UNIVERSITY OF CALIFORNIA SAN DIEGO

Intestinal CD4 Cytotoxic T Lymphocytes are Generated during Steady State and Confer Protective Immunity during Infection

A dissertation submitted in partial satisfaction of the requirements
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in

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by

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The Dissertation of Angeline Chen is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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DEDICATION

To my family, friends, and mentors.

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LIST OF ABBREVIATIONS

α	alpha
Ag	antigen
APC	antigen presenting cell
β	beta
CD	Celiac disease
CFU	colony forming unit
cKO	conditional knock out
CTL	cytotoxic T lymphocyte
Δ/δ	delta
DC	dendritic cell
DN	double-negative
DP	double-positive
Epi	epithelium
ε	epsilon
FACS	fluorescence-activated cell sorting
γ	gamma
GALT	gut-associated lymphoid tissue
HBSS	Hanks's balanced salt solution
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFNγ	interferon γ
IET	intraepithelial T lymphocyte

KO	knock out
LP	lamina propria
MHC	major histocompatibility complex
μ	micro
NK	natural killer cell
PBS	phosphate buffered saline
RA	retinoic acid
RPMI	Roswell Park Memorial Institute 1640 medium
SCV	Salmonella-containing vacuole
SP	single-positive
SPF	specific pathogen free
STM	Salmonella *enterica* serovar Typhimurium
TCR	T cell receptor
Th	T helper cell
TL	thymus-leukemia antigen
Treg	T regulatory cell
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
UC	ulcerative colitis
WT	wild type

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ABSTRACTS

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ABSTRACT OF THE DISSERTATION

Intestinal CD4 Cytotoxic T Lymphocytes are Generated during Steady State and Confer Protective Immunity during Infection

by

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Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2022

Professor Mitchell Kronenberg, Chair

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During steady state, CD4 T helper (Th) cells reprogram to cytotoxic T lymphocytes (CTLs) in the small intestinal epithelium. In the present study, CD4 CTLs were evaluated for their potential to promote tolerance during steady state as well as their ability to fend off enteric pathogens during infection.

The CD4 T cell adoptive transfer model of colitis was used to determine if CTLs may represent a strategy of avoiding CD4 Th-mediated inflammation. Using both an *in vitro* differentiation model as well as transgenic models, where CD4 T cell fates are forced into predetermined phenotypes, the conversion of Th to CTL was found to mitigate disease pathology and promote tolerogenic conditions.

After defining the role of CD4 T cells during steady state, we then assessed the ability of CD4 CTLs to respond to enteric pathogens. Pre-existing CD4 CTLs were found to have the capacity to kill Salmonella *enterica*-infected cells, thereby preserving the integrity of the barrier and preventing bacterial dissemination. Furthermore, we identified IL-15/IL-15Rα complex trans-presentation as a mechanism of inciting CD4 CTLs to become active killers. Functionally, CD4 CTLs represent a strategy of the immune system to fortify the epithelium with quiescent but primed CTLs that can provide rapid immunity during enteric infection. The generation of CD4 CTLs during steady state and their functional relevance during infection is a novel strategy of mucosal immunity that can be defined as 'protective tolerance'.

CHAPTER 1: Introduction

CD4 cytotoxic T lymphocytes (CTLs) naturally arise in the small intestine epithelium. However, as with all T cells, their origin begins in the thymus. The following will cover CD4 T cell development from their early beginnings in the thymus cortex, to their intermission in secondary lymphoid organs, and finally their differentiation into CTLs in the small intestine epithelium.

1.1 CD4 T Cell Development in the Thymus

Cells that have the potential to eventually mature into T cells derive from hematopoietic progenitors that enter the thymus through the corticomedullary junction (Bhandoola, von Boehmer, Petrie, & Zúñiga-Pflücker, 2007). These intrathymic progenitors develop and differentiate following interactions with thymic stromal cells that elicit signaling pathways. Among these pathways, Notch signaling is especially important for T cell lineage commitment

Through interactions with thymic stromal cells involving Notch, among other signaling pathways, these intrathymic progenitors commit to the T cell lineage. During thymic development and differentiation, thymocytes go from a CD4-CD8αβ- double-negative (DN) stage into a CD4+CD8αβ+ double-positive (DP) stage. During this transition, thymocytes also begin the fundamentally stochastic process of T cell receptor (TCR) rearrangement, a critical event that distinguishes lymphocyte biology.

The initial rearrangement of their *Tcrb* locus begins at the DN4 (CD25-CD44-) stage and results in the expression of a variable TCRβ chain that together with an invariant Pre-α appear at the cell surface as a variable Pre-TCR. At the DP stage rearrangements of the *Tcra* locus lead to

the expression of a complete unique αβTCR. If this ontogenically determined αβTCR then weakly recognizes a major histocompatibility complex (MHC) class I or II, the T cell will then further differentiate into either a CD8αβ single-positive (SP) or CD4 SP thymocyte, respectively. These CD8αβ SPs are characterized by the expression of transcription factor Runt domain-related factor 3 (RUNX3). In contrast, CD4 SPs are characterized by the expression of transcription factor T-helper-inducing POZ/Krueppel-like factor (ThPOK). After the audition in the thymus, mature CD4 and CD8αβ T cells will then travel into a secondary lymphoid tissues and further differentiate and potentially migrate largely dependent on their antigen specificity (Hogquist & Jameson, 2014; Wang et al., 2008).

A naïve CD4 T cell can migrate to the gut-draining mesenteric lymph nodes (mLNs) or Peyer's patches (PP) and will then await cognate antigen stimulation. This signal is provided by MHC class II-expressing specialized gut-associated lymphoid tissue (GALT)-derived antigen presenting cells (APCs), such as CX3CR1+ macrophages and migratory CD103+ dendritic cells, which have the capability to phagocytose luminal antigens and cross-present them to naïve T cells. The antigen-experienced CD4 T cells will then migrate to the small intestine epithelium (Campbell & Butcher, 2002). When the recently TCR-activated CD4 T cells reach the epithelium, they will continue tissue-specific differentiation and development.

1.2 Imprinting by the Small Intestine Epithelium

A primary function of the small intestine is to digest macromolecules and absorb nutrients and fluids to nutritionally support the host. This duty is complicated by the presence of opportunistic microbiome members, also known as pathobionts, in addition to enteric pathogens that can potentially invade or bypass the epithelium and cause systemic infection or even sepsis.

Immunity at this barrier requires effective elimination of infected cells while simultaneously minimizing the risk for immunopathology that could compromise the single-cell epithelial layer. As such, the single-cell epithelial layer is fortified with a vast number of heterogeneous effector lymphocytes. These intraepithelial T lymphocytes (IETs) include both natural and induced lymphocyte populations (Cheroutre, Lambolez, & Mucida, 2011). The natural IET populations include CD8α-CD4- DN αβT cells and γδT cells that are thought to directly migrate to the small intestine epithelium. The induced IET populations arise from mature T cells that migrate from the thymus and into GALT. Generally, the IETs have many shared features and functions that distinguish them from systemic effector cells. Among these include the expression of integrin αE, also known CD103, which allows the IETs to interact with the E-cadherin expressed on intestinal epithelial cells (IECs). Another direct interaction between lymphocyte and epithelium is mediated by the expression of CD8αα on the IETs and thymic leukemia antigen (TL) on the IECs. This interaction has further implications due to evidence that CD8αα increases the stimulation threshold by suppressing TCR signaling (Cheroutre & Lambolez, 2008; Denning et al., 2007). Other markers that the IETs tend to share include effector molecule expression, such as activation markers (e.g. CD69), NK markers, and cytotoxic machinery (e.g. granzymes).

Given the heterogeneous nature of the IET populations, there are certainly numerous developmental and functional differences that distinguish the individual populations. Among these populations is a population of MHC class II-restricted CD4 T cells that are unique to the small intestine epithelium.

1.3 Conversion of CD4 T helper cells into Cytotoxic T Lymphocytes

Previously characterized by Mucida & Husain et al., there is a major population of CD4 T cells present during steady state within the small intestine epithelium that express CD8αα homodimers (Mucida et al., 2013). The CD8αα expression is induced and corresponds with the transition from transcription factor ThPOK expression to RUNX3 expression. Given that RUNX3 is a master transcription factor for CTLs, the RUNX3 expression also corresponds with the production of cytotoxicity proteins, such as Granzyme B and perforin, as well as NK-related cytotoxicity factors, such as 2B4. Furthermore, these CD4 CTLs were found to have similar killing capacity as their CD8αβ counterparts in an *in vitro* redirected cytotoxicity assay (Mucida et al., 2013).

The transcriptional conversion from ThPOK+ T helper (Th) to RUNX3+ CTL in the epithelium is a tightly controlled and dynamic process. ThPOK and RUNX3 are thought to mutually repress one another (Egawa, Tillman, Naoe, Taniuchi, & Littman, 2007; Naito, Muroi, Taniuchi, & Kondo, 2018). In addition to ThPOK as a master transcription factor for Th lineage, GATA-binding protein 3 (GATA3) also has a fundamental role in the differentiation of CD4 T cells, both in the thymus and in the periphery (Cheroutre & Husain, 2013). GATA3 both induces ThPOK as well as represses RUNX3 expression. Therefore, in order for ThPOK to be downregulated, GATA3 must first be turned off. Small intestine-specific factors may favor GATA3 downregulation, and consequently ThPOK downregulation, that allow for the subsequent upregulation of TBET and RUNX3 associated with CTL differentiation. CD4 T cells demonstrate profound transcriptional and functional plasticity in the periphery. The heterogeneity of CD4 Th cells as well as their ability to convert into other Th fates in systemic immunity has been well-characterized (Murphy & Stockinger, 2010). This study seeks to elucidate the small intestine

epithelium-specific mechanism of CD4 Th conversion to RUNX3+ CTLs and their functional roles in the context of steady state and infectious conditions.

1.4 Summary and Specific Aims

CD4 T cells are classically thought as T 'helper' cells with the primary function of activating and recruiting other cells. However, in the small intestine epithelium, CD4 T cells demonstrate remarkable transcriptional plasticity to convert their entire ThPOK-driven 'helper' programming towards a RUNX3-driven CTL fate. The following aims seek to examine the developmental requirements of CD4 CTL and determine a potential non-redundant role in combating enteric pathogens without compromising the epithelial barrier.

Aim 1: Identify the cytokine and antigenic requirements, including the nature of the antigen and antigen presenting cell, that lead to CD4 CTL development.

The tissue-specific environment of the small intestine epithelium during steady state is important for CD4 CTL development. Due to the presence of CD4 CTLs prior to enteric infection, the antigen specificity is unlikely to be pathogen-derived. The combination of cellular signals through cytokines and antigenic stimulation will be investigated, including the characterization of the source of cognate antigen and the type of antigen presenting cell responsible for reprogramming T helpers into CTLs.

Aim 2: Assess the potential of CD4 CTLs to either induce or circumvent immunopathology.

CD4 CTLs are typically found in the small intestine epithelium and indicate a normal composition of intraepithelial lymphocytes. However, it is unknown if this conversion process promotes or inhibits the potential of CD4 CTLs to be pathological. Using colitis models, CD4 CTLs and the

underlying factors of transcriptional reprogramming will be assessed for disease induction or prevention.

Aim 3: Assess the protective capacity of non-pathogen-specific CD4 CTLs against the food-borne pathogen Salmonella *enterica* serovar Typhimurium.

The presence of CD4 CTLs prior to pathogen exposure implies the potential for a quiescent role during steady state but active cytotoxicity when required. This aim will seek to determine a non-redundant protective role for MHC class II-restricted CD4 CTLs against a pathogen that is typically not associated with a CD8αβ CTL response. Furthermore, the mechanism of how typically quiescent CD4 CTLs may be induced into active killers will also be addressed.

CHAPTER 2: Generation of Cytotoxic CD4 T Cells in the Epithelium

2.1 Introduction

CD4 cytotoxic T lymphocytes (CTLs) exist during steady state in the small intestine epithelium of specific-pathogen free mice, prior to exposure to infectious agents. Logically, CD4 CTLs must therefore have specificity for non-pathogen-derived antigens, such as those derived from diet or microbiome. Using both monoclonal transgenic and polyclonal WT mouse models, the antigenic requirements of CD4 CTLs will be explored in this chapter.

After determining the role of cognate luminal antigens on CD4 CTL development, we then investigated the type of antigen presenting cell (APC) that drives the conversion of CD4 CTLs at steady state. Intestinal epithelial cells (IECs) represented an intriguing tissue-specific APC due to their ability to absorb luminal antigens, constitutive expression of MHC class II, and their proximity to CD4 CTLs (Hershberg et al., 1998; Wosen, Mukhopadhyay, Macaubas, & Mellins, 2018). Furthermore, IECs have functional differences from systemic myeloid APCs that may be conducive towards CD4 CTL reprogramming, including the lack of costimulatory molecules, such as B7-1/CD80 or B7-2/CD86. In this study, we have investigated the effects of dietary antigen, the mode of antigen presentation, and the site-specific cytokine milieu that allow for CD4 CTL generation in the small intestine epithelium.

2.2 Materials and Methods

Mice

Animal care and experimentation were consistent with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the La Jolla Institute for Immunology. In general, mice were sex- and age-matched and compared with WT littermates where applicable.

Both male and female mice were used, unless otherwise stated. Experiment specific details are included in the figure legends. Transgenic, monoclonal OT-II *Rag2*$^{-/-}$ (#1896) were purchased from Taconic. *Il12rb2*^{tm1Jm} (#003248; IL-12Rb2 KO) and *Il27ra*^{tm1Mak}*/J* (#018078; IL-27Ra KO*)* were purchased from Jackson Labs and crossed with in-house C57BL/6J mice for heterozygous WT controls. The colony was maintained in-house at the La Jolla Institute for Immunology rodent vivarium.

Naive CD4 T Cell Isolation

Spleens from OT-II *Rag2*$^{-/-}$ mice were harvested into HBSS supplemented with 5% FBS and pushed through 70μm cell strainers atop a 50mL conical tube. The single cell suspension was then washed, centrifuged, and the resulting cell pellet was lysed with lysis buffer (Sigma, cat #R7757). The resulting lymphocyte population was then enriched for naïve CD4 T cells using a magnetic separation kit (Stemcell, cat #19852) or FACS. Remaining cells were then counted using a hemocytometer, washed and resuspended in sterile 1X PBS at 5x10^5 cells/100μL.

CD4 T Cell Adoptive Transfer

Naive OT-II CD4 T cells were isolated, as previously described, and 5x10^5 cells were transferred via retro-orbital injection into *Rag*$^{-/-}$ host mice. Host animals were then either fed with an ovalbumin-containing diet with normal compositions of nutrients and macromolecules or with a standard diet that lacks ovalbumin.

Alternative Diet Feeding

Rodent chow supplemented with ovalbumin or casein (Research Diets, Inc, NJ, USA) is a modified version of AIN-76A diet with 20kcal% ovalbumin or casein. Amino acid diet was purchased from Envigo RMS LLC (TD 140168). In general, mice were weaned onto a NIH-31 rodent diet (Taconic) and then switched to the alternative diets for 4-5 weeks, beginning at age 6-8 weeks old.

Lymphocyte Isolation from Thymus, Spleen, Lymph Node

Single cell suspensions were prepared by homogenizing tissues through a 70μm nylon cell strainer in complete RPMI 1640 media supplemented with 5% FBS. If there was a heavy presence of red blood cells, cell suspensions were lysed using Red Blood Cell Lysis Buffer (Sigma, cat #R7757).

Lymphocyte Isolation from Small Intestine

Small intestines were quickly removed, using forceps and scissors, within 5-10 min post-euthanasia. Fecal contents and Peyer's Patches were removed using curved scissors. Intestines were then cut into approximately 0.1 cm pieces and transferred into 50mL conical tubes with 30mL complete RPMI 1640 media supplemented with 5% FBS containing 1mM DTT (Sigma) and 5mM EDTA (Sigma). Cell suspensions were then shaken at 220 RPM for 20 min at 37 degrees Celsius and then passed through a 70um nylon mesh into a fresh 50mL conical tube and pelleted by centrifugation. The cell pellets were then resuspended in 8mL 40% Percoll (Cytiva), then layered over 2mL 70% Percoll (Cytiva) to form a gradient in 15mL conical tubes. Percoll mixes were then centrifuged at room temperature for 20 min. Cells were collected from the 40%/70% interface, washed, and resuspended in complete RPMI 1640 media supplemented with 5% FBS.

***In Vitro* Culture with IL-12 Family of Cytokines**

Naive CD4 T cells were cultured in RPMI 1640 supplemented with 10% FBS, penicillin-streptomycin, and β-mercaptoethanol with 1μg/mL anti-CD28, 5ng/mL TGFβ, and 100nM retinoic acid on high-bind 96 well plates coated with 1μg/mL anti-CD3ε. Depending on experimental condition, 25ng/mL IL-27, 25ng/mL IL-12, or 1μg/mL IFNγ were added.

Flow Cytometry

Cells were suspended in staining buffer (1X PBS with bovine serum albumin and sodium azide) and blocked with 1:500 concentration of anti-Fc receptor antibody 2.4G2. Cells were stained

according to standard fixation and permeabilization protocols for surface and intracellular markers, respectively. Briefly, for detection of transcription factors, the eBioscience™ Intracellular Fixation and Permeabilization kit was used; for the detection of cytokines, the BD™ Cytofix/Cytoperm kit was used. All samples were analyzed using an LSRII or Fortessa flow cytometer (BD Biosciences).

Absolute Cell Numbers

Total cell numbers were acquired using a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter). Absolute numbers of cell populations were then calculated using frequencies determined by flow cytometric analysis.

Statistical Analysis

Figure-specific statistical analyses are provided in figure legends. In summary, all data were analyzed using GraphPad Prism 8 software and data are shown as mean and the standard deviation. Statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or ns (not significant).

2.3 Results

Small intestinal CD4 T cells require cognate antigen to develop into CTLs in the epithelium

To determine if CD4 CTLs can have specificity for non-pathogen-derived antigens, we performed an adoptive transfer of naive OT-II CD4 T cells, which have a specificity for an epitope within the protein ovalbumin (OVA), into *Rag*$^{-/-}$ recipients that were fed OVA or a control diet lacking OVA for 4 weeks. In the context of this lymphopenic host fed OVA, an expansion of OVA-responding OT-II CD4 T cells was observed across all analyzed tissues, including spleen, small intestine (SI) lamina propria (LP), and SI-epithelium (Epi) (*Figure 1*A, B). There was a significant expansion of FOXP3+ Treg cells in the spleen and SI-LP (*Figure 1*C, D).

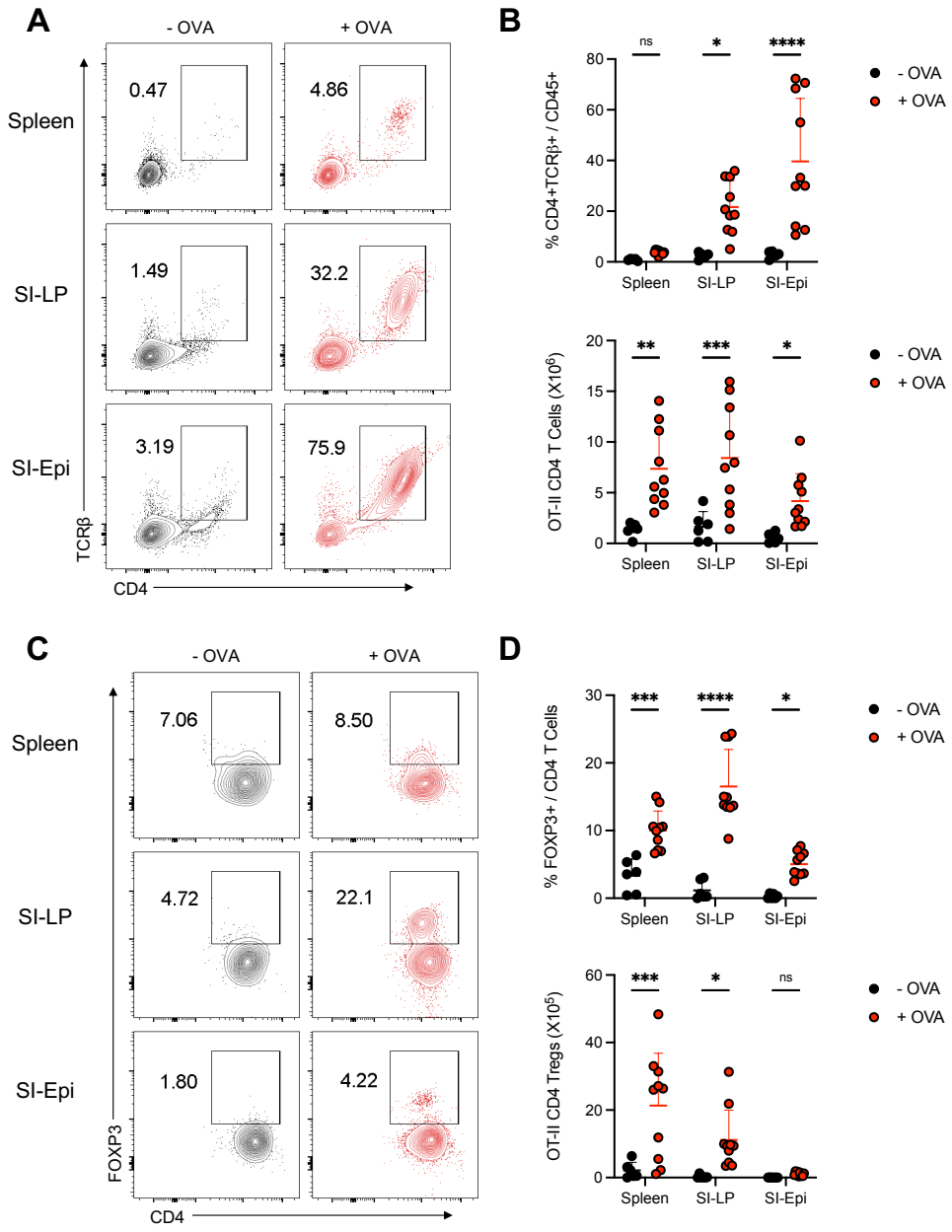

Figure 1: Diet-specific CD4 T cells expand across all tissues in response to cognate antigen in diet.

Representative flow cytometry plots (A, C) and summary statistics (B, D) describing adoptively transferred OT-II CD4 T cells into *Rag*-/- recipients after 4 weeks on Ovalbumin (OVA+) diet or a control (OVA-) diet. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; One-way ANOVA statistical test was used to compare multiple groups. Data are representative results from one of at least two independent experiments with pooled results from at least two independent experiments with at least three mice per group in each experiment.

This phenomenon of FOXP3+ Tregs expanding in the SI-LP was proposed as a mechanism of immune modulation in the intestine to suppress aberrant reactivity towards harmless diet-derived antigens (K. S. Kim et al., 2016). However, this phenomenon was notably less present in the SI-Epi. In particular, the frequency of FOXP3+ Treg cells among total CD4 T cells was not significantly increased, in contrast to the spleen and SI-LP (Figure 1C, D).

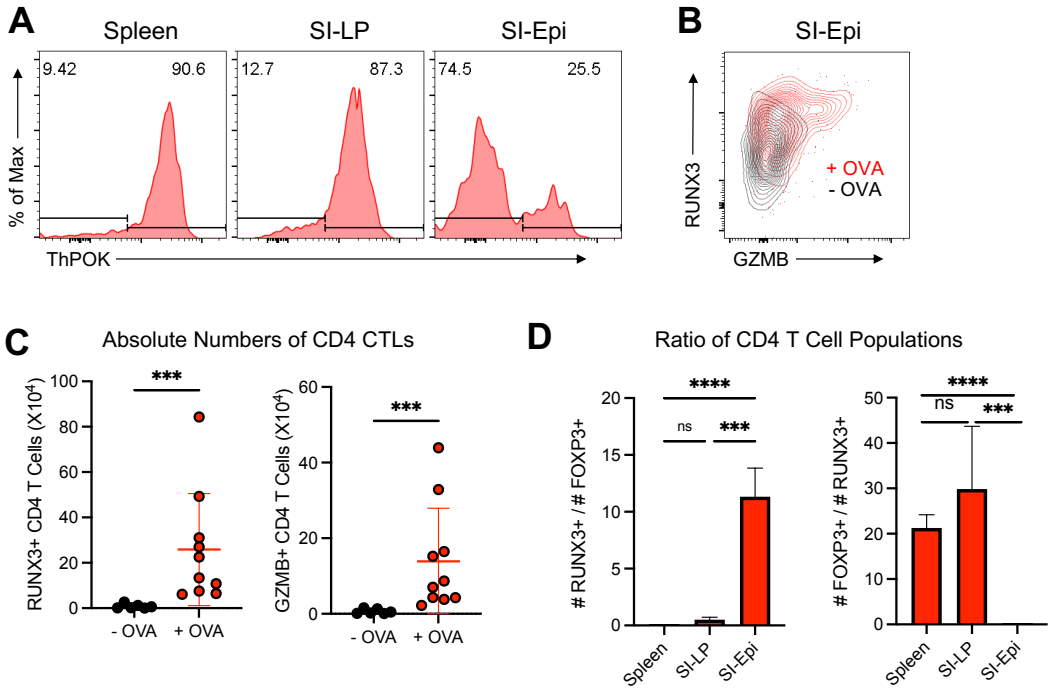

Figure 2: Diet-specific CD4 T cells uniquely convert to CTLs in the small intestine epithelium.

(A) Representative histogram plots of ThPOK expression in the stated tissues. (B) Representative flow cytometry overlay plots comparing epithelial CD4 T cells from the OVA+ and OVA- conditions. (C) Absolute numbers of CD4 CTLs, as defined by RUNX3+ or GZMB+ expression. (D) Ratios of Tregs to CTLs in the stated tissues. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups, One-way ANOVA statistical test was used to compare multiple groups. Data represents pooled results from at least two independent experiments with at least three mice per group in each experiment.

In lieu of FOXP3+ Tregs developing, the majority of the OVA-responding OT-II CD4 T cells in the epithelium had downregulated ThPOK and subsequently upregulated RUNX3 and gained cytolytic machinery, such as Granzyme B (Figure 2A, B, C). RUNX3+ and GZMB+ CD4

CTLs were found to have significantly expanded in the epithelium instead (Figure 2C). The proportion of CTLs to Tregs was significantly higher in the SI-Epi with the proportions inversed in the spleen and SI-LP (Figure 2D). Conversely, the ratio of FOXP3+ Treg to CTLs was significantly higher in the spleen and SI-LP as compared to the SI-Epi (Figure 2D).

Dietary antigens can shift whole body TCR repertoire

After determining that monoclonal OT-II TCR transgenic CD4 T cells convert to CTLs in response to cognate OVA-antigen present in the diet, we sought to recapitulate these findings in a polyclonal *Rag*-competent mouse model.

OT-II transgenic Rag^{WT} mice were fed with either an ovalbumin-containing diet or a control diet that lacks ovalbumin and the resulting populations were assessed (Figure 3A). Whereas the splenic population of Vα2β5+ OT-II CD4 T cells decreased (Figure 3B, C) in response to 4 weeks of ovalbumin feeding, the frequencies and total cell numbers of polyclonal Vα2β5- non-OT-II TCR became more dominant in the spleen (Figure 3D). Conversely, in the small intestine epithelium, the presence of cognate dietary antigen led to the accumulation or expansion of the ovalbumin-responding OT-II CD4 T cells alongside a decrease in the frequency of polyclonal non-transgenic CD4 T cells (Figure 3E, F).

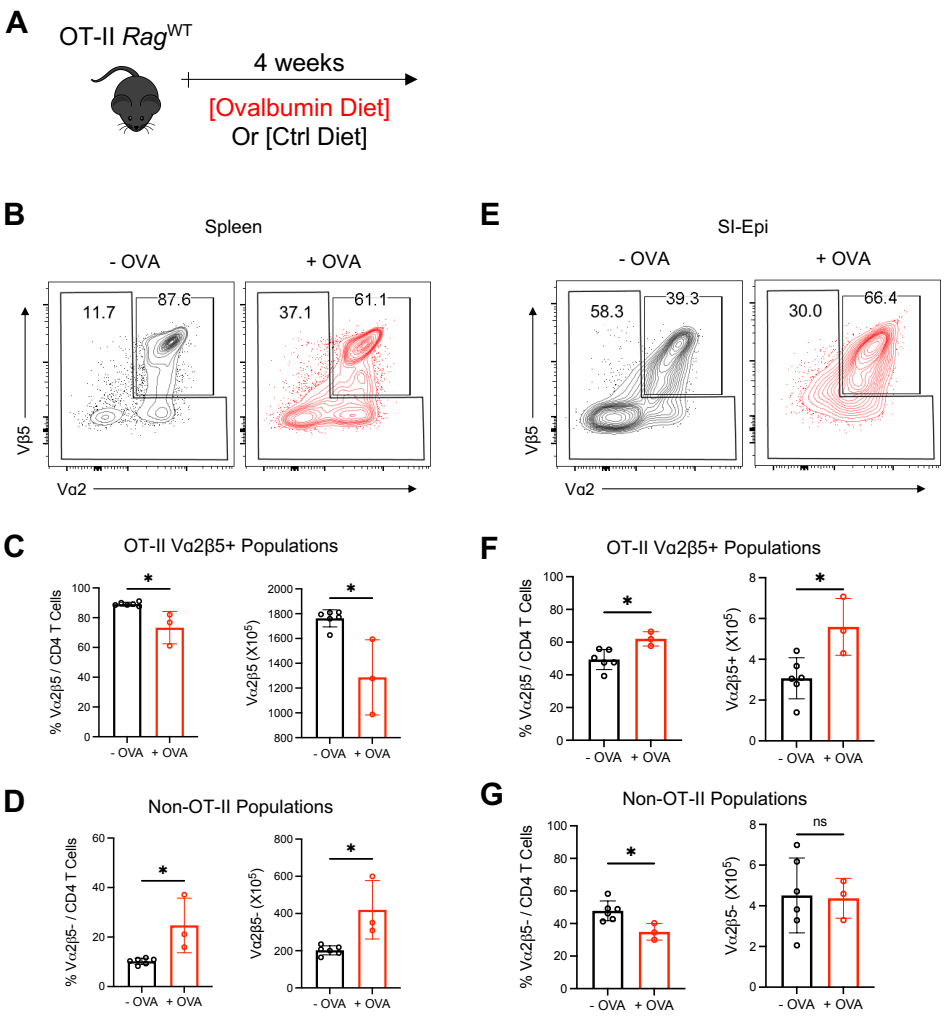

Figure 3: Dietary antigens can shift systemic and epithelial T cell repertoire.

(A) Schema of experimental design. (B, E) Representative flow cytometry plots and (C, D, F, G) summary statistics comparing OT-II CD4 T cells from the OVA+ and OVA- conditions. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

That a single dietary antigen can shift the T cell repertoire of the peripheral immune system and "resident" tissue immune cells in opposite directions is a testament to how influential antigens can be in shaping and directing immunity in the whole body.

CD4 CTLs must be repeatedly stimulated with luminal antigen to persist in the epithelium

The next question we sought to answer was if the CD4 CTLs require continuous stimulation by luminal antigens to remain the small intestinal epithelium. Using both the monoclonal OT-II adoptive transfer model and polyclonal WT C57BL/6 mice, dietary antigen was removed and the resulting CD4 T cell populations were evaluated.

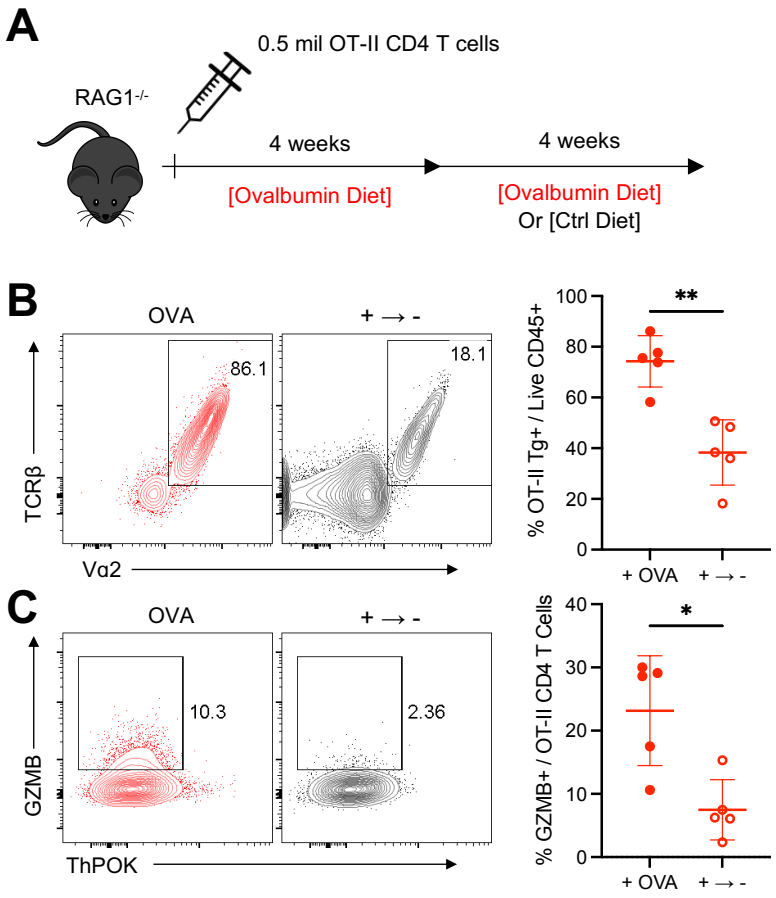

Figure 4: CD4 CTLs depend on repeated dietary antigen stimulation to persist.

(A) Schema of experimental design. (B, C) Representative flow cytometry plots (left) and summary statistics (right) comparing OT-II CD4 T cells from the OVA+ and the OVA-removal (+ -> -) conditions. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data represents pooled results from at least two independent experiments with at least two mice per group in each experiment.

When OT-II CD4 T cells were adoptively transferred and the mice were fed OVA diet for 4 weeks and then OVA diet was either removed or continued for another 4 weeks (Figure 4A), frequencies of the OT-II transgenic TCR Vα2 were found to have significantly decreased in the condition that had ovalbumin diet removed (Figure 4B). Furthermore, the Granzyme B-expressing portion of those OT-II cells were also significantly decreased (Figure 4C). Therefore, we concluded that dietary antigen specific CD4 T cells require a continuous source of antigen to persist with cytotoxic machinery in the small intestine epithelium.

We then addressed the nature of the antigens that drive the conversion of conventional MHC class II restricted CD4 Th cells to CTL. WT polyclonal C57BL/6 mice were initially weaned onto a standard diet, which contains various types of antigens, including complex carbohydrates, glycolipids, and proteins. At the adult stage, the mice were switched over to an amino acid (AA)-containing diet, which consists of normal quantities of carbohydrates and glycolipids but proteins were replaced by individual amino acids (*Figure* ***5***A). Four weeks later mice were analyzed for the systemic and small intestine epithelial T cells. The result was a striking decrease in overall CD4 T cells in the SI-Epi of the mice that were fed an AA-containing diet that lacked whole proteins (*Figure* ***5***B). Among the CD4 T cells that decreased, the CTL compartment of RUNX3+ and GZMB+ cells were the most significantly impacted (*Figure* ***5***C, D). Following this loss of CD4 CTLs, the proportion of ThPOK+ Th cells compared to RUNX3+ CTLs was skewed in favor of the Th cells (*Figure* ***5***E).

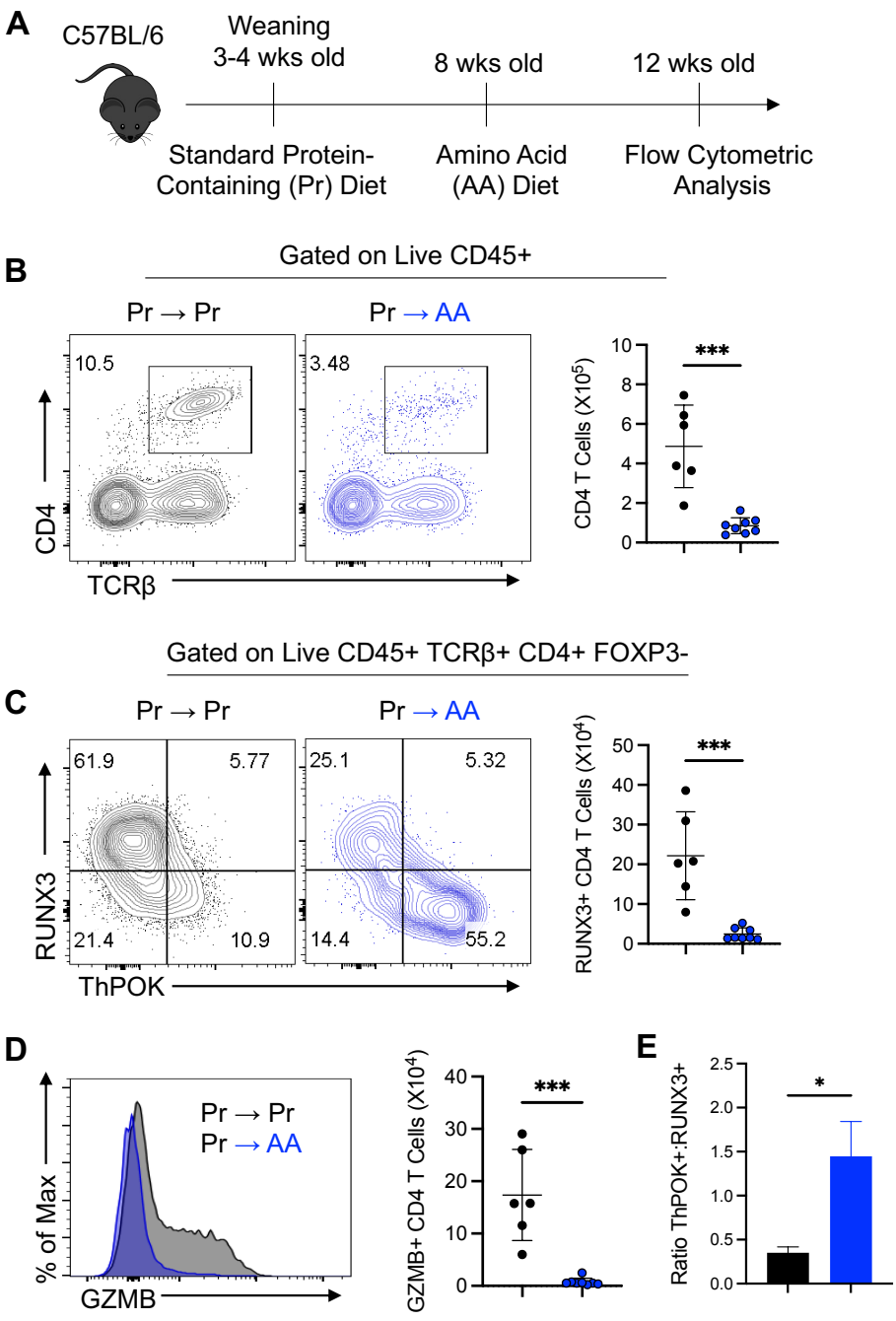

Figure 5: Loss of dietary proteins leads to decrease in small intestine epithelial CD4 CTLs.

(A) Schema of experimental design. (B, C, D) Representative flow cytometry plots (left) and summary statistics (right) comparing the stated small intestine epithelial CD4 T cell populations from the standard protein-containing (Pr) and the amino acid diet (AA) conditions. (E) Ratio of total numbers of ThPOK+ Th to RUNX3+ CTLs. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data represents pooled results from at least two independent experiments with at least three mice per group in each experiment.

Feeding the WT mice with an AA-containing diet did not impact the systemic CD4 T cell populations in terms of frequency of CD4+ TCRβ+ among the live CD45+ population or their total CD4 T cell numbers (Figure 6A). Furthermore, splenic CD4 T cells typically do not have a RUNX3+ CTL compartment and this was the case in either the standard protein-containing diet fed condition or for mice switched to an AA-containing diet for 4 weeks (Figure 6B).

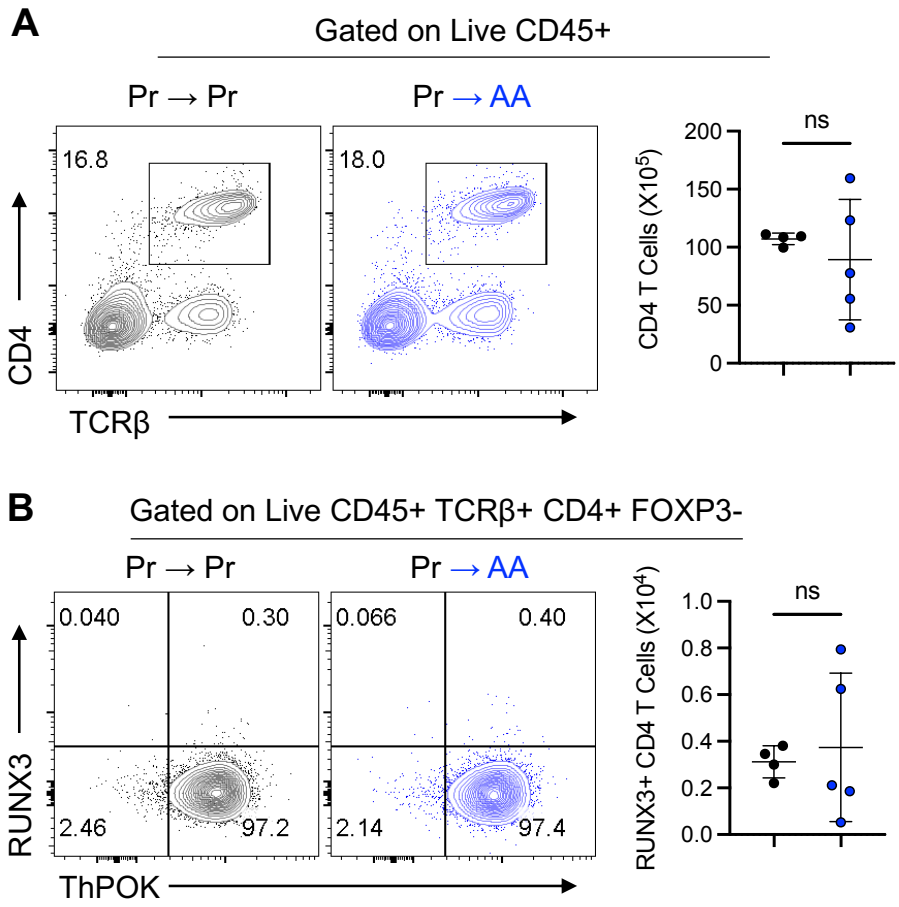

Figure 6: Loss of dietary proteins does not affect systemic CD4 T cells.

(A, B) Representative flow cytometry plots (left) and summary statistics (right) comparing stated CD4 T cell populations from the standard protein-containing (Pr) and the amino acid diet (AA) conditions. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group (panels) or pooled results from at least two independent experiments with at least three mice per group in each experiment (panels).

After determining that the proteinaceous nature of the antigen is important for the CD4 CTL conversion in the small intestine epithelium, we then addressed if the simple presence of any protein is enough to generate and maintain the CD4 CTL population in the intestine. C57BL/6 mice fed a normal protein-containing diet were switched onto a nutritionally identical novel protein-containing diet (NPD), where the protein compartment consisted solely of casein, which the mice had not encountered previously (Figure 7A). Intriguingly, we observed that the overall CD4 T cell population was significantly decreased in the SI-Epi following the loss of the original repertoire of protein peptides after 4 weeks (Figure 7B). Among the remaining CD4 T cells, their RUNX3 and Granzyme B expression was also significantly decreased, both in terms of frequency as well as total cell numbers (Figure 7C, D). The loss of the original repertoire of proteins or peptides in the diet that the mice were weaned onto also skewed ratio of Th cells to CTLs towards Th-dominance in the SI-Epi, which is a departure from the site-specific prevalence of CD4 CTLs (Figure 7E). Similar to the AA-containing diet switch, there was virtually no difference in terms of overall CD4 T cells or the continued absence of CD4 CTLs in the spleen (Figure 8A, B).

Taken together, using both a monoclonal adoptive transfer model and a polyclonal WT model, the presence of a consistent source and repertoire of specific cognate protein antigens in the diet is important to generate and maintain CD4 CTL at steady state in the small intestine. In contrast to conventional resident memory T cells, the CD4 CTLs require continuous or at least intermittent stimulation by cognate antigen to remain functional with cytotoxic machinery in the epithelium.

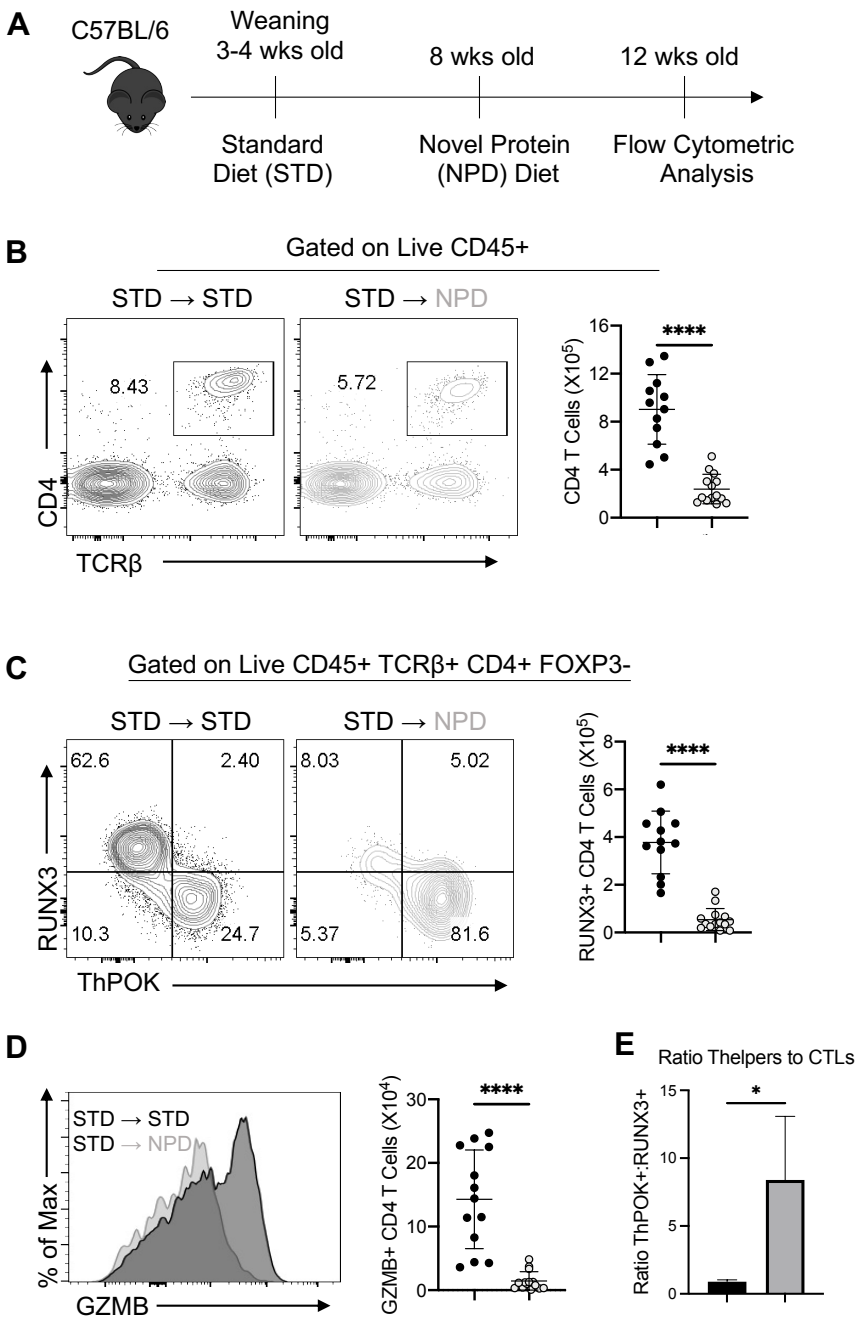

Figure 7: Loss of original dietary protein repertoire leads to a decrease in epithelial CD4 CTLs.

(A) Schema of experimental design. (B, C, D) Representative flow cytometry plots (left) and summary statistics (right) comparing the stated small intestine epithelial CD4 T cell populations from the standard diet (STD) and the novel protein diet (NPD) conditions. (E) Ratio of total numbers of ThPOK+ Th to RUNX3+ CTLs. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data represents pooled results from at least two independent experiments with at least three mice per group in each experiment.

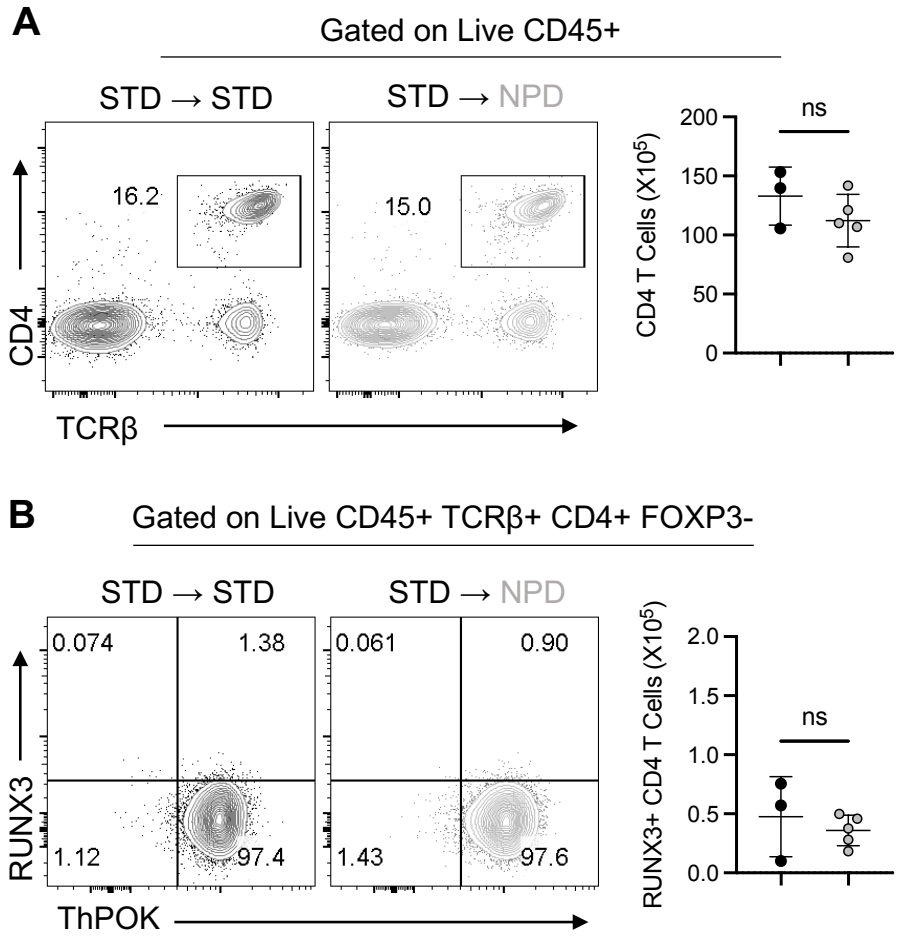

Figure 8: Loss of original dietary proteins does not affect systemic CD4 T cells.

(A, B) Representative flow cytometry plots (left) and summary statistics (right) comparing stated CD4 T cell populations from the standard diet (STD) and the novel protein diet (NPD) conditions. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

Antigen presentation IECs through MHC class II is important for generating and maintaining the intestinal CD4 CTL population

After determining that continuous antigen-stimulation of intraepithelial CD4 T cells is important for the reprogramming and maintenance of CD4 CTLs, we evaluated the various antigen presenting cells that may have a role in presenting antigens via MHC class II.

The prevalence of CD4 CTLs at the epithelium suggests that, besides the diet protein antigens, the local environment may be important for reprogramming CD4 T helper cells into CTLs. IECs express MHC class II on their basolateral side, which is directly adjacent to intraepithelial CD4 T cells. The potential crosstalk between MHC class II-expressing IECs and resident CD4 T cells thus represents a possible mucosa-specific mechanism of generating site-specific CD4 CTL that respond to diet-derived protein antigens. MHC class II-expressing IECs provide a potentially unique means of immune regulation in the small intestine due to their differences from a classic APC, such as a dendritic cell. First, the site-specific features of IECs are compelling in their relationship to local CD4 T cells. With MHC class II constitutively expressed on their basolateral side, there is a potential mode of antigenic presentation to CD4 T cells that are situated within the epithelium (Ménard, Cerf-Bensussan, & Heyman, 2010). Furthermore, IECs do not express known co-stimulatory molecules, in contrast to myeloid cells, that could potentially incite immediate effector functions from antigen-responding CD4 T cells. IECs are also physiologically situated and responsible for the absorption and processing of luminal antigens.

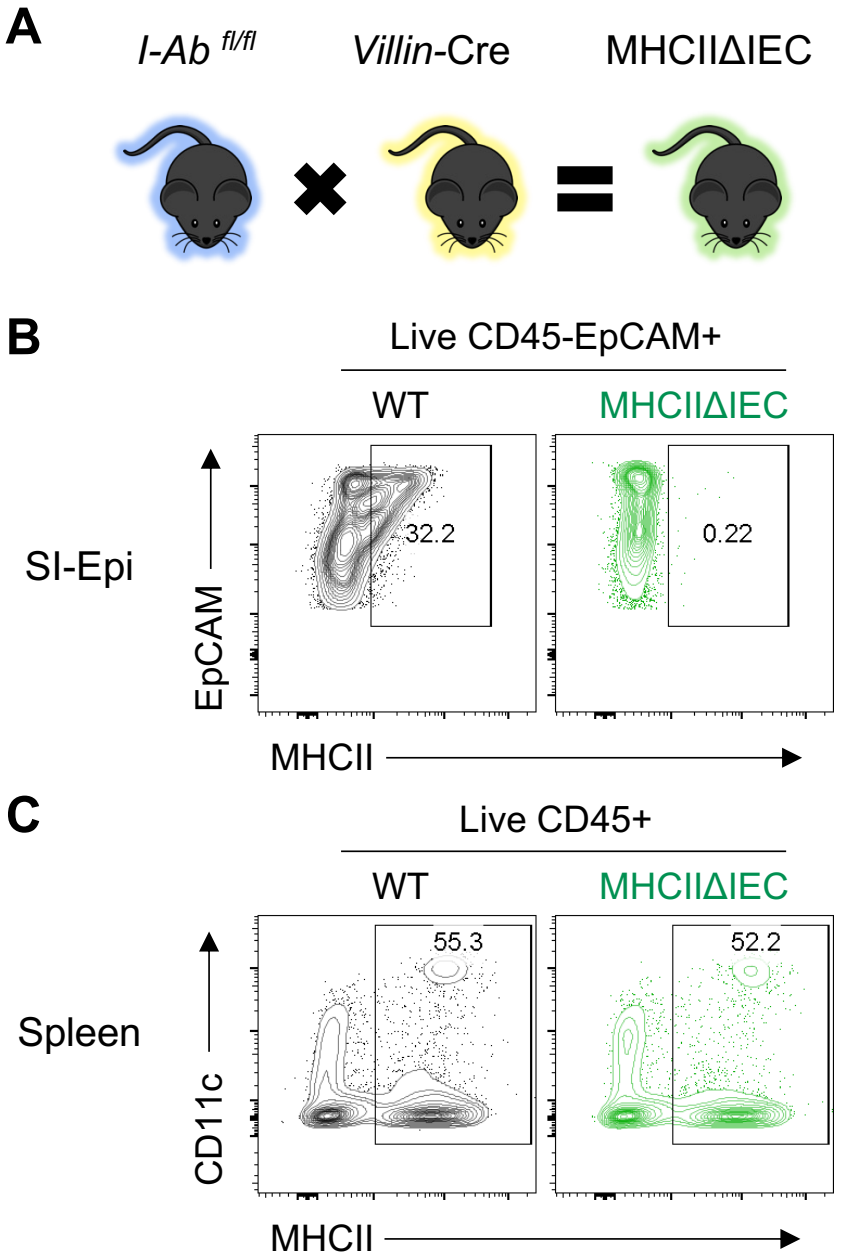

Figure 9: Murine model of IEC-specific deletion of MHC class II

(A) Schema of conditional gene deletion set-up. Representative flow cytometry plots comparing MHC class II expression on (B) IECs in the small intestine epitheliums and (C) hematopoietic stem cells in the spleens of WT littermate and MHCIIΔIEC mice. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

We generated mice that had MHC class II ablated from their IECs by crossing *I-A*^{b}-floxed mice with *Villin*-Cre (MHCIIΔIEC) (Figure 9A). In these MHCIIΔIEC mice, we found that there

was a significant decrease in overall CD4 T cells (Figure 10A). Of the decreased CD4 T cell population, the RUNX3+ and GZMB+ CD4 CTL population exhibited the most significant loss (Figure 10B, C).

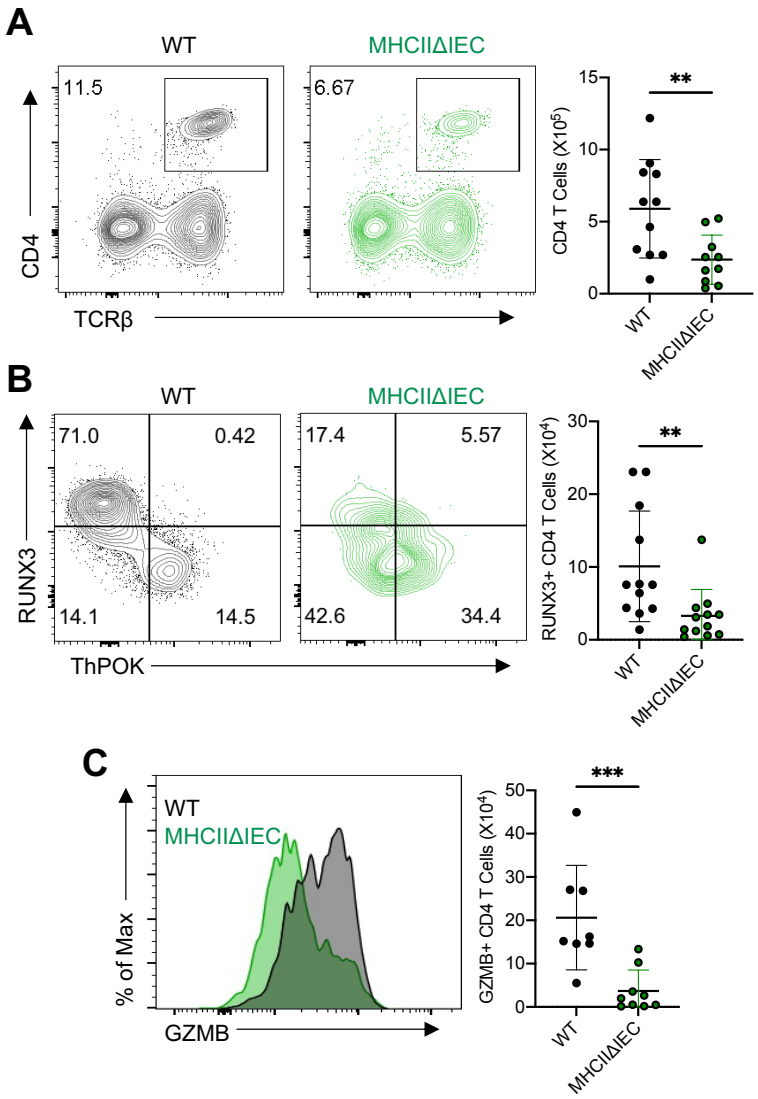

Figure 10: MHC class II-expressing IECs are important for CD4 CTL generation.

(A) Schema of conditional gene deletion set-up. (B, C) Representative flow cytometry plots (left) and summary statistics (right) comparing epithelial CD4 T cell populations in the WT and the MHCIIΔIEC mice. (D) Overlay of histograms (left) and summary statistics (right) comparing GZMB expression in the WT and MHCIIΔIEC mice. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

The effect of the loss of MHC class II from the intestinal epithelial cells was specific to the small intestine epithelium (Figure 9B, C). In the spleen, the CD4 T cell population was not statistically different from WT littermates (Figure 11A, B).

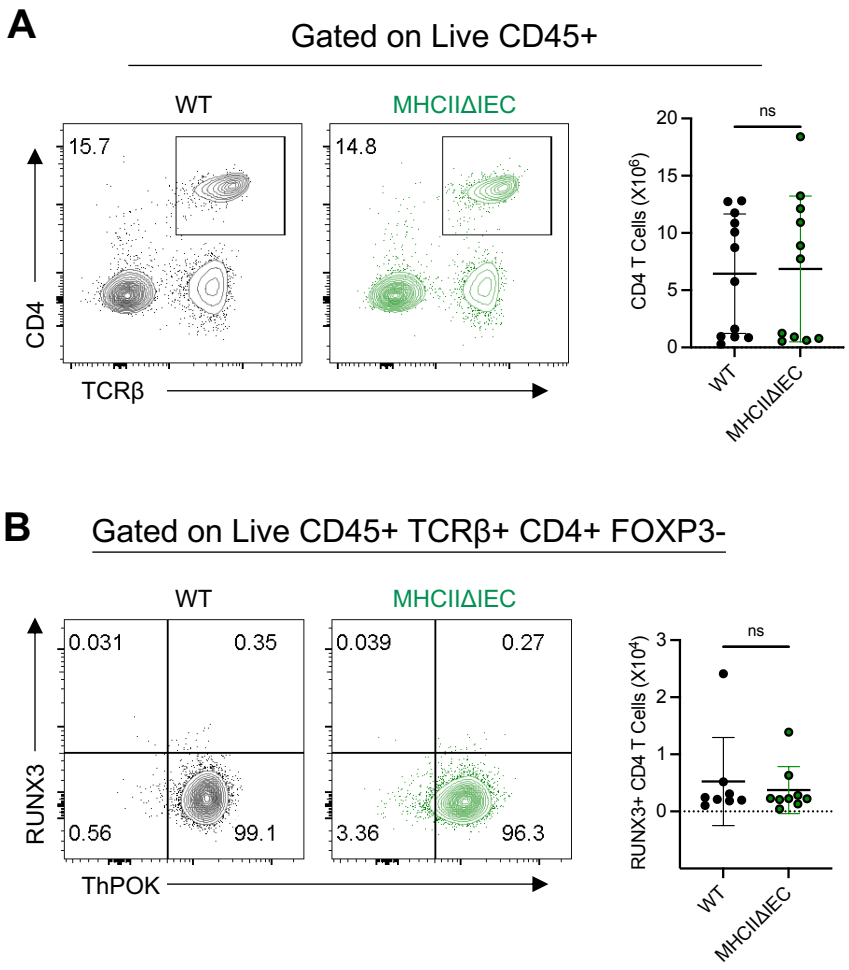

Figure 11: Loss of MHC class II expression on the IECs does not impact systemic CD4 T cells.

(A, B) Representative flow cytometry plots (left) and summary statistics (right) comparing stated CD4 T cell populations from the spleens of WT and MHCIIΔIEC mice. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

Diet-specific CD4 CTLs require MHC class II expression on IECs to be maintained

We then addressed if diet-specific OT-II CD4 T cells, known to be specific for an ovalbumin-derived peptide, would also be affected.

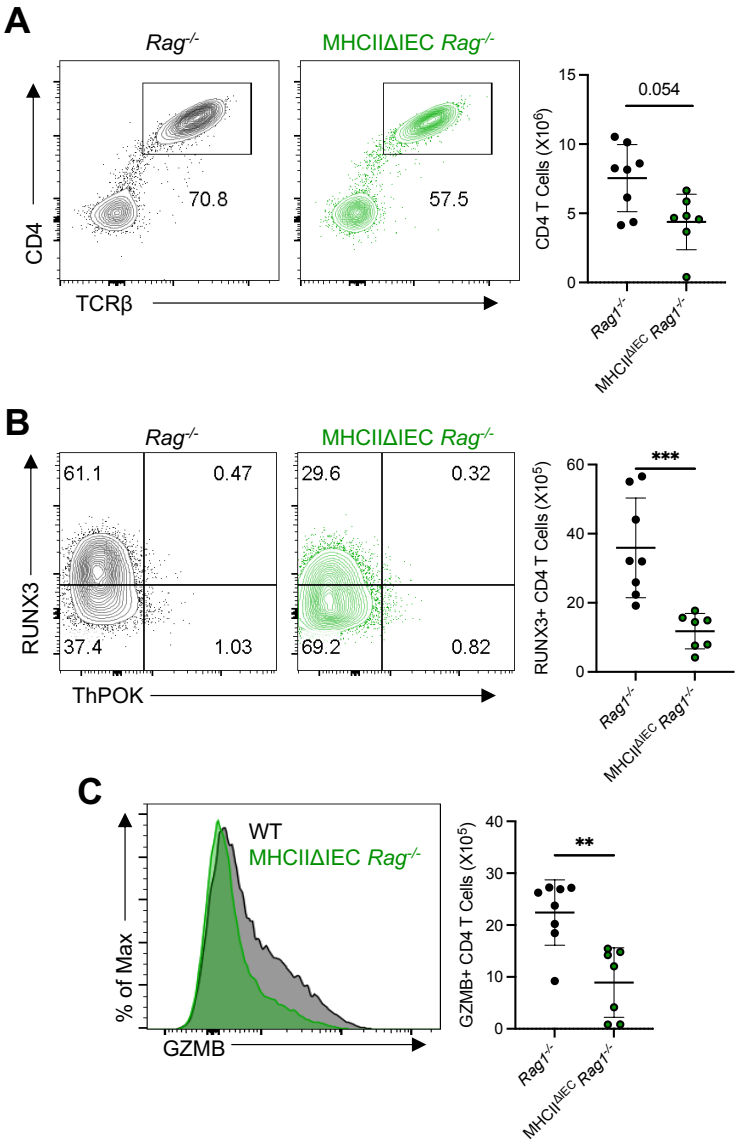

Figure 12: MHC class II-expressing IECs generate diet specific OT-II CD4 CTLs

(A, B, C) Representative flow cytometry plots (left) and summary statistics (right) comparing stated CD4 T cell populations from the small intestine epitheliums of *Rag*$^{-/-}$ or MHCIIΔIEC *Rag*$^{-/-}$ mice. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data represents pooled results from at least two independent experiments with at least three mice per group in each experiment.

Naïve OT-II CD4 T cells, isolated and enriched from the spleens of OT-II *Rag*$^{-/-}$, were adoptively transferred via retro-orbital injection into *Rag*$^{-/-}$ or MHCIIΔIEC *Rag*$^{-/-}$ recipient mice. Following adoptive transfer and ovalbumin feeding for 4 weeks, the lymphocyte populations of the small intestine epithelium were analyzed (Figure 12A-C). While there was only trending evidence that the OT-II CD4 T cells were less capable of occupying the small intestine epithelium (Figure 12A), there was a significant decrease in the CD4 CTL population, as defined by RUNX3 expression (Figure 12B) and GZMB expression (Figure 12C). Therefore, the ablation of MHC class II expression on the IECs resulted in a loss of diet-specific CD4 CTLs.

The role of IL-27 in generating CD4 CTLs in the small intestine epithelium

After determining the small intestine-specific mode of antigen presentation by MHC class II-expressing IECs, we then investigated additional factors that may contribute to the generation of epithelial CD4 CTLs.

In order for CD4 T cells in the small intestine epithelium to convert to CTLs, ThPOK must first be downregulated followed by an upregulation of RUNX3. Furthermore, the transcription factor TBET has a synergistic role with RUNX3 in inducing the cytotoxic fate (Reis, Hoytema van Konijnenburg, Grivennikov, & Mucida, 2014). We therefore investigated the role of the IL-12 family of cytokines, which are known inducers of TBET, in CD4 CTL development.

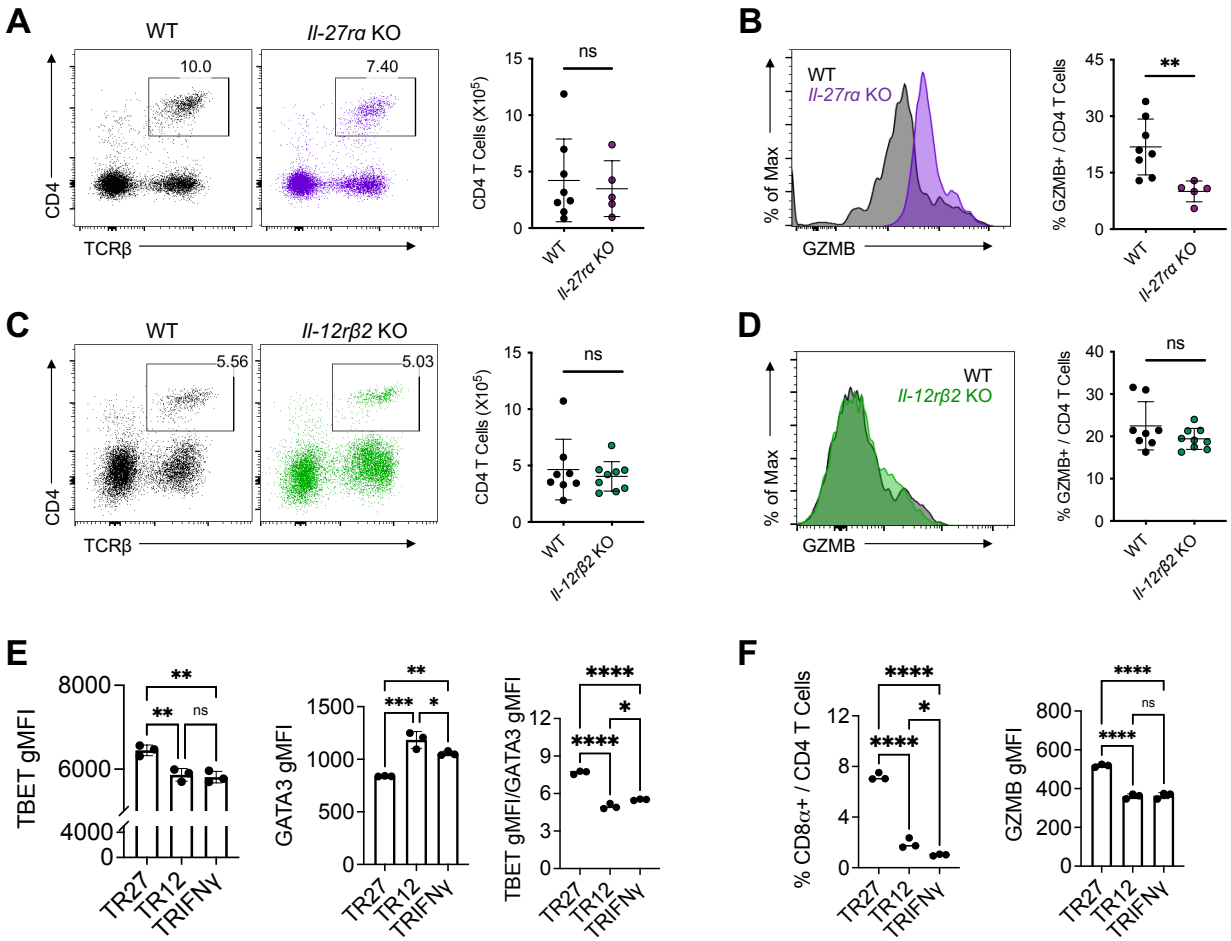

Figure 13: IL-27, not IL-12, is important for CD4 CTL development

(A-D) *Ex vivo* analysis of *Il-27Rα*^{KO} (A, B) and *Il-12Rβ2*^{KO} (C, D). (A, C) Representative flow cytometry plots (left) and summary statistics (right) of the total CD4 T cell population in the small intestine epithelium. (B, D) Histogram overlays (left) and summary statistics (right) of granzyme B expression from the small intestine epithelial CD4 T cells. (E, F) *In vitro* cell culture of naïve CD4 T cells cultured with TGFβ (T), retinoic acid (R), and IL-27 (27), IL-12 (12) or IFNγ. (E) Summary statistics of TBET, GATA3, and the ratio of TBET to GATA3 expression. (F) Summary statistics of CTL parameters CD8α and GZMB. Data represents pooled results from at least two independent experiments with at least three mice per group in each experiment (A-D) or are representative results from one of at least two independent experiments with at least three mice in each experimental group (E, F).

When the small intestine epithelial lymphocytes of *Il-12Rβ2*^{KO} and *Il-27Rα*^{KO} mice were analyzed ex vivo, both the *Il-12Rβ2*^{KO} and *Il-27Rα*^{KO} mice did not exhibit a loss of total CD4 T cells in the small intestine epithelium (Figure 13A, C). However, the *Il-27Rα*^{KO} mice had significantly less CD4 CTLs (Figure 13B), while the CD4 CTL population in the *Il-12Rβ2*^{KO} mice

was not affected (Figure 13D). IL-27 was therefore implicated as an important cytokine in the development of CD4 CTLs in the small intestine epithelium.

To corroborate the in vivo findings, naïve CD4 T cells were incubated in vitro with IL-27, IL-12, or IFNγ, in addition to the presence of TGFβ and retinoic acid (RA), TBET expression was the highest in the condition that contained IL-27. This increase in TBET expression coincided with the downregulation of GATA3, which is a known inducer of ThPOK (Figure 13E) (Cheroutre & Husain, 2013). Therefore, in order for ThPOK to become downregulated, GATA3 must be downregulated first. Following the downreulation of GATA3 and ThPOK, CD4 Th cells are then able to convert into CTLs, as evidenced by the increase in CD8α and GZMB expression in the presence of IL-27, rather than IL-12 or IFNγ (Figure 13F).

2.4 Discussion

The classical definition of oral tolerance is a purposeful non-response by the systemic immune system against antigens that were initially presented via the oral-gastrointestinal route. in In addition to a lack of immune response, FOXP3 suppressor Treg cells also arise in the lamina propria in response to dietary antigen (Kim et al., 2016). In this study, we identified an alternative adaptation of responding conventional CD4 T cells to the dampened immune response associated with oral tolerance with the characterization of an active immune response that leads to the conversion of CD4 T helper cells to CD4 CTLs that express cytotoxic machinery. The resulting intraepithelial CD4 CTLs were also found to be dependent on repeated antigenic stimulation, with the loss of specific peptides in the diet causing significant decreases in CD4 CTL population. It is worth speculating, however, that CD4 CTLs specific for the new repertoire of proteins will eventually arise.

In the OT-II Rag^{WT} model, where the vast majority of T cells express the OT-II transgene, dietary ovalbumin antigen was found to have a dramatic effect in both the spleen and the intestine. While this systemic effect was not seen in the WT C57BL/6 models that had their diets changed to either an amino acid-containing diet or a novel protein-containing diet, this difference can be explained that, in polyclonal mice, the proportion specific for diet antigen in the spleen may not be perceptible. Furthermore, WT C57BL/6 mice are polyclonal and therefore continuously renewing naïve T cells that can migrate to the spleen. Therefore, the OT-II Rag^{WT} model is a reductionist approach that conceptually shows the importance of specific dietary antigens on systemic and intestinal T cell populations, while the WT C57BL/6 demonstrate how the systemic effect may be diluted in a polyclonal context.

As resident intraepithelial T lymphocytes, CD4 CTLs require TCR stimulation to convert in the epithelium. Furthermore, we characterized the requirement for a consistent repertoire of cognate proteins in the diet for responding CD4 CTLs to be maintained, both in terms of numbers and cytolytic potential. In addition to TCR stimulation, other cytokines such as IL-15 have been proposed to have a role in the maintenance of intraepithelial T lymphocytes (Jabri & Abadie, 2015).

Among these tissue-specific factors is the presence of MHC class II-expressing intestinal epithelial cells (IECs). Given that IECs lack co-stimulatory molecules such as CD80/B7-1 or CD86/B7-2, which are typical for myeloid cells, the unique ability for IECs to present antigen via MHC class II without co-stimulation to nearby CD4 T cells represents a mucosa-specific mode of antigen presentation that might be conducive to the conversion of CD4 T helper cells towards CTL. Mucosal CD4 T cells that interact with IECs through the TCR:Ag-MHC class II signal alone have been theorized to be an adaptation for tolerance of harmless luminal antigens, such as those from

the diet (Robert M. Hershberg & Mayer, 2000). The study here provides direct evidence in support of this novel concept in oral tolerance.

The ability for IECs to signal and functionally educate conventional CD4 T cells is a feature that is vaguely reminiscent of CD4 T cell early development in the thymus. Initially, naïve CD4 T cells are positively selected through a communication between the developing thymocyte and MHC class II expressing cortical thymic epithelial cells (Breed, Lee, & Hogquist, 2018). MHC class II conditionally knocked-out of intestinal IECs demonstrate how epithelial cells can provide a local antigen-specific TCR signal that further directs CD4 T cell maturation and differentiation in the periphery.

In addition to the interaction between MHC class II-expressing IECs, intraepithelial CD4 T cells are further differentiated towards the CTL phenotype in the presence of IL-27. We have shown here that IL-27 is capable of downregulating GATA3, which then allows for the downregulation of ThPOK, thus suppressing the T helper fate. IL-27 is also capable of inducing TBET expression, which enables the cytotoxic program and results in greater CD8α and Granzyme B expression.

IL-27 has been identified to have pleiotropic effects ((Hunter & Kastelein, 2012)). Notably, IECs have been identified as a major producer of IL-27, and the ablation of IL-27 production by IECs has also been observed to decrease the CD8αα+ CD4 CTL population in the small intestine epithelium (Lin et al., 2021). IL-27 also has a role in preventing activation induced CD4 T cell death (G. Kim, Shinnakasu, Saris, Cheroutre, & Kronenberg, 2013).Taken together, IECs have two modes of signaling to the intraepithelial CD4 T cells: MHC class II expression of luminal antigens and the production of IL-27.

The combination of FOXP3+ Treg development in the lamina propria and the RUNX3+ CTL development in the epithelium are two relatively recent amendments to the concept of oral tolerance as it pertains to lymphocyte generation and adaptation in the small intestine. Taken together, we would like to put forth the concept of 'protective tolerance'; whereby homeostatic conditions in the intestines are maintained, per the original definition of oral tolerance, but instead of suppression or weakening of the immune response, an alternative tolerance process operates that prevents the generation of potentially harmful inflammatory cells and simultaneously fortifies the mucosal barrier with a distinctive MHC class II-restricted class of CTLs that display the potential for protective immunity. The alternative tolerance portion of this new concept will be discussed in Chapter 3 and the active immunity or 'protection' portion of this definition will be discussed in Chapter 4.

2.5 Acknowledgements

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CHAPTER 3: CTL Conversion is an Alternative Form of Tolerance

3.1 Introduction

Inflammatory bowel disease (IBD) is a chronic and potentially life-threatening condition characterized by periods of intestinal distress, including abdominal pain, bloody stools, and diarrhea (Hou, Lee, & Lewis, 2014). Patients with IBD are generally thought to have genetic predispositions that have been triggered by some environmental cues. However, elucidation of pathogenesis is further complicated by the diversity of symptoms and pathologies in the different types of IBD, including Crohn's disease and ulcerative colitis.

One factor that is consistent in IBD is the imbalance of intestinal CD4 T cell subsets (Imam, Park, Kaplan, & Olson, 2018). There is also evidence that CD4 T cells have the potential to initiate or perpetuate IBD. This theory has been corroborated by studies showing that blocking antibodies against CD4 T cells have caused disease remission in some Crohn's disease and ulcerative colitis patients (Imam et al., 2018).

CD4 CTLs develop post-thymically and tend to increase systemically in numbers and frequencies with age. Therefore, the presence of CD4 CTLs may be interpreted as an adaptation associated with healthy aging. At steady state, CD4 CTLs are frequently found in the small intestine epithelium and, as we showed in Chapter 1, are generated in response to harmless food-derived antigens that are constantly absorbed by the intestinal epithelium. In parallel to the CD4 CTL conversion in the epithelium, CD4 T cells in the lamina propria that are responding to the same diet-derived antigens convert to the suppressive Treg fate (Kim et al., 2016). Based on these findings, we proposed that the conversion process to CD4 CTL may be an alternative process of tolerance that not only avoids aberrant inflammatory Th responses to those diet-derived antigens but also promotes the protective capacity at the mucosal border.

CD4 CTLs have the potential for immunopathology because they contain cytotoxic granules, including granzyme and perforin, in addition to being able to express potentially inflammatory cytokines, such as IFNγ. Therefore, in this chapter, we evaluated the consequences of the conversion process, or defects thereof, for maintenance of homeostatic quiescence or induction of inflammatory disease. Using *in vitro* differentiated cells as well as naïve CD4 T cells isolated from genetically manipulated mouse models that predispose CD4 T cells towards either a helper or CTL phenotype, CD4 CTLs were evaluated for their immunopathological potential.

3.2 Materials and Methods

Mice

Animal care and experimentation were consistent with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the La Jolla Institute for Immunology. In general, mice were sex- and age-matched and compared with WT littermates where applicable. Both male and female mice were used, unless otherwise stated. Experiment specific details are included in the figure legends. *Thpok*-flox (009369), Distal-Lck-Cre (012837), IFNγ-YFP (017580), and FOXP3-RFP (008374) mice were purchased from the Jackson Laboratories and maintained in-house. ThPOK-Cre and *Thpok*-Silencer-flox mice were developed by Drs. Ichiro Taniuchi, Hilde Cheroutre, and Akihito Harusatu (RIKEN). Il17F-Thy1.1 reporter mice were developed and generously donated by Dr. Casey Weaver (University of Alabama, Birmingham).

FACS-Isolation of Naive CD4 T Cells

Spleens from OT-II *Rag2*$^{-/-}$ mice were harvested into HBSS supplemented with 5% FBS and pushed through 70um cell strainers atop a 50mL conical tube. The single cell suspension was then washed, centrifuged, and the resulting cell pellet was lysed with lysis buffer (Sigma, cat #R7757).

The resulting lymphocyte population was then enriched for CD4 T cells using an enrichment kit (Stemcell, cat #19852). The resulting CD4 T cells were then sorted for naive CD4 T cells, as defined by CD4+ CD25- CD45RB^{hi} expression.

***In Vitro* Differentiation of CD4 T Cells**

T cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin-streptomycin and β-mercaptoethanol (Gibco). For T cell polarization, 1 x 10^5 cells were seeded onto 96-well plates pre-coated with 1ug/mL anti-CD3ε. Cells were cultured under Th17 (2ug/mL anti-CD28, 30 ng/mL IL-6, 5 ng/mL TGFβ, 1 ug/mL anti-IFNγ and 1ug/mL anti-IL-4 for three days, then 30 ng/mL IL-6, 5 ng/mL TGFβ, 1 ug/mL anti-IFNγ and 1ug/mL anti-IL-4 for four days), Treg (2ug/mL anti-CD28, 5 ng/mL TGFβ, 100 nM all-trans retinoic acid (RA) for three days, then 5 ng/mL TGFβ and 100 nM RA for four days) or CTL (1 ug/mL anti-CD28, 25 ng/mL IL-27, 5 ng/mL TGFβ, and 250 nM RA for one day, then 2 cycles of 25 ng/mL IL-27, 5 ng/mL TGFβ, and 250 nM RA for 3 days) conditions.

CD4 T Cell Adoptive Transfer Colitis Model

Naive CD4 T cells were isolated from the spleen and sorted for CD4+CD25-CD45RB^{hi} expression. 2x10^5 naive CD4 T cells were adoptively transferred via retro-orbital injection into *Rag*$^{-/-}$ recipients. Mice were then weighed and monitored every other day for signs of disease pathology (weight loss, diarrhea, lethargy) until the terminal endpoint of -20% original body weight occurs.

Histology Analysis

Samples were immersion fixed in zinc formalin (Medical Chemical Corporation), processed routinely, cut at 5um thickness and stained with hematoxylin and eosin (H&E). Slides were scanned into whole slide images at 40X using a Zeiss Axio Scan.Z1. Histological scoring was performed by a board-certified pathologist, in a blinded fashion.

Flow Cytometry

Cells were suspended in staining buffer (1X PBS with bovine serum albumin and sodium azide) and blocked with 1:500 concentration of anti-Fc receptor antibody 2.4G2. Cells were stained according to standard fixation and permeabilization protocols for surface and intracellular markers, respectively. Briefly, for detection of transcription factors, the eBioscience™ Intracellular Fixation and Permeabilization kit was used; for the detection of cytokines, the BD™ Cytofix/Cytoperm kit was used. All samples were analyzed using an LSRII or Fortessa flow cytometer (BD Biosciences).

Statistical Analysis

Figure-specific statistical analyses are provided in figure legends. In summary, all data were analyzed using GraphPad Prism 8 software and data are shown as mean and the standard deviation. Statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or ns (not significant).

3.3 Results

In vitro differentiated CD4 CTLs do not induce pathology in an adoptive transfer model

Given their potential for cytotoxicity, we evaluated if CD4 CTLs can induce immunopathology in an adoptive transfer colitis model. We harvested naive CD4 T cells from the spleens of IFNγ YFP, IL-17F-Thy1.1, and FOXP3-RFP triple reporter mice and *in vitro* differentiated them towards a CTL, Treg, Th0, Th1, or Th17 fate (Figure 14A). Following the one week *in vitro* polarization protocol, the resulting populations were sorted with CD4 CTLs based on their CD8α expression; Tregs based on FOXP3-RFP expression; Th0 based on their lack of

IFNγ or IL-17F expression; Th1 based on their IFNγ expression; and Th17 based on their IL-17F expression (Figure 14 A).

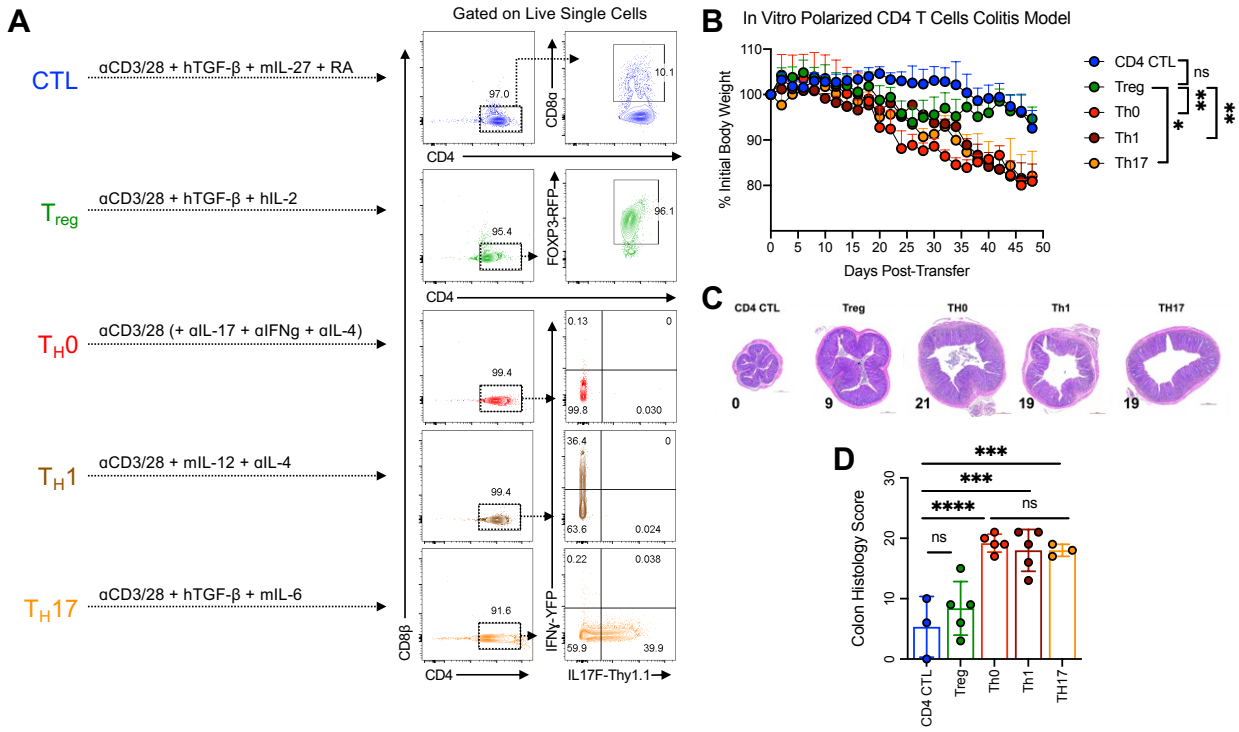

Figure 14: In vitro polarized CD4 CTLs will not induce colitis upon adoptive transfer.

(A) Summary of differentiation protocols (left) towards the stated lineages with resulting FACS-sorted populations (right). (B) Weight loss plot following adoptive transfer. (C) Representative histology H&E staining images and (D) colon histology scores of the transferred conditions at the terminal endpoint. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; One-way ANOVA statistical test was used to compare multiple groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

These individual populations were then adoptively transferred into *Rag*$^{-/-}$ recipients via retro-orbital injection of $2x10^5$ polarized cells per mouse and monitored every other day for weight loss and other signs of colitis pathology (Figure 14 B). Based on weight loss, the CD4 CTL and Treg recipients were not significantly different, in contrast to the recipients of the Th0, Th1, and Th17 conditions, which all exhibited severe weight loss (Figure 14B). Furthermore, the histology

scores of the colon revealed that the CD4 CTL and Treg conditions both had significantly less pathology compared to the T helper conditions (Figure 14C, D).

Similar to naïve CD4 T cells, which are considered a standard model of colitis-induction in mice, CD4 T cells that were differentiated towards Th0, Th1, or Th17 resulted in a strong disease phenotype. These results support the human data that IBD patients tend to have intestinal populations skewed towards Th1 or Th17 (Brand, 2009; Feng et al., 2011). Treg cells are considered the archetypal suppressive, immunoregulatory CD4 Th cell. As such, the mice that were adoptively transferred with Treg cells exhibited less disease compared to the other more inflammatory Th subsets. Interestingly, the recipients that were adoptively transferred CD4 CTLs were more like the Treg condition and had less disease compared to the other Th subsets. Therefore, CD4 CTLs may be similar to Treg cells in their ability to promote homeostasis.

At the terminal endpoint, the spleen, SI-Epi and -LP, and colon-Epi and -LP were analyzed (Figure 15A, B). Most of the *in vitro* differentiated CTLs migrated to the small intestine epithelium and remained ThPOK-negative, with more than 90% having retained their RUNX3 expression (Figure 15A). Some of the transferred CTLs also migrated to other tissues, such as the SI-LP, colon-Epi, and colon-LP (Figure 15A). Interestingly, virtually none of the transferred CTLs gained FOXP3 expression in any of the tissues, implying that CTLs do not convert to the Treg fate as their mode of promoting tissue homeostasis.

Across all analyzed tissues, there is a presence of FOXP3+ Tregs in the Treg adoptive transfer condition, ranging from approximately 3-8% (Figure 15B). However, polarized Treg also converted to ThPOK-RUNX3+ CTL, with the SI-Epi having the most dramatic frequency of conversion at approximately 80% (Figure 15B). The concept of FOXP3+ Tregs converting to CTLs has been previously noted (Sujino et al., 2016). Interestingly, based on ThPOK and RUNX3

expression, in a lymphopenic environment, the conversion of FOXP3+ Treg to CTLs may take place in other tissues too.

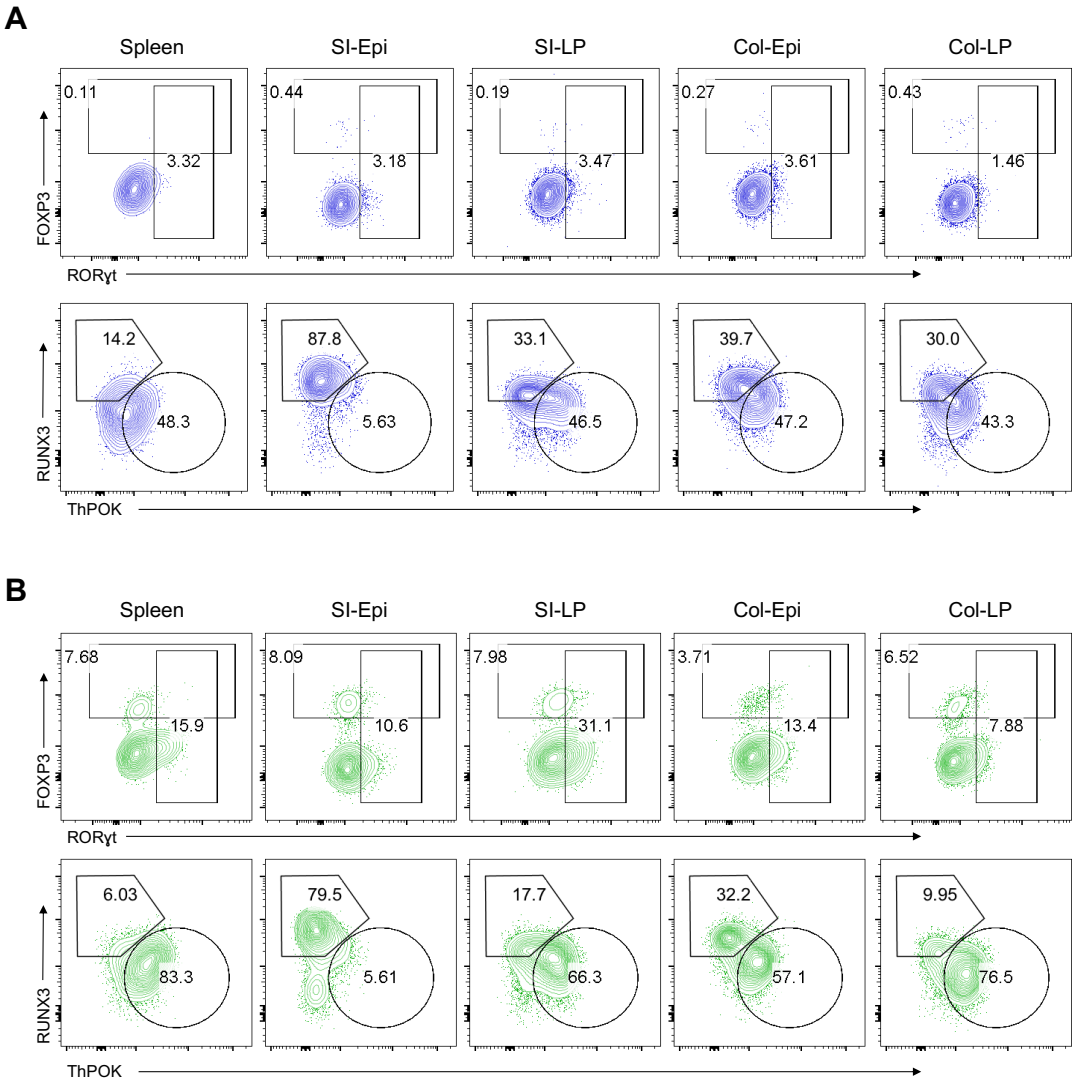

Figure 15: In vitro differentiated CD4 Tregs convert to CTLs, but CTLs do not convert to Tregs.

Representative flow cytometry plots describing transcription factor expression in (A) CTL, marked in blue, and (B) Treg, marked in green, transfer conditions in the stated tissues. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

Polarized diet-responsive CD4 CTLs do not induce pathology in an adoptive transfer model

Polyclonal CD4 T cells that have been polarized towards a Th0, Th1, or Th17 fate were proven to be pathology-inducing (Figure 14B, C, D). However, according to the principle of oral tolerance, dietary antigens are tolerated by the host. To support this concept, we used diet-specific OT-II CD4 T cells that had been polarized towards a CTL, Treg, Th1, or Th17 fate (Figure 16A). The polarized OT-II cells were then adoptively transferred to recipients that were fed with OVA-containing diet.

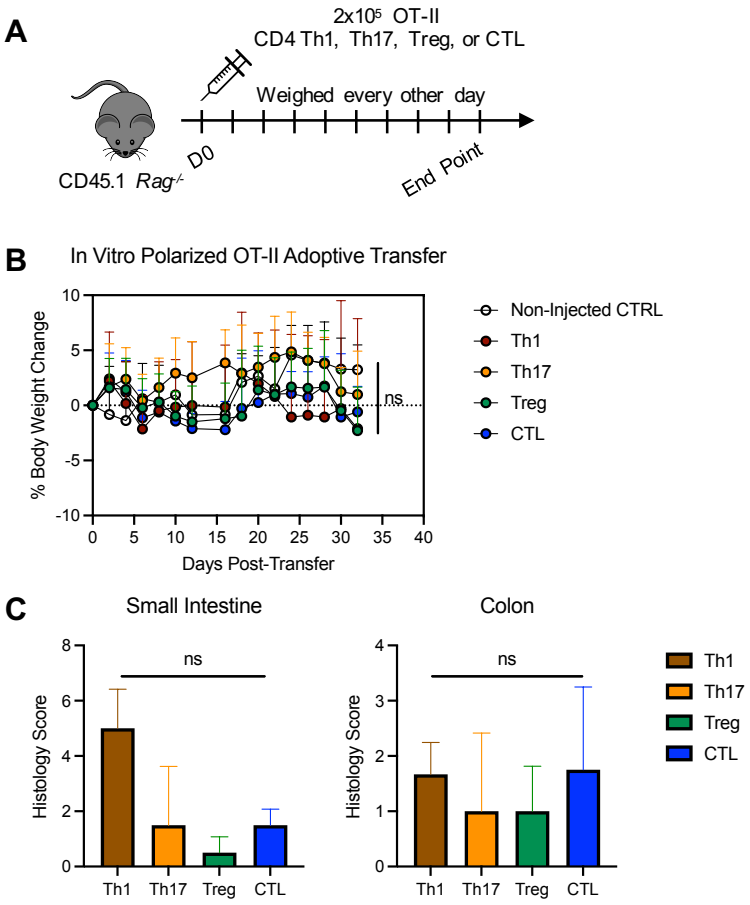

Figure 16: Polarized diet-specific OT-II CD4 T cells do not induce pathology

(A) Schema of experimental design. (B) Body weight loss following adoptive transfer of *in vitro* polarized OT-II CD4 T cells. (C) Small intestine and colon histology scores. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups, One-way ANOVA statistical test was used to compare multiple groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

Interestingly, we found that none of these conditions induced the type of colitis that we were seeing with the polyclonal cells. While this may be another iteration of the concept of oral tolerance, this result was interesting that even when cells were primed towards an inflammatory phenotype (i.e. Th1 or Th17), they did not induce pathology in the form of weight loss nor histology when they are specific for luminal antigens (Figure 16B, C). Therefore, in the case of diet-specific CD4 T cells, *in vitro* polarized CD4 CTL are inherently non-pathogenic while *in vitro* polarized Th cells convert to CTL as a mechanism of avoiding inflammation.

The deviation from T helper fate towards CTL conversion prevents disease induction

Given that ex-CD4 Th cells readily convert to CTLs *in vivo* and that polarized CD4 CTL do not induce colitogenic pathology upon adoptive transfer, the conversion of CD4 Th cells to CTLs in response to luminal antigens may be an essential process to induce tolerance at the mucosal border. To examine this, we generated genetically modified mice in which all CD4 T helper cells are either forced to remain as helper T cells ('CD4 CTL-less' mice) or forced to convert to CTL ('forced-CD4 CTL' mice) (Figure 17A).

To generate 'CD4 CTL-less' mice, *Thpok*-Silencer-floxed mice were crossed with distal-Lck-Cre transgenic mice to force all mature CD4 T cells in these mice to maintain ThPOK expression; these mice are referred to as ΔThPOK$^{\text{Silencer}}$. Conversely, to generate 'forced-CD4 CTL' mice, we crossed *Thpok*-floxed mice with distal-Lck-Cre transgenic mice; to force the mature CD4 T cells in these mice to turn off ThPOK expression and thus to convert to CTL upon activation; these mice are referred to as ΔThPOK.

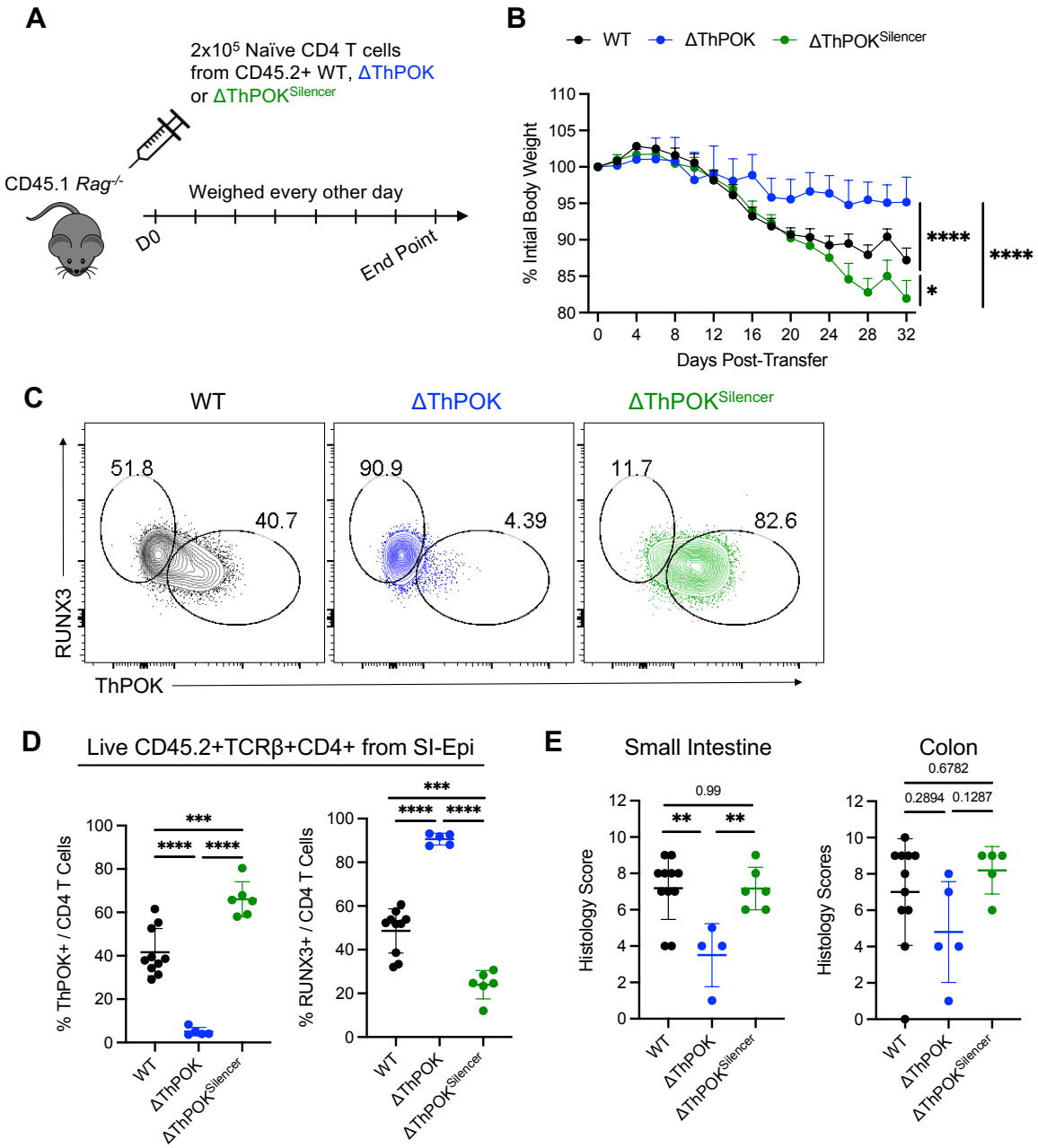

Figure 17: Loss of ThPOK expression ameliorates colitis disease.

(A) Schema of experimental design. (B) Body weigh loss following adoptive transfer of naïve CD4 T cells isolated from the stated mice. (C) Representative flow cytometry plots, gated on Live CD45+TCRβ+CD4+ cells in the small intestine epithelium.(D) ThPOK (left) and RUNX3 (right) expression of the CD4 T cells from the small intestine epithelium. (E) Small intestine and colon histology scores. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups, One-way ANOVA statistical test was used to compare multiple groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group (D, E) or pooled results from at least two independent experiments with at least three mice per group in each experiment (B).

Using the naive CD4 T cells isolated from these 'forced CD4 CTL' or ΔThPOK or 'CD4 CTL-less' or ΔThPOK^{Silencer} mice, we performed adoptive transfer colitis experiments by transferring these cells into *Rag*$^{-/-}$ recipients and monitored them for disease pathology. First, we confirmed that the CD4 ΔThPOK T cells lost ThPOK expression while the ΔThPOK ^{Silencer} CD4 T cells maintained ThPOK expression in the SI-Epi (Figure 17C, D). Consistent with CTL conversion, the ΔThPOK CD4 T cells also exhibited increased RUNX3 expression in the SI-Epi, whereas ΔThPOK^{Silencer} CD4 T cells did not. The resulting weight-loss of the recipients corroborated our initial hypothesis that prevention of CTL conversion (ΔThPOK ^{Silencer} CD4 T cells) results in pathogenic T cells and greater colitis pathology, while the loss of ThPOK and subsequent gain of RUNX3 (ΔThPOK CD4 T cells) results in forced CTL conversion and reduced pathology (Figure 17D, E). The forced CTL-less CD4 T cells caused greater pathology in the small intestine, as opposed to the typical site of colitis pathology, the colon (Figure 17E), further underscoring the importance of CTL conversion as a mechanism to induce tolerance and prevent tissue damage of the fragile single-cell epithelial layer of the small intestine.

3.4 Discussion

CD4 Th conversion to CTL in response to luminal antigens is typical of small intestinal lymphocyte development. However, because they are equipped with cytotoxic granules, CD4 CTLs have the potential to be highly cytolytic and inflammatory. Therefore, we evaluated the potential for CD4 CTLs to be pathogenic through adoptive CD4 T cell transfer models. Naïve CD4 T cells were *in vitro* differentiated towards Th0, IFNγ+ Th1, IL-17F+ Th17, and FOXP3+ Treg, or CTLs, and then adoptively transferred into lymphopenic *Rag*$^{-/-}$ recipients. The Th0, Th1, and Th17 transfer conditions all resulted in immunopathology, as evidenced by weight loss and histology scores. The Treg transfer condition did not result in immunopathology due to the

suppressive nature of Tregs. Interestingly, the CD4 CTL-transferred condition also did not result in colitis-related immunopathology. This was in stark contrast to the Th0, IFNγ+ Th1, and IL-17F+ Th17 polarized CD4 T cell transfer conditions. Notably, excessive IFNγ and IL-17 are cytokines known to compromise the intestinal barrier through the recruitment of inflammatory cell populations (Feng et al., 2011). Because CD4 CTLs do not express IL-17 and only low levels of IFNγ during steady state, the presence of CD4 CTLs may allow for the quiescent differentiation of cells that are primed for cytotoxicity, without necessarily engaged in cytotoxic activity, without the risk of rampant inflammation.

While the suppressive mechanisms of Treg cells have been viewed as the drivers of oral tolerance through the production of suppressive cytokines, such as IL-10 and TGF-β, we have discovered another mechanism of how Treg cells may allow for homeostatic conditions upon transfer into a lymphopenic host. Within the small intestine epithelium, the majority of the transferred Tregs became CTLs. Our study provides further insight that, at the mucosal barrier, Treg cells preferentially convert into CTLs as a mode of tolerance to preserve immune quiescence and prevent inflammation and tissue damage. The lack of CTL conversion to Treg also suggests that the conversion to CTL is an independent immunomodulatory mechanism, separate from Treg-mediated suppression.

To examine if the conversion of potentially inflammatory CD4 Th cells to CTL is an essential process of immune regulation at the mucosal barrier, we analyzed CD4 T cells from the genetically engineered 'CD4 CTL-less' mutant mice that were unable to lose ThPOK expression due to the deletion of the ThPOK-Silencer. The inability to convert to CTL forced CD4 Th cells to become pathogenic and exacerbated immunopathology. Conversely, with the 'forced-CD4 CTL' mutant mice, where ThPOK expression was ablated from T cells, colitis disease was

ameliorated. The results obtained with the 'CD4 CTL-less' or 'forced-CD4 CTL' mice provided evidence demonstrating the critical role of the CTL conversion process as an essential mechanism of tolerance to maintain immune quiescence and preserve the integrity of the mucosal barrier.

Of note, with the *in vitro* differentiated T cell subset transfer, the most significant pathology was found in the colon, based on histology scores. However, with the 'CD4 CTL-less' transfer, tissue damage was most pronounced in the small intestine, as opposed to the colon. These histology results may indicate a greater impact of this alternative regulatory mechanism in the small intestine. In summary, the ability for CD4 T cells in the intestinal epithelium to convert at steady state to CTLs in response to luminal antigens represents a strategy of both mitigating immunopathology while potentially fortifying the barrier with effector MHC class II-restricted CTLs.

3.5 Acknowledgements

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CHAPTER 4: CD4 Cytotoxic T Lymphocytes Confer Protective Immunity against Food-Borne Enteric Infection

4.1 Introduction

After determining that the conversion of CD4 T helper (Th) cells to cytotoxic T lymphocytes (CTLs) is an essential process of tolerance to avoid immune pathology directed towards luminal antigens, we sought to determine their function as cytotoxic MHC class II-restricted cells at the epithelial barrier. Given that CD4 CTLs appear quiescent during steady state, under what circumstances will CD4 CTLs actively kill? Here, we investigated a non-redundant protective role for CD4 CTLs, where they may be more effective to certain pathogenic challenges compared to CD8αβ CTLs.

CD4 CTLs were demonstrated to have equal cytolytic capacity as their CD8αβ counterparts in an *in vitro* redirected cytotoxicity assay in the presence of IL-15 (Mucida et al., 2013). To build upon these initial findings and demonstrate an *in vivo* need for CD4 CTLs against enteric pathogens, we examined oral infections with Salmonella *enterica* serovar Typhimurium (STM), as a model of a food-borne pathogen.

Salmonella *enterica* is a flagellated, gram-negative bacterium with more than 1800 serovars. The vast majority of these serovars are known to cause severe diarrheal disease and even death in humans (Perez-Lopez, Behnsen, Nuccio, & Raffatellu, 2016). As a food-borne pathogen, Salmonella *enterica* is known to infect a variety of cells, including the enterocytes of the intestinal epithelium, through a variety of invasion strategies. With the use of numerous virulence factors, Salmonella *enterica* is capable of manipulating its environment through chemical and biomechanical means to better facilitate invasion into the host cells and tissues (Hume, Singh, Davidson, & Koronakis, 2017). Particular to enterocytes, there is evidence that Salmonella

enterica can identify the apical side of intestinal epithelial sides through their polarized expression of Villin (Hume et al., 2017). This is further corroborated by studies that have shown mice with mutated Villin have increased resistance to Salmonella *enterica* infection (Hume et al., 2017).

Once inside the host cell, Salmonella *enterica* is known for its ability to sequester itself in vacuoles, aptly named Salmonella-containing vacuoles (SCVs). While sequestering in SCVs gives the pathogen some advantages, the SCVs are nevertheless susceptible to intracellular processes such as autophagy (Cemma & Brumell, 2012). Autophagy is a cellular degradative process that is essential for cellular health as well as a defensive mechanism for ridding the cell of intracellular pathogens. By delivering the SCV-contained pathogens directly to be degraded within lysosomes, autophagy is capable of directly killing Salmonella *enterica* or other pathogens as well as facilitating their antigens to be processed and presented by MHC class II molecules on the cell surface. Following autophago-lysosomal digest, pathogen antigens become subject to the MHC class II pathway, which is upregulated and results in both an increase in both endogenous antigens as well as pathogen-derived antigens being displayed on the cell surface. Furthermore, intestinal epithelial cells generally upregulate of MHC class II following an oral STM infection (Lapaque et al., 2009). We hypothesized that CD4 CTL may potentially have a non-redundant role in protecting epithelial cells from intravacuolar pathogens like Salmonella *enterica.*

Understanding the mucosal immunology underlying this barrier is critical to knowing how the mucosal immune system can simultaneously tolerate the microbiome while distinguishing and responding to enteric pathogens. Because CD4 CTLs have a prepared repository of cytolytic machinery during steady state, we sought to investigate if they have a non-redundant purpose in bolstering protection at the small intestine epithelium against food-borne pathogens that cannot be eliminated by MHC class I-specific CD8αβ CTL.

4.2 Materials and Methods

Mice

Animal care and experimentation were consistent with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the La Jolla Institute for Immunology. In general, mice were sex- and age-matched and compared with WT littermates where applicable. Both male and female mice were used, unless otherwise stated. Experiment specific details are included in the figure legends.

Culturing and Preparation of Bacteria

Salmonella *enterica* serovar Typhimurium strain IR715 is a naturally occurring nalidixic acid resistant derivative of ATCC 14028, generously donated by Dr. Manuella Raffatellu (UCSD), and was used in the infectious studies. The overnight culture was aerobically grown with shaking at 37°C degrees C in Luria-Bertani broth for at least 12 hrs. The day culture was produced by diluting the overnight culture 1:20 in fresh Luria-Bertani broth and aerobically grown with shaking at 37°C for 4hr. Bacteria was then washed in cold 1X PBS and diluted 1:10 in cold PBS.

Gastroenteritis Model of Salmonellosis

24hr prior to infection, mice were fasted without food for 4hr and then administered 20mg of streptomycin via oral gavage (Barthel et al., 2003). Food was returned *ad libitum* following streptomycin treatment. On the day of infection, mice were weighed and then fasted again for 4hr. At the end of the fasting period, mice were administered 100uL of washed day culture in 1X PBS via oral gavage, which equated to approximately $1x10^7$ CFU per mouse. Confirmation of CFU was performed by plating out dilutions of the washed day culture. Mice were assessed for weight loss and signs of pathology in the days post-infection. When mice had lost approximately 10% of their original body weight, typically day 3 or 4 post-infection, mice were euthanized and tissues were

dissected for CFU counts and lymphocyte analysis, where applicable. Mice that reached 20% below original body weight were immediately euthanized. For CFU counts, several tissues including spleen, liver, small intestine ileum, and colon were weighed, homogenized in 1X PBS, serially diluted, and plated on LB agar plates supplemented with nalidixic acid (Sigma).

Tamoxifen Treatment

Mice were treated with 2mg tamoxifen (Sigma), dissolved in corn oil, via the intraperitoneal route for three consecutive days followed by a booster dose 3-4 days later.

Antibody against IL-15/15Rα administration

Mice were injected with 20mg functional grade IL-15/15Rα complex monoclonal antibody clone GRW15PLZ (ThermoFisher) or Rat IgG1 kappa isotype control antibody clone eBRG1(ThermoFisher) via the intraperitoneal route once a day, beginning 1 day prior to STM infection until the terminal end point.

Flow Cytometry

Cells were suspended in staining buffer (1X PBS with bovine serum albumin and sodium azide) and blocked with 1:500 concentration of anti-Fc receptor antibody 2.4G2. Cells were stained according to standard fixation and permeabilization protocols for surface and intracellular markers, respectively. Briefly, for detection of transcription factors, the eBioscience™ Intracellular Fixation and Permeabilization kit was used; for the detection of cytokines, the BD™ Cytofix/Cytoperm kit was used. All samples were analyzed using an LSRII or Fortessa flow cytometer (BD Biosciences).

Absolute Cell Numbers

Total cell numbers were acquired using a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter). Absolute numbers of cell populations were then calculated using frequencies determined by flow cytometric analysis.

Statistical Analysis

Figure-specific statistical analyses are provided in figure legends. In summary, all data were analyzed using GraphPad Prism 8 software and data are shown as mean and the standard deviation. Statistical significance was deterred by unpaired t-test for cell numbers and by Mann-Whitney test for bacterial burden assays. Statistical significance is indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or ns (not significant).

4.3 Results

Epithelial MHC class II expression and CD4 CTL TCR signaling increase following STM infection

Following oral STM infection, MHC class II is upregulated on small intestinal IECs, analyzed 3 days post-infection (Figure 18A, B). Corresponding with the increase in MHC class II expression, there is also an increase in TCR signals in CD4 CTLs, as represented by *Nur77*-GFP staining in these TCR reporter mice (Figure 18C). The TCR response seems unique to the CD4 CTLs, given that the CD8αβ CTLs did not exhibit this same response (Figure 18C). STM infection has been closely linked to autophagy and activation of the MHC class II pathways for processing and presentation; therefore, the CD4 CTL-specific response to this intravacuolar pathogen may represent a non-redundant role for this class of intraepithelial CTL.

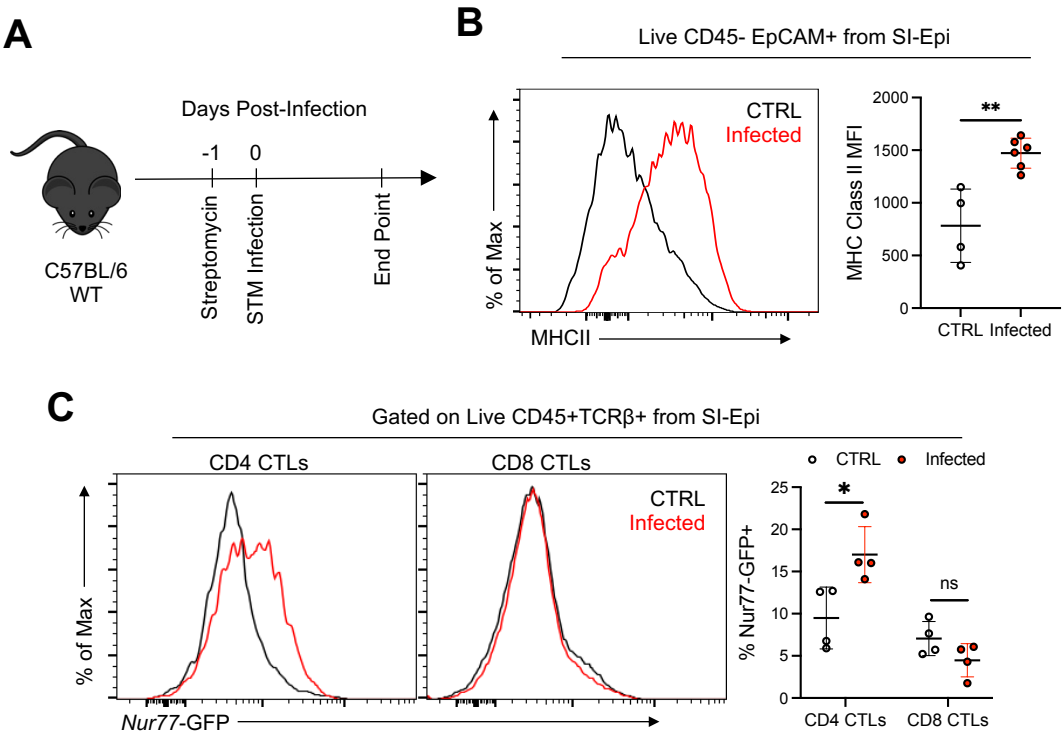

Figure 18: Both IECs and CD4 CTLs are responsive post-STM infection

(A, C) Schema of experimental design. (B) Representative flow cytometry histogram overlays (left) and summary statistics (right) of (B) MHC class II expression by IECs and (C) Nur77-GFP signal in intraepithelial T cells following STM infection. Each dot in the summary statistics represents an individual mouse; data is shown as mean ± standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare two groups. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

CD4 CTLs exhibit heightened effector function following STM infection

At steady state, CD4+CD8α+ and CD4+ CD8α- IETs express similar levels of the degranulation marker LAMP-1, also known as CD107, following PMA and ionomycin stimulation for 4 hours (Figure 19A). However, in STM infected mice, the level of CD107 is much higher in the CD8α+ CD4 CTL population, as compared to the CD8α- population (Figure 19A). Similar with CD107/LAMP-1 expression, IFNγ and perforin expression are also increased among the mature CD4 CTL in comparison to the CD8α- CD4 T cell population following STM infection

(Figure 19 B, C) indicating that in the context of an oral infection with STM, CD4 CTLs become activated as functional killer cells.

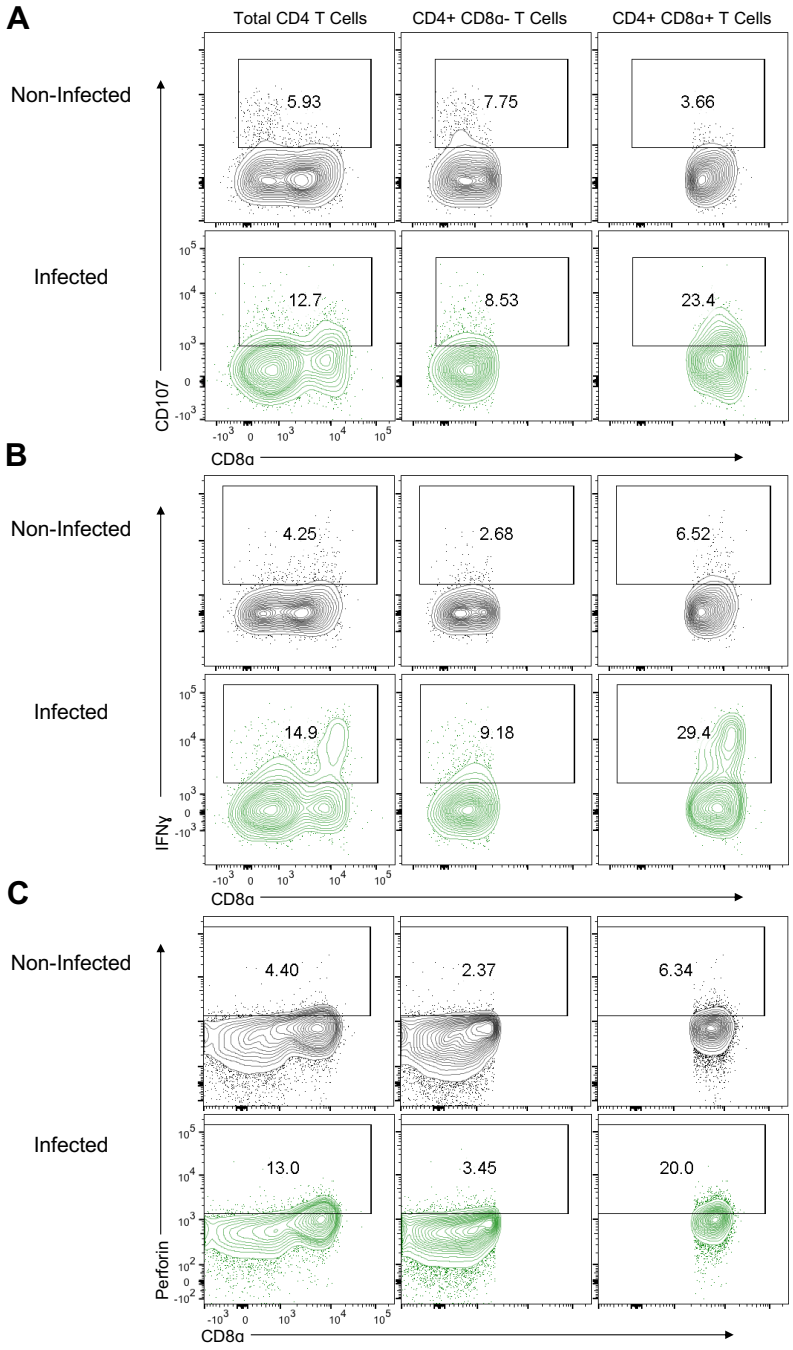

Figure 19: CD4 CTLs exhibit heightened effector function post-STM infection.

Representative flow cytometry plots comparing CD4 T cells in the small intestine epithelium in terms of expression of (A) LAMP/CD107, (B) IFNγ, and (C) Perforin in non-infected (top, black) and STM-infected (bottom, green) mice following PMA and ionomycin stimulation. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

Loss of epithelial MHCII class II results in greater susceptibility to STM infection

To investigate if the MHC class II restricted CD4 CTL in the intestinal epithelium play a non-redundant protective role against oral infection with a food-borne pathogen like Salmonella *enterica*, we used the previously described MHCIIΔIEC mice, which exclusively lack MHC class II expression on the IECs and consequently have decreased CD4 CTL populations. We challenged these mice with oral STM infection (Figure 20A).

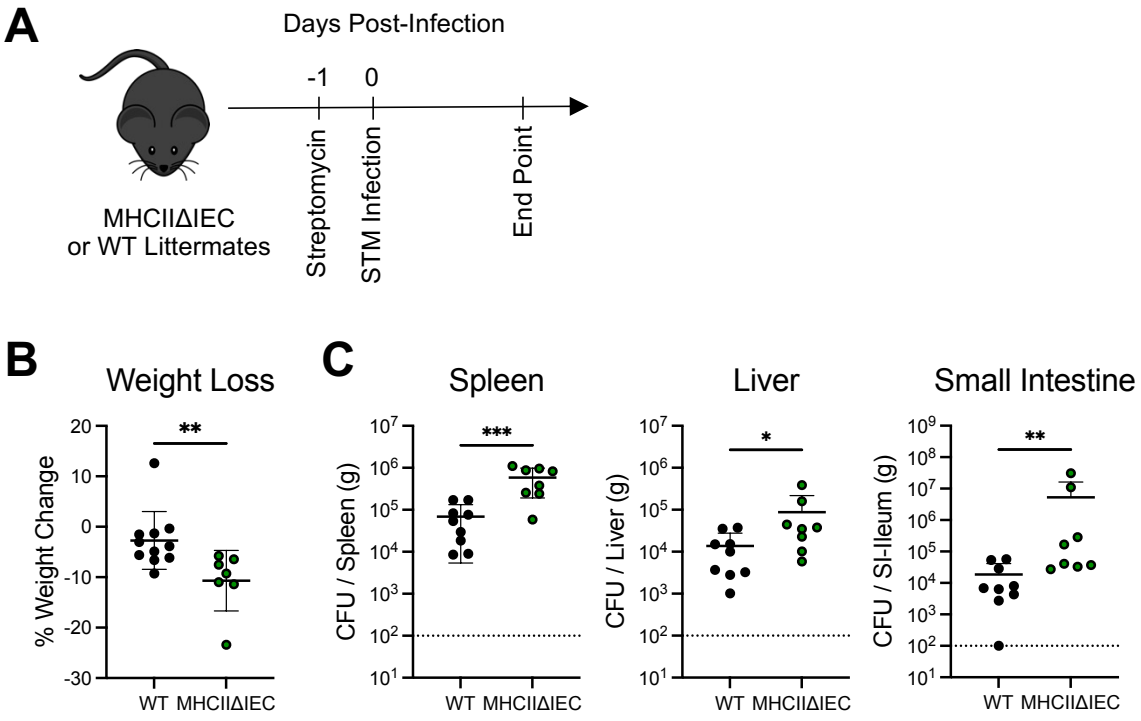

Figure 20: CD4 CTL generated during steady state protect against STM

(A, C) Weight loss and (B, D) bacterial burdens in the stated tissues. Each dot in the summary statistics represents an individual mouse. Data for weight loss is shown as mean ± standard deviation. Data for bacterial burdens is shown as geometric mean ± geometric standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare weight loss data, Mann-Whitney test was used to compare bacterial burden data. Data represent pooled results from at least two independent experiments with at least three mice per group in each experiment.

There are two mechanisms defective in these mice that may lead to greater susceptibility to infection with intravacuolar pathogens. First, these mice have a drastically reduced CD4 CTL

population in the intestinal epithelium (Figure 10C, D). The second defect is the loss of MHC class II-mediated antigen processing and presentation by the IECs, which precludes any TCR:MHC communication between the resident CD4 IETs and IECs. Consequently, orally infected MHCIIΔIEC mice showed much greater weight loss and significantly more bacterial burden in the spleen, liver, and small intestine compared to infected WT littermates (Figure 20B, C).

These results underscore an important non-redundant role for the MHC class II-dependent CD4 CTLs in providing protection against intravacuolar food-borne pathogens that cannot be sensed by MHC class I restricted CD8 CTL. Moreover, because CD4 CTL are pre-existing and generated at steady state in response to harmless luminal antigens, they are not dependent on pathogen specificity to carry out their protective function.

To further isolate this effect to the CTL compartment of the intraepithelial CD4 T cells, we analyzed the protective capacity of CD4 T cells isolated from monoclonal OVA-specific OT-II TCR transgenic mice that had been crossed onto a *Perforin*^{KO} background and from control mice on a *Perforin*^{WT} background. OT-II TCR transgenic spleen CD4 T cells on a *Perforin*^{WT} or *Perforin*^{KO} background were transferred to *Rag*$^{-/-}$ recipient mice that were fed for 4 weeks with either a regular standard diet or a standard diet that contained ovalbumin (Figure 21A). Following the 4-week period, which is adequate to generate OT-II CD4 CTLs in the intestinal epithelium in response to OVA in the diet, the mice were orally infected with STM. The mice on a *Perforin*^{WT} background that were fed an ovalbumin-containing diet were significantly better at resisting an oral infection with STM, based on the degree of weight-loss and bacterial burden as compared to the mice that had been fed an ovalbumin-free diet and therefore, lacked OT-II TCR transgenic CD4 CTL in the small intestine epithelium (Figure 21C, D).

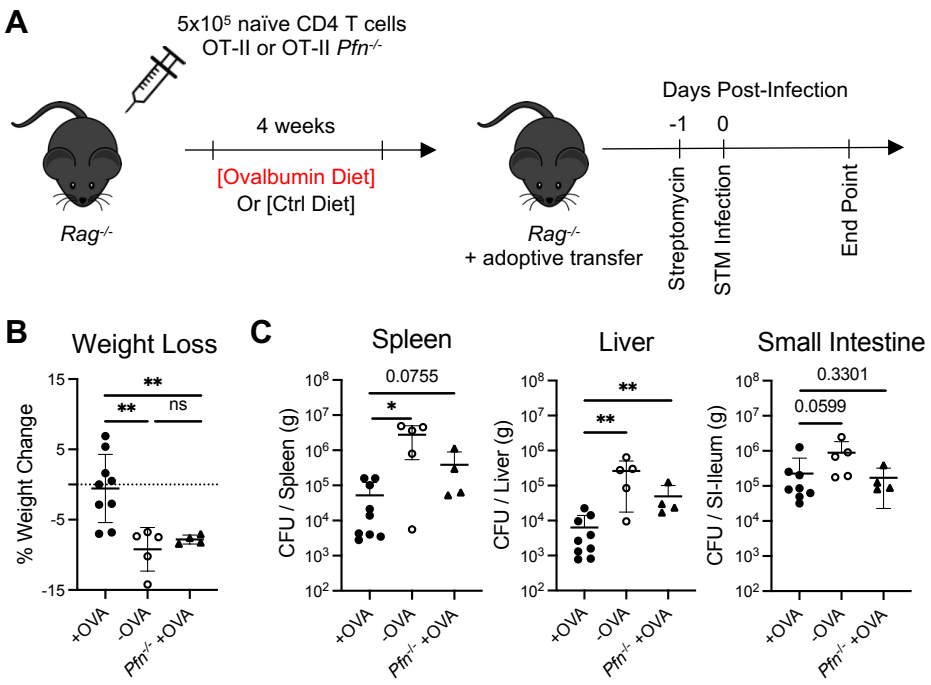

Figure 21: CD4 CTLs are protective against STM infection.

(A, C) Weight loss and (B, D) bacterial burdens in the stated tissues. Each dot in the summary statistics represents an individual mouse. Data for weight loss is shown as mean ± standard deviation. Data for bacterial burdens is shown as geometric mean ± geometric standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare weight loss data, Mann-Whitney test was used to compare bacterial burden data. Data represent pooled results from at least two independent experiments with at least three mice per group in each experiment.

In contrast, the recipient mice that received OT-II TCR transgenic CD4 T cells on a *Perforin*^{KO} background showed severe weight-loss and increased bacterial burden regardless of the presence OVA antigen in the diet (Figure 21B, C). Overall, these results demonstrated the essential protective immunity that CD4 CTLs confer against certain food-borne pathogens. Moreover, the results also demonstrate that CD4 CTL, which are pre-existing and generated in response to non-pathogen antigens, can provide protection independently of pathogen-specificity.

CD4 CTLs do not require IEC-mediated MHC class II:Ag presentation during infection

While the requirement for IEC expression of MHC class II during steady state is important for CD4 CTL development during steady state, we then sought to address if CD4 CTLs require MHCII:TCR signal during infection.

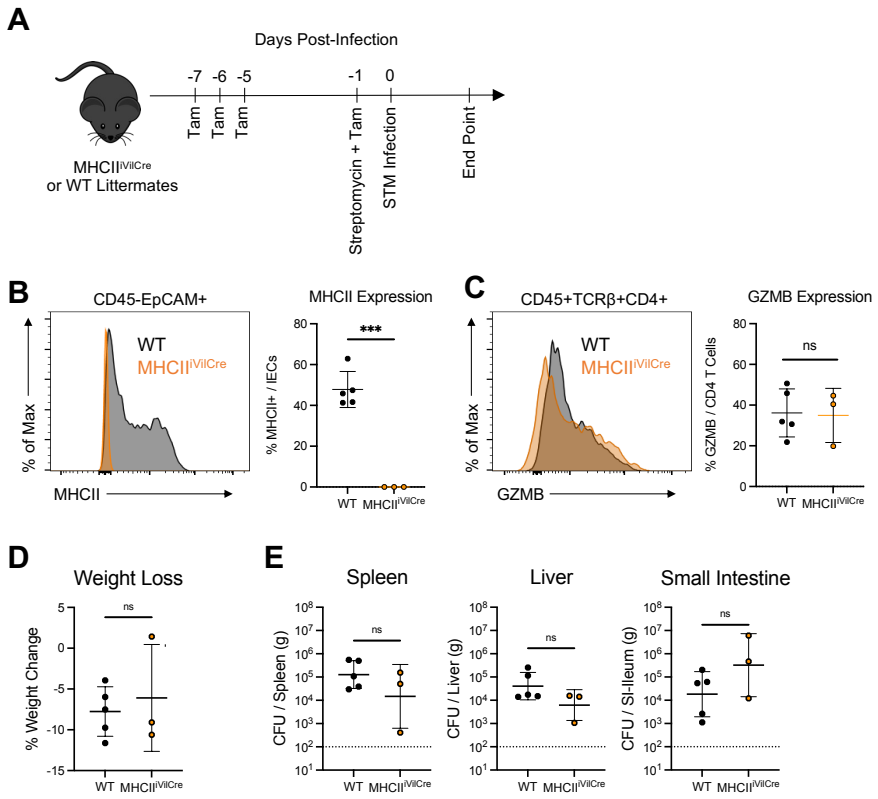

Figure 22: MHC class II-expression on IECs is not required for CD4 CTL killing

(A, C) Weight loss and (B, D) bacterial burdens in the stated tissues. Each dot in the summary statistics represents an individual mouse. Data for weight loss is shown as mean ± standard deviation. Data for bacterial burdens is shown as geometric mean ± geometric standard deviation; $p > 0.05$, ns; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Unpaired t-test was used to compare weight loss data, Mann-Whitney test was used to compare bacterial burden data. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

To address this question, we generated an inducible model of MHC class II deletion from the IECs by crossing *I-A*^{b}-floxed mice with *Villin*-Cre^{ERT2} mice (MHCII^{iVilCre}), whereby the presence of tamoxifen induces *Villin*-Cre expression that excises *I-A*^{b} in a small intestine epithelium-specific fashion. Mice were intraperitoneally injected with tamoxifen for three

consecutive days, beginning a week prior to infection, with a final dose of tamoxifen injected 1 day before infection alongside an orally gavaged dose of streptomycin. Mice were then analyzed 3-4 days post-infection and assessed for small intestine epithelial populations (Figure 22 B, C), body weight loss (Figure 22D) and bacterial burden (Figure 22E). MHC class II was absent on the IECs following the 1 week period of tamoxifen treatment (Figure 22B), confirming the efficacy of our murine model. Interestingly, there was not a significant decrease in Granzyme B expression on the intraepithelial CD4 T cells (Figure 22C). Furthermore, the weight loss and bacterial burdens were not significantly different between the $MHCII^{iVilCre}$ mice and their WT littermates (Figure 22E, F). Therefore, we concluded that CD4 CTLs do not require MHCII:TCR signal during infection to be protective against enteric infection.

IL-15/IL-15Rα trans-presentation is required for active killing by CD4 CTLs

In Mucida and Hussain et al, monoclonal OT-II TCR transgenic CD4 T cells as well as polyclonal CD4 T CTLs, defined by either ThPOK- or CD8α+, that were activated *in vitro* in the presence of rIL-15 expressed significantly more CD107 at the cell surface and secreted significantly more IFNγ and TNF, suggesting a costimulatory role for IL-15 to enhance the protective capacity of CD4 CTL (Meresse et al., 2006; Mucida et al., 2013).

Soluble IL-15 (sIL-15) produced at the mucosal border is essential for the survival of intraepithelial T cells, including CD4 CTL, which sense sIL-15 by the constitutive expression of CD122 (IL-2Rβ/IL-15Rβ) and CD132 (γ-c) at their cell surface (Ebert, 1998; Schluns et al., 2004). Under stress conditions, IECs induce cell surface expression of IL-15Rα that can capture sIL-15 and trans-present IL-15/IL-15Rα complexes to IL2Rβ/Cγ on the interacting IET thereby signaling distress and promoting a cytotoxic response (Jabri & Abadie, 2015).

We have previously shown that non-pathogen-specific CD4 CTLs, generated at steady state in response to antigens present during steady state, can protect against enteric pathogens. We then reasoned that stress-induced trans-presentation of IL-15/IL-15Rα might provide a signal that can induce quiescent CD4 CTLs into active killer cells.

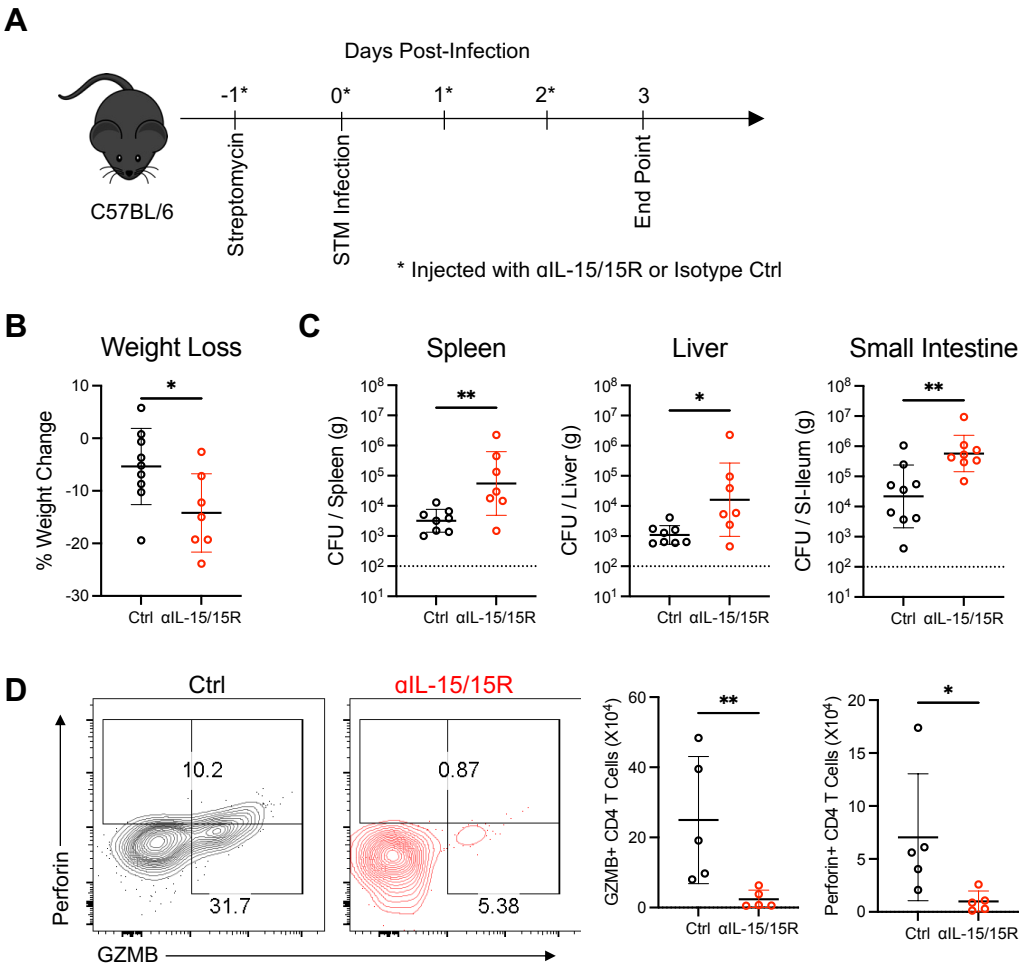

Figure 23: IL-15/IL-15Rα trans-presentation is essential for active killing by CD4 CTLs

(A) Schema of experimental design. (B) Bacterial burden in small intestine; each dot in the summary statistics represents an individual mouse; data is shown as geometric mean ± geometric standard deviation; $p > 0.05$, ns; $p <x 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; Mann-Whitney test was used to compare groups. Representative flow cytometry plots from the stated conditions of (C) CD8α and CD103 co-expression, (D) FOXP3 and RORγt co-expression, (E) Perforin and Granzyme B co-expression, and (F) 2B4 and Granzyme B co-expression. Data are representative results from one of at least two independent experiments with at least three mice in each experimental group.

To test this hypothesis, we interfered with IL-15/IL-15Rα trans-presentation by using an antibody that specifically detects and neutralizes IL-15/IL-15Rα complexes. WT C57BL/6 mice were intraperitoneally injected with anti-IL-15/IL-15Rα antibody 1 day prior to oral STM infection (Figure 23A). The anti-IL-15/IL-15Rα antibody administration continued daily until the infected mice were dissected for analysis (Figure 23A). Orally infected mice that were also treated with antibody against IL-15/IL-15Rα showed a much greater weight loss and bacterial burden in the spleen, liver, and small intestine compared to isotype control-injected mice (Figure 23B, C). Intraepithelial CD4 T cells isolated from the anti-IL-15/IL-15Rα treated mice also failed to upregulate cytotoxic markers like Granzyme B and Perforin, and the CD4 CTL population was decreased (Figure 23D). Overall, these results show that cytokine signals released by the infected or stressed epithelial cells can trigger the killing capacity of non-pathogen specific CD4 CTL that are generated at steady state in response to harmless luminal antigens and provide critical nonredundant protective immunity against any pathogen that invades the mucosal borders of the intestine.

4.4 Discussion

Enteric food-borne pathogens, alongside opportunistic microbiome pathobionts, challenge the intestine with constant risk of infection. To contend with these diverse threats, the epithelium hosts several modes of immunity that are unique to the mucosal immune system. Specific to the intestinal epithelium, CD4 CTLs are among the lymphocytes that acquire or maintain their functional capacity in the epithelium. As MHC class II-restricted CTLs, CD4 CTLs may be more receptive to certain pathogens compared to their CD8αβ CTL counterparts. In particular, the CD8αβ CTLs are hypothesized to be less sensitive to pathogen antigens that are preferentially

processed and presented by the MHC class II pathway. One such pathogen is Salmonella *enterica* serovar Typhimurium (STM), where the ablation of MHC class II (and lacking CD4 T cells) dramatically increased disease pathology; as opposed to the ablation of MHC class I (and lacking CD8αβ T cells), which did not have a significant effect (Hess, Ladel, Miko, & Kaufmann, 1996). The presence of TBET-expressing CD4 T cells is also essential for protection against systemic STM infection (Ravindran, Foley, Stoklasek, Glimcher, & McSorley, 2005). As such, we hypothesized that intraepithelial TBET+ CD4 CTLs have a non-redundant role in combatting STM.

In this study, we have demonstrated a unique, protective role for CD4 CTLs in combatting intravacuolar food-borne pathogens. STM infection causes MHC class II upregulation in the IECs and a subsequent TCR in the CD4 CTLs. Furthermore, mice with MHC class II conditionally knocked out of the IECs and orally challenged with STM infection performed considerably worse at eliminating the pathogen threat as compared to their WT littermates. The result of this experiment could be attributed to both the loss of MHC class II signaling that fails to alert the resident CD4 T cell population and the original significant decrease in CD4 CTLs associated with the absence of MHC class II expression on the IECs.

To further dissect the importance of pre-existing non-pathogen-specific CD4 CTL, we analyzed the importance of OVA-responsive OT-II CD4 T cells to protect against an oral infection with STM. The presence of OVA-specific OT-II CD4 CTL at the mucosal border resulted in considerably better protection, with less weight-loss and less bacterial burden locally and in the systemic tissues. When compared to the condition where the OVA-specific OT-II CD4 CTL lack perforin expression, which have defunct killing capacity. In the presence of non-functional perforin-deficient OVA-specific CD4 CTL colitis was as severe as the condition where CD4 CTL

were totally lacking (i.e. adoptively transferred OT-II cells into a host that was not fed OVA). Taken together, pre-existing diet-specific CD4 CTLs were found to provide protective immunity against an oral infection with a food-borne pathogen like STM.

Interestingly, while CD4 CTLs are generated in response to MHC class II-expressing IECs during steady state, the inducible deletion of MHC class II from IECs immediately before and during infection did not influence disease susceptibility. The presence of intraepithelial Granzyme B-expressing CD4 CTLs was also at normal frequencies and unaffected by the short-term loss of MHC class II-signaling from the IECs. Therefore, the MHC class II:TCR signaling between IEC and CD4 CTL is dispensable during infection, and CD4 CTLs must rely on a different cue to become active killers.

Cytokines within the environmental milieu are known to substantially contribute to the development and functions of intraepithelial T lymphocytes. Among these cytokines, secreted IL-15 is known for its role in intraepithelial T lymphocyte survival, maturation, and function (Fehniger & Caligiuri, 2001). IL-15 shares common traits with IL-2, including shared receptor components composed of CD122 (IL-2Rβ/IL-15Rβ) and CD132 (γ-c). In addition, when trans-presented by stressed epithelial cells that induce IL-15Rα, IL-15/IL-15Rα can function as a "danger signal" in the intestine that is able to activate normally quiescent resident T lymphocytes (Jabri & Abadie, 2015). Here we show that when IL-15/IL-15Rα signaling was blocked through antibody administration, there was an increase in bacterial burden that corresponded with a loss in effector molecules, including Perforin and Granzyme B, among the intraepithelial CD4 CTLs.

The interplay between TCR and cytokine signaling is complex. In summary, IL-15/IL-15Rα trans-presentation acts as a licensing mechanism, whereas TCR signaling may function as

more of a costimulatory role, for CD4 CTLs to become active killers and confer protective immunity against enteric infection.

4.5 Acknowledgements

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CHAPTER 5: Conclusions

5.1 Dual-Purposed CD4 CTLs: Quiescent during Steady State and Active during Infection

CD4 cytotoxic T lymphocyte (CTL) are the majority population of CD4 T cells in normal, healthy small intestine epithelial tissue during steady state. While this provides circumstantial evidence that CD4 CTLs are indicative of a homeostatic environment, we investigated further through colitis models. Not only did we find that CD4 CTLs did not induce pathology, but we also found that the prevention of CD4 Th cell conversion to CTL exacerbates autoimmune disease.

Because CD4 CTLs are generated and remain quiescent during steady state, we then investigated under what context will CD4 CTLs actively kill. Using an oral gastroenteritis model of infection, the presence of CD4 CTLs in the small intestine epithelium was found to be instrumental in resisting pathogenic Salmonella *enterica* serovar Typhimurium (STM) challenge. Because intraepithelial CD4 CTLs are antigen-experienced prior to pathogenic encounter, CD4 CTLs are ready with granzyme and perforin granules when active cytotoxicity could be beneficial.

The complete ablation of MHC class II from IECs significantly decreases the presence of intraepithelial CD4 CTLs and resulted in greater susceptibility to STM oral infection. Surprisingly, there was not a difference in disease susceptibility when MHC class II was ablated from IECs during infection. Instead, we found that IL-15/IL-15Rα trans-presentation, which occurs on the surface of damaged or infected intestinal epithelial cells (IECs), mediates active killing by CD4 CTLs. TCR signaling is critical for CD4 CTL conversion, maintenance, and retention in the small intestine epithelium, but IL-15/IL-15Rα trans-presentation acts as the ‘danger signal’ that activates killing by the non-pathogen-specific CD4 CTLs.

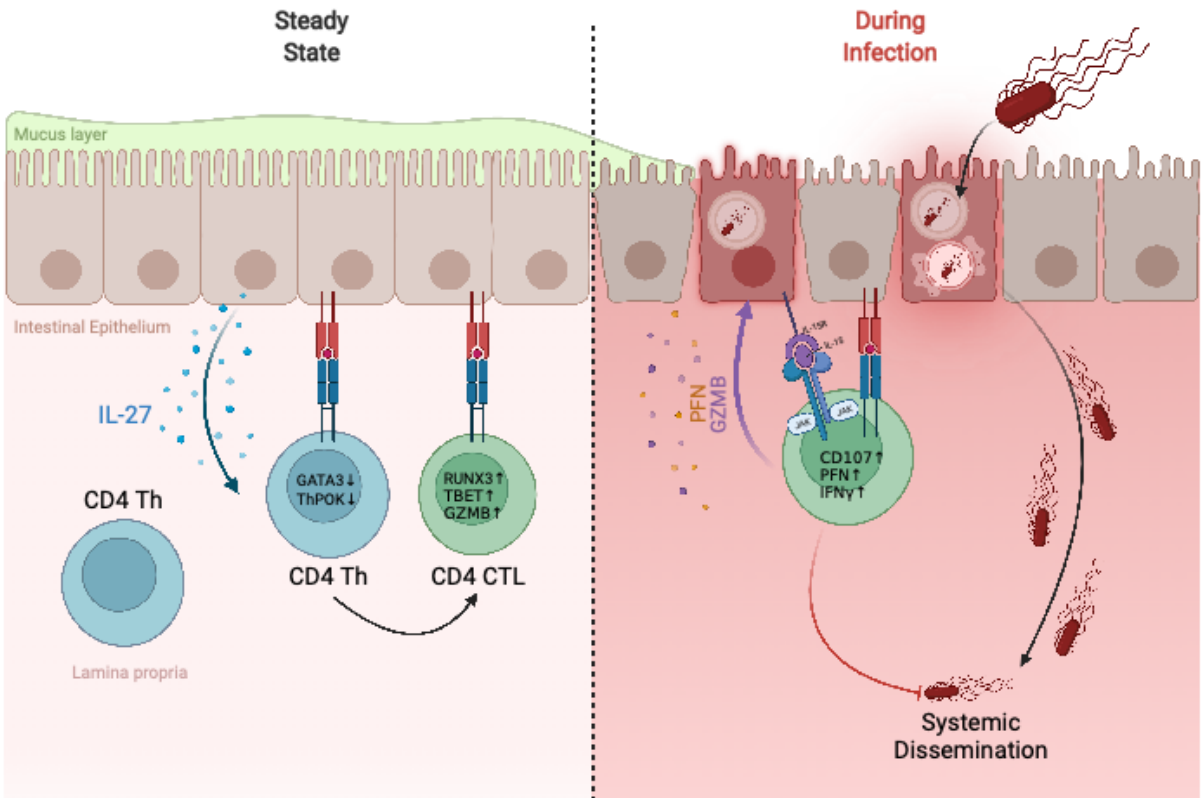

Figure 24: Graphical abstract

Small intestine intraepithelial CD4 CTLs are generated in response to MHC class II presentation of luminal antigens by the IECs and the presence of IL-27 in the environmental milieu. During infection, CD4 CTLs may become active killers in response to danger signals, such as the trans-presentation of IL-15/IL-15Rα by IECs. In summary, CD4 CTLs represent a mucosal strategy of arming the small intestine with MHC class II-restricted CTLs that prevents immunopathology during steady state while conferring broad protection against novel pathogens.

Intraepithelial T lymphocytes express several inhibitory molecules that increase the threshold for activation, including CD8αα (Denning et al., 2007). Notably, the presence of IL-15 has been theorized to reduce T cell receptor (TCR) activation threshold and promote cytolytic functions of CTLs (Jabri & Abadie, 2015). In the case of CD4 CTLs and their role in killing STM-infected cells, IL-15/IL-15Rα trans-presentation appears to play a greater role as compared to TCR:MHCII signaling from the IECs. For T cells that are generated and adopt a cytotoxic phenotype during steady state, cytokine signaling may be the primary signal for active effector

function while TCR signaling may act as more of a costimulatory role. Taken together, the existence of CD4 CTLs represents an elegant strategy by the mucosal immune system to fortify the small intestinal barrier with non-redundant MHC class II-restricted CD4 CTLs that confer broad, non-pathogen specific protection when given the appropriate 'danger' cues.

5.2 CD4 CTLs Arise from Oral Tolerance Conditions as a Form of "Protective Tolerance"

The classic definition of oral tolerance is a lack of systemic immune response against orally-introduced antigens. Here we describe an alternative mechanism of homeostasis in the small intestine epithelium, where CD4 T cells have specificity for a non-pathogen-derived antigen presented by MHC class II-expressing IECs that directs the reprogramming from the T helper (Th) lymphocyte towards a cytotoxic fate. The resulting CD4 CTL population is conducive to a homeostatic environment and confers non-pathogen specific protection during gastrointestinal infection. As such, the generation of CD4 CTLs during steady state and their functional relevance during infection is a novel strategy of the mucosal immune system that can be described as 'protective tolerance'.

An exemplar of a disease where oral tolerance mechanisms are dysfunctional is celiac disease. Celiac disease is a condition in which patients exhibit a pathologic, aberrant CD4 T cell response to dietary gluten (Jabri & Sollid, 2017; Stamnaes & Sollid, 2015). As a complex, multifactorial disease, celiac disease is characterized by prominent inflammation that coincides with the presence of gluten-reactive CD4 T cells in the proximal small intestine (Stamnaes & Sollid, 2015). Celiac disease patients are also frequently diagnosed based on serology, as the patients develop IgA and IgG antibodies against gluten peptides as well as to the autoantigen transglutaminase 2 (TG2) (Jabri & Sollid, 2017).

However, for the majority of people who do not have celiac disease, gluten is tolerized and potentially a source of epitopes for human CD4 CTLs in the small intestine. As with any autoimmune disease, celiac disease is a case where immunity is defective due to both genetic and environmental factors. It is interesting to think of CD4 CTLs developing in the context of healthy tissue, i.e. “protective tolerance”, as opposed to in the context of diseased tissue, e.g. celiac disease, where normal CD4 T cell differentiation towards cytotoxicity may be compromised.

The mucosal immune system is challenged to operate in a microbial and antigen-rich environment. As compared to the relatively sterile environment of systemic immunity, the mucosa is challenged to develop alternative mechanisms that promote both tolerance and protection. The development of resident T lymphocyte populations in the small intestine epithelium as a form of “protective tolerance” presents an intriguing demonstration of how immunity has adapted to the needs of the host and the local environment.

5.3 Broader Impacts and Future Directions

In this dissertation, CD4 CTLs were hypothesized to serve multiple functions depending on the requirements of the environment. During steady state, the reprogramming of CD4 T help cells into CD4 CTLs represents a mechanism of tolerance that prevents inflammatory bowel disease by diverting CD4 Th from a pathological fate towards a quiescent cytotoxic fate. However, when the small intestine epithelium is under attack by an enteric pathogen, the normally quiescent CD4 CTLs may become actively cytolytic. The findings of this study characterized the antigenic requirements, including the source of antigen presentation, that lead to CD4 CTL conversion; the homeostasis-promoting function of CD4 CTLs during steady state; and the active cytotoxicity that

CD4 CTLs exhibit during infection. Taken together, CD4 CTLs represent a versatile and epithelium-specific immune modality of protection.

Cytotoxic CD4 T cells have become more appreciated in recent years, as their presence has been repeatedly noted in several studies, especially in terms of cancer biology and virology (Cenerenti, Saillard, Romero, & Jandus, 2022; Juno et al., 2017; Oh & Fong, 2021). While the mechanisms by which tumor-specific CD4 CTLs and virus-specific CD4 CTLs arise and develop remain an intriguing field of study, there have been some findings that bear resemblance to the diet-specific CD4 CTLs. Notably, chronic viral infections have been shown to generate CD4 CTLs (Juno et al., 2017). Due to the chronic nature of these viral infections, it is presumed that the virus-specific CD4 CTLs are also generated in response to multiple rounds of TCR stimulation, like the diet-specific CD4 CTLs. However, a fundamental difference between diet-specific CD4 CTLs and virus-specific CD4 CTLs is the context in which their antigens are presented. As described previously, diet-specific CD4 CTLs are dependent on MHC class II-expressing IECs for repeated TCR signaling that allows for their retention in the small intestine epithelium and their cytolytic capability. Virus-specific CD4 CTLs, however, likely have their antigens presented to them through an infected MHC class II-expressing cell in the periphery, such as myeloid cells. Tumor-specific CD4 CTLs may have some similarities to both the diet-specific and the virus-specific CD4 CTLs. Like the diet-specific CD4 CTLs, tumor-specific CD4 CTLs may be developing in the immunosuppressive, non-inflammatory environment of a ‘cold’ tumor. Like the virus-specific CD4 CTLs, tumor-specific CD4 CTLs may receive MHC class II antigen presentation from non-epithelial cells that co-express costimulatory molecules.

Future directions for this study may involve general categories of therapy: against infections, tumors, and inflammatory diseases. Conceptually in the *in vitro* polarized adoptive

transfer model shown earlier, we have seen that Treg cells may convert into CTLs. This ability may be taken advantage of in the concept of tumor models, where immunosuppressive Treg cells can be polarized towards a CTL phenotype to elicit better anti-tumoral effects. Likewise for the more inflammatory T helper subsets, the ability for pathogenic Th1 or Th17 cells to be converted into CTLs also represents an intriguing therapeutic avenue to treat autoimmune and other inflammatory diseases. Finally, the conversion of CD4 T helper cells to CTL, adds another avenue to boost protective immunity against viral and bacterial pathogens. While the contents of this dissertation contribute most directly to the field of mucosal immunology and fundamental T cell biology, there is still much to learn about CD4 CTLs and their therapeutic potential.

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