sup> populations were collected (3.5 X 10<sup>3</sup>-2 X 10<sup>4</sup> cells/sample; Table 1). FACS-sorted populations were then spiked with 5 X 10<sup>2</sup> 2B4.11 <sup>122</sup> cells as a normalization control. Populations with < 2 X 10<sup>4</sup> cells were supplemented with LB27.4 <sup>123</sup> cells to bring the total cell number to 2 X 10<sup>4</sup>. Samples were then lysed and RNA was extracted using the Direct-Zol RNA Isolation Kit (Genesee Scientific, San Diego, CA, USA) per the manufacturer's protocol. RNA (total sample amount) was reverse-transcribed into single-stranded cDNA using the qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Inc., Gaithersburg, MD, USA) and 1  $\mu$ M TCR $\beta$ -specific primer (ATCTCTGCTTCTGATGGCTCA). The PCR program was as follows: 65°C (5 minutes (mins)), 25°C (5 mins), 42°C (60 mins), 70°C (5 mins). Whole-TCR $\beta$  sequencing<sup>124</sup> was performed using the Ion Torrent PGM platform (Life Technologies), and sequencing analyses were conducted using MATLAB 2013b (MathWorks, Natick, MA, USA) with manual scripts <sup>124</sup>.

### 2.2.8 qPCR

T cell samples ( $2 \times 10^4$ - $2 \times 10^6$  cells) were enriched by (1) magnetic separation using the Dynabeads Untouched Mouse CD4/CD8 Cells Kits or Dynabeads Flowcomp Mouse CD4/CD8 Kits per the manufacturer's protocol (ThermoFisher Scientific) or (2) FACS-sorting (Flow cytometry method). Cell lysis and RNA extraction were performed using the Direct-Zol RNA Isolation Kit (Genesee Scientific) or the RNAqueous-Micro Total RNA Isolation Kit (ThermoFisher Scientific) per the manufacturer's protocol. RNA (200-500 ng) was reverse-transcribed into single-stranded cDNA using oligo-dTs/random primers and the qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Inc.) per the manufacturer's protocol. qPCR was then conducted using primers designed with Vector NTI (ThermoFisher Scientific) and the SYBR Green Perfecta Supermix (Quanta Biosciences, Inc.) on a Mastercycler ep *realplex*<sup>2</sup> S real-time PCR system (Eppendorf North America, Hauppauge, NY, USA). Primer sequences are listed in Table 2. The PCR reaction was as follows: 95°C (2 mins) followed by 40-50 cycles at 95°C (15 secs), 50-70°C (30 secs) and 68°C (25 secs). Melting curve analysis was performed to confirm that primer-dimer amplification did not occur. Relative expression analysis was conducted by the delta-delta Ct method using R (The R Foundation) with manual scripts.

### **2.2.9 Statistics**

Mann-Whitney U tests were conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Pearson product-moment correlation coefficients and all statistical tests for TCR $\beta$  sequencing analyses were performed using MATLAB 2013b (MathWorks, Natick, MA, USA) with manual scripts <sup>124</sup>.

### **2.2.10 Study approval**

All husbandry and experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and protocols approved by the Duke University Institutional Animal Care and Use Committee (IACUC).

## 2.3 Results

### 2.3.1 Activation of Vps34-deficient CD4<sup>+</sup> T cells produces a CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cell population

My investigation began with an unexpected observation while studying Vps34 function during CD4<sup>+</sup> T cell activation. Vps34 is the sole Class III PI3 kinase, which converts phosphatidylinositol to phosphatidylinositol 3-phosphate (PI<sub>3</sub>P) (Fig. 10) <sup>125</sup>. Via this enzymatic activity, Vps34 catalyzes a range of vesicle trafficking processes in the cell, including endocytosis and autophagy (Fig. 10) <sup>125</sup>. In order to investigate the effect of Vps34 on effector CD4<sup>+</sup> T cell function, I generated LLO118 $\alpha\beta$  Vps34<sup>f/f</sup> CD4-Cre<sup>+</sup> (LLO Vps34<sup>KO</sup>) mice with T cell-specific deletion of functional Vps34 and transgenic expression of the LLO118 $\alpha\beta$  TCR (V $\alpha$ 2<sup>+</sup>V $\beta$ 2<sup>+</sup>). The LLO118 $\alpha\beta$  TCR mediates MHCII (I-A<sup>b</sup>)-restricted recognition of the listeriolysin O protein (LLO190-205), the major epitope of *Listeria monocytogenes* <sup>111</sup>. I found that *in vitro* LLO190-205 stimulation of either total lymph node cells or FACS-purified TCR V $\alpha$ 2<sup>+</sup>CD4<sup>+</sup> T cells resulted in the loss of CD4 surface expression (Fig. 11A). Although antigen engagement initiated CD4 downregulation with similar kinetics in both LLO118 $\alpha\beta$  Vps34<sup>f/f</sup> CD4-Cre<sup>-</sup> (LLO WT) and LLO Vps34<sup>KO</sup> TCR V $\alpha$ 2<sup>+</sup> cells, LLO Vps34<sup>KO</sup> cells failed to recover normal CD4 surface levels (Fig. 11B). CD4 protein did not accumulate intracellularly in activated LLO Vps34<sup>KO</sup> cells (Fig. 11C), indicating that downregulated CD4 surface display was not due to abrogation of Vps34-mediated intracellular vesicle trafficking <sup>125</sup>. Loss of functional Vps34 also did not enhance proliferation of CD4<sup>-</sup> cells, because CD4<sup>-</sup> and CD4<sup>+</sup> LLO Vps34<sup>KO</sup> T cells proliferated

comparably (Fig. 12). In addition, CD4<sup>-</sup> cells were markedly more susceptible to activation-induced cell death (AICD): LLO Vps34<sup>KO</sup> CD4<sup>-</sup> cells survived more poorly than either LLO WT CD4<sup>-</sup> cells or LLO Vps34<sup>KO</sup> CD4<sup>+</sup> cells (Fig. 12). Therefore, neither loss of functional Vps34 nor CD4 protein conferred a proliferative/survival advantage to T cells, making less likely the possibility that pre-existing, mature naïve/memory LLO Vps34<sup>KO</sup> CD4<sup>-</sup> T cells preferentially accumulated over CD4<sup>+</sup> T cells upon TCR activation.

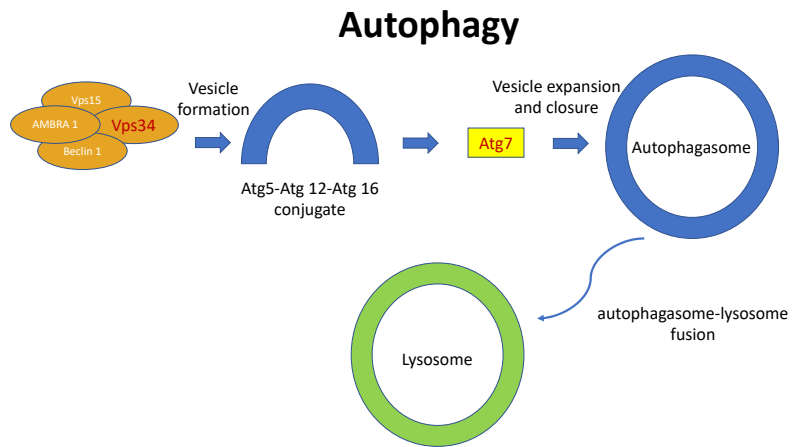
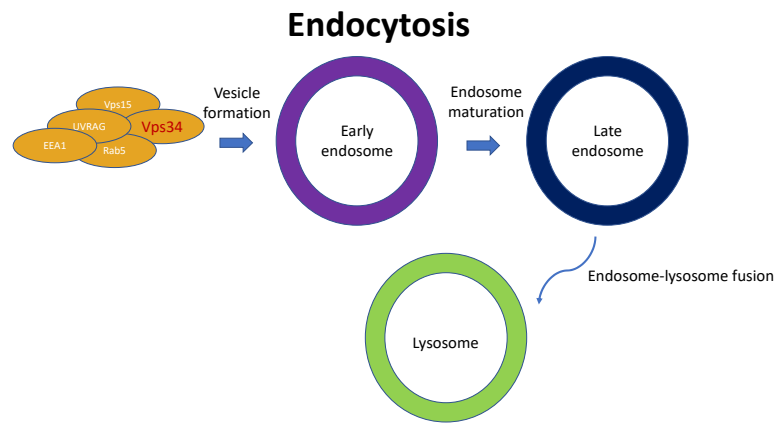
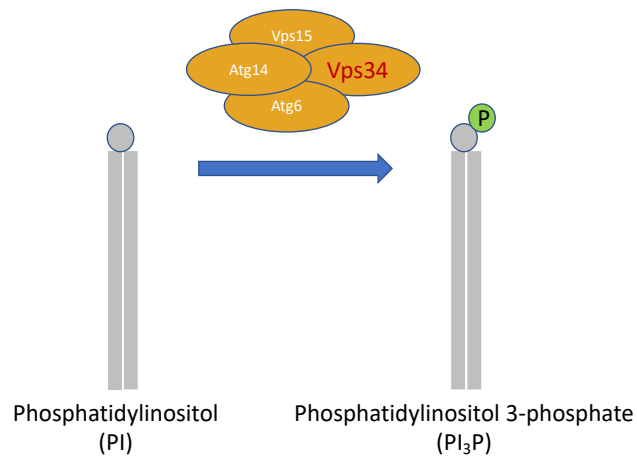
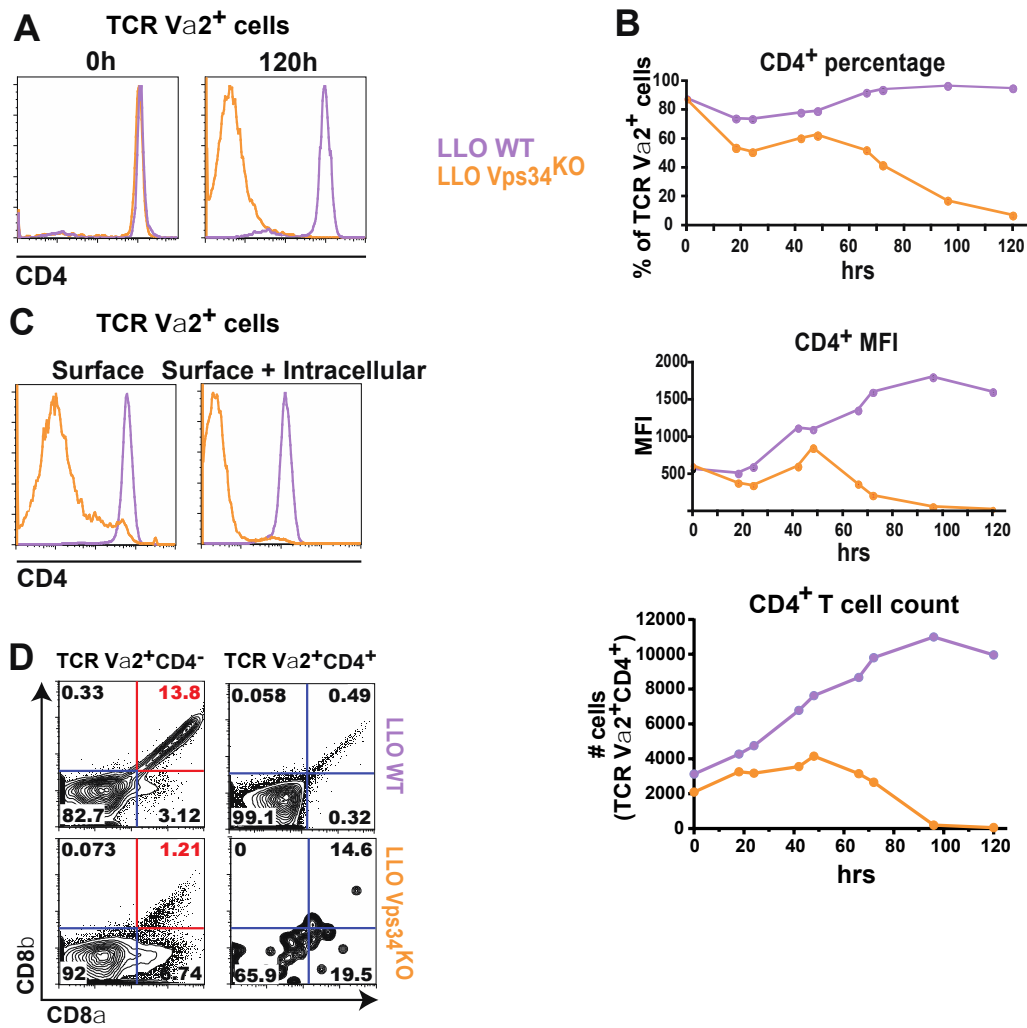
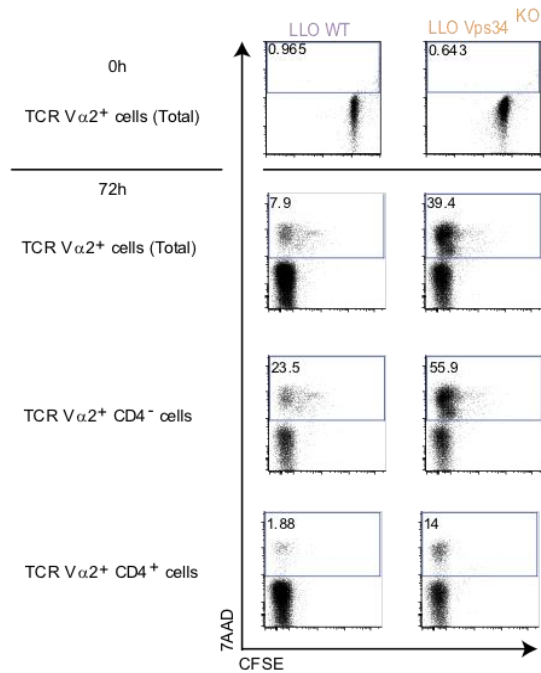


Figure 10. The role of Vps34 in vesicle trafficking.



**Figure 11. *In vitro* stimulation of LLO Vps34<sup>KO</sup> CD4<sup>+</sup> T cells produces a CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cell population.**

Total cells or FACS-sorted CD4<sup>+</sup> TCRV $\alpha$ 2<sup>+</sup> cells from the lymph nodes of LLO118 $\alpha\beta$  Vps34<sup>fl/fl</sup> CD4-Cre<sup>-</sup> (LLO WT) or LLO118 $\alpha\beta$  Vps34<sup>fl/fl</sup> CD4-Cre<sup>+</sup> (LLO Vps34<sup>KO</sup>) mice were stimulated *in vitro* with 10  $\mu$ M LLO190-205 peptide for 120 hours. Surface expression of CD4 is shown at 120 hours (A) and at 24-hour intervals from 0-120 hours (B). (C) LLO WT and LLO Vps34<sup>KO</sup> T cells were analyzed for intracellular CD4 protein expression after *in vitro* peptide stimulation for 120 hours. (D) CD8 $\alpha/\beta$  expression was analyzed in LLO WT and LLO Vps34<sup>KO</sup> T cells stimulated with LLO190-205 *in vitro* for 120 hours. Expression is shown in TCR V $\alpha$ 2<sup>+</sup> CD4<sup>-</sup> and TCR V $\alpha$ 2<sup>+</sup> CD4<sup>+</sup> cells. The CD4-CD8 $\alpha\beta$ <sup>+</sup> population is indicated in red. n = 16 from 9 independent experiments for (A), n = 3 from 3 independent experiments for (B), n = 7 from 7 independent experiments for (C) and n = 8 from 5 independent experiments for (D).



**Figure 12. Generation of LLO Vps34<sup>KO</sup> effector CD4<sup>-</sup> T cells cannot be merely attributed to defects in proliferation, survival or Vps34 function.**

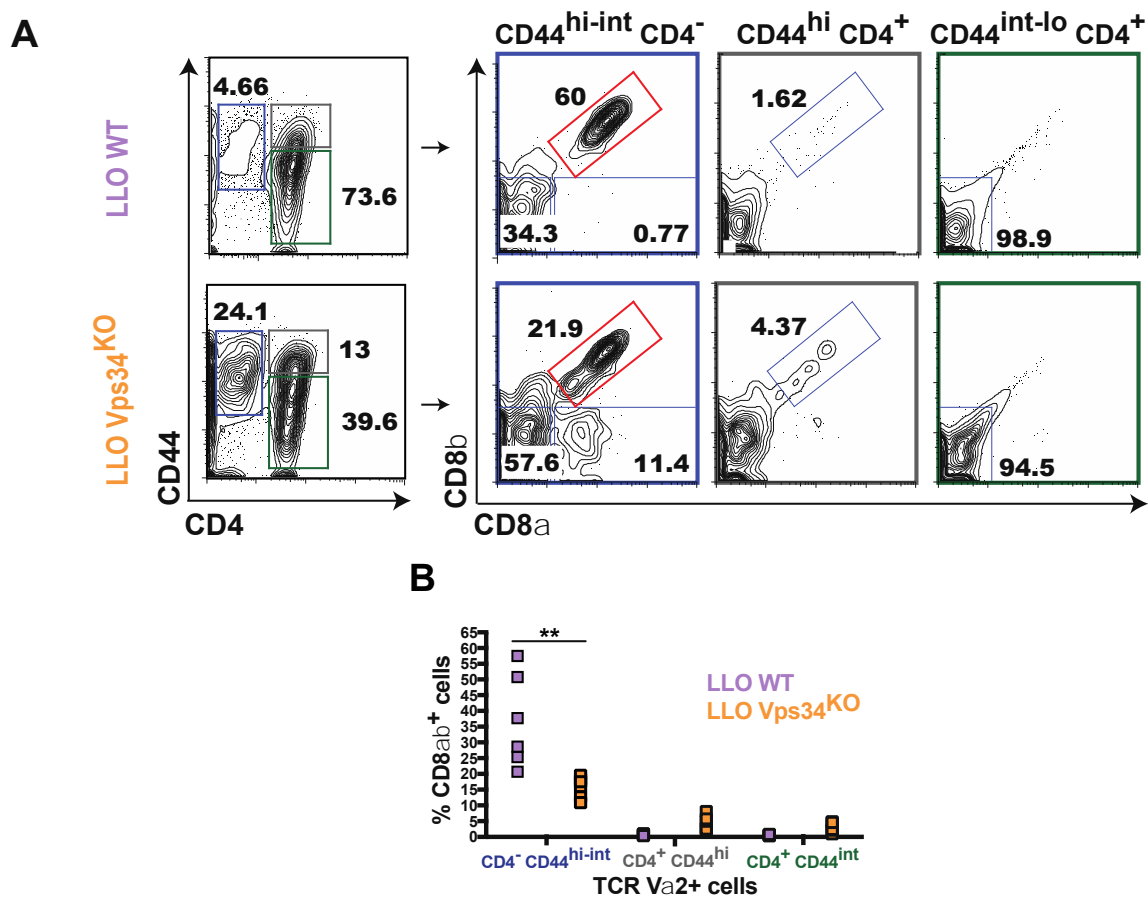
Proliferation/survival of *in vitro*-stimulated LLO Vps34<sup>KO</sup> T cells. Total lymph node cells from LLO WT or LLO Vps34<sup>KO</sup> mice were labeled with CellTrace™ Violet proliferation dye and cultured with LLO190-205 (10  $\mu$ M) for 120 hours. Cell proliferation and activation-induced cell death (AICD) of activated LLO T cells were then assessed by flow cytometry analysis of CellTrace™ Violet and 7AAD staining. n = 7 from 7 independent experiments for CFSE data and n = 9 from 9 independent experiments for 7AAD data.

Furthermore, the CD4 downregulation I found in activated LLO Vps34<sup>KO</sup> CD4<sup>+</sup> T cells was accompanied by multiple CD8 expression phenotypes (Fig. 11D). The majority of activated LLO Vps34<sup>KO</sup> TCR V $\alpha$ 2<sup>+</sup> cells were CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>-</sup>, paralleling populations previously found in both animals and humans <sup>20</sup>. I observed a smaller CD4<sup>-</sup>CD8 $\alpha\alpha$ <sup>+</sup> population, reminiscent of CD4<sup>+</sup>CD8 $\alpha\alpha$ <sup>+</sup> intraepithelial lymphocytes (IELs) associated with mucosal inflammation <sup>56</sup>, as well as a CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> population, which has been previously observed during HIV infection and other human chronic diseases <sup>68, 71, 127</sup>. Surprisingly, a CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> subset was also generated. Given that my LLO Vps34<sup>KO</sup> T cells expressed an MHCII-restricted transgenic TCR, I noted that this might be a previously unreported CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cell population in mice <sup>27, 56, 58</sup>.

In fact, I found that CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-restricted T cells could also be generated from *in vitro*-stimulated LLO WT CD4<sup>+</sup> T cells (Fig. 11D). While loss of CD4 expression occurred at a much lower frequency among activated LLO WT cells as compared to LLO Vps34<sup>KO</sup> cells, a higher proportion of TCR V $\alpha$ 2<sup>+</sup> CD4<sup>-</sup> cells expressed both CD8 $\alpha$  and CD8 $\beta$  in the LLO WT versus LLO Vps34<sup>KO</sup> subset. This observation suggested that, while Vps34 could sustain CD4 molecule expression in effector CD4<sup>+</sup> T cells, the generation of a CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cell population was not dependent on loss of Vps34 function.

### 2.3.2 CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-restricted T cells are generated *in vivo* during acute infection

To determine whether CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-restricted T cells could be generated during an *in vivo* effector response, I infected LLO WT and LLO Vps34<sup>KO</sup> mice with *Listeria monocytogenes*, their transgenic TCR cognate pathogen <sup>111</sup>. In *Listeria*-infected LLO Vps34<sup>KO</sup> mice, over 20% of splenic effector (CD44<sup>hi-int</sup>) TCR V $\alpha$ 2<sup>+</sup> T cells lost CD4 expression (Fig. 13A). Among those, I identified multiple lineage-intermediate populations, including CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells. Albeit at a much lower frequency, CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells were also identified in *Listeria*-infected LLO WT mice (Fig. 13A). Paralleling my *in vitro* observations, a higher proportion of LLO WT CD4<sup>-</sup> cells exhibited the CD8 $\alpha\beta$ <sup>+</sup> phenotype than their LLO Vps34<sup>KO</sup> counterparts (Fig. 13A and B).



**Figure 13. CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing effector T cells are generated during acute infection.**

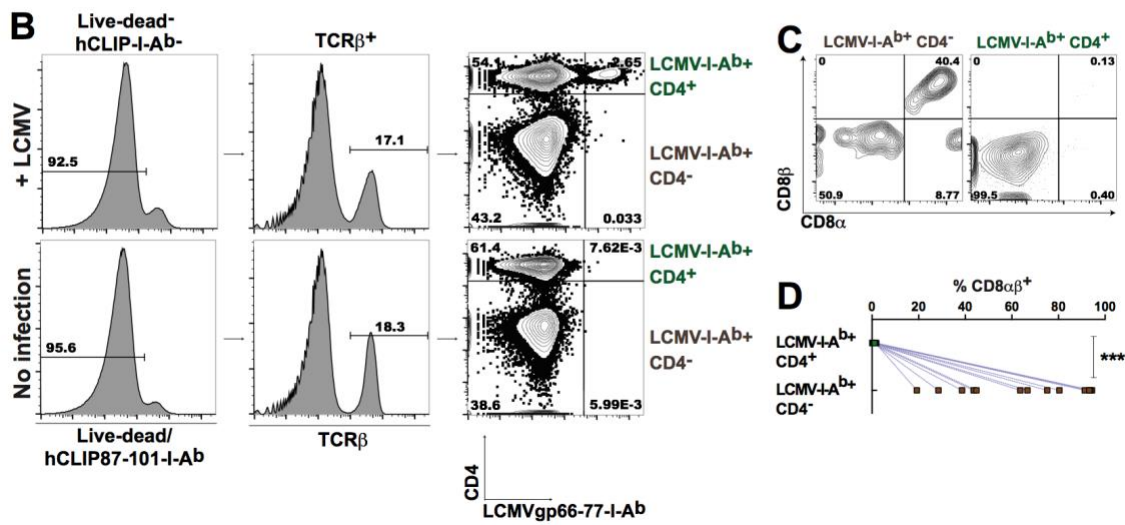
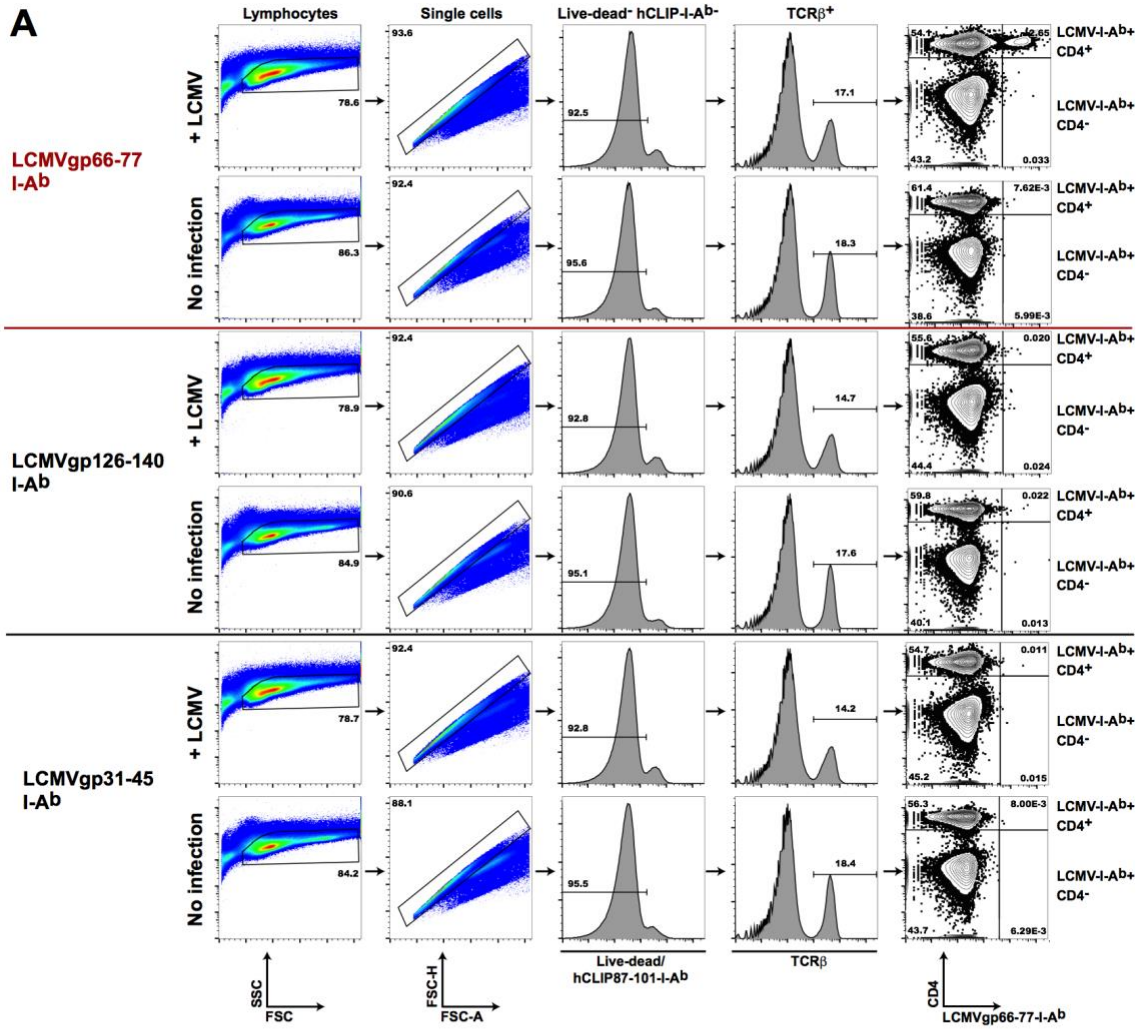
*Acute Listeria monocytogenes infection*

LLO WT and LLO Vps34<sup>KO</sup> mice were infected with 1 X 10<sup>7</sup> cfu *Listeria monocytogenes* by intravenous (i.v.) injection. **(A)** Four days post-infection, splenic lymphocytes were harvested and analyzed for the presence of naïve CD4<sup>+</sup> T cells (CD44<sup>int-lo</sup> TCR V $\alpha$ 2<sup>+</sup> CD4<sup>+</sup>, marked in green), effector CD4<sup>-</sup> T cells (CD44<sup>int-hi</sup> TCR V $\alpha$ 2<sup>+</sup> CD4<sup>-</sup>, marked in blue) and effector CD4<sup>+</sup> T cells (CD44<sup>hi</sup> TCR V $\alpha$ 2<sup>+</sup> CD4<sup>+</sup>, marked in gray) (left panel). CD8 $\alpha\beta$  expression was analyzed in each of these populations (right panel). The CD4-CD8 $\alpha\beta$ <sup>+</sup> population is indicated in red. **(B)** Frequency of CD8 $\alpha\beta$  expression within naïve CD4<sup>+</sup>, effector CD4<sup>-</sup> and effector CD4<sup>+</sup> T cells from *Listeria*-infected LLO WT and LLO Vps34<sup>KO</sup> mice. n = 6 from 1 experiment. Statistical significance was determined by the Mann-Whitney U Test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, n.s. = not significant.

To demonstrate that CD4-CD8 $\alpha\beta$ <sup>+</sup> T cells were not specific to (1) the Vps34<sup>KO</sup> context, (2) *Listeria* infection or (3) the LLO118 TCR transgene, I employed WT Thy1.1<sup>+</sup> C57BL/6 mice infected with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV Armstrong) as an alternative *in vivo* acute infection model for analyzing CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing effector T cells. Because C57BL/6 mice have an open TCR repertoire, I sought to develop and optimize an LCMV-MHCII (LCMV-I-A<sup>b</sup>) tetramer system that (1) minimally detected conventional effector CD4<sup>+</sup> MHCII-restricted T cells in uninfected controls, and (2) maximally detected conventional effector CD4<sup>+</sup> MHCII-restricted T cells in LCMV-infected mice. To accomplish this, I identified 5 LCMV-I-A<sup>b</sup> tetramers, each bearing a different bona fide LCMV epitope that could elicit a CD4<sup>+</sup> T cell response (LCMVgp6-20-I-A<sup>b</sup>, LCMVgp31-45-I-A<sup>b</sup>, LCMVgp66-77-I-A<sup>b</sup>, LCMVgp126-140-I-A<sup>b</sup>, LCMVnp309-328-I-A<sup>b</sup>)<sup>128, 129, 130, 131, 132</sup>. All 5 tetramers mediated detection of conventional CD4<sup>+</sup> LCMV-I-A<sup>b</sup>-recognizing effector T cells from LCMV-infected mice, at frequencies ranging from ~0.01-2% of live splenic T cells. (Representative results for 3 out of the 5 tetramers are shown in Fig. 14A.) However, for several of the tetramers, the frequency of CD4<sup>+</sup> LCMV-I-A<sup>b</sup>-recognizing T cells from infected mice nearly matched the background frequency found in uninfected mice. LCMVgp66-77-I-A<sup>b</sup>, a commonly-used major LCMV tetramer epitope, had the

highest avidity for LCMV-binding CD4<sup>+</sup> TCRs, and therefore provided the best detection of LCMV-I-A<sup>b</sup>-specific T cells above background binding (Fig. 14A).

Using LCMVgp66-77-I-A<sup>b</sup>, I observed that a substantial population of LCMV-I-A<sup>b</sup>-specific effector T cells generated during acute infection were CD4<sup>-</sup> (Fig. 14B). Among CD4<sup>-</sup> LCMV-I-A<sup>b</sup>-recognizing cells, at least 25% were CD8αβ<sup>+</sup> (Fig. 14C & D). Taken together, the results of my two *in vivo* infection models indicated that the production of CD4<sup>-</sup>CD8αβ<sup>+</sup> effector T cells could be facilitated by—but did not require—loss of Vps34, and might be underpinned by a general CD4<sup>+</sup> lineage destabilization process induced by diverse pathogens in a range of TCR repertoires.



**Figure 14. CD4CD8 $\alpha\beta$ <sup>+</sup> MHCII (I-A<sup>b</sup>)-recognizing T cells can be detected during acute LCMV infection using an I-A<sup>b</sup> tetramer.**

*I-A<sup>b</sup> tetramer optimization*

**(A)** C57BL/6 mice were infected with 1 X 10<sup>5</sup> cfu lymphocytic choriomeningitis virus-Armstrong strain (LCMV Armstrong) by intraperitoneal (i.p.) injection. Eight days post-infection, splenic lymphocytes were harvested and analyzed for the presence of LCMV-MHCII (I-A<sup>b</sup>)-specific T cells using 5 I-A<sup>b</sup> tetramers bearing different established CD4<sup>+</sup> T cell epitopes: LCMVgp6-20-I-A<sup>b</sup>, LCMVgp31-45-I-A<sup>b</sup>, LCMVgp66-77-I-A<sup>b</sup>, LCMVgp126-140-I-A<sup>b</sup>, LCMVnp309-328-I-A<sup>b</sup>. Tetramer binding was analyzed in effector CD4<sup>+</sup> T cells (Live-dead<sup>-</sup> hCLIP87-101-I-A<sup>b</sup>- TCR $\beta$ <sup>+</sup> LCMV-I-A<sup>b</sup><sup>+</sup> CD4<sup>+</sup>) and CD4<sup>+</sup> T cells (Live-dead<sup>-</sup> hCLIP87-101-I-A<sup>b</sup>- TCR $\beta$ <sup>+</sup> LCMV-I-A<sup>b</sup><sup>+</sup> CD4<sup>+</sup>). Analysis is shown for LCMVgp31-45-I-A<sup>b</sup>, LCMVgp66-77-I-A<sup>b</sup> and LCMVgp126-140-I-A<sup>b</sup>, with LCMVgp66-77-I-A<sup>b</sup> highlighted in red as the optimal tetramer. n = 6 from 1 experiment.

*Acute LCMV Armstrong infection*

Thy1.1<sup>+</sup> C57BL/6 mice were infected with 1 X 10<sup>5</sup> cfu lymphocytic choriomeningitis virus-Armstrong strain (LCMV Armstrong) by intraperitoneal (i.p.) injection. **(B)** Seven-8 days post-infection, splenic lymphocytes were harvested and analyzed for the presence of LCMV-I-A<sup>b</sup>-specific effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells (Live-dead<sup>-</sup> huCLIP87-101-I-A<sup>b</sup>- LCMVgp66-77<sup>+</sup>-I-A<sup>b</sup><sup>+</sup> CD44<sup>hi</sup> TCR $\beta$ <sup>+</sup> CD4<sup>+</sup>, marked in brown) and LCMV-I-A<sup>b</sup>-specific effector CD4<sup>+</sup> T cells (Live-dead<sup>-</sup> huCLIP87-101-I-A<sup>b</sup>- LCMVgp66-77<sup>+</sup>-I-A<sup>b</sup><sup>+</sup> CD44<sup>hi</sup> TCR $\beta$ <sup>+</sup> CD4<sup>+</sup>, marked in green). Percentage of CD8 $\alpha\beta$ <sup>+</sup> cells in these sub-populations from LCMV-infected mice is shown in **(C)** and enumerated in **(D)**. n = 13 from 2 independent experiments. Statistical significance was determined by the Mann-Whitney U test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, n.s. = not significant.

### **2.3.3 Conventional effector CD4<sup>+</sup> T cells give rise to CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells**

I deemed it crucial to definitively demonstrate that CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells could originate from mature effector CD4<sup>+</sup> T cells, and that they did not merely represent the expansion of mature, aberrant CD8<sup>+</sup> MHCII-restricted T cells. I employed two independent tracking strategies to pursue this aim.

#### **2.3.3.1 TCR $\beta$ repertoire sequencing**

The first strategy was a comprehensive assessment of the TCR spectrum of virus-specific effector CD4<sup>-</sup> versus CD4<sup>+</sup> T cells in acutely-infected WT mice with an open TCR repertoire. I reasoned that, if CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells were produced by the expansion of a pre-existing CD8<sup>+</sup> T cell pool generated during thymic selection, CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells probably would not share TCR sequences with CD4<sup>+</sup> T cells. On the other hand, if CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells were generated from conventional effector CD4<sup>+</sup> T cells, I expected that CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> TCRs should substantially overlap with conventional effector CD4<sup>+</sup> TCRs.

From the spleens of 4 C57BL/6 mice 8 days post-LCMV Armstrong infection, I sorted LCMVgp66-77-I-A<sup>b</sup>-recognizing CD44<sup>hi</sup> CD4<sup>-</sup> and CD4<sup>+</sup> T cells and performed TCR $\beta$  sequencing to reach saturation depth (Table 1). Out of  $\sim 6.0 \times 10^5$  total effective TCR reads analyzed across all mice, I detected  $\sim 5.3 \times 10^3$  LCMV-I-A<sup>b</sup>-recognizing CD44<sup>hi</sup>CD4<sup>+</sup> clonotypes and  $\sim 1.5 \times 10^3$  LCMV-I-A<sup>b</sup>-recognizing CD44<sup>hi</sup>CD4<sup>-</sup> clonotypes. Collectively, a substantial number of these clonotypes ( $\sim 4 \times 10^2$ ) were found in both the CD4<sup>+</sup> and CD4<sup>-</sup>

T cell populations, representing ~8% of CD4<sup>+</sup> clonotypes and ~26% of CD4<sup>-</sup> clonotypes (Fig. 15A). These shared clonotypes were present at a broad range of frequencies within each repertoire pool, indicating that TCR affinity might not play a strong role in lineage destabilization of effector CD4<sup>+</sup> T cells. I noted that a portion of these shared clonotypes might represent TCRs that could recognize both MHCI and MHCII, and therefore be expressed by both mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However, in order for this to be the major reason for the clonotype sharing I observed, CD4<sup>+</sup> and CD8<sup>+</sup> T cells with identical TCRs would need to develop simultaneously in the thymus, bypassing standard selection processes, and subsequently reside in the naïve T cell pool with similar life spans. This scenario would be somewhat improbable. Furthermore, I did note that the majority of CD4<sup>-</sup> clonotypes were not found among CD4<sup>+</sup> clonotypes. Although this observation raised the possibility that the unique CD4<sup>-</sup> clonotypes represented pre-existing CD8<sup>+</sup> MHCII-recognizing T cells, it likely reflected a major limitation of TCR sequencing technology—the subsampling effect in an open repertoire. Nevertheless, the substantial number of TCRs that overlapped the CD4<sup>-</sup> and CD4<sup>+</sup> effector T cell populations suggested that a mechanism exists for effector CD4<sup>+</sup> T cells to produce a CD4<sup>-</sup> phenotype.

**Table 1. LCMV-I-A<sup>b</sup>-recognizing effector CD4<sup>-</sup> and CD4<sup>+</sup> T cell populations analyzed by TCR $\beta$  repertoire sequencing.**

Cells were FACS-sorted from 4 Thy1.1<sup>+</sup> C57BL/6 mice directly *ex vivo* 8 days post-LCMV Armstrong infection. nt: nucleotide sequence, aa: amino acid sequence.

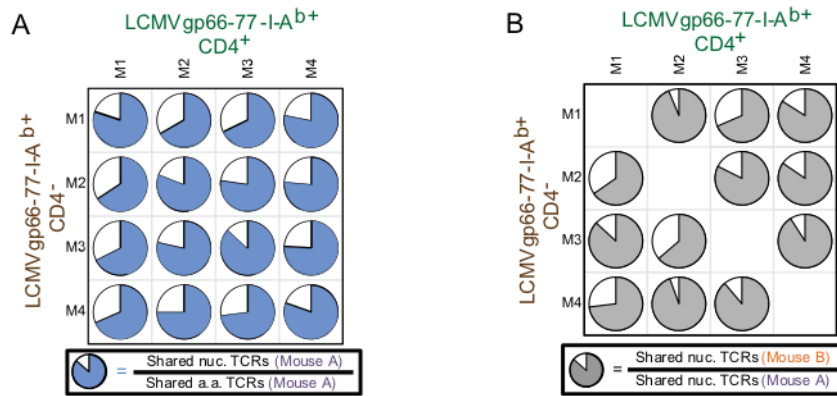
	Mouse	Cell input	Effective reads (nt)	Clonotypes (nt)	Clonotypes (aa)
<b>LCMV<sub>gp66-77-I-A<sup>b</sup> CD4<sup>-</sup></sub></b>	1	3600	62535	728	470
	2	8100	22977	488	317
	3	5000	13852	398	284
	4	9600	36628	542	328
<b>LCMV<sub>gp66-77-I-A<sup>b</sup> CD4<sup>+</sup></sub></b>	1	20000	290588	3007	1646
	2	20000	42853	864	525
	3	20000	59224	976	582
	4	20000	62286	1092	653



**Figure 15. Lineage tracing by TCR $\beta$  repertoire sequencing indicates the generation of CD4 $^-$  T cells from effector CD4 $^+$  T cells during an antigen-specific effector response.**

Splenic lymphocytes were harvested from WT Thy1.1 $^+$  C57Bl/6 mice 7 days post-LCMV Armstrong infection. Live LCMV-I-A $^b$ -recognizing effector CD4 $^-$  T cells (Live/Dead $^-$  huCLIP87-101-I-A $^b$ - LCMVgp66-77-I-A $^b$  CD44 $^{\text{hi}}$  CD4 $^-$ ) or effector CD4 $^+$  T cells (Live/Dead $^-$  huCLIP87-101-I-A $^b$ - LCMVgp66-77-I-A $^b$  CD44 $^{\text{hi}}$  CD4 $^+$ ) were collected ( $3.5 \times 10^3$ - $2 \times 10^4$  cells/sample). FACS-sorted populations were then spiked with  $5 \times 10^2$  2B4.11 cells to provide an absolute reads:cell number ratio for effective reads calculation. Populations with  $< 2 \times 10^4$  cells were supplemented with LB27.4 cells to facilitate RNA extraction. cDNA libraries were prepared from total RNA by reverse transcription using an in-house-designed TCR $\beta$ -specific primer. Whole-TCR $\beta$  sequencing was performed using the Ion Torrent PGM platform. Cell numbers, effective reads and clonotype counts are listed in Table 1. **(A)** Pairwise comparison of nucleotide clonotype frequencies between LCMV-I-A $^b$ -recognizing effector CD4 $^+$  and CD4 $^-$  T cells across all mice. The proportion of shared clonotypes among total clonotypes for each population is listed above the plot. Clonotypes were placed into 3 frequency categories: (1) High-frequency among CD4 $^-$  TCRs, (2) High-frequency among CD4 $^+$  TCRs and (3) Low frequency among all TCRs. **(B)** Schematic representation of intra- and inter-mouse clonotype comparisons. **(C)** UpSet plot representation of intra-mouse and inter-mouse shared nucleotide clonotypes. The histogram (upper panel) enumerates the number of clonotypes shared by the indicated populations (lower panel) out of the 100 most-frequent ("top 100") clonotypes across all mice and populations. The histogram in the left panel enumerates the number of clonotypes in the indicated population found among the 150 most-frequent ("top 150") clonotypes in the M4-CD4 $^+$  population. Shared clonotypes are indicated by color as intra-mouse (found in CD4 $^-$  and CD4 $^+$  populations within the same mouse; shown in blue), inter-mouse (found in  $\geq 2$  populations in  $\geq 2$  mice; shown in black) or public TCRs (found in all mice and  $\geq 1$  CD4 $^+$  population; shown in red). All sequencing analyses were conducted by Ming Zheng using MATLAB 2013b with manual scripts. n = 4 from 1 experiment.

For analysis of individual mouse repertoires, I conducted a comprehensive search for either intra- or inter-mouse clonotype sharing (Fig. 15B). At the nucleotide level, the highest-frequency clonotype sharing took place between CD44<sup>hi</sup>CD4<sup>+</sup> and CD44<sup>hi</sup>CD4<sup>-</sup> T cells within individual mice, which suggested that these two populations might share the same origin (Fig. 15C). This was also supported by my observation that, at the amino acid level, most of the intra-mouse shared TCRs were encoded by identical nucleotide sequences (Fig. 16A). In addition, 8 of the LCMVgp66-77-I-A<sup>b</sup>-recognizing CD4<sup>+</sup> clonotypes were identified in all 4 mice (i.e., “public” clonotypes) (Fig. 15C). Five out of these 8 CD4<sup>+</sup> clonotypes were shared with a CD4<sup>-</sup> clonotype in the same individual mouse, respectively; the other 3 CD4<sup>+</sup> public clonotypes were shared with CD4<sup>-</sup> clonotypes in at least 2 mice (Fig. 15C, Fig. 16B-C). I considered that, if these public TCR $\beta$  clonotypes did indeed indicate identical TCRs, they were unlikely to have been produced by simultaneous thymic development of mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells with the same TCR, which were subsequently maintained in the naïve T cell pool with the same life span. On the other hand, the lack of TCR $\alpha$  sequences prevented me from excluding the possibility of non-identical TCRs containing identical TCR $\beta$  chains. Nevertheless, it was most likely that, upon infection, bona fide naïve, LCMV-recognizing CD4<sup>+</sup> T cell clonotypes lineage-converted to acquire the CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> effector phenotype in each mouse. Taken together, these findings provided clonal lineage evidence to suggest that a broad spectrum of effector CD4<sup>+</sup> T cells could become CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing cells during an *in vivo* effector response.



**C**  
Ranks of Public nucleotide TCRs

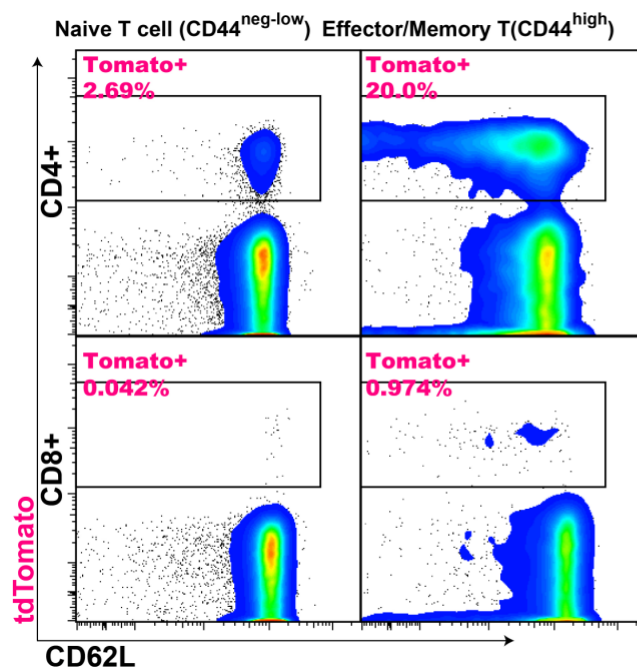
CDR3 nucleotide sequence	CDR3 amino acid sequence	V gene	J gene	M1		M2		M3		M4	
				CD4 <sup>-</sup>	CD4 <sup>+</sup>	CD4 <sup>-</sup>	CD4 <sup>+</sup>	CD4 <sup>-</sup>	CD4 <sup>+</sup>	CD4 <sup>-</sup>	CD4 <sup>+</sup>
TGTGCCAGCGGTGATGCTAGATGGGACAGGGGGCCCTAGATGTTCTGGAAATACGCTCTATTTT	CASGDARWDRPRCSGNTLYF	TRBV13-2	TRBJ1-3	3	3	3	3	1	4	3	2
TGTGCCAGCAGCCAGATAAAGTAGTAACCAAGACACCCAGTACTTT	CASSQITSNQDTQYF	TRBV5	TRBJ2-5	9	70	8	66	8	76	8	37
TGTGCCAGCGGTGATGTGGAGACACCCAGTACTTT	CASGDVEDTQYF	TRBV13-2	TRBJ2-5	14	60	15	29	9	54	9	112
TGTGCCAGCAGCTCTGAAGCTGGAGCCAAAGACACCCAGTACTTT	CASSLNWSQDTQYF	TRBV24	TRBJ2-5	32	37	9	49	5	115	10	54
TGTGCCAGCAGCTCTGAAGCTGGAGCCAAAGACACCCAGTACTTT	CASSLNWSQDTQYF	TRBV22	TRBJ2-5	109	80	32	182	16	212	61	112

**Figure 16. Effector CD4<sup>-</sup>/CD4<sup>+</sup> MHCII-recognizing TCR clonotypes are shared within and between mice.**

TCR $\beta$  sequencing of LCMVgp66-77-I-A<sup>b</sup>-recognizing effector CD4<sup>-</sup> T cells and CD4<sup>+</sup> T cells from 4 LCMV-infected Thy1.1<sup>+</sup> C57BL/6 mice (M1-M4) was performed as described in Figure 2. **(A)** Pairwise comparison of inter- and intra-mouse shared effector CD4<sup>-</sup> and CD4<sup>+</sup> TCR $\beta$  clonotypes. Data represent the proportion of shared nucleotide clonotypes among shared amino acid clonotypes within an individual mouse or between 2 mice. **(B)** Pairwise comparison of inter-mouse shared effector CD4<sup>-</sup> and CD4<sup>+</sup> TCR $\beta$  clonotypes. Data are represented as the proportion of shared nucleotide clonotypes between 2 mice. **(C)** Table identifying 5 public clonotypes found in both the effector CD4<sup>-</sup> and CD4<sup>+</sup> populations of all 4 mice. Two clonotypes with identical nucleotide/amino acid sequences but different V-gene usage are highlighted in red. All sequencing analyses were performed by Ming Zheng using MATLAB 2013b with manual scripts. Cell numbers, effective reads and clonotype counts are listed in Table 1. n = 4 from 1 experiment.

### 2.3.3.2 Genetic lineage tracing

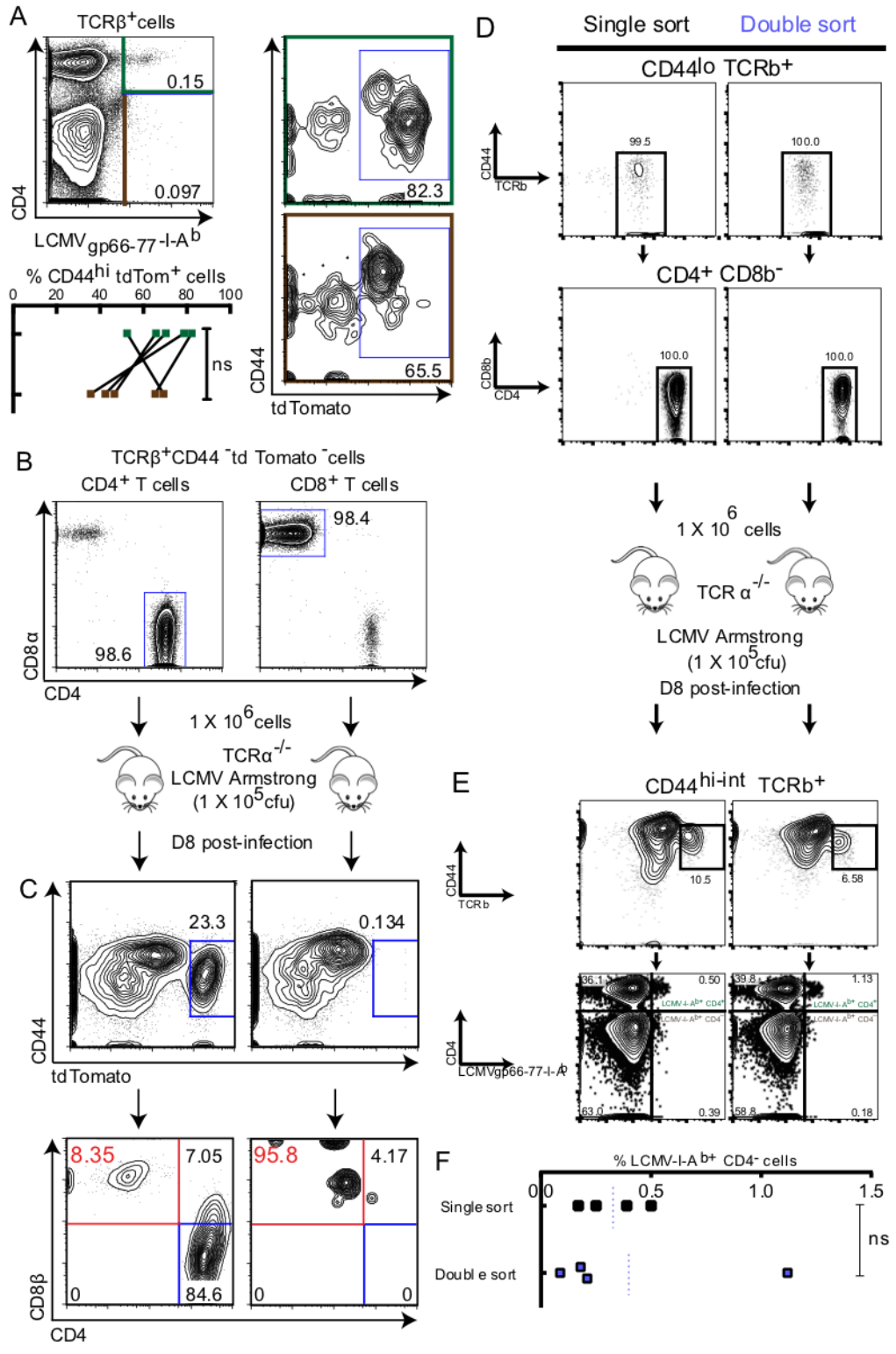
My second approach made use of the Ox40-Cre Rosa26<sup>tdTomato</sup> (OxTom)<sup>115</sup> murine strain. By the genetic strategy of this strain, tdTomato (tdTom) expression is controlled by the promoter for *Ox40*, which encodes a co-stimulatory surface receptor on CD4<sup>+</sup> T cells. Because *Ox40* is generally silent in naïve T cells, and its expression is preferentially induced in CD4<sup>+</sup> T cells versus CD8<sup>+</sup> T cells after strong antigen stimulation<sup>115</sup>, I reasoned that this system would allow us to preferentially mark activated CD4<sup>+</sup> T cells, regardless of changes in CD4 surface expression. In adult, unchallenged OxTom mice raised in a standard pathogen-free environment, tdTom expression was approximately 10-fold higher in splenic effector/memory (CD44<sup>hi</sup>CD62L<sup>lo</sup>) CD4<sup>+</sup> cells than in naïve (CD44<sup>lo</sup>CD62L<sup>hi</sup>) CD4<sup>+</sup> cells (Fig. 17). Equally important, the frequency of tdTom expression in effector/memory CD4<sup>+</sup> cells was more than 20-fold higher than in effector/memory CD8<sup>+</sup> cells and nearly 500-fold higher than in naïve CD8<sup>+</sup> cells. Therefore, I concluded that preferential tdTom labeling of effector CD4<sup>+</sup> T cells using the OxTom system would allow us to trace the generation of CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> cells from effector CD4<sup>+</sup> T cells.



**Figure 17. tdTomato expression in mature T cell subsets of uninfected OxTom mice.**

Lymphocytes were harvested from the spleens of Rosa26<sup>tdTomato +/-</sup> O<sub>x</sub>40-Cre<sup>+/-</sup> (OxTom) mice raised in a standard pathogen-free facility. Naïve (CD44<sup>neg-low</sup>CD62L<sup>hi</sup>) and effector/memory (CD44<sup>high</sup>CD62L<sup>lo</sup>) T cells were analyzed based on surface expression of CD4/CD8 and tdTomato labeling. n = 6 from 4 independent experiments. 2 of the experiments were performed by Baojun Zhang.

In OxTom mice infected with LCMV Armstrong, I detected conventional LCMV-I-A<sup>b</sup>-recognizing, CD44<sup>hi-int</sup>CD4<sup>+</sup> T cells (Fig. 18A). ~50-80% of this population were tdTom<sup>+</sup>, indicating that my genetic strategy efficiently marked effector/memory CD4<sup>+</sup> T cells, albeit to a varying degree. Meanwhile, an LCMV-I-A<sup>b</sup>-specific CD44<sup>hi</sup>CD4<sup>+</sup> population was also generated, ~30-70% of which were tdTom<sup>+</sup> (Fig. 18A). This suggested that the CD4<sup>+</sup>CD8αβ<sup>+</sup> LCMV/I-A<sup>b</sup>-recognizing T cells I detected during the acute anti-LCMV response had originated from activated CD4<sup>+</sup> T cells.



**Figure 18. Lineage tracing indicates lineage conversion of CD4<sup>+</sup> T cells to CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> T cells during an antigen-specific effector response.**

*Genetic labeling of effector CD4<sup>+</sup> T cells*

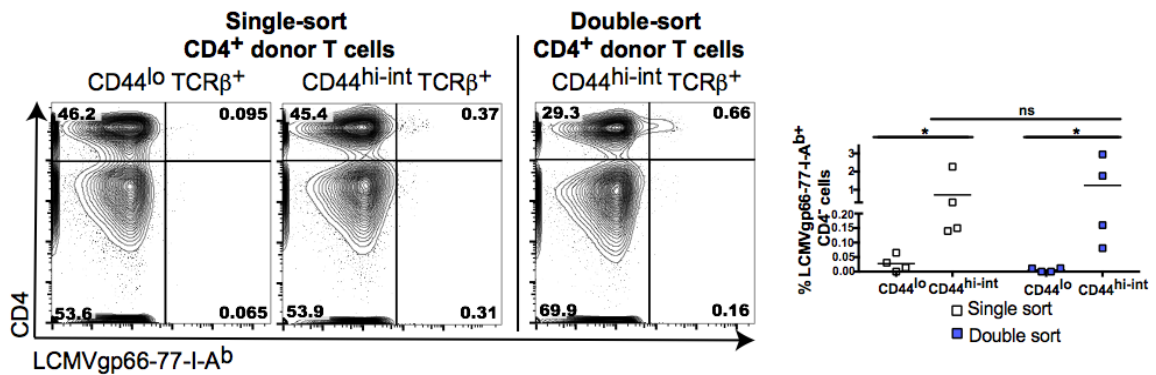
**(A)** Rosa26<sup>tdTomato/+</sup> O<sub>x</sub>40-Cre<sup>+/-</sup> (OxTom) mice were infected with 1 X 10<sup>5</sup> cfu LCMV Armstrong by i.p. injection. Eight days post-infection, splenic lymphocytes were harvested and analyzed for tdTomato (tdTom) expression among LCMV-specific effector CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> T cells (Live/Dead<sup>-</sup> huCLIP87-101-I-A<sup>b</sup>- LCMVgp66-77-I-A<sup>b</sup>+ CD44<sup>hi</sup> TCR $\beta$ <sup>+</sup> CD4<sup>+</sup>, marked in brown) and LCMV-specific effector CD4<sup>+</sup> T cells (Live/Dead<sup>-</sup> huCLIP87-101-I-A<sup>b</sup>- LCMVgp66-77-I-A<sup>b</sup>+ CD44<sup>hi</sup> TCR $\beta$ <sup>+</sup> CD4<sup>+</sup>, marked in green). **(B-C)** Naïve CD4<sup>+</sup> T cells (TCR $\beta$ <sup>+</sup> CD44<sup>lo</sup> tdTom<sup>-</sup> CD4<sup>+</sup>) and naïve CD8<sup>+</sup> T cells (TCR $\beta$ <sup>+</sup> CD44<sup>lo</sup> tdTom<sup>-</sup> CD8<sup>+</sup>) were FACS-sorted from the splenic lymphocytes of OxTom mice. **(B)** 1 X 10<sup>6</sup> FACS-sorted CD4<sup>+</sup> or CD8<sup>+</sup> OxTom T cells were adoptively transferred to TCR $\alpha$ <sup>-/-</sup> recipient mice by i.v. injection, and recipient mice were infected with 1 X 10<sup>5</sup> cfu LCMV Armstrong by i.p. injection 24 hours post-adoptive transfer. **(C)** Eight days post-infection, splenic lymphocytes were harvested and analyzed for tdTom expression among effector (CD44<sup>hi</sup>) cells (middle panel). CD8 $\alpha$  $\beta$  expression was assessed among CD44<sup>hi</sup> tdTom<sup>+</sup> cells (bottom panel). n = 5 from 2 independent experiments for (A), and n = 2 from 1 experiment for (B) and (C). Statistical significance was determined by the Mann-Whitney U test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, n.s. = not significant.

*Adoptive transfer of serially-enriched CD4<sup>+</sup> T cells*

**(D)** Naïve CD4<sup>+</sup> T cells (TCR $\beta$ <sup>+</sup> CD44<sup>-</sup> CD4<sup>+</sup>) were FACS-sorted once (single sort; shown in black) or twice (double sort; shown in periwinkle) from the splenic lymphocytes of Rosa26<sup>mT/mG</sup> (mT/mG) mice. Cells were enriched from the live donor population (Live-dead<sup>-</sup> tdTom<sup>+</sup>; tdTom is a constitutive, ubiquitous label). 1 X 10<sup>6</sup> naïve CD4<sup>+</sup> T cells were adoptively transferred to TCR $\alpha$ <sup>-/-</sup> recipient mice by i.v. injection, and recipient mice were infected with 1 X 10<sup>5</sup> cfu LCMV Armstrong by i.p. injection. **(E)** Eight days post-infection, splenic lymphocytes were harvested and analyzed for CD4/CD8 expression among LCMV-I-A<sup>b</sup>-binding donor effector T cells (LCMVgp66-77-I-A<sup>b</sup>+ CD44<sup>hi</sup> TCR $\beta$ <sup>+</sup>). Percentage of LCMVgp66-77-I-A<sup>b</sup>+ CD4<sup>-</sup> cells among CD44<sup>hi</sup> TCR $\beta$ <sup>+</sup> live donor cells from single- vs. double-sort recipients is shown in **(F)**. n = 4 from 1 experiment. Statistical significance was determined by the Mann-Whitney U test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, n.s. = not significant.

I did note that  $\geq 30\%$  of LCMV-I-A<sup>b</sup>-recognizing CD4<sup>-</sup> cells were tdTom<sup>-</sup> (Fig. 18A). This population likely resulted from poor labeling efficiency in weakly-stimulated CD4<sup>+</sup> T cells<sup>115</sup>. However, the simultaneous generation of CD44<sup>hi</sup>CD4<sup>-</sup>tdTom<sup>-</sup> and CD44<sup>hi</sup>CD4<sup>-</sup>tdTom<sup>+</sup> cells could arise from the combination of 2 other possibilities: (1) a population of developmentally-derived, CD8<sup>+</sup> MHCII-restricted T cells was present, which did not efficiently induce Ox40 promoter-controlled transcription upon activation (tdTom<sup>-</sup> cells), and (2) another population of developmentally-derived, CD8<sup>+</sup> MHCII-restricted T cells was present, which was permissive to OX40 expression and tdTom labeling upon activation, perhaps due to the expression of high affinity TCRs (tdTom<sup>+</sup> cells). To exclude these possibilities, I adoptively transferred FACS-purified naïve (CD44<sup>lo</sup>tdTom<sup>-</sup>) CD4<sup>+</sup> or CD8<sup>+</sup> T cells from OxTom mice into T cell-deficient TCR $\alpha^{-/-}$  hosts and subsequently challenged the hosts with LCMV Armstrong (Fig. 18B). Among transferred CD4<sup>+</sup> T cells, ~20% of CD44<sup>hi</sup> cells were tdTom<sup>+</sup>. The majority of these tdTom<sup>+</sup> cells exhibited the conventional CD4<sup>+</sup> T cell phenotype, but a significant proportion exhibited either a CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8 $\alpha\beta^{+}$  phenotype (Fig. 18C). On the other hand, while a large portion of transferred CD8<sup>+</sup> T cells were efficiently activated by LCMV (CD44<sup>hi</sup>), they were rarely tdTom<sup>+</sup> (Fig. 18C). I therefore reasoned that, even if developmentally-derived CD8<sup>+</sup> LCMV-I-A<sup>b</sup>-recognizing T cells were present and could permit OX40 expression upon activation, they could not completely account for the CD4<sup>-</sup>tdTom<sup>+</sup> cells I detected in CD4<sup>+</sup> T cell recipient mice. To completely exclude the potential confounding effect of contamination by pre-existing CD8<sup>+</sup> T cells, I employed a more rigorous purification

procedure by conducting serial FACS enrichment<sup>133</sup> of donor CD4<sup>+</sup> T cells. Naïve CD4<sup>+</sup> T cells, enriched to 100% purity by either single- or double-sorting (Fig. 18D), were adoptively transferred into TCR $\alpha^{-/-}$  recipient mice in parallel. After LCMV infection, CD4<sup>+</sup> CD8 $\alpha\beta^{+}$  donor T cells were detected at a similar frequency in singly-sorted versus doubly-sorted CD4<sup>+</sup> T cell recipients (Fig. 18E & F). This population was found specifically among CD44<sup>hi-int</sup> LCMV-I-A<sup>b+</sup> T cells (Fig. 19). This suggested that contamination from pre-existing CD8<sup>+</sup> T cells was unlikely to be the source of the LCMV-I-A<sup>b</sup>-recognizing CD4<sup>+</sup>CD8 $\alpha\beta^{+}$  T cell population. These 3 independent lineage tracing approaches strengthened my conclusion that effector differentiation of CD4<sup>+</sup> T cells could indeed drive the generation of CD4<sup>+</sup>CD8 $\alpha\beta^{+}$  MHCII-recognizing T cells.

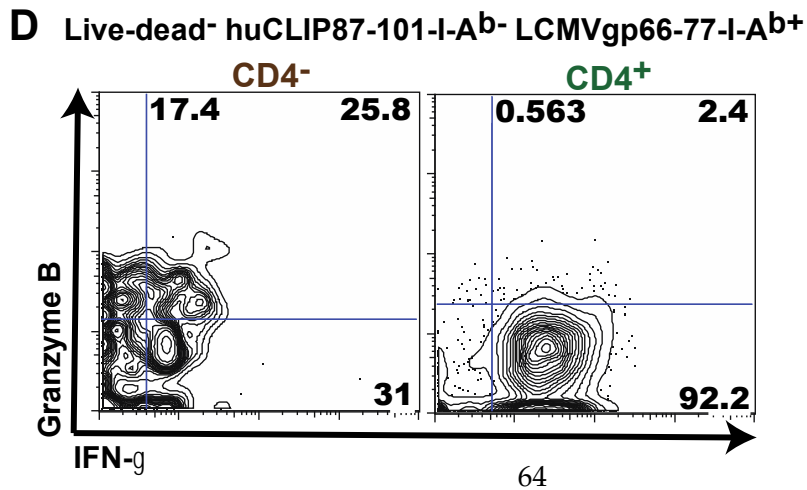
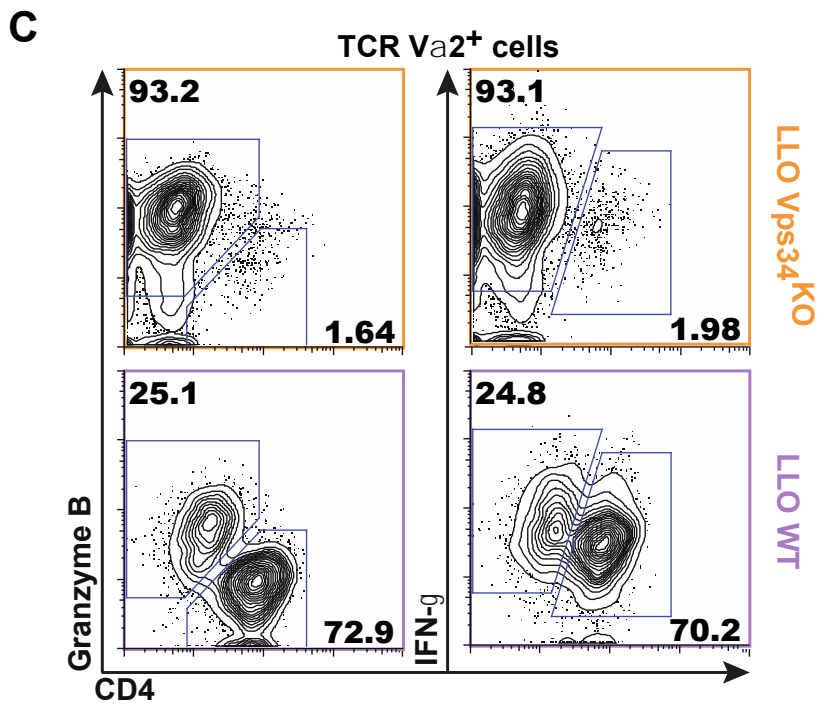
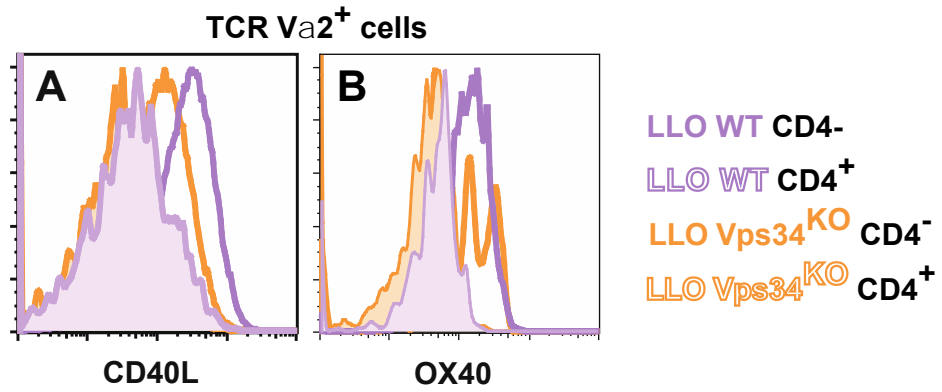


**Figure 19. Donor-derived CD4<sup>+</sup> T cells are found among LCMV-specific effector T cells.**

Naïve CD4<sup>+</sup> T cells from the splenic lymphocytes of Rosa26<sup>mT/mG</sup> (mT/mG) mice were FACS-enriched to 100% purity by either single- or double-sorting. 1 X 10<sup>6</sup> singly- or doubly-sorted donor cells were adoptively transferred in parallel into TCRα<sup>-/-</sup> recipient mice infected with LCMV Armstrong. Eight days post-infection, recipient splenocytes were analyzed for the presence of donor-derived LCMV-I-A<sup>b+</sup> CD4<sup>+</sup>CD8αβ<sup>+</sup> T cells. n = 4 from 1 experiment. Statistical significance was determined by the Mann-Whitney U test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, n.s. = not significant.

### **2.3.4 Cytotoxic function is enhanced in CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells**

I next examined whether the shift in CD4/CD8 surface marker expression corresponded with a shift in T cell function. Using *in vitro*-generated effector T cells, I found that both LLO WT and LLO Vps34<sup>KO</sup> TCRV $\alpha$ 2<sup>+</sup> CD4<sup>-</sup> T cells downregulated surface expression of the costimulatory molecules CD40L and Ox40 (Fig. 20A-B), indicating loss of Th cell function. Concomitantly, these cells upregulated production of granzyme B and IFN- $\gamma$ , which are hallmark cytotoxic mediators of CD8<sup>+</sup> T cells (Fig. 20C). Indeed, I also found that LCMV-I-A<sup>b</sup>-recognizing CD4<sup>-</sup> effector T cells generated in C57BL/6 mice during LCMV Armstrong infection expressed elevated levels of granzyme B and IFN- $\gamma$  (Fig. 20D). These results indicated that CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells shifted towards the functional phenotype of conventional effector CD8<sup>+</sup> T cells.



**Figure 20. CD4<sup>-</sup>CD8αβ<sup>+</sup> T cells suppress T helper function and gain enhanced cytotoxic function.**

(A-C) Total lymph node cells or FACS-enriched CD4<sup>+</sup> TCR Vα2<sup>+</sup> cells from LLO WT or LLO Vps34<sup>KO</sup> mice were stimulated *in vitro* with LLO190-205 for 120 hours. Surface expression of CD40L (A) and O×40 (B) and intracellular expression of granzyme B/IFN-γ (C) was analyzed in TCR Vα2<sup>+</sup> CD4<sup>-</sup> and TCR Vα2<sup>+</sup> CD4<sup>+</sup> cells. (D) Splenic lymphocytes from C57BL/6 mice infected with LCMV Armstrong for 7-12 days were harvested and analyzed for intracellular granzyme B/IFN-γ levels among LCMVgp66-77-I-A<sup>b+</sup> CD44<sup>hi</sup> CD4<sup>-</sup> and CD4<sup>+</sup> T cells. n = 2 from 2 independent experiments for (A) and (B), n = 8 from 3 independent experiments for (C) and n = 12 from 3 independent experiments for (D).

### **2.3.5 CD4<sup>-</sup>CD8αβ<sup>+</sup> MHCII-recognizing T cells exhibit a CD8<sup>+</sup> T cell-like transcriptional program**

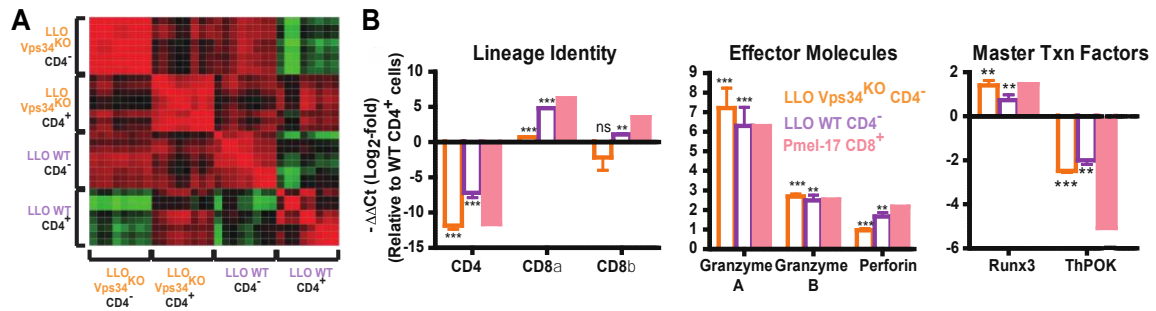
I then proceeded to determine whether the CD4<sup>-</sup>CD8αβ<sup>+</sup> T cell phenotype was underpinned by alterations in lineage transcriptional programming. I analyzed *in vitro*-generated LLO WT/LLO Vps34<sup>KO</sup> CD4<sup>-</sup> and CD4<sup>+</sup> effector T cells for the expression of 16 signature genes specifying the CD4<sup>+</sup> versus CD8<sup>+</sup> lineage (Table 2). With samples from 8 independent experiments, unsupervised clustering analysis of overall similarity profiles indicated that the transcriptional programs of CD4<sup>-</sup> vs. CD4<sup>+</sup> effector T cells were determined by lineage identity rather than by Vps34 expression (Fig. 21A). That is, the profiles of LLO WT vs. LLO Vps34<sup>KO</sup> CD4<sup>-</sup> T cells correlated more closely than LLO WT CD4<sup>-</sup> T cells vs. LLO WT CD4<sup>+</sup> T cells or LLO Vps34<sup>KO</sup> CD4<sup>-</sup> T cells vs. LLO Vps34<sup>KO</sup> CD4<sup>+</sup> T cells. Using *in vitro*-primed pMel-1 effector CD8<sup>+</sup> T cells as a CD8<sup>+</sup> lineage expression benchmark, and using LLO WT effector CD4<sup>+</sup> T cells as a normalization standard, I analyzed these genes in three categories (Fig. 21B): (1) Lineage identity: In LLO Vps34<sup>KO</sup> CD4<sup>-</sup> T cells, CD4 expression was profoundly silenced; in LLO WT CD4<sup>-</sup> cells, CD4

expression was downregulated more modestly, while suppression of the CD8  $\alpha/\beta$  loci was released; (2) Effector molecules: Expression of granzyme A, granzyme B and perforin in both LLO WT and LLO Vps34<sup>KO</sup> CD4<sup>+</sup> T cells reached levels similar to those found in effector CD8<sup>+</sup> T cells; (3) Master transcription factors: Both LLO WT and LLO Vps34<sup>KO</sup> CD4<sup>+</sup> T cells acquired a unique ThPOK<sup>lo-int</sup>Runx3<sup>hi</sup> profile, reflecting both their CD4<sup>+</sup> origin and CD8<sup>+</sup>-oriented lineage transition. Taken together, my transcriptional data demonstrated that the production of CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells was associated with a fundamental lineage switch at the transcription level.

**Table 2. Primer sequences for qPCR analysis of CD4<sup>+</sup>/CD8<sup>+</sup> T cell-specific transcript expression.**

Some primer sequences designed by Regina Lin.

GENE NAME	GENE SYMBOL	FORWARD PRIMER	REVERSE PRIMER
<b>18S rRNA</b>	<i>Rn18s</i>	GGCCGTTCTTAGTTGGTGAG	TGCTCAATCTCGGGTGGCT
<b>Bcl6</b>	<i>Bcl6</i>	ACTCGCTTCCGGCACCTTCA	CGCGGTATTGCACCTTGGTGT
<b>Beta-actin</b>	<i>Actb</i>	ATCTGGCACCACACCTTCTACA	ACGTACATGGCTGGGGTGTG
<b>Blimp-1</b>	<i>Prdm1</i>	TGTGCCAAGACGTTCCGGTCAG	GGTCTTGAGATTGCTTGTGCTGC
<b>CD4</b>	<i>Cd4</i>	CAGACAGTGTTCCCTGGCTTGCG	GGGGCACTGGCAGGTCTTCTTC
<b>CD8, alpha chain</b>	<i>Cd8a</i>	TGCGAACTCCCTCACCTGTGCA	AGCAGAAGGGCCACGCAGATT
<b>CD8, beta chain</b>	<i>Cd8b1</i>	CAGCCTTACCACCCTCAGCCTG	GGCTATCAGTGTTGTGGGCGCT
<b>Eomes</b>	<i>Eomes</i>	CAAAGGTGCAAACAACAACAACAC	GGTTATGGTCGATCTTTAGCTGGG
<b>Granzyme A</b>	<i>Gzma</i>	AACATGATTTGTGCAGGGGACC	TTAATTAACGCCAGCAGTATAGACAC CAGGCCATCG
<b>Granzyme B</b>	<i>Gzmb</i>	TCGAGAGGACTTTGTGCTGACTGC	TTAATTAACGCCAGCGCCTCACAGCT CTAGTCCTCTTG
<b>IFN-gamma</b>	<i>Ifng</i>	CCTTCTCAGCAACAGCAAGGCG	CCGCTTCCTGAGGCTGGATTCC
<b>IL-2</b>	<i>Il2</i>	CAAGCTCTACAGCGGAAGCACAG	AATTCTGTGGCCTGCTTGGG
<b>Perforin</b>	<i>Prf1</i>	TTCAACTGGCTTCTCCCTGG	TACGCTTCGTGGCAGTAGTTG
<b>Runx3</b>	<i>Runx3</i>	ACCTACCACCGAGCCATCAAG	GTGCCTTGATTGGGGTCTG
<b>SDHA</b>	<i>Sdha</i>	TTATTGCTACTGGGGGTACGGG	AGGCAGCCAGCACCGTATATAACC
<b>T-bet</b>	<i>Tbx21</i>	ATCGACAACAACCCCTTTGCCA	GGGGTAGAAACGGCTGGGAACA
<b>Vps34</b>	<i>Pik3c3</i>	CAGTGCTACACAGCCTTCTCCTCC	CTGACAGGTCCAGGCGGAAC



**Figure 21. Effector CD4<sup>+</sup> MHCII-recognizing T cells shift towards CD8<sup>+</sup> lineage transcriptional programming.**

Total lymph node cells from LLO WT and LLO Vps34<sup>KO</sup> mice were stimulated with LLO190-205 *in vitro* for 120 hours. *In vitro*-stimulated LLO WT/Vps34<sup>KO</sup> TCR V $\alpha$ 2<sup>+</sup> CD4<sup>-</sup> and TCR V $\alpha$ 2<sup>+</sup> CD4<sup>+</sup> were FACS-sorted and the expression of 16 T cell lineage-specific transcripts was analyzed by qPCR. **(A)** Heatmap representation of Pearson product-moment correlation coefficients for the 4 sample groups. **(B)** mRNA expression levels for each target are presented relative to expression levels in *in vitro*-stimulated LLO WT CD4<sup>+</sup> T cells. Targets are grouped based on function (Lineage Identity, Effector Molecules or Master Txn Factors). Expression levels in *in vitro*-stimulated pMel-1 CD8<sup>+</sup> T cells were also assessed for a CD8<sup>+</sup> lineage reference. Primer sequences are listed in Table 2. n = 9 from 4 independent experiments. Y-axis label shown for Lineage Identity also applies to Effector Molecules and Master Txn Factors. Relative transcript expression was calculated by the delta-delta Ct method using R with manual scripts. Pearson product-moment correlation coefficients were calculated by Qi-Jing Li using MATLAB 2013b with manual scripts. Statistical significance was determined by the Mann-Whitney U Test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, n.s. = not significant.

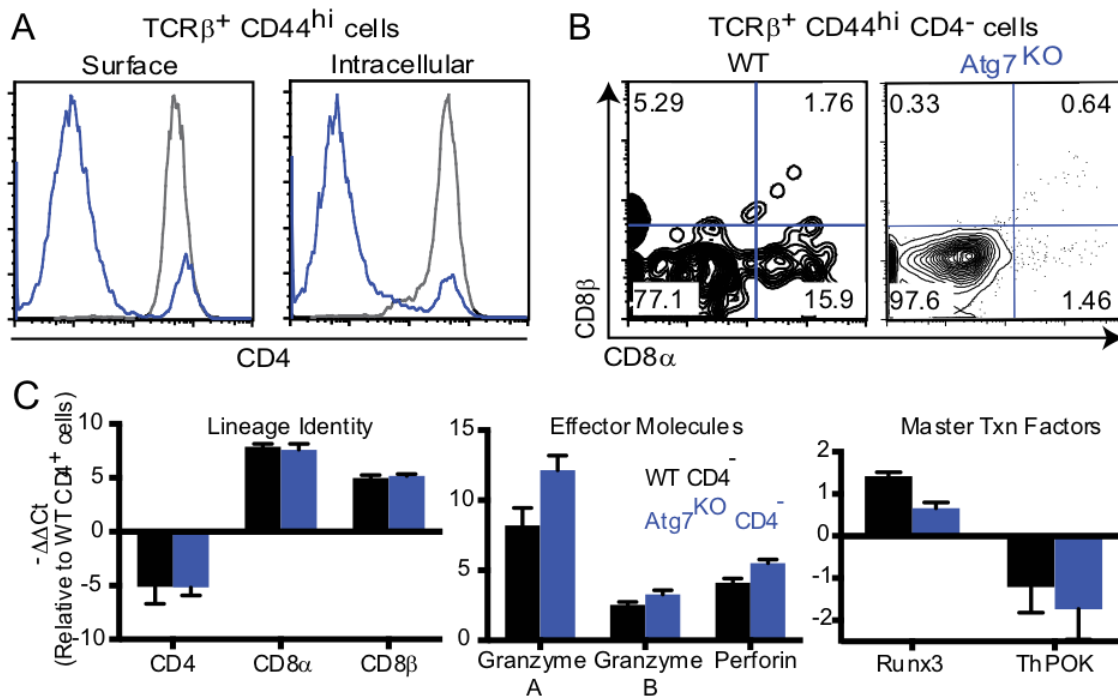
### 2.3.6 Autophagosome formation regulates the generation of effector CD4<sup>-</sup> CD8αβ<sup>+</sup> T cells

While Vps34 is an important facilitator of vesicle trafficking, it is particularly well-known as a key enzyme during the early stages of autophagy<sup>134</sup>. Specifically, Vps34 forms a complex with Beclin-1, AMBRA-1 and Vps15, in which it catalyzes the formation of PI<sub>3</sub>P (Fig. 10)<sup>135</sup>. PI<sub>3</sub>P in turn facilitates the initialization of autophagosome membrane formation.

Because (1) CD4<sup>-</sup> T cells were generated more frequently from LLO Vps34<sup>KO</sup> vs. LLO WT effector CD4<sup>+</sup> T cells, and (2) CD4 transcription was more profoundly suppressed in LLO Vps34<sup>KO</sup> vs. LLO WT effector CD4<sup>-</sup> T cells, I speculated that the general machinery of autophagy could regulate lineage destabilization of effector CD4<sup>+</sup> T cells towards the CD4<sup>-</sup> phenotype. To test this hypothesis, I FACS-purified naïve CD4<sup>+</sup> T cells from Atg7<sup>fl/fl</sup> Lck-Cre (Atg7<sup>KO</sup>) mice<sup>108</sup> that harbored a T cell-specific deletion of Atg7, a critical enzyme that operates downstream of Vps34 during autophagy. Like LLO Vps34<sup>KO</sup> cells, nearly all Atg7<sup>KO</sup> CD4<sup>+</sup> T cells lost CD4 surface expression upon *in vitro* stimulation (Fig. 22A). Again, the absence of intracellular CD4 accumulation indicated that downregulated CD4 expression was not due to impaired recycling. I noted, however, that unlike LLO Vps34<sup>KO</sup> cells, most Atg7<sup>KO</sup> CD4<sup>-</sup> effector T cells remained CD4<sup>-</sup>CD8αβ<sup>-</sup>, with very few cells acquiring CD8αα<sup>+</sup> or CD8αβ<sup>+</sup> expression (Fig. 22B).

Furthermore, the lineage skewing profile of Atg7<sup>KO</sup> CD4<sup>-</sup> effector T cells was slightly different at the transcription vs. surface expression level. Like CD4 surface

expression, CD4 transcription was markedly suppressed (Fig. 22C). However, CD8 $\alpha/\beta$  transcription was enhanced, which was not reflected in the surface phenotype of most Atg7<sup>KO</sup> CD4<sup>-</sup> effector T cells. Transcription of other lineage-specifying genes, nevertheless, did match the pattern I observed in LLO Vps34<sup>KO</sup> CD4<sup>-</sup> effector T cells, namely, (1) upregulated expression of effector molecules granzyme A, granzyme B and perforin, and (2) a ThPOK<sup>lo-int</sup>Runx3<sup>hi-int</sup> master transcription factor signature (although Runx3 expression was lower in Atg7<sup>KO</sup> vs. WT CD4<sup>-</sup> T cells, while it had been higher in Vps34<sup>KO</sup> vs. WT CD4<sup>-</sup> cells) (Fig. 21B, Fig. 22C). From these results, I concluded that autophagic activity stabilized CD4 transcription while suppressing transcription and permitting protein expression of CD8 $\alpha/\beta$  during effector CD4<sup>+</sup> T cell differentiation, thereby regulating the generation of CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells.



**Figure 22. Autophagy regulates the effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cell phenotype.**

CD4<sup>+</sup> T cells were enriched from the total lymph nodes of Atg7<sup>fl/fl</sup> Lck-Cre (Atg7<sup>KO</sup>) mice by magnetic separation or FACS sorting, then stimulated *in vitro* using plate-bound anti-CD3/CD28 (1  $\mu$ g/ml) for 120 hours. **(A)** Surface/intracellular expression of CD4 was assessed in unstimulated Atg7<sup>KO</sup> T cells (0h, shown in gray) vs. stimulated Atg7<sup>KO</sup> T cells (120h, shown in blue). **(B)** CD8 $\alpha\beta$  surface expression was assessed in the TCR $\beta^+$ CD44<sup>hi</sup>CD4<sup>-</sup> sub-population of *in vitro*-stimulated WT and Atg7<sup>KO</sup> cells. **(C)** WT and Atg7<sup>KO</sup> effector CD4<sup>-</sup> T cells (TCR $\beta^+$ CD44<sup>hi</sup>CD4<sup>-</sup>) and effector CD4<sup>+</sup> T cells (TCR $\beta^+$ CD44<sup>hi</sup>CD4<sup>+</sup>) were generated by *in vitro* stimulation for 120 hours and enriched by magnetic separation or FACS sorting. Expression of 16 CD4/CD8 T cell lineage-specific transcripts was analyzed by qPCR, and relative expression was assessed by the delta-delta-Ct method using R with manual scripts. Data presented are from 1 of 2 independent experiments, with error bars representing standard deviations of experimental triplicates. Y-axis label shown for Lineage Identity also applies to Effector Molecules and Master Txn Factors. n = 3 from 3 independent experiments for **(A)** and **(B)**, and n = 2 from 2 independent experiments for **(C)**.

## **2.4 Conclusion**

The experimental evidence presented in this chapter defines a novel murine effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T lineage. Using both *in vitro* and *in vivo* approaches, I have demonstrated that (1) this population exhibits CD8<sup>+</sup> lineage-skewed phenotype, function and transcriptional programming; (2) this population can be generated from conventional effector CD4<sup>+</sup> T cells; and (3) the generation of this population is suppressed by the molecular mechanisms of autophagy. Building upon reports of other lineage-intermediate effector populations <sup>27, 56, 57, 58</sup>, the existence of effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells could further challenge our conventional assumptions about T cell lineage identity and its stability during effector differentiation.

### 3. Discussion

This study has produced several provoking clues to the origin, molecular regulation and physiological significance of effector CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells. Using multiple *in vitro* and *in vivo* strategies, I have demonstrated that this population exhibits a CD4/CD8 lineage-intermediate phenotype, can be generated from bona fide effector CD4<sup>+</sup> cells and is regulated by the key autophagy molecules Vps34 and Atg7. These findings may have implications not only for current paradigms concerning effector CD4<sup>+</sup> T cell differentiation, but also for the origin and function of a CD4-CD8<sup>+</sup> MHCII-recognizing T cell population found in human patients during a range of chronic diseases<sup>20, 33, 34, 35, 36, 37</sup>. Nevertheless, several unanswered questions arise as a result of this work. I seek to address some of the most outstanding ones here.

#### **3.1 Do CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells exclusively originate from effector CD4<sup>+</sup> T cells?**

With various lineage tracking methods, I have demonstrated that the CD4-CD8 $\alpha\beta$ <sup>+</sup> T cell can originate from mature naïve CD4<sup>+</sup> T cells. However, it should be noted that my demonstration of the CD4<sup>+</sup> origin of CD4-CD8 $\alpha\beta$ <sup>+</sup> T cells does not exclude the possibility that CD8<sup>+</sup> MHCII-restricted T cells can be derived during thymic development, or even that conventional CD8<sup>+</sup> MHCI-restricted T cells can exhibit cross-reactivity with MHCII peptide epitopes. Indeed, it has been shown that T cells with MHCI-restricted TCRs may enter the CD4<sup>+</sup> lineage with certain positive selecting ligands<sup>136</sup> or impaired function of certain transcription factors<sup>28, 29, 32, 90</sup>, and that CD8<sup>+</sup> MHCII-restricted T cells can be

generated in the context of abrogated ThPOK expression<sup>30</sup> or enforced Runx3 expression<sup>31</sup>. In addition, (1) human CD8<sup>+</sup> MHCII-recognizing T cells have been identified in ankylosing spondylitis patients (unpublished data), and (2) in the context of herpes virus infection, human CD8<sup>+</sup> T cells with bona fide MHCI recognition capacity have been shown to proliferate upon recognition of MHCII, in an MHC haplotype- and TCR clonotype-specific manner<sup>137</sup>.

Furthermore, I acknowledge that my conclusions on CD4<sup>-</sup>CD8 $\alpha\beta$ <sup>+</sup> lineage conversion are somewhat technologically restricted: (1) for *in vitro* and transfer experiments, FACS-sorting could not guarantee 100% cell purity, although I aimed for or attained this high level of technical stringency; (2) for *in vivo* tracking experiments, the efficiency of genetic marking was not 100%; and (3) for TCR repertoire sequencing, I could not recover every tetramer-positive cell from each animal.

However, for each of the experimental strategies I employed, I propose that the probability that aberrant development could completely account for the majority of CD8<sup>+</sup> MHCII-restricted T cells is extremely low. This probability is even lower when all of my strategies are considered together. Specifically, in my adoptive-transfer, lineage tracking experiments starting with purified naïve TdTomato<sup>-</sup>CD4<sup>+</sup> T cells, 8% of TdTomato<sup>+</sup> T cells were CD4<sup>-</sup>CD8<sup>+</sup> after LCMV infection. Before transfer, contamination from CD8<sup>+</sup> T cells during FACS-sorting was less than 2%, and expression of Cre recombinase was driven by the OX40 promoter, which is strongly preferred in T cells of CD4<sup>+</sup> origin<sup>115</sup>. In addition, CD4<sup>-</sup> MHCII-recognizing T cells did not have a proliferative or survival advantage in

comparison to bona fide CD4<sup>+</sup> effector T cells. Furthermore, repertoire sequencing results showed that a large portion of LCMV/I-A<sup>b</sup>-specific CD4<sup>+</sup> T cells shared an identical TCR sequence with LCMV/I-A<sup>b</sup>-specific CD4<sup>+</sup> T cells. Therefore, it is quite statistically improbable that all CD4<sup>+</sup> MHCII-recognizing clonotypes could be amplified from pre-existing populations that were completely distinct from CD4<sup>+</sup> ones. Taken together, I assert that, while mature, aberrant CD8<sup>+</sup> MHCII-restricted T cells may have contributed to my observations during this investigation, their potential presence does not negate the existence of CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells generated from effector CD4<sup>+</sup> T cells.

### ***3.2 How does autophagy suppress lineage conversion? For what physiological purpose?***

My findings indicate that Vps34 and Atg7, members of the autophagy machinery, regulate effector CD4<sup>+</sup> T cell lineage stability by a yet unknown mechanism. Others have reported that transcriptional regulators, including the NF- $\kappa$ B signaling components, can be processed by the autophagosome<sup>138, 139, 140, 141, 142</sup>, and also that CD4<sup>+</sup> T cell activation induces the preferential autophagosomal encapsulation of non-organelle cytosolic material<sup>100</sup>. I hypothesize that abrogated autophagosome formation inhibits autophagy-mediated sequestration/degradation of Runx3. This could in turn permit the accumulation of Runx3, upsetting the ThPOK/Runx3 concentration equilibrium that is necessary to maintain the stability of the CD4<sup>+</sup> lineage. In turn, ThPOK-mediated suppression of its silencer could be abrogated, allowing Runx3 to suppress ThPOK expression and CD4<sup>+</sup> lineage transcriptional programming<sup>30, 47, 89, 91, 93, 143, 144</sup>.

In addition, autophagy-mediated regulation of CD4<sup>+</sup> MHCII-recognizing T cell differentiation may be facilitated by other mechanisms. Specifically, preliminary evidence indicates that CD4 expression is regulated by the co-receptor activity of CD4 itself. Abrogation of CD4 co-receptor activity resulted in suppression of CD4 expression in CD4<sup>+</sup> T cells activated *in vitro* (unpublished data). It is therefore possible that suppression of autophagic activity could inhibit CD4 transcription by permitting increased intracellular levels of Runx3 protein, while suppression of CD4-pMHC engagement could also inhibit CD4 expression in tandem.

### **3.3 What is the physiological significance of CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells?**

Based on the findings produced by myself and others, I surmise that effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells can indeed be generated during the effector differentiation of conventional Th cells, particularly Th1 cells. Nevertheless, this population may play a key role in facilitating conventional CTL function within the specific context of chronic viral infection. More specifically, I consider 2 reasons why CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells may make an important contribution to the control or elimination of HIV infection. Firstly, due to lack of CD4 expression, CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells should be impervious to CD4-mediated viral infection <sup>145, 146, 147</sup>. Secondly, APCs that provide intracellular reservoirs for HIV and allow the virus to persist in a latent state should be efficiently targeted by effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells, due to their MHCII-mediated recognition capacity <sup>148</sup>. Indeed, a previous study in African green monkeys infected with

simian immunodeficiency virus (SIV), an HIV ortholog, indicated that downregulation of CD4 expression in memory T cells is associated with antiviral protection.<sup>145</sup> In addition, I note that autophagy plays a significant but evadable role in controlling HIV infection<sup>149, 150</sup>, which is not yet completely understood in T cells<sup>151, 152</sup>. Therefore, further investigations on CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells may indicate potential vaccine strategies for the prevention of HIV-1 infection that can circumvent previous obstacles to the development of T cell-based HIV vaccines. The promise of this research direction may be indicated by a CD4<sup>+</sup>CD8<sup>+</sup> MHCII-recognizing T cell population in HIV patients, identified by myself and others (unpublished data,<sup>110</sup>), which exhibits a lineage phenotype that is similar to the phenotype of the murine effector CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells defined during this study. Given that these cells exist at a higher frequency in HIV virus controllers, it is worth determining whether this effector phenotype can contribute to the prevention and control of HIV infection.

No doubt, the success of these hypothetical advances would depend on the capacity of CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells to form a long-lived memory population, and in turn, to generate robust immune recall responses. The experimental results I have produced evidence that CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells can acquire effector status and function. While I have not determined the memory capacity of this population, I hypothesize that CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells can indeed form an effective memory compartment for three reasons. Firstly, many of my experimental approaches depended on the LLO118 TCR transgenic system, which has previously been

shown to generate superior primary vs. secondary antigen-specific CD4<sup>+</sup> T cell responses<sup>111, 153</sup>. Secondly, in C57BL/6 mice infected with LCMV Armstrong for 30 days, I identified LCMV-I-A<sup>b</sup>-recognizing CD4<sup>+</sup>CD8 $\alpha\beta$ <sup>+</sup> T cells in the bone marrow (unpublished data), which is considered to be a niche that sustains memory T cells <sup>154</sup>. Thirdly, I identified a central memory phenotype among CD4<sup>+</sup>CD8<sup>+</sup> HIV-MHCII-recognizing T cells from virus controller PBMCs (unpublished data). Considering these observations together, the possibility that CD4<sup>+</sup>CD8<sup>+</sup> MHCII-recognizing T cells can form a robust memory population should be investigated.

## 4. Conclusion

What now remains is the application of what I have discovered to future directions for investigation. There are two main areas that I would like to address: (1) molecular mechanisms that facilitate lineage destabilization of conventional effector CD4<sup>+</sup> T cells to produce the CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cell lineage; and (2) HIV vaccine strategies designed to elicit an HIV-specific, memory population of CD4-CD8<sup>+</sup> MHCII-recognizing T cells.

### ***4.1 Molecular mechanisms of lineage conversion***

Based on work performed by myself and others, two mechanisms are apparent for harnessing the lineage conversion of CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells. Firstly, autophagy clearly plays a role in restricting the generation of this population. Secondly, CD4 co-receptor activity may inhibit CD4 expression. Therefore, CD4 vs. CD8 identity may be regulated during effector differentiation by autophagy or CD4 co-receptor activity, in a manner that may not be mutually exclusive. It will therefore be important to conduct the following investigations:

- 1) Determine the autophagy-mediated mechanism of sequestration/degradation of Runx3.

Initial studies may use immunofluorescent confocal imaging to trace the autophagosomal trafficking of Runx3 during effector CD4<sup>+</sup> T cell differentiation.

Localization of Runx3, autophagosome/lysosome markers, and nuclear markers

may be compared among (A) Naïve vs. *in vitro*-stimulated CD4<sup>+</sup> T cells: WT, Vps34-deficient and Atg7-deficient and (B) Effector CD4<sup>+</sup> vs. effector CD4-CD8αβ<sup>+</sup> MHCII-recognizing T cells: WT, Vps34-deficient and Atg7-deficient. If Runx3 levels are indeed suppressed by autophagosomal sequestration/degradation, then I would expect the following results: (A) Runx3 would co-localize with autophagosomal and lysosomal markers in *in vitro*-stimulated WT CD4<sup>+</sup> T cells. Co-localization would be abrogated in *in vitro*-stimulated Vps34-deficient and Atg7-deficient CD4<sup>+</sup> T cells. (B) Runx3 would co-localize with autophagosomal and lysosomal markers in *in vitro*-stimulated CD4<sup>+</sup> T cells, but not in CD4-CD8αβ<sup>+</sup> MHCII-recognizing T cells. Furthermore, higher levels of Runx3 would be found in the nucleus of Vps34-deficient and Atg7-deficient CD4-CD8αβ<sup>+</sup> MHCII-recognizing T cells vs. WT cells. These results would indicate that autophagy regulates Runx3 activity by decreasing intracellular Runx3 levels via protein sequestration/degradation.

2) Determine how abrogation of CD4 co-receptor engagement enhances the production of CD4-CD8αβ<sup>+</sup> MHCII-recognizing T cells.

The investigation may begin by generating CD4-CD8αβ<sup>+</sup> MHCII-recognizing T cells from WT CD4<sup>+</sup> T cells by antibody-mediated *in vitro* stimulation, in the presence of Th1-skewing cytokines, and in the presence/absence of a CD4 antagonist (e.g., GK1.5). Effector CD4-CD8αβ<sup>+</sup> MHCII-recognizing T cells may then be analyzed for (A) CD4/CD8 surface expression, (B) Functional molecule

expression (i.e., Th molecules: CD40L and Ox40, CTL molecules: granzyme B and IFN- $\gamma$ ) and (C) Transcript expression of lineage-specific genes (e.g., CD4, CD8 $\alpha/\beta$ , granzymes A & B, perforin, ThPOK, Runx3). If abrogation of CD4 co-receptor engagement does indeed promote the generation of CD4-CD8 $\alpha\beta^+$  MHCII-recognizing T cells, then I would expect the following results: (A) A range of CD4/CD8 phenotypes would be expressed in WT CD4 $^+$  T cells upon *in vitro* stimulation, in both the presence and absence of GK1.5. However, CD4-phenotypes, including CD4-CD8 $\alpha\beta^+$ , would be generated at an increased frequency in the presence of GK1.5. (B) CD40L and Ox40 surface expression would be decreased in the presence of GK1.5, while granzyme B and IFN- $\gamma$  expression would be increased. (C) CD4/ThPOK transcript levels would be decreased and CD8/cytotoxic molecule/Runx3 transcript levels would be increased in the presence of GK1.5. These results would suggest that abrogation of CD4 co-receptor engagement destabilizes the CD4 $^+$  T cell lineage program, allowing lineage-intermediate populations such as effector CD4-CD8 $\alpha\beta^+$  T cells to arise. On the other hand, if CD4 surface expression is decreased, but expression of all other lineage markers analyzed is not significantly changed in the presence of GK1.5, this would suggest that CD4 antagonism merely affects CD4 protein trafficking, rather than shifting the entire lineage program at the transcriptional level.

## **4.2 HIV vaccine development**

The potential utility of the molecular mechanisms underlying effector CD4<sup>+</sup> T cell lineage plasticity becomes prominent when considered in the arena of vaccine development, particularly against HIV. Future studies should include the following:

- 1) Develop an *in vitro* system for the differentiation of CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells from human PBMCs.

System development can be initiated by activating PBMCs from healthy patients by antibody-mediated *in vitro* stimulation, under the following differentiation conditions (separately as well as combined): (A) Th1-skewing cytokines/antibodies, (B) autophagy inhibition (e.g., treatment with the PI3K inhibitor 3-Methyladenine) and (C) CD4 antagonism (e.g., treatment with the anti-CD4 antagonistic antibody GK1.5). I would expect that conditions A-C would increase the frequency of effector CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells generated *in vitro*, and might have an additive effect when combined. This would indicate that (1) human CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells can be obtained at adequate numbers for *in vivo* studies and (2) abrogated autophagy and CD4 co-receptor engagement do play a mechanistic role in lineage destabilization of effector CD4<sup>+</sup> T cells to the CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing phenotype.

2) Develop an *in vitro* system for generating HIV-specific effector CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells.

Preliminary steps towards system development may include co-culture of human PBMCs or *in vitro*-derived human CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells with antigen-presenting cells loaded with various MHCII-restricted HIV-gag and HIV-env peptides. Activated, HIV-specific CD4<sup>+</sup> and CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells may then be analyzed for antiviral capacity using a virus inhibition assay (VIA).<sup>155</sup> If this system does indeed generate HIV-specific human effector CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells, then I would expect *in vitro*-stimulated effector CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells to exhibit antiviral activity against HIV, perhaps more potent than the antiviral activity exhibited by HIV-specific effector CD4<sup>+</sup> T cells. These results would indicate that a robust system for the generation of HIV-specific effector CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells had been developed, in order to produce this population at sufficient numbers for *in vitro* and *in vivo* studies. Furthermore, the results would suggest that CD4-CD8 $\alpha\beta$ <sup>+</sup> T cells exhibit enhanced potential to detect and eradicate HIV-infected cells vs. CD4<sup>+</sup> T cells.

3) Determine the potential of CD4-CD8 $\alpha\beta$ <sup>+</sup> MHCII-recognizing T cells for memory formation and function.

Initial studies could focus on LCMV-I-A<sup>b</sup>-recognizing memory CD4-CD8 $\alpha\beta$ <sup>+</sup> T cells from LCMV Armstrong-infected WT mice. Memory LCMV-I-A<sup>b</sup>-recognizing

CD4<sup>+</sup> and CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> T cells could be harvested and FACS-enriched from WT mice 30 days post-LCMV Armstrong infection. CD4<sup>+</sup> and CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> donor cells could then be transferred in parallel to T cell-deficient recipient mice infected with LCMV Armstrong. Eight days post-transfer, viral load could be assessed in recipient mice. If functional memory CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells can indeed be generated, I would expect that this population will be isolated in sufficient numbers from donor mice, and viral load will be lower in CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> recipients vs. CD4<sup>+</sup> recipients.

Performing these investigations could elucidate remaining important questions, and could allow us to fully understand, realize and harness the clinical potential of effector CD4<sup>+</sup>CD8 $\alpha$  $\beta$ <sup>+</sup> MHCII-recognizing T cells.

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## **Biography**

Elizabeth Robins received her Ph.D. in Immunology from Duke University in March 2019. She received her B.S. in Biology with Department Honors and a Specialization in Microbes and Immunity from Stanford University. Meanwhile, Elizabeth conducted research as an intern at the National Heart, Lung, and Blood Institute (NHLBI). She then trained in the Immunology Ph.D. program at Duke University, under the mentorship of Dr. Qi-Jing Li, authoring or contributing to 4 publications in peer-reviewed journals. Elizabeth has presented her work at numerous Immunology research conferences, including the Southeastern Immunology Symposium and the American Association of Immunologists (AAI) Annual Meeting. Elizabeth is the recipient of an AAI Careers in Immunology Fellowship, an AAI Young Investigator Award and a Duke University Graduate School James B. Duke Fellowship. She is also a member of the Society of Duke Fellows.