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Functional Consequences of CD4+ T Cell Receptor Ligation in the Immune Response to Listeria monocytogenes
by
Stephen Phillip Persaud

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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List of Abbreviations

7AAD 7-amino actinomycin D
APC Antigen presenting cell
CBA Cytokine bead array
CD Cluster of differentiation
CD4SP CD4 single positive
CFA Complete Freund’s adjuvant
CFSE Carboxyfluorescein diacetate succinimidyl ester
CLIP Class II-associated invariant chain peptide
DN Double negative
DP Double positive
ELISA Enzyme-linked immunosorbent assay
ERK Extracellular signal-related kinase
FACS Fluorescence-activated cell sorting
FPLC Fast protein liquid chromatography
IFNγ Interferon gamma
IL-2 Interleukin 2
IκBα NFκB inhibitor alpha
k a Association rate constant (“on-rate”)
K D Dissociation (affinity) constant
k d Dissociation rate constant (“off-rate”)
LLO Listeriolysin O
LM Listeria monocytogenes
MACS Magnetic activated cell sorting
MHC Major histocompatibility complex
NFAT Nuclear factor of activated T cells
NFκB Nuclear factor of kappa light polypeptide gene enhancer in B-cells
P+I PMA + Ionomycin
pERK Phosphorylated extracellular signal-related kinase
PMA Phorbol 12-myristate 13-acetate
pMHC Peptide-bound Major histocompatibility complex
RAG Recombinase activating gene
scTCR Single-chain T cell receptor
SEC Size exclusion chromatography
SPR Surface plasmon resonance
TCR T cell receptor
TNFα Tumor necrosis factor alpha
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Interactions between the αβ T cell receptor (TCR) and peptide-bound MHC molecules (pMHC) are highly specific and sensitive despite low affinities. The basis of TCR ligand specificity and its functional manifestations during protective immune responses are incompletely understood. To study this, we have generated two TCR transgenic mice called LLO56 and LLO118, which bear CD4+ T cells that recognize the same dominant epitope from the virulence factor Listeriolysin O (LLO) of Listeria monocytogenes. These TCRs were cloned from Listeria-infected B6 mice, and thus represent two solutions to recognizing the same pathogen-derived pMHC. LLO118 T cells expanded better than LLO56 during primary responses to Listeria, due to LLO56 cells’ greater propensity to undergo cell death. Both TCRs utilize an overall similar constellation of contacts with the LLO peptide, but differ dramatically in their contacts with peptide residues flanking the MHC binding groove. Nevertheless, LLO56 and LLO118 bind cognate pMHC with identical affinities. Unexpectedly, LLO56 T cells showed greater IL-2 responses in vitro than LLO118. This was true even when cells were stimulated nonspecifically downstream of the TCR, suggesting intrinsic differences in their responses to stimulation. LLO56’s stronger IL-2 responses were associated with greater TCRζ phosphorylation at baseline and ERK phosphorylation upon activation. Interestingly, the strength of these responses, in both
the transgenic and polyclonal CD4$^+$ and CD8$^+$ T cells, tracked with the expression of CD5, which reflects TCR reactivity to self-pMHC. Consistently, LLO56 and LLO118 T cells acquired their respective sensitivities, basal signaling and propensities for cell death during positive selection, paralleling the strength of signal received from selecting self-pMHC. Notably, withdrawal of self-pMHC reduced LLO56 and LLO118 IL-2 and pERK responses, and compromised the LLO56 response to *Listeria*. We conclude that thymic education is a crucial inflection point about which the functional properties of CD4$^+$ T cells are determined, ultimately impacting their performance in antipathogen responses.
Chapter 1: Introduction to Recognition of Peptide-MHC by the TCR

Many of the experiments, methods and writing presented here have been published by our laboratory in *Molecular Immunology* in 2010\(^1\).

1.1 The function and importance of CD4\(^+\) T cells

In the context of the immune response to a pathogen, CD4\(^+\) T cells bind to foreign peptides bound to Class II molecules of the major histocompatibility complex (pMHC). This interaction with pMHC is mediated by the \(\alpha\beta\) T cell receptor (TCR), and is crucial for the onset of adaptive immunity\(^2\). The primary roles of these cells include cytokine secretion and the coordination, or “help,” of immune responses mediated by other cell types, including macrophages, cytotoxic T cells and B cells \(^3\). The vast importance of CD4\(^+\) T cell responses to immunity is perhaps best demonstrated by the Acquired Immunodeficiency Syndrome, in which CD4\(^+\) cells are destroyed by the Human Immunodeficiency Virus and patients succumb to a wide range of opportunistic infections. Due to failures of central and peripheral tolerance, CD4\(^+\) T cells can also contribute to the pathogenesis of numerous inflammatory and autoimmune disorders, including Type I diabetes mellitus, inflammatory bowel disease and rheumatoid arthritis\(^4\). Finally, alloreactivity of CD4\(^+\) T cells plays an important role in the setting of transplantation, mediating the undesirable effects of graft rejection and graft versus host disease, as well as the desirable graft versus leukemia effect in cancer patients receiving bone marrow transplants.
Though the importance of CD4$^+$ T cells and their effector functions are well understood, our understanding of the exquisite specificity of TCR-pMHC interactions is incomplete. Specifically, while it is clear that ligation of the TCR by Class II pMHC is an essential first step in CD4$^+$ T cell responses, it is not clear how this receptor biochemically recognizes particular pMHC ligands and how signals of different strength transmitted via the TCR are manifest during immune responses. Greater comprehension of CD4$^+$ T cell antigen recognition and subsequent orchestration of immune responses has far reaching biological and clinical relevance, including vaccine development for infection and cancer, and improved basic understanding of the responses to self-peptides bound to syngeneic and allogeneic pMHC in the contexts of autoimmunity and transplantation, respectively.

1.2 Biochemistry of TCR-pMHC interactions

The TCR is a fascinating receptor, showing the exquisite specificity that typifies adaptive immunity but interacting with cognate ligand in the micromolar affinity range$^5$. Despite this, T cells are also exquisitely sensitive to their cognate antigen, showing the remarkable ability to flux intracellular calcium in response to a single cognate pMHC molecule amidst an overwhelming background of self-pMHC$^6$. The binding kinetics and thermodynamics for a number of TCR-pMHC interactions have been probed using surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). These methods have helped us gain insight to some of the general biophysical features of how TCR distinguishes different ligands to various functional outcomes.
Kinetically, most SPR measurements show that a TCR binds pMHC with fast dissociation ($k_{off}$) and slow association ($k_{on}$) rate constants. Ours and other laboratories have pursued connections between binding kinetics and activity using altered peptide ligands (APLs), which have single residue substitutions generally at positions expected to contact the TCR. With our well-characterized, hemoglobin-responsive 3.L2 TCR system, we have identified APLs with a wide range of potencies, from null agonists and antagonists to full agonists, allowing correlations between kinetics and ligand potency to be made (Figure 1.1). These studies have revealed that more potent T cell activation correlates with higher affinity, most strikingly with $k_{off}$.

However, deviations from this basic model have been identified. Despite its importance as a contributor to overall binding affinity, fewer studies have focused on the importance of $k_{on}$. Recently, we have identified TCR-pMHC interactions in the 3.L2 TCR system where a full agonist and weak agonist differed kinetically by a 9-fold different $k_{on}$ but only 1.5-fold in $k_{off}$ (Figure 1.1). It is remarkable that over such a narrow kinetic range such the distinction between full and weak agonist can be made. By and large, however, kinetics analyses using SPR have not yielded rules of TCR triggering that apply to all interactions with pMHC.

Thermodynamic studies have been done directly with isothermal titration calorimetry or indirectly with van’t Hoff analysis and SPR. They have revealed a variety of binding strategies, both entropy- and enthalpy-driven, by which pMHC ligand recognition is achieved by the TCR. Partly because the flexible CDR loops were predicted to become less flexible on binding pMHC, it was thought that TCR-pMHC interactions would reliably be entropically unfavorable, though this is not uniformly the case. The exquisite
thermodynamic sensitivity of TCR-pMHC interactions is clear from work done by our lab and others, in which it has been shown that a TCR can bind APLs differing from the cognate pMHC by as little as a single methylene group with drastically different thermodynamics (Figure 1.2). This can be the case if the methylene difference is at a TCR or MHC contact position, the latter likely due to indirect conformational effects on residues interacting with the TCR\textsuperscript{1,15,26}. Changes in surface hydrophobicity could be responsible for changes in binding thermodynamics, but this is unlikely given such subtle changes to the pMHC surface.

Another interesting thermodynamic measurement that can be examined in TCR-pMHC interactions is the heat capacity change ($\Delta C_p$), which measures the temperature dependence of the enthalpy change. It has been estimated that each 0.2-0.3 kcal/mol-K $\Delta C_p$ reflects 1000 Å\textsuperscript{2} of hydrophobic surface area obscured by a protein-protein interaction\textsuperscript{27,28}. $\Delta C_p$ value for some TCR-pMHC interactions examined suggests burial of much more than the 1000-2500 Å\textsuperscript{2} of surface area possibly buried in a typical TCR-pMHC interaction (Figure 1.2)\textsuperscript{1,15}. This, taken with $k_{on}$ values several orders of magnitude lower than predicted by simple diffusion, has been interpreted as an indication of molecular flexibility of the TCR. A two-step model has been described based upon the observation that MHC mutations most dramatically affect the association rate of the reaction, while peptide mutants mostly affect dissociation\textsuperscript{29}. Due to the flexible CDR3 loops, a TCR may only occupy the correct pMHC-binding conformation a fraction of the time, resulting in a slow $k_{on}$. Upon initial engagement of pMHC, conformational changes in the TCR surface could better accommodate the peptide and stabilize the interaction. Binding certain peptides could require substantial conformation change, leading to more burial
of surface area than would be predicted if the two interacting surfaces simply came together and obscured only the hydrophobicity at the protein contact area. One study has combined these parameters to develop a model focusing on both binding half-life and molecular flexibility, which improved the fit for interactions that were outliers in a simple $k_{\text{off}}$-based model\textsuperscript{15}.

Most recently, several groups have considered TCR-pMHC interactions in the 2D environment of the plasma membrane. Unlike in an SPR apparatus, where one binding partner is immobilized and the other is soluble, the mobility of both binding partners at a cell-cell contact is restricted primarily to lateral diffusion limited by the fluidity of the membrane. This environment may impose vastly different binding properties than what we might expect from SPR studies. Consistent with this, a “confinement model” has been proposed which accounts for rebinding of TCR to pMHC following dissociation at the cell surface\textsuperscript{30}. That TCR molecules would be able to rebind pMHC is compatible with the notion that agonist pMHC molecules are concentrated in the immunological synapse. Furthermore, a recent report found that TCR molecules are constitutively localized to clusters termed “protein islands,” even in the absence of ligand\textsuperscript{31}. This type of TCR oligomerization increases the likelihood that a dissociated pMHC will rebind, if not to the identical TCR then another TCR within a given cluster. An important consequence of a confinement time model is a critical importance of $k_{\text{on}}$, as this governs the ability to rebind recently unbound pMHC before separated by diffusion.

Two recent reports have lent considerable credence to this perspective by making novel kinetic measurements of TCR-pMHC interactions in the plasma membranes\textsuperscript{32, 33}. These reports found
higher affinity interactions occurred in situ compared to in an SPR apparatus, with faster \( k_{\text{off}} \) values but dramatically faster \( k_{\text{on}} \) values. The primacy of \( k_{\text{on}} \) in those studies likely reflects greater spatial confinement and concentration of TCR and pMHC within a small area of plasma membrane.

### 1.3 Signal transduction through the TCR

The TCR lacks intrinsic signaling capability, and thus requires accessory molecules to transduce signal from the cell surface upon pMHC ligation. This is accomplished by the CD3 signaling complex, composed of one each of the \( \gamma\varepsilon, \delta\epsilon, \) and \( \zeta\zeta \) dimers. Together, the CD3 complex contains a total of ten conserved sequences known as immunomodulatory tyrosine-containing activation motifs (ITAMs) that are the targets of the Src family of kinases; in T cells, these include the tyrosine kinases Lck and Fyn. Phosphorylation of both tyrosines in each ITAM leads to recruitment and subsequent activators of various downstream effectors, including the molecules ZAP-70, SLP-76, LAT and PLC\( \gamma^4 \).

Two major models have been proposed to link the binding of TCR and pMHC to initiation of proximal T cell signaling. First, the kinetic proofreading model states that TCR ligation must occur for sufficiently long that ITAM phosphorylation by Src kinases can overcome the high baseline activity of tyrosine phosphatases like SHP-1 that would otherwise suppress CD3 signaling\(^{34, 35}\). A key prediction of this model is that more stable interactions (slower \( k_{\text{off}} \)) will give rise to stronger proximal signaling. Consistent with this, our laboratory has found a pattern between CD3\(\zeta \) phosphorylation and binding kinetics. Whereas fast \( k_{\text{off}} \) APLs induce no further
CD3ζ phosphorylation than the baseline level, slow $k_{\text{off}}$ APLs induce fuller extents of phosphorylation and T cell activation\(^9\). Consistent with this, partial CD3ζ phosphorylation was induced by APLs with antagonistic effects on TCR signaling\(^36\). Anergy-inducing APLs also have been shown to have specific, incomplete patterns of CD3ζ phosphorylation and reduce CD3 association with ZAP70\(^37\).

A second, non-mutually exclusive model draws from the observation that a large fraction of the TCRs on the cell surface are downregulated during T cell activation. As cognate pMHC levels are low on the cell surface and binding pMHC is necessary for TCR downregulation, it was thought that normal T cell signaling occurs by consistent cycles of binding and unbinding of many TCR molecules by a limiting number of cognate pMHC complexes\(^38\). This repeated binding, or “serial triggering,” would lead to accumulation and maintenance of phosphorylated ITAMs at the synapse sufficient to recruit downstream effectors, despite the low affinity of the individual binding events. This model predicts both the importance of $k_{\text{on}}$ and, curiously, an optimal $k_{\text{off}}$ or “dwell time”. A very fast $k_{\text{off}}$, much like in kinetic proofreading, would not sufficiently induce CD3 phosphorylation above the background of cytoplasmic phosphatases, whereas a very slow $k_{\text{off}}$ would prevent the rapid occupancy and phosphorylation of enough CD3 ITAMs at the cell surface to maintain the signal. In silico studies modeling signaling at the immunologic synapse also predict a maximally slow $k_{\text{off}}$, beyond which signaling becomes less effective\(^39\). TCR-pMHC binding measurements taken in situ and the confinement model are compatible with this signaling hypothesis. Finally, in mathematical modeling studies, a TCR-pMHC interaction with fast $k_{\text{on}}$ but fast $k_{\text{off}}$ could be stimulatory due to rebinding before the
binding partners diffuse apart. Importantly, these studies suggest a type of kinetic proofreading wherein the ability to serially trigger many TCRs gives rise to a prolonged effective half-life which governs the stimulatory potency of a given interaction\textsuperscript{16}.

Recent evidence from membrane FRET and NMR spectroscopy experiments has demonstrated that the ITAMs of the CD3\(\varepsilon\) complex associate with the plasma membrane in the absence of cognate pMHC and are released from the membrane in its presence\textsuperscript{40}. The release of the CD3\(\varepsilon\) ITAMs renders key activating tyrosines accessible for phosphorylation by Src family kinases. This provides a mechanism that enforces specificity in activation via the TCR, whereby proximal signaling is initiated only in the presence of cognate pMHC. It is unclear how precisely pMHC induces this conformational change, or to what extent this occurs with weak and null agonists or ligands binding with varying kinetic properties.

### 1.4 TCR signal strength from thymic and peripheral self-pMHC

While TCR signal strength is most often considered in the context of immune responses upon recognition of cognate pMHC, the strength of signal through the TCR from ligation of self-pMHC complexes is of crucial importance in a variety of contexts. During T cell development in the thymus, positive selection is necessary to ensure that T cells can recognize peptide bound to self-MHC molecules. This is accomplished by a very low affinity interaction between a thymocyte TCR with self-pMHC molecules presented by cortical thymic epithelial cells (cTECs). Using M15, a high-affinity TCR engineered by \textit{in vitro} evolution of the 3.L2 TCR\textsuperscript{41}, we have measured an affinity value for an interaction with a positively selecting APL that lies in
the micromolar range (Figure 1.3). Assuming the fold-difference in affinity between M15 binding the wild type ligand and the positively selecting APL were the same for the 3.L2 TCR, we estimated that the wild type 3.L2 TCR interaction with the positively selecting ligand would occur with a millimolar affinity range\(^1,42\). Failure to detect any binding signal by SPR at TCR concentrations as high as 200 μM is consistent with a millimolar range interaction. Despite having such low affinity, the biological relevance of the interaction with positively selecting pMHC is clear, as its presence or absence dictates thymocyte survival or death by neglect, respectively. The process of negative selection, occurring primarily in the thymic medulla, is necessary to eliminate selected TCR specificities that too strongly engage self-pMHC, as these can lead to autoimmunity. A potent stimulus delivered to a developing thymocyte will lead to its death by apoptosis\(^43\).

The strength of the signal a developing thymocyte receives from selecting self-pMHC molecules can be read out by expression of the C-type lectin CD69. However, as CD69 expression is lost before cells leave the thymus, it does not report on the strength of self-pMHC interactions among peripheral cells. Expression of the cell surface protein CD5, a member of the SRCR (scavenger receptor, cysteine-rich) family of proteins, is more informative in this regard. The expression of CD5 is set during positive selection in proportion to the strength of signal perceived by a developing thymocyte\(^44\). Once set in the thymus, the relative expression level of CD5 between different T cells is maintained in the periphery, though the absolute expression levels are reduced. The expression of CD5 appears to be dynamically regulated by the interaction with self-pMHC, as deprivation of self-pMHC leads to a reduction in CD5 expression\(^45\). Recently, a
transgenic reporter was developed to monitor the expression of the orphan nuclear receptor Nur77, which correlates well with the strength of antigen receptor signaling. Using this system, it was confirmed that agonist selected T cells like Tregs and iNKT cells do perceive a stronger signal from self-ligands via the TCR during their development\textsuperscript{46}.

TCR:self-pMHC interactions also occur in the periphery, with important implications for T cell homeostasis\textsuperscript{47, 48, 49, 50}. Notably, during responses to cognate pMHC, self-pMHC molecules can be found within the immunological synapse and contribute as coagonists to T cell activation\textsuperscript{51, 52}. Work from our laboratory suggested that while positive selection is highly specific for a particular self-pMHC ligand, coagonism is apparently less stringent; while many APLs of an endogenous positively selecting peptide can act as coagonists, far fewer of these APLs can drive progression of DP thymocytes through selection\textsuperscript{53}.

In the absence of cognate pMHC, TCR:self-pMHC interactions regulate basal signaling in naive T cells, such that cells are kept at a tonically stimulated yet still inactive state. It has been shown that self-pMHC maintain basal CD3\(\zeta\) phosphorylation and polarization of proximal signaling components, thus impacting T cell responses to cognate pMHC\textsuperscript{54}. This notion has held true in memory T cells as well, in that TCR:self-pMHC interactions were shown to sustain memory gene expression, metabolism and effector function\textsuperscript{55, 56}. Using a CD11c-DTR mouse model, where CD11c\(^{+}\) cells could be eliminated by treatment with diphtheria toxin, it was argued that classical dendritic cells (cDCs) were uniquely important APCs for maintaining the tonic T cell signals from self-pMHC\textsuperscript{57}. However, an important caveat to this work is that CD11c is not a
specific marker for cDCs. Mice lacking the transcription factor ZBTB46, which do specifically lack cDCs\textsuperscript{58, 59}, would be a useful model system in which to clarify this issue.

1.5 The immune response to \textit{Listeria monocytogenes}

\textit{Listeria monocytogenes} (LM) is a facultative intracellular gram-positive organism that has provided a relatively safe yet powerful infectious mouse model for studying cellular immunity. Clinically, it is a food-borne pathogen that generally causes a mild, self-limiting gastroenteritis. In immunocompromised patients and pregnant women, it causes a disease called listeriosis, which is manifest by septicemia, meningitis and/or encephalitis, and uterine infections. Due to the latter disease manifestation, pregnant women are a uniquely susceptible host; ascending \textit{Listeria} infection can cause neonatal sepsis, abortion or stillbirth in the child, or puerperal sepsis in the mother\textsuperscript{60}.

LM expresses proteins called internalins, which bind target proteins on host target cells, namely E-cadherin and the hepatocyte growth factor receptor, to facilitate invasion\textsuperscript{61}. Indeed, this interaction is the basis for LM crossing the gut epithelium in human food-borne infection. Mice are not easily susceptible to oral LM infection due to a single amino acid difference that prevents high-affinity binding of murine E-cadherin by internalin A\textsuperscript{60}. Consistently, gastrointestinal LM infection models in mice generally prescribe oral gavage of an incredibly large inoculum (>10\textsuperscript{10} CFU)\textsuperscript{62}. Routine introduction of LM to mice is therefore done by either intravenous or intraperitoneal injection, thus bypassing the intestinal epithelium. Once administered, the bacteria invade a variety of cell types, principally fibroblasts, endothelial cells, hepatocytes and
macrophages. The internalized organisms are directed to the lysosome to be destroyed by the host’s lytic enzymes. However, LM escapes to the cytosol via expression of a pore-forming protein, the cytolysin Listerialysin O (LLO). LM lacking LLO is completely avirulent and fails to evoke an effective CTL response from the host, highlighting the essential role of this protein in both virulence and immunity.\textsuperscript{63, 64} Once the organism has entered the cytosol, it utilizes a second essential protein, called ActA, which jettisons it through the cytoplasm using the host’s actin cytoskeleton. Polymerization of actin pushes LM against the host plasma membrane and into adjacent cells, which themselves become infected. This cell-to-cell movement is immunologically relevant, as it evades extracellular effectors of innate immunity.\textsuperscript{65}

The immune response to LM is primarily cell-mediated, with little role for antibody generation.\textsuperscript{66} LM expresses several pathogen-associated molecular patterns (PAMPs) that stimulate macrophages and dendritic cells via Toll-like receptors and other molecules to express innate immune cytokines and upregulate MHC and costimulatory molecules. Neutrophils respond rapidly to infection, playing a crucial early role in controlling LM by executing the acute inflammatory phase and possibly via production of IFNγ.\textsuperscript{67} NK cells are also important early responders, reacting to IL-12 generated by activated APCs by secreting IFNγ, which activates macrophages and polarizes activated CD4\textsuperscript{+} T cells towards a Th1 phenotype. Adaptive immunity is primarily T cell based, with CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells expanding rapidly over the 7 days following infection and rapidly contracting by 14 days post-infection.\textsuperscript{68}
Due to the intracellular tropism of LM, an effective CD8\(^+\) T cell responses is essential for sterilizing immunity and rapid clearance of a subsequent challenge\(^{68}\). CD4\(^+\) T cells, in theory, can help CD8\(^+\) responses by a number of mechanisms, including cytokine production, provision of costimulatory molecules or by facilitating entry into infected tissues\(^3,69\). Primary murine LM infection, however, has been shown to be independent of CD4\(^+\) help, demonstrated by infecting Class II KO (“helpless”) mice that lack CD4\(^+\) T cells\(^{70}\). In another study, post-expansion CD8\(^+\) T cells were transferred from mice 7 days post infection to WT or Class II KO mice\(^{71}\). Whether these CD8\(^+\) T cells had expanded in a WT or Class II KO mouse, antigen-specific cells were less long lasting and less responsive on rechallenge in the Class II KO recipient. This shows that CD4\(^+\) T cells themselves do not program memory characteristics or recall responsiveness early in the response, but instead are necessary for maintenance during the memory phase. Finally, using CD25\(^{-/-}\) mixed bone marrow chimeras, it was found that a CD8\(^+\) recall response requires IL-2 signals during initial activation\(^{72}\). As CD4\(^+\) T cells were found to be unnecessary for recall responsiveness, presumably another source of IL-2 is able to compensate during priming. It is not yet known how altering the signal through the CD4\(^+\) TCR affects the CD8\(^+\) response in vivo.

### 1.6 Methods

#### Proteins and peptides

T cell receptors analyzed include the 3.L2 single chain TCR (scTCR) and M15, a hemoglobin (Hb) specific, high-affinity scTCR engineered from 3.L2 by \textit{in vitro} evolution\(^{41}\). The peptides used in this study and their sequences are as follows: Hb, residues 64–76, GKKVITAFNEGLK; Q72, Asn to Gln substitution of Hb at the P5 position, GKKVITAFQEGLK; D73, Glu to Asp
substitution of Hb at P6, GKKVITAFNDGLK; MCC, moth cytochrome C, residues 88–103, ANERADLIAYLKQATK. Boldfaced residues are those occupying the I-E\(^k\) peptide binding groove, P1 to P9. Peptides were synthesized in-house using a Protein Technologies Symphony automated solid-phase peptide synthesizer via Fmoc chemistry. Peptides were purified by high performance liquid chromatography and confirmed by mass spectrometry.

**Soluble protein preparation**

Soluble, single-chain 3.L2 and M15 single-chain TCRs, designed as V\(\beta\)-linker-V\(\alpha\) constructs, were engineered by error prone mutagenesis and conformational selection of stable mutants by yeast display as described\(^41\). The scTCR genes were cloned into pET28a, placing them in-frame with a 6x-His tag. Protein expression in *E. coli* was induced overnight with 1mM IPTG, thus generating large amounts of insoluble scTCR inclusion bodies (approximately 1.2-1.4g per 1L *E. coli* culture) which were harvested as described\(^23\). Inclusion bodies were then refolded under oxidative conditions and purified by nickel bead batch purification (Qiagen) followed by S200 fast protein liquid chromatography (FPLC) with size exclusion chromatography (SEC). Purified protein was concentrated using Amicon centrifugal filters and quantified by A280 using an extinction coefficient of 1.264. Monomeric pMHC complexes were generated by refolding I-E\(^k\) from *E. coli* inclusion bodies in the presence of Hb or APL (D73 and Q72) peptides as described\(^73\). I-E\(^k\) bound to moth cytochrome c (MCC) peptide residues 88-101 was also generated for use as a negative control in surface plasmon resonance experiments. Prior to purification, refolded I-E\(^k\) was biotinylated at a C-terminal BirA site by incubation with BirA.
ligase plus excess biotin at room temperature overnight. Subsequent purification by S200 FPLC-SEC effectively separated the biotinylated protein from free biotin.

**T cell hybridoma assay**

Stimulation of the 3.L2 and M15 T cell hybridomas was performed as described\(^42\). Briefly, \(10^5\) T hybridoma cells in a 96-well tissue culture plate were incubated with \(5 \times 10^4\) CH27 antigen presenting B cells, along with the Hb, Q72, or D73 peptides (0.001–100 \(\mu\)M) for 24 h. The level of T cell stimulation was determined by a bioassay for IL-2 involving \(^3\)H-TdR incorporation of the IL-2 dependent cell line, CTLL-2.

**Structural figures**

The molecular graphics program MacPyMol (DeLano Scientific LLC, San Carlos, CA) was used to make the structural figures, using the PDB deposited coordinates of Hb/I-Ek (1FNG) and D73/I-Ek (1FNE)\(^26\).

**Surface plasmon resonance**

scTCR–pMHC binding kinetics and thermodynamics were analyzed using a Biacore 2000 surface plasmon resonance instrument as described\(^1\). CM5 sensor chips (Biacore AB, Uppsala, Sweden) were activated with a 1:1 mixture of 100 mM N-hydroxysuccinimide and 75 mg/mL 1-ethyl- 3-[3-dimethylaminopropyl] carbodiimide hydrochloride (Biacore AB, Uppsala, Sweden). Amine coupling of activated chips was accomplished using 1mg/mL Neutravidin (Pierce, Rockford, IL) diluted 1:15 in 20 mM sodium citrate pH 4.5. Activated but non-Neutravidin-
bound moieties on the chip surface were blocked with 1M ethanolamine pH 8.5 (Biacore AB, Uppsala, Sweden). Biotinylated pMHC monomer was immobilized to the chip surfaces to a total response level of 250–500 resonance units (RU) as described\textsuperscript{29}. For all experiments, injection of sample entailed flowing 80 μL scTCR in HEPES-buffered saline (10 mM HEPES, 3 mM EDTA, 150 mM NaCl and 0.005% Tween 20) over the chip surface with the KINJECT command at a flow rate of 30 μL/min. The flow rate and ligand coupling levels have shown no indication of mass transport or rebinding artifacts. All sensorgrams were corrected for bulk flow effects and nonspecific binding by subtracting the response from a surface bound with MCC/I-E\textsuperscript{k} monomer, or from a blocked, Neutravidin-bound surface with no immobilized pMHC. All subtraction methods produced equivalent results.

**Binding kinetic analysis**

Concentration series of scTCR covering at least two orders of magnitude were injected in duplicate or triplicate at 25°C over surfaces coupled with monomeric peptide–I-E\textsuperscript{k}. Sensorgrams from the concentration series were fitted to a 1:1 Langmuir binding model using BiaEvaluation version 4.1 (Biacore AB, Uppsala, Sweden) to calculate the $k_{off}$, $k_{on}$, and $K_D$ ($=k_{off}/k_{on}$). $K_D$ and maximum response ($R_{Max}$) values for equilibrium binding analysis were obtained by plotting the equilibrium response ($R_{eq}$) at each concentration and fitting these data to a one-step binding model using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA). Scatchard plots were generated to confirm 1:1 binding stoichiometry by graphing $R_{eq}/[\text{scTCR}]$ versus $R_{eq}$. The Gibbs free energy change of binding was calculated from the $K_D$ using Equation 1.1 as follows:
\[ \Delta G = RT \ln(K_D) \] (1.1)

\[ \Delta G \] is the binding free energy change in kcal/mol; \( R \) is the universal gas constant, 1.987 cal/mol K; and \( T \) is temperature in Kelvin.

**van’t Hoff analysis of binding thermodynamics**

scTCR was injected at temperatures in the range of 10–30°C in 2°C increments with 2–4 replicates taken per temperature. Sensorgrams were individually fit to a 1:1 Langmuir model using BiaEvaluation version 4.1 (Biacore AB, Uppsala, Sweden) to obtain multiple \( K_A (=k_{on}/k_{off}) \) values at each temperature. The natural logarithm of each \( K_A \) was then plotted against inverse absolute temperature. Data were fitted to a nonlinear van’t Hoff model, as shown in Equation 1.2 below, and weighted by \( 1/SD^2 \) as described\(^{18,20} \) using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA):

\[
\ln K_A = -\frac{\Delta H(T_0)}{RT} - \left( \frac{\Delta C_P}{R} \right) \left( 1 - \frac{T_0}{T} \right) + \frac{\Delta S(T_0)}{R} - \left( \frac{\Delta C_P}{R} \right) \ln \left( \frac{T_0}{T} \right) \] (1.2)

\( \Delta H \) is the enthalpy change in kcal/mol; \( \Delta S \) is the entropy change in kcal/mol-K; \( \Delta C_P \) is the heat capacity change in kcal/mol-K; and \( T \) and \( T_0 \) are temperatures in Kelvins. All calculations were done using \( T = 298.15 \) K. The nonlinear van’t Hoff model was fitted to the data using Equation (1.3), and \( \Delta H \) and \( \Delta S \) were calculated from the fit using Equations 1.4 and 1.5, respectively\(^{74} \):

\[
\ln K_A = a + b \left( \frac{1}{T} \right) + c(\ln T) \] (1.3)

\[
\Delta H = R(cT - b) \] (1.4)

\[
\Delta S = \frac{\Delta H - \Delta G}{T} \] (1.5)
\( a, b \text{ and } c \) are constants to be determined. Equation 1.2 assumes a constant \( \Delta C_P \) that may be calculated using Equation 1.6:

\[
\Delta C_P = R c \quad (1.6)
\]
**Figure 1.1. Altered peptide ligands demonstrate that overall affinity and dissociation kinetics most strongly correlate with T cell activity, though exceptions exist.** (A) Molecular model of Hb\textsuperscript{d} peptide residues 64-76 within binding groove of I-E\textsuperscript{k}. Note that N72 (P5) is a TCR contact position, with the Asn side chain exposed to solvent, and E73 (P6) is a MHC contact position, with the Glu side chain contacting the floor of the groove. **(B and C)** Proliferative response of T hybridomas expressing 3.L2 (B) or M15 TCR (C) to WT Hb(64-76) peptide, or the altered peptide ligands Q72 and D73. Q72 and D73 differ from WT peptide by a single methylene group at a TCR or MHC contact position, respectively. **(D)** SPR binding kinetic analysis of purified 3.L2 and M15 single-chain TCRs with purified Hb- or APL-bound I-E\textsuperscript{k} molecules. Notably, the binding kinetics of 3.L2 TCR for Hb and D73 (highlighted in red) demonstrates one case where that ligand potency correlates more strongly with $k_{on}$ than $k_{off}$. 
3.1.2 TCR Binding Kinetics

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<td>(K_D) (nM)</td>
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<td>70.4</td>
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Figure 1.1. Altered peptide ligands demonstrate that overall affinity and dissociation kinetics most strongly correlate with T cell activity, though exceptions exist.
Figure 1.2. Subtle changes to pMHC can have dramatic effects on TCR binding thermodynamics. (A) van’t Hoff analysis of M15 TCR binding to purified Hb-, Q72-, and D73-bound I-E\(^k\) monomers, which derives binding thermodynamic parameters based on the temperature dependence of the binding affinity. (B) Binding thermodynamic parameters calculated from van’t Hoff analysis. \(\Delta G\) = Gibbs free energy change; \(\Delta H\) = enthalpy change; \(T\Delta S\) = energy contribution from entropy change; \(\Delta C_p\) = heat capacity change.
Figure 1.2. Subtle changes to pMHC can have dramatic effects on TCR binding thermodynamics.
Figure 1.3. Extrapolation of affinity for 3.L2 TCR binding the positively selecting altered peptide ligand Q72/I-E^k using high affinity TCR as a surrogate probe. (A and B) SPR sensorgrams used to derive binding affinities of 3.L2 and M15 TCR binding to pMHC. Note that due to the incredibly weak interaction of the 3.L2 TCR and Q72/I-E^k, no binding affinity could be calculated. (C) Extrapolation of the difference in affinity between M15 interaction with Hb/I-E^k and Q72/I-E^k to the 3.L2 TCR to estimate the unknown 3.L2-Q72/I-E^k affinity.
Affinity: 17.3 μM

3.L2-Q72/I-E<sup>k</sup>

Affinity: 4.94 nM

M15-Q72/I-E<sup>k</sup>

Affinity: undetectable

Affinity: 1220 nM

Estimate of unknown 3.L2-Q72/I-E<sup>k</sup> affinity

- Fold-difference in affinity of M15 binding Q72 vs Hb:
  - 1220 nM/4.94 nM = 247 → M15 binds Q72 247-fold weaker than to Hb

- Assuming 3.L2 also binds Q72 247-fold more weakly than to Hb:
  - 3.L2-Q72 affinity = 3.L2-Hb affinity x fold-difference in Q72 affinity
  - = 17.3 μM * 247 = 4.3 mM

Figure 1.3. Extrapolation of affinity for WT TCR binding positively selecting altered peptide ligand using high-affinity M15 TCR as a surrogate probe.
Chapter 2: Generation of a novel pathogen-responsive TCR transgenic system and molecular dissection of its antigen specificity

Some of the experiments presented here were performed by Scott Weber, a former postdoctoral fellow in our laboratory, and Jennifer Racz, a former technician in our laboratory. They are credited for their contributions as indicated in each figure legend. Much of the work regarding the in vivo responses of LLO56 and LLO118 T cells to Listeria monocytogenes has been published by Scott Weber in a 2012 manuscript in PNAS; some material from that manuscript is presented here with his permission. Also, experiments and text presented here discussing the differences in LLO56 and LLO118 T cell responses to extended LLO peptides are currently being used to generate a second manuscript.

2.1 Introduction

Indeed, much remains to be learned about how the TCR recognizes cognate- and self-pMHC ligands. Many systems designed to study TCR:pMHC interactions in CD4+ T cells have used TCRs that recognize non-pathogenic model antigens (i.e., hen egg lysozyme, ovalbumin). While these systems have provided invaluable insights into many aspects of T cell biology, it remains unclear how the biochemical features of the TCR:pMHC interaction give rise to TCR signals of different strength, and how this is manifest in the context of infection with a pathogen. Further, it is unclear how TCR interactions with self-pMHC, including those involved in positive selection itself, impact the functional behavior of mature CD4+ T cells. Herein, we describe the generation
of a pair of TCR transgenic mice that recognize a common, dominant epitope derived from Listeriolysin O. These TCRs were cloned from T hybridoma lines generated from LM-infected mice, and therefore represent two distinct solutions to recognizing the same cognate pMHC in a pathogenic context. With these reagents at hand, we can better understand what features of CD4⁺ T cells make for effective helper responses \textit{in vivo}, and how these owe to the quality of the TCR interaction with its ligands. These studies would also provide valuable insights to basic questions about TCR ligand specificity, particularly how distinct biology might be elicited from two T cells upon recognition of the same cognate pMHC.

2.2 Identification of LLO56 and LLO118, two Listeriolysin-O specific TCRs

Using a panel of LM-responsive T cell hybridomas generated in the laboratory of Emil Unanue, we sought to identify candidate TCR clones for the generation of TCR transgenic mice. These hybridomas were generated using CD4⁺ T cells isolated from LM-infected B6 mice, and so contain I-Aᵇ restricted TCRs that had expanded in response to the infection. Since we wanted to understand how different functionality might arise from recognition of a common ligand, we were particularly interested in clones that showed different responses to stimulation with LLO(190-201) peptide, which seemed a logical screening tool given that this LLO epitope is the one most frequently discussed in the literature. From a practical standpoint, clones that could be identified by available anti-TCR antibodies were also desirable, as this would obviate the generation of clonotypic reagents to specifically detect cells expressing the TCRs. Based on these criteria, we identified two suitable clones called LLO56 and LLO118.
The LLO56 and LLO118 TCRs both utilize Vα2 and Vβ2 gene segments, which are easily detectable by commercially available monoclonal antibodies. In terms of primary sequence, LLO56 and LLO118 differ by a total of 15 amino acids; with 13 of these differences residing within or near the CDR3 region of the TCRβ chain (Figure 2.1). As structural studies have shown that the CDR3 regions of the TCR make considerable contacts with the peptide\textsuperscript{76}, we expected that two clones differing so much in this region should differ in their recognition of LLO peptide. Indeed, when stimulated with LLO(190-201) peptide, LLO118 responds strongly whereas LLO56 responds very poorly (Figure 2.2). Thus, despite their high degree of structural similarity, the LLO56 and LLO118 TCRs showed markedly different responses, certainly suitable for further study. Based on these results, we proceeded to generate TCR transgenic mice using the LLO56 and LLO118 TCRs to provide a source of primary T cells in order to carry out greater \textit{in vitro} characterization and \textit{in vivo} experiments.

### 2.3 Generation of LLO56 and LLO118 TCR transgenic mice

The LLO56 and LLO118 TCRα and TCRβ variable regions (assembled VJ or VDJ sequences, respectively) were cloned into shuttle vectors by Qi-Jing Li, then a postdoctoral fellow in the laboratory of Mark Davis, which would place them in frame with TCR constant regions after transcription and mRNA splicing. These vectors were then co-injected into B6 oocytes and implanted to a receptive mother, yielding a cohort of potential founder mice. From these animals, LLO56 and LLO118 founders were identified by FACS analysis of peripheral blood for Vα2\textsuperscript{+}Vβ2\textsuperscript{+} CD4\textsuperscript{+} T cells. Both LLO56 and LLO118 founders showed excellent allelic exclusion, with greater than 90% CD4\textsuperscript{+} T cells expressing the transgenic TCR in the periphery (Figure 2.3).
Importantly, the frequency of TCR transgenic CD4+ T cells in whole spleen was similar in LLO56 and LLO118 mice. This allowed us to directly compare responses in T cell assays done by simply adding LLO peptide to whole LLO splenocytes. Were the frequency of TCR transgenic CD4+ not equal, responses of different magnitudes could be due to differences in total responding cells. *In vitro* analysis of the LLO56 and LLO118 TCR transgenic cells’ response to LLO(190-201) peptide confirmed our findings with the LLO56 and LLO118 hybridoma lines, with LLO118 showing stronger responses than LLO56 by proliferation assay, IL-2 ELISA, and activation marker upregulation (Figure 2.4).

Since these animals were designed to be a source of pure primary TCR transgenic cells with no contaminating CD4+ T cells of other specificities, complete allelic exclusion was desirable. Thus, the LLO56 and LLO118 TCR transgenic mice were crossed with mice deficient in recombinase activating gene 1 (RAG1), an essential component of the enzyme complex that catalyzes the somatic rearrangement of T- and B-cell antigen receptors. As expected, all CD4+ T cells from LLO56 and LLO118 peripheral blood and lymphoid organs mice lacking RAG1 expressed the transgenic TCR (Figure 2.3).

Finally, it was imperative for adoptive transfer and LM infection experiments that the LLO56 and LLO118 T cells could be distinguished from the cells of B6 recipient mice. For this reason, RAG1-deficient LLO56 and LLO118 cells were crossed with animals expressing unique congenic variants of the proteins CD45 (Ly5) and CD90 (Thy1). Both proteins are highly expressed cell-surface molecules that exist in distinct allelic forms that can be distinguished by
available monoclonal antibodies. B6 mice are homozygous for the CD45.2 and CD90.2 allelic variants of CD45 and CD90, respectively; as such, they do not express the CD45.1 and CD90.1 alleles. Thus, we engineered the LLO56 line to express CD90.1 (Thy1.1) and the LLO118 line to express CD45.1 (Ly5.1). In so doing, both transgenic T cells could be transferred at once to B6 recipients and unequivocally identified amidst a background of recipient-derived cells.

2.4 LLO56 has markedly higher expression of CD5 than LLO118

An important early priority upon generation of these mice was a thorough cell surface phenotyping of the LLO56 and LLO118 T cells, as this could provide important clues to findings made in our in vitro and in vivo studies (Figure 2.5). Both LLO56 and LLO118 CD4+ T cells had the expected phenotype for a naive cell: CD62L^hi CD44^lo TCR^hi CD3^hi CD25^lo. LLO118 had slightly but reproducibly higher expression of TCR compared to LLO56. CD5 expression showed the greatest difference, with markedly higher levels in LLO56 cells compared to LLO118. As CD5 correlates with TCR self-reactivity, we considered that LLO56 and LLO118 might make ideal models with which to study the impact of TCR:self-pMHC avidity on T cell responses in vitro and in vivo. Alternatively, as CD5 has been reported to negatively impact T cell responses, further studies with the LLO56 and LLO118 TCR transgenic mice might reveal phenotypes that are influenced by CD5 itself.
2.5 LLO56 and LLO118 primary T cell responses to live LM in vivo

With the RAG-deficient, congenically-marked LLO56 and LLO118 lines prepared, we could proceed with adoptive transfer and infection studies in B6 mice. During design of this experimental system, we considered that naive T cells are normally found at low frequencies. Using highly-sensitive tetramer based enrichment, it was estimated that a single mouse contains 20-200 cells of a given specificity for CD4$^+$ T cells, and 80-1200 cells for CD8$^+$ T cells.$^{77,78}$ T cell clones tended to give stronger responses when kept at such low frequencies compared to the higher frequencies ($>10^5$ cells) often used in adoptive transfer models.$^{79}$ In line with these observations, we decided to transfer 3x10$^3$ LLO T cells in our infection model; assuming 10% of these cells persist in recipients following transfer, there would be approximately 300 cells remaining, which is within range of a physiologic naive precursor frequency. Recipient mice would then be infected with LM the following day, and analyzed for expansion of the LLO T cells 7 days post-infection, at the peak of the T cell response.$^{75}$ Assessment of memory responses was also of interest, which we tested with a 4-day rechallenge with a 100-fold greater inoculum at day 35. This late time point is well into memory phase of the response, following weeks after the rapid T cell expansion and contraction that typifies the first and second weeks of the response, respectively.

As we might have predicted given the responses to LLO(190-201) peptide, LLO118 showed markedly greater expansion than LLO56 (Figure 2.6). Unexpectedly, however, LLO56 T cells prevailed when mice were rechallenged. Upon confirming this result, we first determined the
extent to which the primary response of our two T cells was driven by cell proliferation versus cell death. Surprisingly, CFSE-labeled LLO56 and LLO118 T cells showed similar in vivo proliferation by day 4 post-infection (Figure 2.7). Furthermore, LLO56 cells showed a markedly higher frequency of apoptotic cells than LLO118 following the response. Thus, the relative in vivo expansions of LLO56 and LLO118 T cells tracked with differences in cell death rather than proliferative differences, an unexpected finding given our initial observation that LLO56 proliferated poorly in response to LLO(190-201) peptide.

2.6 Processing of LLO protein generates ligands that more potently stimulate LLO56 T cells than LLO(190-201) peptide

Using LM-infected B6 splenocytes as APCs in an in vitro proliferation assay, we confirmed that LLO56 and LLO118 do proliferate similarly well in response to live LM (Figure 2.8). One possible explanation for the difference between stimulation with peptide versus live LM is that stronger epitopes are generated when LLO protein is processed by an APC, ones that are equally stimulatory for both LLO T cells. To test this possibility, we synthesized LLO peptides with additional LLO residues through residue 205 added to the C-terminus and tested them in in vitro stimulation assays. While LLO118 proliferative responses were mildly improved when LLO residues 202 and 203 were present, LLO56 showed marked gains in the potency of its response. The presence of these additional residues equalized the LLO56 and LLO118 T cell proliferative responses, which is in line with our findings with live LM. Notably, addition of amino acids past residue 203 did not improve the responses of either LLO56 or LLO118. Therefore, C-terminal
LLO residues 202 and 203 are critical for maximal proliferation of the LLO56 and LLO118 T cells.

A key feature of *in vivo* infection with live LM is that LLO56 cells accumulate less than LLO118, despite seemingly identical proliferative capacity. As longer LLO peptides induced similarly strong *in vitro* proliferative responses as did live LM, we wondered whether these peptides nevertheless induced poor expansion of LLO56 T cells *in vivo*, as we had seen with live LM. We therefore transferred LLO56 and LLO118 T cells to B6 mice, then immunized them the following day with LLO(190-201) or LLO(190-203) peptide emulsified in complete Freund’s adjuvant (CFA) (Figure 2.9). As expected, immunization with LLO(190-201)/CFA elicited very weak expansion, which was improved by 11-fold by the presence of LLO residues 202 and 203. Still, LLO118 showed greater expansion to either peptide than did LLO56 by at least 7-fold. These results solidify our finding that different proliferative capacity does not explain the disparate *in vivo* responses of the LLO T cells, further suggesting that differences in propensity to undergo cell death are responsible.

### 2.7 LLO56 and LLO118 cytokine responses diverge upon stimulation with C-terminal extended peptides

That longer LLO peptides could elicit such strong responses from LLO56 T cells led us to test them as stimuli in our activation marker and cytokine assays. Consistent with the results of our proliferation assays, LLO56 and LLO118 showed similar upregulation of CD25 and CD69 when stimulated with LLO(190-203) peptide (Figure 2.10). Unexpectedly, LLO(190-203) peptide
elicited significantly stronger IL-2 responses from LLO56 and weaker IL-2 responses from LLO118, as measured by ELISA. To confirm this, we analyzed the production of IL-2 as well as a number of other cytokines using a cytokine bead array (CBA) assay (Figure 2.11). Indeed, higher amounts of not only IL-2, but IL-17A, IL-6, and TNFα could be elicited from LLO56 than LLO118 upon stimulation with LLO(190-203) peptide. Intriguingly, nonspecific stimulation using αCD3+αCD28, which bypasses the TCR, could also elicit stronger cytokine responses from LLO56.

The difference in IL-2 production was not apparently due to differences in activation of IL-2 secretion kinetics, as both LLO56 and LLO118 activated and had detectable IL-2 production after 4 and 6 hours of peptide stimulation (Figure 2.12). Since only the magnitude of secretion over time seemed to differ between LLO56 and LLO118, we considered that this might be due to different frequencies of cells being induced to secrete cytokine by our two T cells. Indeed, using a sensitive, single-cell cytokine capture assay, we detected a higher frequency of LLO56 T cells were secreting IL-2 compared to LLO118 in response to stimulation with LLO(190-203) and even αCD3+αCD28 stimulation (Figure 2.13). Thus, despite the fact that LLO56 cells were poor responders in vivo, they showed superior ability to produce cytokines compared to LLO118.

2.8 Development of a cellular peptide-MHC binding assay

Given the critical importance of LLO residues 202 and 203 for the activity of LLO56 T cells, we set out to determine the biochemical basis for the difference. Presumably, that fact that LLO118 did not share LLO56’s sensitivity to the presence of these residues suggests that the difference is
TCR-driven. However, several other possibilities exist, including altered interaction with Class II MHC, which needed to be considered. This motivated our efforts to conduct a thorough molecular characterization of the LLO epitope, using peptide mutagenesis to determine how LLO(190-203) interacts with the LLO56 and LLO118 TCRs and with I-A\(^b\).

While the effect of peptide mutagenesis on T cell activity can be easily measured by standard T cell assays, any observed effects could be confounded by effects on peptide binding and presentation by I-A\(^b\). We therefore began by setting up a cellular, competition-based peptide-MHC binding assay, based on assays reported in the literature\(^{80,81}\) (Figure 2.14). In this assay, a fixed concentration of a known I-A\(^b\) binding peptide (the “indicator” peptide) is mixed with a concentration series of a peptide whose binding we want to measure (mutant LLO peptides, in this case). These mixes of peptides are incubated with splenocytes from mice deficient in the Class II peptide editor H2-M (also called H2-DM). The absence of H2-M does not affect I-A\(^b\) expression, but causes the repertoire of I-A\(^b\) bound self-peptides to be limited predominantly to a single species derived from invariant chain, CLIP. Thus, H2-M\(^{-}\) splenocytes provide a homogenous source of I-A\(^b\) onto which peptide loading can be measured, without interference from peptide editing.

The indicator peptide used in these studies was E\(\alpha\)(52-68), which is derived from the alpha chain of the Class II MHC molecule I-E\(^b\). In strains expressing I-E\(^b\), this peptide is abundantly presented on APCs; B6 mice, however, do not express this protein or E\(\alpha\)(52-68)-loaded I-A\(^b\), due to a mutation in the E\(\alpha\) promoter. Loading of this peptide can be readily identified using the
YAe antibody, which specifically recognizes Eα(52-68)-I-A^b complexes but does not bind the free peptide or I-A^b alone^82,83. The presence of LLO peptides would then compete with Eα(52-68), reducing the binding signal from YAe in a concentration dependent manner. In this way, binding curves can be generated and IC50 values (the concentration at which the YAe signal is reduced by half) values derived for each LLO peptide, with lower IC50 values indicating more strongly binding peptides (i.e., less peptide is required to effectively compete with Eα(52-68), indicating stronger binding to MHC).

2.9 Presence of C-terminal LLO peptide residues does not improve peptide activity via stronger binding to I-A^b

We first used this peptide-MHC binding assay to ask whether the presence of LLO residues 202 or 203 affected the peptide binding to I-A^b. While the IC50 increased by about 2-fold with residue 202 present, this increase was mostly lost by the addition of residue 203 (Figure 2.15). Importantly, no binding was detected from Hb(64-76) peptide, which binds I-E^k but not I-A^b. Thus, the stimulatory activity of LLO peptides of different length does not track with their relative ability to bind I-A^b.

2.10 LLO Residues 202 and 203 flank the I-A^b peptide binding groove

A unique feature of MHC Class II molecules is an open-ended peptide binding groove^84. The closed binding pocket of Class I MHC makes defined contacts with the N- and C-termini of the peptide, and restricts peptide length to 8 or 9 residues (though there are known exceptions of
longer peptides that bind to Class I in a “bulged” configuration\textsuperscript{85, 86}. Class II binding peptides are longer, with 9 residues occupying the binding pocket in extended conformation (numbered P1 through P9 from N- to C-terminus) and the rest extending out of the pocket. In this configuration, residues occupying the P2, P3, P5, and P8 positions have solvent-accessible side chains which can contact the TCR, while residues in the P1, P4, P6 and P9 positions have side chains able to contact the MHC binding groove. Some peptides have side chains which act as dominant MHC anchors, clearly defining the peptide register, which is the group of 9 residues within the binding groove. Many peptides, however, may interact with Class II MHC via interactions with the peptide backbone atoms, relaxing the stringency on specific side chains to act as MHC anchors. Such peptides can in theory exhibit MHC binding in multiple registers, with each register potentially providing a unique surface for TCR recognition. This raises the possibility that the LLO residues 202 and 203 make possible alternate binding registers that are differentially recognized by LLO56 and LLO118.

Based on the properties of the I-A\textsuperscript{b} binding pocket and the MHC contact residues of a panel of known I-A\textsuperscript{b} binding peptides\textsuperscript{87}, we noted that the LLO peptide contains a set of canonical MHC contact residues which predicts a dominant 9-mer register consisting of residues 193-201, which is present in the original LLO(190-201) epitope (Figure 2.16). In this register, residues 202 and 203 occupy peptide flanking positions outside the peptide binding groove. To determine whether this register might be correct, we began by carrying out N-terminal truncation mutagenesis on the LLO(190-205) peptide, reasoning that this approach would narrow down the list of possible binding registers.
While loss of residues 190 and 191 had little effect, removal of amino acids through residue 192 led to a complete loss of LLO56 and LLO118 T cell responses (Figure 2.17). The loss of activity seen in the LLO(193-205) peptide appeared to be due to a marked loss in peptide binding to I-A\textsuperscript{b}. Since the LLO(193-205) peptide was completely unstimulatory, any possible binding register contained within it is similarly unstimulatory. This includes any binding registers made possible by the addition of residues 202 through 205. Based on these data, we conclude that the addition of C-terminal LLO residues does not induce greater responses from LLO56 via presentation of alternative binding registers.

Inasmuch as truncation mutagenesis effectively ruled out alternative binding registers made possible by C-terminal LLO residues, it was essential to specifically test whether LLO residues 193-201 comprise the register recognized by the LLO56 and LLO118 TCRs. We therefore conducted scanning mutagenesis of the LLO peptide, by making lysine substitutions (or glutamic acid, if the WT residue was already a lysine) at each position in the LLO peptide, then measuring the effect of these mutations on LLO T cell responses and binding to I-A\textsuperscript{b}. This analysis would thus determine what positions are TCR and MHC contacts, and whether these are consistent with recognition of the same or different binding registers by the LLO56 and LLO118 TCRs (Figure 2.18).

Mutation of predicted TCR contact residues P2, P3 and P5 (which correspond to LLO peptide residues 194, 195, 197) led to a complete loss of T cell stimulatory potency without affecting
binding to I-A^b; P7 (residue 199) behaved in a similar manner when mutated. Interestingly, mutation of the predicted TCR contact at the P8 position (LLO peptide residue 200) caused a marked reduction of the LLO56, but not LLO118 response, with a modest reduction I-A^b binding. Mutation of the predicted MHC contact positions P1, P4 and P9 (LLO peptide residues 193, 196, and 201) led to similarly modest reductions in the LLO56 and LLO118 T cell responses, associated with mild reductions in I-A^b binding. Mutation of the predicted P6 position (LLO residue 198) caused a complete loss of LLO56 and LLO118 responses linked to a complete loss of peptide binding to I-A^b. Therefore, the experimentally determined TCR and MHC contacts mapped essentially as expected to the predicted LLO peptide binding register, with LLO56 and LLO118 making an overall similar constellation of contacts. Importantly, the proline in the P6 position was the only MHC anchor in the binding groove crucial for MHC binding. Taken together, these data establishes LLO peptide residues 202 and 203 as peptide flanking residues, the presence of which are differentially recognized by LLO56 and LLO118.

### 2.11 LLO56 shows exquisite sensitivity to acidic LLO peptide C-termini

That LLO56 and LLO118 differed so markedly in their dependence on peptide residues outside of the peptide binding groove led us to probe the interaction between the TCRs and the residues 202 and 203. We therefore performed more extensive peptide mutagenesis studies at these positions, hoping to identify particular residue changes differently tolerated by the two TCRs. Interestingly, LLO56 responded poorly to peptides with acidic residues occupying positions 202 and 203, while LLO118 was relatively unaffected (Figure 2.19). This effect on LLO56 T cells
was exacerbated if both residues 202 and 203 were acidic. We wondered whether the negative charge of the residues might contribute to this, so we analyzed the LLO56 and LLO118 response to peptides mutated to Asn or Gln at positions 202 and 203, which have essentially the same structure as Asp or Glu, respectively, but lack negative charge. Indeed, removal of the charge improved the LLO56 T cell response.

Based on these data, it was clear that LLO56 interacted unfavorably with peptides whose C-termini are negatively charged. We next considered that since the C-terminus of all peptides is itself a negatively charged group, this could explain why LLO(190-201) stimulates the LLO56 T cell so poorly. To test this, we generated LLO(190-201) peptides whose C-terminal charge was nullified by amide capping (Figure 2.20). While LLO118 was unresponsive to this change, the response of LLO56 to amide-capped LLO(190-201) was as strong as it was to wild-type LLO(190-202). When we next tested amide-capped LLO(190-202), we made similar findings; LLO56 responded nearly as well to the capped LLO(190-202) as it did to wild-type LLO(190-203), while the LLO118 response was unchanged. No change in activity was noted for either T cell in response to amide-capped LLO(190-203) peptide. These experiments suggested that for LLO56, neutralizing the charge of the C-terminus of LLO(190-201) and LLO(190-202) was as effective at increasing the response as adding an entire amino acid.

### 2.12 Conclusions

We generated two novel, LLO-responsive CD4⁺ TCR transgenic mouse lines, LLO56 and LLO118, to better understand what features of ligand recognition by the TCR give rise to
effective helper responses. The two TCRs are highly structurally similar, differing primarily in their CDR3β regions. These mice were bred onto RAG1<sup>-/-</sup> backgrounds with unique congenic markers to facilitate their use in adoptive transfer and LM infection studies. The LLO118 T cell shows markedly greater expansion during primary <i>in vivo</i> responses to LM than LLO56, a property associated with a greater degree of cell death among LLO56 T cells.

Both <i>in vitro</i> and <i>in vivo</i>, LLO56 and LLO118 show equivalent proliferative responses to live LM, a finding which was initially at odds with the observation that LLO118 proliferated better in response to LLO(190-201) peptide. The discrepancy between the proliferative responses to LM versus peptide was likely due to more potent LLO epitopes being generated when LLO protein was processed by an APC. Consistent with this idea, the addition of LLO residues 202 and 203 to the LLO(190-201) peptide elicited identical proliferative responses from LLO56 and LLO118, mirroring the findings with live LM. Strikingly, the presence of LLO residues 202 and 203 could induce markedly stronger cytokine responses from LLO56, which was the weaker responder during primary LM infection <i>in vivo</i>. For IL-2, this was due to a higher frequency of LLO56 T cells being induced to secrete cytokine compared to LLO118, and not due to a difference in secretion kinetics.

It was predicted based on previous studies that residues (193-201) comprise a canonical 9-mer for binding to I-A<sub>b</sub>. Scanning and truncation mutagenesis studies determined that the LLO(190-205) peptide contains a single dominant binding register stabilized by anchors at the P-1 (residue 192) and P6 (residue 198) positions; loss or mutagenesis at these positions markedly reduced
peptide binding to I-A<sup>b</sup>. In this register, LLO56 and LLO118 utilize an overall similar set of TCR contacts, with mutagenesis of the P2, P3, P5 or P7 positions completely abrogating both LLO56 and LLO118 responses. In such a model, residues 202 and 203 occupy flanking positions which lie outside of the peptide binding pocket. Mutagenesis at these positions revealed that LLO56 is not only sensitive to the presence of these residues, but also to their charge, showing weaker T cell responses when one or both of these residues are acidic. Neutralization of the charge of the carboxy terminus of the LLO(190-201) and LLO(190-202) peptides by amide-capping was as effective at increasing the LLO56 T cell response as adding the next C-terminal amino acid.

In considering potential models for how LLO56 and LLO118 might differ in their ability to sense the presence of negative charge at the LLO peptide C-terminus, we focused on the CDR3β region, the primary locus of difference between the two T cells. Notably, LLO56 has an aspartic acid in CDR3 that LLO118 lacks, which is the only acidic residue difference between the two TCRs. This is particularly interesting given that the CDR3β footprint lies over the C-terminal aspect of the peptide in available TCR:pMHC co-crystal structures. Our data suggest a model whereby charge repulsion between the LLO(190-201) peptide C-terminus and LLO56 CDR3β Asp contributes to the poor activation we see. As amide capping the peptides were functionally equivalent to adding an entire amino acid, we propose that one role for the C-terminal amino acids is to separate the charge of the C-terminus from LLO56 CDR3β Asp. One notion supporting this is that residue 205 is an Asp, but LLO56 and LLO118 respond similarly well with or without it, suggesting that LLO56’s sensitivity to negative charge may show distance dependence. It is possible that LLO56 simply responded to the presence of the amide nitrogen
rather than the loss of charge. Even so, it is remarkable that this TCR was so responsive to such a
minute difference at a position outside the MHC binding pocket.

Taken together, we conclude that the TCR is at the heart of the disparate *in vitro* and *in vivo*
responses of the LLO56 and LLO118 T cells to LM and LLO peptides are largely TCR driven.
This distinct biology owes to differential recognition of a similar constellation of TCR contacts,
as presented by a single, dominant binding register consisting of LLO residues 193-201 in the
context of I-A\textsuperscript{b}.

### 2.13 Methods

**Mice**

LLO56 and LLO118 TCR transgenic mice were generated as described in the main text with
unique congenic markers both on RAG1-sufficient and deficient backgrounds. These mice were
maintained as heterozygous for the TCR transgenes. B6 mice and MHC Class II\textsuperscript{−/−} mice were
obtained from Jackson Laboratories. H2-M\textsuperscript{−/−} β2m\textsuperscript{−/−} mice were provided by Jenny Ting’s
laboratory; these mice were backcrossed to B6 to restore the WT β2m alleles, thus generating the
H2-M\textsuperscript{+/+} mice used in this study. Breeding, housing and care of all mice was done in specific
pathogen-free facilities under a protocol approved by the Washington University Animal Studies
Committee.
Peptides

The peptides used in this study and their sequences were all derived from the dominant LLO epitope comprising residues 190-201. Variants of this peptide, including C-terminally extended or N-terminally truncated, or peptides mutated at specific positions, were utilized in these studies as described in the main text and figures. All peptides were synthesized in-house using a Symphony automated solid-phase peptide synthesizer via Fmoc chemistry. Peptides were purified by high performance liquid chromatography and confirmed by mass spectrometry.

T cell hybridoma assay

Stimulation of the LLO56 and LLO118 T cell hybridomas was performed essentially as described42. Briefly, T hybridoma cells in a 96-well tissue culture plate were incubated with irradiated B6 splenocytes, along with the LLO(190-201) peptide (top concentration of 100 μM, with half-log dilutions down to 10^{-5} μM) for 24 h. The level of T cell stimulation was determined by a bioassay for IL-2 involving ^3H-thymidine incorporation of the IL-2 dependent cell line, CTLL-2.

Primary T cell assays

Proliferation assays were conducted by plating LLO56 or LLO118 CD4^+ T cells magnetically purified from pooled spleen and lymph node with APCs presenting LLO-derived antigen. For peptide stimulation experiments, the APCs were irradiated B6 splenocytes treated with varying concentrations of peptide; in live LM stimulation experiments, the APCs were B6 splenocytes infected 6 hours with varying titers of *Listeria monocytogenes* strain 10403S, then cultured in the
presence of gentamicin to eliminate any extracellular LM. In either case, T cells were cocultured with APCs in 96-well tissue culture plates for 72 hours at 37\(^\circ\)C/5\% CO\(_2\) in complete D10 medium (DMEM + 10\% FCS and 2 mM GlutaMAX II), with a \(^3\)H-thymidine pulse added during the final 24 hours. Plates were harvested onto filter mats, which were dried overnight then immersed in scintillation fluid and read on a Micro-beta scintillation counter.

IL-2 ELISA, cytokine bead array, activation marker upregulation, and cytokine capture assays were conducted using LLO56 or LLO118 splenocytes maintained on a non-RAG background. LLO56 and LLO118 splenocytes from these mice had identical frequencies of V\(\alpha_2^+\) CD4\(^+\) cells, and had ample B cells to act as APCs (Figure 2.3). Thus, with these mice, assays could be initiated simply by adding peptide. Cytokine capture assays were done with LLO56 and LLO118 splenocytes stimulated for 4 hours, then stained as per the manufacturer (Miltenyi Biotec) protocol. For all other assays, 18 hour stimulation timepoints were routinely taken. Cytokine bead array assays were done as per the manufacturer (BD Biosciences) protocol, except one-fifth the recommended amount of anti-cytokine beads was used for detection. For all assays, either peptides or \(\alpha\)CD3 (clone 145-2C11, BioLegend) plus \(\alpha\)CD28 (clone 37.51, BioLegend) were used as stimuli.

**In vivo Listeria infection and adoptive T cell transfer model**

LM infection and T cell adoptive transfer were done as described\(^{75}\). LLO56 or LLO118 TCR transgenic cells were purified by negative selection using magnetic isolation (MACS), and 3x10\(^3\) T cells of each were transferred via retroorbital plexus to B6 mice. The following day, frozen
glycerol stocks of *Listeria monocytogenes* strain 10403S were thawed at room temperature and serially diluted to $10^4$ CFU/mL in PBS. 100μL of this solution was injected via the retro-orbital plexus, giving an inoculum of $10^3$ CFU/mouse. Approximate injection titers were determined by plating injection solution onto brain-heart infusion agar, incubating overnight at 37°C, and colony counting. 7 days later, spleens and lymph nodes were harvested, and transferred cells were enriched via MACS positive selection via their unique congenic markers. The expansion of the LLO56 and LLO118 T cells during the infection was determined by counting the enriched congenic marker$^+$ CD4$^+$ Vα2 TCR$^+$ cells by FACS. For rechallenge experiments, mice received LLO56 and LLO118 T cells, were given a primary LM infection as per the above protocol, then re-infected with $10^5$ CFU LM at d35 post primary infection. Expansion of the LLO T cells was enumerated at day 4 post rechallenge (Day 35+4).

**CFSE labeling**

LLO56 and LLO118 T cells were stained with carboxyfluorescein diacetate-succinimidyl ester (Molecular Probes) at a final concentration of 5 μM in PBS + 0.1% BSA. Cells were then used for experiments after three washes in PBS + 0.1% BSA.

**Peptide-MHC binding assay**

The assay for detecting peptide binding to MHC was adapted from previously published protocols$^{80,81}$. It is described in detail in the main text and depicted in Figure 2.12. Biotinylated YAe antibody, recognizing Ea(52-68)/I-A$^b$, was obtained from eBioscience. Phycoerythrin-conjugated streptavidin was obtained from Molecular Probes.
Peptide Immunizations

LLO(190-201) and LLO(190-203) peptides in 1X PBS were emulsified with Complete Freund’s adjuvant by loading peptide and adjuvant solution in separate glass syringes, connecting the syringes with a luer fitting, and mixing by injecting the solution back and forth between the syringes for 10 minutes. 50 μL of this emulsion, containing 25 nmol peptide, was injected to each rear footpad of B6 mice, giving 50 nmol total peptide per mouse. T cell responses were assessed 7 days after immunization.

Statistics

Statistical comparisons were made using Student’s t tests for all experiments. p < 0.05 was set as the criterion for significance.
Figure 2.1. Gene segment usage and amino acid sequence data for the LLO56 and LLO118 TCRs. (A) V region usage of LLO56 and LLO118 TCRs. (B) Primary sequence differences in the CDR3β regions of the LLO56 and LLO118 TCRs. Note that of the 15 amino acid differences between LLO56 and LLO118, 13 of these differences lie in this region (highlighted in pink).
(A) LLO56 vs LLO118 V region usage

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(B) LLO56 vs LLO118 TCRβ C-terminus

Figure 2.1. Gene segment usage and amino acid sequence data for the LLO56 and LLO118 TCRs.
Figure 2.2. LLO56 and LLO118 hybridoma responses to LLO(190-201) peptide. LLO56 and LLO118 hybridoma cells were cocultured with irradiated B6 splenocytes plus a concentration series of LLO(190-201) peptide for 24 hours. T cell stimulation was assessed by measuring $^{3}$H-Thymidine incorporation of CTLL cells incubated with supernatant from the T hybridoma cultures. These experiments were done by Jennifer Racz.
Figure 2.2. LLO56 and LLO118 hybridoma responses to LLO(190-201) peptide.
Figure 2.3. Primary LLO56 and LLO118 T cells from TCR transgenic mice show excellent allelic exclusion on both RAG1-sufficient and RAG1-deficient backgrounds. (A) Staining for CD4+ Vα2 TCR+ transgenic cells in LLO56 and LLO118 splenocytes on RAG1-sufficient (top panels) or RAG1-deficient (bottom panels). Plots are gated on CD4+ T cells. (B) Frequencies of CD4+ Vα2 TCR+ transgenic cells in among total viable splenocytes. Please note that the Vα2 TCR fluorescence intensity in (A) and (B) cannot be directly compared, as they were collected on different days with different instrument settings.
Figure 2.3. Primary LLO56 and LLO118 T cells from TCR transgenic mice show excellent allelic exclusion on both RAG1-sufficient and RAG1-deficient backgrounds.
Figure 2.4. LLO56 and LLO118 TCR transgenic T cell responses to LLO(190-201) peptide.

(A) Proliferation assay of LLO(190-201) treated LLO56 and LLO118 splenocytes. $^3$H-thymidine was added to each well at $t = 48$ hours, then cultures were harvested 24 hours later. (B) IL-2 ELISA analysis of LLO56 and LLO118 splenocytes treated with LLO(190-201) peptide, measured after 18 hours of stimulation. (C) Upregulation of CD69 and CD25 by LLO56 and LLO118 T cells, measured after 18 hours stimulation with LLO(190-201) peptide.
(A) Proliferation response to LLO(190-201) peptide

(B) IL-2 response to LLO(190-201) peptide

(C) Activation marker upregulation response to LLO(190-201) peptide

Figure 2.4. LLO56 and LLO118 TCR transgenic T cell responses to LLO(190-201) peptide.
Figure 2.5. LLO56 and LLO118 show expected naive T cell phenotype, but differ markedly in their expression of CD5. Expression analysis of various common T cell markers on LLO56 and LLO118 peripheral CD4\(^+\) T cells. Solid lines indicate LLO118 T cells, dotted lines indicate LLO56 T cells. These experiments were conducted by Scott Weber and are reproduced here from his manuscript (Weber et al (2012) PNAS).
Figure 2.5. LLO56 and LLO118 show expected naive T cell phenotype, but differ markedly in their expression of CD5.
Figure 2.6. LLO118 shows significantly greater *in vivo* expansion in response to *Listeria monocytogenes* than LLO56, but LLO56 prevails during rechallenge. (A) Primary response of LLO56 and LLO118 T cells to LM. 3x10^3 LLO56 and LLO118 T cells were transferred retro-orbitally to B6 mice, then infected with 10^3 CFU LM strain 10403S the following day. Seven days post-infection, spleens and lymph nodes were harvested and the total numbers of LLO56 and LLO118 T cells were determined by FACS. (B) Secondary response of LLO56 and LLO118 T cells to LM. B6 mice received 3x10^3 LLO56 and LLO118 T cells, were infected with 10^3 CFU LM the following day, then rechallenged with 10^5 CFU LM 35 days later. Four days following rechallenge (Day 35+4), spleens and lymph nodes were harvested and LLO56 and LLO118 cell counts were enumerated by FACS. These experiments were done by Scott Weber, as presented in his manuscript (Weber et al. (2012) *PNAS*).
Figure 2.6. LLO118 shows significantly greater *in vivo* expansion in response to *Listeria monocytogenes* than LLO56, but LLO56 prevails during rechallenge.
Figure 2.7. LLO56 and LLO118 show similar *in vivo* proliferation, but LLO56 shows a significantly greater degree of cell death. *(A)* Proliferation of adoptively transferred, CFSE-labeled LLO56 and LLO118 T cells at day four post-LM infection. Please note that a considerably larger T cell transfer was done in order to get enough cells to analyze at this early timepoint, which is before the explosive proliferation that normally occurs between day 4 and day 7. *(B)* Annexin V staining for apoptotic cells on LLO56 and LLO118 cells day 7 post-LM infection. These experiments were conducted by Scott Weber, and are reproduced here from his manuscript (Weber et al (2012) *PNAS*).
Figure 2.7. LLO56 and LLO118 show similar in vivo proliferation, but LLO56 shows a significantly greater degree of cell death.
Figure 2.8. LLO56 and LLO118 proliferate identically in response to LM-infected splenocytes, similarly as stimulation with C-terminally extended LLO peptides. (A) LM-infected B6 splenocytes were incubated 48 hours with LLO56 or LLO118 T cells, then pulsed with $^3$H-thymidine for another 24 hours to assess cell proliferation. (B) LLO56 and LLO118 proliferation response to LLO peptides with additional C-terminal residues through residue 205 after 72 hours stimulation, with a $^3$H-thymidine pulse at 48 hours. These experiments were conducted by Scott Weber; panel (B) was reproduced from his manuscript (Weber et al (2012) PNAS).
(A) LLO56 and LLO118 response to LM-infected B6 splenocytes

(B) LLO56 and LLO118 response to C-terminally extended peptides

Figure 2.8. LLO56 and LLO118 proliferate identically in response to LM-infected splenocytes, similarly as stimulation with C-terminally extended LLO peptides.
Figure 2.9. LLO(190-203) peptide induces stronger in vivo expansion from LLO118 than LLO56 despite equivalent proliferation, mirroring the response to live LM. (A and B)

3x10^3 LLO56 (A) or LLO118 (B) T cells were transferred to B6 recipients, which were then immunized the following day with either LLO(190-201) or LLO(190-203) peptide emulsified in complete Freund’s adjuvant. 7 days post-immunization, expansion of the transferred T cells was enumerated by FACS.
Figure 2.9. LLO(190-203) peptide induces stronger *in vivo* expansion from LLO118 than LLO56 despite equivalent proliferation, mirroring the response to live LM.
Figure 2.10. C-terminally extended LLO peptides reveal stronger magnitude IL-2 response from LLO56 compared to LLO118, despite receipt of a similarly activating stimulus. (A and B) Activation marker upregulation (A) and IL-2 secretion (B) of LLO56 and LLO118 splenocytes incubated with the indicated peptides for 18 hours.
Figure 2.10. C-terminally extended LLO peptides reveals stronger magnitude IL-2 response from LLO56 compared to LLO118, despite receipt of a similarly activating stimulus.
Figure 2.11. LLO56 shows greater magnitude responses of several cytokines besides IL-2 in response to LLO(190-203) peptide. Cytokine bead array analysis of IL-2, IL-17A, TNFα and IL-6 secretion in response to 10 μM LLO peptide, 10 μM moth cytochrome C (MCC) peptide (negative control), or 10 μg/mL αCD3+αCD28 (positive control). IL-4, IL-13, IL-1β, IFNγ, and IL-22 were also measured in the same assay, with no cytokine detected for either LLO56 or LLO118.
Figure 2.11. LLO56 shows greater magnitude responses of several cytokines besides IL-2 in response to LLO(190-203) peptide.
Figure 2.12. LLO56 and LLO118 show similar kinetics of activation and IL-2 secretion in response to LLO(190-203) peptide. (A and B) LLO56 and LLO118 splenocytes were incubated with 10 μM LLO peptide and cultured in 96-well plates. At the indicated timepoints, supernatant and cells were collected from sample wells; the cells were used to analyze activation marker upregulation by FACS (A), while the supernatants were analyzed for IL-2 by ELISA (B).
Figure 2.12. LLO56 and LLO118 show similar kinetics of activation and IL-2 secretion in response to LLO(190-203) peptide.
Figure 2.13. LLO56 has a higher frequency of IL-2 secreting cells than LLO118 upon stimulation. Single-cell IL-2 production was assessed using a cytokine capture assay. LLO56 and LLO118 splenocytes were stimulated 4 hours with 10 μM LLO peptide, 10 μM moth cytochrome C (MCC) peptide (negative control), or 10 μg/mL αCD3+αCD28 (positive control).
Figure 2.13. LLO56 has a higher frequency of IL-2 secreting cells than LLO118 upon stimulation.
Figure 2.14. A cellular, competition-based assay to measure peptide binding to I-A<sup>b</sup>. (A)

Schematic of the assay workflow. DM<sup>−/−</sup> refers to splenocytes deficient in the MHC Class II peptide editor H2-M. (B) Controls demonstrating the specificity of the YAe antibody for E<sub>α</sub>(52-68) peptide bound to I-A<sup>b</sup>. Neither free peptide nor endogenous peptide-loaded I-A<sup>b</sup> alone were detectable by YAe.
Figure 2.14. A cellular, competition-based assay to measure peptide binding to I-A<sup>b</sup>.
Figure 2.15. C-terminally extended LLO peptide binding to I-A\textsuperscript{b} does not track with its greater stimulatory activity. H2-M\textsuperscript{-/-} splenocytes were incubated 24 hours with 2.25 \textmu M E\alpha(52-68) peptide along with a concentration series of LLO peptides, or Hb(64-76) as a negative control. Competition of E\alpha(52-68) binding I-A\textsuperscript{b} was read out using the YAe antibody, with the response at each concentration of competitor peptide normalized to the maximum response. The table below the graph contains IC50 values for LLO peptide binding to MHC, along with summary statistics.
Figure 2.15. C-terminally extended LLO peptide binding to I-A<sup>b</sup> does not track with its greater stimulatory activity.

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Figure 2.16. Predicted I-A\textsuperscript{b} binding register for LLO(190-205) peptide, based on structural features of I-A\textsuperscript{b} peptide binding groove. Upward arrows indicate positions where residue side chains are solvent-exposed and facing the TCR, downward arrows indicate residues whose side chains would be oriented toward the floor of the binding pocket. The nine residues highlighted in green represent the canonical I-Ab binding residues of the LLO peptide. The yellow box indicates positions 202 and 203, which are residues flanking the peptide binding pocket in this predicted register.
Figure 2.16. Predicted I-A<sup>b</sup> binding register for LLO(190-205) peptide, based on structural features of I-A<sup>b</sup> peptide binding groove.
Figure 2.17. Loss of LLO N-terminal residues 190-192 leads to a complete loss of stimulatory activity, linked to a marked reduction of peptide binding to I-A\textsuperscript{b}. (A and B) LLO peptides lacking one or more N-terminal residues were tested for their ability to stimulate LLO56 and LLO118 T cells in IL-2 ELISA experiments (A), or for their ability to bind to I-A\textsuperscript{b} (B).
(A) Stimulatory activity of N-terminal truncated LLO peptides

![Graph showing LLO56 and LLO118 Truncation Mutagenesis with IL-2 levels.]

(B) MHC binding of N-terminal truncated LLO peptides

![Graph showing MHC binding to I-A^b with competitor levels.]

Figure 2.17. Loss of LLO N-terminal residues 190-192 leads to a complete loss of stimulatory activity, linked to a marked reduction of peptide binding to I-A^b.
Figure 2.18. Scanning mutagenesis of LLO epitope reveals that LLO56 and LLO118 make a common set of critical TCR contacts within a common peptide binding register. (A and B) LLO peptides singly substituted with lysines at every position were analyzed for their ability to induce LLO56 and LLO118 T cell responses, which were read out by IL-2 ELISA (A), or their ability to bind I-A^b (B). Each residue in the predicted binding register, from residues 193-201, is indicated by a colored square; the color shows the effect of each mutation on the measured response, as indicated in the key. The predicted positions of each residue in the binding register, P1 through P9, are indicated directly below the squares; positions in red are predicted TCR contacts, positions in blue are predicted MHC contacts.
(A) Effect of single Lys-substitution on LLO56 and LLO118 T cell responses

![Diagram showing effect of single Lys-substitution on LLO56 and LLO118 T cell responses]

(B) Effect of single Lys-substitution on peptide binding to I-A^b

![Diagram showing effect of single Lys-substitution on peptide binding to I-A^b]

Figure 2.18. Scanning mutagenesis of LLO epitope reveals that LLO56 and LLO118 make a common set of critical TCR contacts within a common peptide binding register.
Figure 2.19. LLO56 is relatively unresponsive to LLO peptides with acidic residues substituted at the flanking positions occupied by residues 202 and 203. (A) The effect of single mutations to LLO peptide residues 202 and 203 on LLO56 and LLO118 T cell responses was measured by IL-2 ELISA. Mutant peptides with the acidic residues (Asp and Glu) and their neutrally charged counterparts (Asn and Gln) at positions 202 and 203 are presented. (B) LLO peptide mutants doubly substituted at positions 202 and 203 with acidic residues were tested for their effect on LLO T cell stimulation.
(A) T cell responses to LLO peptide single mutants at 202 and 203 positions

(B) T cell responses to LLO peptide double mutants at 202 and 203 positions

Figure 2.19. LLO56 is relatively unresponsive to LLO peptides with acidic residues substituted at the flanking positions occupied by residues 202 and 203.
Figure 2.20. Amide-capping C-terminus of LLO(190-201) and LLO(190-202) peptides is as effective in enhancing LLO56 responses as addition of the next amino acid. LLO(190-201), LLO(190-202), and LLO(190-203) peptides were synthesized with their C-terminal carboxyl groups modified to amide groups, thus removing the negative charge. These modified peptides and their WT counterparts were tested for their ability to stimulate LLO56 and LLO118 T cells. For reference, the structural difference between WT and amide-capped C-termini are provided in the images below the T cell response graphs.
Figure 2.20. Amide-capping C-terminus of LLO(190-201) and LLO(190-202) peptides is as effective in enhancing LLO56 responses as addition of the next amino acid.
Chapter 3:
Intrinsic LLO CD4⁺ T cell sensitivity and response to pathogen are set and sustained by avidity for thymic and peripheral self-pMHC

This material in this chapter draws extensively from a manuscript published by our laboratory in Nature Immunology."}

3.1 Introduction

Initiation of CD4⁺ T cell responses requires productive interaction between the αβ T cell receptor (TCR) and Class II peptide-MHC (pMHC)²⁻⁵. These interactions are highly sensitive and specific despite their micromolar-range binding affinities⁸⁹. Even weaker interactions between the TCR and self-pMHC ligands serve critical roles in T cell development, survival, and peripheral function⁴⁷, ⁴⁸, ⁴⁹, ⁵⁰, ⁵¹, ⁵³, ⁵⁶, ⁹⁰. It is evident from this that the TCR can discriminate between pMHC ligands, even subtly different ones²⁶, to signal distinct functional outcomes. It remains an important pursuit to understand the molecular features of TCR:pMHC interactions that promote effective, appropriate CD4⁺ T cell responses to pathogens.

TCR affinity for cognate pMHC and clonal frequency in the preimmune repertoire have proven to be important factors governing the magnitude of in vivo CD4⁺ and CD8⁺ T cell responses to pathogen⁷⁷, ⁷⁸, ⁷⁹, ⁹¹, ⁹². High-affinity CD4⁺ and CD8⁺ T cells, with greater functional avidity for antigen, were more prevalent after infection than before, demonstrating clear evolution of the
anti-pathogen repertoire towards more effective clones. Low-affinity interactions could also lead to generation of effector and memory CD8$^+$ populations, albeit more slowly and to a lesser extent than their higher affinity counterparts$^{93}$.

How TCR recognition of self-pMHC affects CD4$^+$ T cell responses has received less attention. Functionally, the degree of TCR self-reactivity has been correlated with cell surface expression of the negative regulator CD5$^{44}$. However, the incredibly weak affinities of TCR:self-pMHC interactions has impeded direct functional and biochemical characterization, which has complicated efforts to understand their role in protective immune responses.

To gain further insight into how TCR:pMHC interactions impact CD4$^+$ T cell responses to pathogen, we employed two CD4$^+$ TCR transgenic mouse lines called LLO56 and LLO118. Both lines have CD4$^+$ T cells that recognize residues 190-205 of the *Listeria monocytogenes* virulence factor Listeriolysin O in the context of I-A$^b$. The LLO56 and LLO118 TCRs were cloned from T cell hybrids generated using *Listeria*-infected B6 mice, and so represent two solutions to recognizing the same pMHC in an infectious context. These reagents allow us to investigate at a clonal level how disparate T cell biology can be elicited by recognition of the same pathogen-derived epitope.

Previously, using a low number T cell transfer (3x10$^3$ cells) and *Listeria* infection model in B6 mice, we showed that LLO118 T cells expanded more than LLO56 during primary responses to *Listeria* in vivo, even though both T cells proliferated similarly to peptide in vitro and to live
Listeria both in vitro and in vivo (see Figure 2.6). Upon rechallenge, however, LLO56 T cells expanded more than LLO118. LLO56’s poor primary response was associated with greater cell death, and potentially with its significantly greater expression of CD5 (Figure 3.1a). The basis for the disparate behavior of our two T cells, particularly with respect to CD5, remains unclear.

In this study, we set out to shed light on the basis for the in vivo biology of the LLO56 and LLO118 T cells via a thorough in vitro functional characterization. In the process, we made the unexpected finding that stimuli bypassing the TCR could elicit stronger IL-2 responses from LLO56 than LLO118. This stronger IL-2 response was associated with greater phospho-TCRζ at baseline and phospho-ERK upon activation. These features emerged during positive selection in proportion to the strength of the TCR signal from selecting self-pMHC, and required active maintenance by peripheral self-pMHC. Notably, self-pMHC deprivation markedly reduced the ability of LLO56 T cells to expand during Listeria infection in vivo. Despite the expectation that the more responsive LLO56 would expand better than LLO118 during Listeria infection, it does not because it more readily undergoes cell death. We show that LLO56’s greater propensity to undergo cell death is itself a property that emerges following positive selection.

3.2 Distinct IL-2 responses from TCR transgenic cells to stimuli bypassing the TCR

Consistent with our previous findings that LLO56 and LLO118 proliferated similarly in vitro (Figure 2.8), these T cells showed similar upregulation of the activation markers CD69 and CD25 in response to LLO peptide and αCD3+αCD28 stimulation (Figure 3.2a). However,
LLO56 T cells produced more IL-2 than LLO118 over the same peptide dose range (Figure 3.2b). This could not be explained by differences in expression of the TCR, CD3, CD4 or the costimulatory molecules CD28, CTLA-4, PD-1, or PD-L1 (Figure 3.1b). The affinities of the LLO56 and LLO118 TCRs for LLO(190-205)/I-A\textsuperscript{b} were identical, suggesting that the distinct IL-2 responses were not related to differences in binding to LLO/I-A\textsuperscript{b} (Figure 3.2c). Thus, in response to a similarly activating stimulus, LLO56 and LLO118 diverged in their abilities to produce IL-2; notably, the stronger IL-2 responder \textit{in vitro} was the weaker primary responder \textit{in vivo}.

Unexpectedly, a stronger IL-2 response could also be elicited from LLO56 T cells by stimulation with $\alpha$CD3+$\alpha$CD28 (Figure 3.2b and 3.2d). This was also true when cells were stimulated with PMA and ionomycin (P+I), which act intracellularly downstream of the TCR (Figure 3.2e). LLO56 and LLO118 did not differ markedly in their ability to produce IFN$\gamma$ or TNF$\alpha$ in response to P+I, indicating that LLO56’s stronger IL-2 response could not be generalized to all cytokine responses (Figure 3.2e). These findings contrasted with our presumption that two T cells with different TCRs would respond equally to stimuli bypassing the TCR. Rather, the data suggested that LLO56 and LLO118 bore intrinsic differences governing the strength of their IL-2 responses.
3.3 Greater ERK and basal TCRζ phosphorylation underlie intrinsic LLO56 response

To mechanistically understand how nonspecific stimuli could elicit distinct IL-2 responses from two TCR transgenic cells, we investigated the signaling pathways activated by P+I expression, including the NFAT, NFκB, and Ras/ERK pathways. Using phosphoflow cytometry, we found that nonspecific stimulation induced higher levels of phospho-ERK from LLO56 than LLO118, with similar results obtained by immunoblot (Figure 3.3a, Figure 3.4a). PMA-induced IκBα degradation (Figure 3.3b) and ionomycin-induced calcium flux (Figure 3.3c) were similar between LLO56 and LLO118, with LLO118 showing somewhat stronger responses in both assays. Thus, greater activation of ERK most clearly tracked with the stronger IL-2 response to P+I stimulation in LLO56 T cells.

As peptide and antibody stimulation also elicited stronger IL-2 responses from LLO56 than LLO118, we considered that there might also be differences in proximal signaling. Several studies have linked TCR self-reactivity to the extent of basal TCRζ phosphorylation\textsuperscript{54, 57, 94} Indeed, upon examination, LLO56 had higher basal levels of p21 phospho-TCRζ than LLO118 (Figure 3.3d).
3.4 Strength of polyclonal T cell IL-2 responses correlates with CD5 expression

Based on their respective expression of CD5 and basal TCRζ phosphorylation, we would predict that the LLO56 T cell receives a stronger signal from self-pMHC than LLO118. We hypothesized that such a signal might underlie the stronger LLO56 response to P+I stimulation. However, the observed biology could be an artifact of the TCR transgenic cells. We therefore tested our hypothesis by asking whether TCR self-reactivity, as gauged by CD5 expression, correlated with the strength of the response to nonspecific stimulation in polyclonal B6 T cells. If this were true, CD5hi T cells (like LLO56) should be more responsive to P+I stimulation than CD5lo cells (like LLO118).

Indeed, CD5hi CD4+ and CD8+ T cells more readily produced IL-2 in response to P+I (Figure 3.5a) or αCD3+αCD28 (Figure 3.6a) than CD5lo cells. Stimulation of the cells did not markedly alter the distribution of CD5 expression (Figure 3.6b), which we confirmed using cells that were FACS-sorted by CD5 expression prior to stimulation (Figure 3.6c). Furthermore, CD5hi CD4+ and CD8+ T cells had higher levels of pERK on activation and higher basal levels of p21 phospho-TCRζ than their CD5lo counterparts (Figure 3.5b and 3.5c). As these experiments were done with bulk CD4+ and CD8+ cells, they would include memory phenotype and nonconventional αβ T cells (i.e., Tregs, NKT cells). We therefore repeated these analyses using magnetically- or FACS-sorted naive conventional αβ T cells (CD44lo CD25- NK1.1+), and obtained essentially identical results (Figure 3.5d-f). Taken together, these data demonstrate a
clear link between CD5 expression, intrinsic strength of IL-2 and pERK responses, and basal signaling in polyclonal T cells, validating the findings made with the LLO56 and LLO118 mice.

3.5 Intrinsic LLO56 responses and proclivity to cell death emerge upon positive selection

Since T cell development is predicated on interactions with self-pMHC, we reasoned that analysis of thymic selection would yield important insights into the origin of the LLO T cells’ respective biology. LLO56 thymi had markedly higher frequencies and numbers of CD4SP thymocytes than LLO118 (Figure 3.7a), but fewer total thymocytes. This, taken together with the considerably larger population of TCR hiCD69+ post-selection thymocytes (Figure 3.8a), suggested that LLO56 DP thymocytes were more efficiently positively selected than LLO118. Consistently, post-selection LLO56 thymocytes expressed higher levels of CD5 and CD69, markers upregulated in response to the strength of the selecting signal (Figure 3.7b). That LLO118 received a weaker signal from self-pMHC than LLO56 suggests that the lower frequency of LLO118 CD4SP thymocytes was not due to negative selection.

We next considered that selecting TCR interactions with the thymic self-pMHC milieu might be what sets the respective sensitivities of the LLO56 and LLO118 T cells. To explore this, we stimulated LLO56 and LLO118 thymocytes with P+I and analyzed their IL-2 production at each developmental stage. DN thymocytes produce IL-2 in response to P+I stimulation95, which we detected at similarly high frequencies among LLO56 and LLO118 DN thymocytes (Figure 3.7c). Any contribution of NK, NKT, and γδ T cells to this IL-2 response was negligible given their
low frequencies (Figure 3.8b). LLO56 and LLO118 DP thymocytes were similarly refractory to stimulation by P+I. However, as LLO56 and LLO118 DP thymocytes transitioned to the CD4SP stage, we observed the emergence of the difference in IL-2 response, ERK phosphorylation, and basal p21 TCRζ seen in mature LLO56 and LLO118 T cells (Figure 3.7c-e, Figure 3.8c).

To further test the concept that the selecting MHC environment impacts the intrinsic IL-2 responses of mature CD4$^+$ T cells, we took advantage of the fact that the AND TCR is selected strongly in H-2$^k$ mice and more weakly in H-2$^b$ mice$^{96}$. This allowed us to investigate whether different intrinsic functionality could be observed from T cells expressing the same TCR, but which developed on different MHC backgrounds. Indeed, AND T cells selected on H-2$^k$ MHC had markedly stronger IL-2 responses than cells selected on H-2$^b$ MHC (Figure 3.8d). Taken together with the data from the LLO56 and LLO118 mice, our results demonstrate that intrinsic T cell responsiveness is set during T cell development in proportion to the strength of the selecting signals from self-pMHC.

Finally, we considered that other relevant phenotypic features of the LLO T cells might emerge upon selection. We therefore asked whether differences in cell death, which associate with LLO56 and LLO118’s disparate in vivo responses to *Listeria*, might also emerge during this transition. Indeed, when stimulated with αCD3+αCD28 in culture, pre-selection LLO118 cells died more than LLO56, but post-selection LLO56 cells died more than LLO118 (Figure 3.7f). Developmentally acquired differences in IL-7Rα and Bcl-2 expression could not explain this abrupt shift in cell death behavior (Figure 3.8e). While the expression of Bim was higher in
LLO56 CD4SP thymocytes, this difference did not persist in peripheral cells. However, the greater cell death response of stimulated LLO118 DP thymocytes did associate with higher average per cell p21-TCRζ (Figure 3.7e), a pattern that reversed in CD4SP cells and persisted in mature CD4+ T cells. Thus, the respective propensities of the LLO56 and LLO118 T cells to undergo cell death were acquired upon positive selection, paralleling the emergence of their respective sensitivities to stimulation.

### 3.6 Peripheral self-pMHC maintains intrinsic LLO T cell responsiveness

After leaving the thymus, T cells continue to receive tonic self-pMHC signals in the periphery. We next tested whether removal of this tonic signal compromised CD4+ T cell responses to P+I, which would directly demonstrate that self-pMHC impact TCR signaling beyond the most proximal components. To do this, we functionally assessed LLO56 and LLO118 T cells that were adoptively transferred to B6 and MHC Class II−/− recipients (Figure 3.9). Phenotypically, cells transferred to MHC Class II−/− mice had similar expression of CD3, CD4, or TCR as cells transferred to B6 mice, but they did have reduced expression of CD5, as might be expected for a molecule dynamically regulated by TCR:self-pMHC signals45,46 (Figure 3.10a).

LLO56 and LLO118 T cells transferred to B6 mice showed similar IL-2 responses to P+I as seen with freshly isolated cells. However, transfer to MHC Class II−/− recipients rendered both T cells more poorly responsive to P+I, particularly LLO56 (Figure 3.9a). To exclude the possibility that the lack of CD4+ T cells in MHC Class II−/− recipients contributed to the reduced IL-2 responses,
we transferred LLO T cells to Cα−/− mice, which have normal MHC Class II but lack αβ T cells, or H2-M−/− mice, which have αβ T cells but whose self-peptide repertoire is largely restricted to CLIP peptide. The IL-2 responses of LLO56 and LLO118 cells transferred to Cα−/− recipients mirrored those seen with transfers to B6 mice, while the IL-2 responses of cells transferred to H2-M−/− mice mirrored those seen with transfers to MHC Class II−/− mice (Figure 3.10b). Timecourse experiments done with H2-M−/− recipients revealed that the IL-2 responses of LLO56 T cells declined sharply by day 1 post transfer, then more gradually thereafter (Figure 3.10c). Taken together, these results indicate that the intrinsic strength of LLO56 and LLO118 IL-2 responses are actively maintained by TCR:self-pMHC interactions, and decay rapidly in their absence.

We next asked whether deprivation of self-pMHC ligands affected ERK activation. Indeed, LLO56 cells transferred to MHC Class II−/− recipients showed a marked reduction in ERK phosphorylation, while the reduction in the LLO118 cells’ pERK response was more modest (Figure 3.9b). These experiments provide evidence that the signal from self-pMHC impacts TCR signaling as far downstream as ERK.

Finally, we sought to test whether deprivation of self-pMHC signals affected the LLO T cell response to Listeria in vivo. However, the MHC Class II in B6 mice, while necessary to present LLO peptide, could also present self-ligands and restore tonic signaling to cells that had been deprived of self-pMHC. To mitigate this, we devised an infection strategy in which LLO T cells deprived or not deprived of MHC Class II were transferred into cohorts of Listeria-infected B6
mice (Figure 3.9c). The timing of the T cell transfer to the infected mice coincided with a period of abundant *Listeria* antigen presentation in the spleen, which is where the intravenously transferred LLO T cells would home first. Overall, this scheme would promote encounter of LLO-A\(^b\) complexes rapidly upon T cell transfer, encouraging the cells to activate “as is” before regaining tonic signal from self-pMHC. Since self-ligands would be among the species present on any APCs bearing cognate antigen, they could participate in responses to LLO-A\(^b\). Thus, our experimental system allowed us to test the effect of withdrawing tonic self-pMHC signals without disrupting the ability of self-ligands to act as coagonists.

Strikingly, LLO56 T cells deprived of MHC Class II expanded significantly worse than those that were not deprived (Figure 3.9d). LLO118 cells, however, responded similarly well whether they were deprived of self-pMHC or not. Importantly, the modified adoptive transfer and infection schedule did not affect the relative abilities of the LLO56 and LLO118 T cells to expand in response to *Listeria*, suggesting that the cell death-driven *in vivo* behavior of LLO56 T cells was unaltered by the model. Overall, these data demonstrate that deprivation of TCR:self-pMHC interactions can compromise CD4\(^+\) T cell responses to pathogen *in vivo*.

### 3.7 CD5 feedback inhibition of LLO56 TCR self-reactivity and IL-2 responses

It remains unclear thus far whether CD5 itself influences the intrinsic strength of IL-2 responses or is merely a marker for the TCR:self-pMHC avidity underlying them. There are conflicting data in the literature regarding whether CD5 augments or interferes with TCR signaling, and in
what contexts. To address this issue in our system, we generated LLO56 and LLO118 mice deficient in CD5. Post-selection CD5−/− LLO56 thymocytes perceived a stronger signal from self-pMHC than WT LLO56 as judged by their elevated CD69 expression but this greater self-reactivity was not manifest as activation of peripheral T cells (Figure 3.11a). This may be accomplished partly by compensatory reductions in post-selection expression of the TCR and CD3 in the CD5−/− LLO56 T cells. Conversely, CD5−/− LLO118 thymocytes did not show increased CD69 upregulation self-pMHC post-selection compared to WT, and peripheral CD5−/− LLO118 T cells did not show reduced surface expression of TCR or CD3 (Figure 3.11b).

Upon stimulation, CD5−/− LLO56 and LLO118 T cells had dramatically higher IL-2 responses than WT cells (Figure 3.11c), which was associated with moderate increases in ERK signaling (Figure 3.11d). These findings support the view that CD5 antagonizes self-pMHC signals from the TCR, reducing the intrinsic IL-2 responsiveness maintained by TCR:self-pMHC interactions. That the cells with the strongest IL-2 responses had the highest CD5 expression suggests that this molecule does not impose a dominant inhibitory tone. Rather, since the expression level of CD5 is set and maintained based on the strength of TCR:self-pMHC interactions (Figure 3.7b, Figure 3.10a), CD5 is positioned to impose feedback inhibition on TCR signaling to restrain the most strongly self-reactive cells.

3.8 Conclusions

Studies of the LLO56 and LLO118 mice revealed that the intrinsic strength of CD4+ T cell cytokine responses are determined by TCR:self-pMHC interactions. Intriguingly, we show that
greater self-reactivity and intrinsic IL-2 responses in vitro need not correlate with T cell expansion in vivo. We suggest that a T cell’s propensity to undergo cell death as a mechanism by which in vitro reactivity might be decoupled from in vivo response. Furthermore, our data demonstrate a crucial role for thymic education in determining these functional attributes of CD4+ T cells. Based upon our results, we propose a TCR-instructive model whereby selecting TCR:self-pMHC interactions establish CD4+ T cell function centrally and maintain this functionality in the periphery, ultimately shaping how a given T cell will perform during pathogen challenge.

3.9 Methods

Mice

The LLO56/B6.Thy1.1/RAG1−/− and LLO118/B6.Ly5.1/RAG−/− TCR transgenic mice (referred to simply as LLO56 and LLO118, respectively) were described previously75. Briefly, the CD4+ T cells in the LLO56 and LLO118 mice express a single, distinct Vα2-Vβ2 TCR that recognizes Listeriolysin O (LLO) residues 190-205 bound to I-A^b. The LLO56 and LLO118 mice were maintained heterozygous for the TCR transgenes. B6 and MHC Class II−/− mice were obtained from The Jackson Laboratory. CD5−/− mice were obtained as part of the NIAID Exchange Program from the transgenic mouse repository maintained by Taconic. H2-M^−/− β2m−/− mice were provided by Jenny Ting’s laboratory; these mice were backcrossed to B6, and the F1 progeny intercrossed to restore the WT β2m alleles, thus generating the H2-M^−/− mice used in this study. AND RAG^kk, AND^vb, and TCR Cα−/− mice from our colony were also used in some of the described experiments. All mice were between 4 and 12 weeks of age at the beginning of each
experiment, with all experimental comparisons done without blinding between age- and sex-matched cohorts. As they are extensively backcrossed, all age- and sex-matched mice of a given strain were treated as identical and assigned randomly to treatment groups. Breeding, housing and care of all mice was done in specific pathogen-free facilities under a protocol approved by the Washington University Animal Studies Committee.

Antibodies and other reagents

For flow cytometry, the following antibodies (clones indicated in parentheses) were purchased from either BD Biosciences, Biolegend, eBioscience, or Invitrogen: CD4 (clones RM4-4, RM4-5 and GK1.5), CD8 (53-7.8), CD5 (53-7.3), CD69 (H1.2F3), CD25 (PC61), B220 (RA3-6B2), F4/80 (BM8), CD11b (M1/70), CD11c (N418), IL-2 (JES6-5H4), PD-1 (J43), PD-L1 (MIH5), CD28 (37.51), CTLA-4 (UC10-4F10-11), Thy1.1 (His51), Ly5.1 (A20), CD3ε (145-2C11), Vα2 TCR (B20.1), γδ TCR (GL3), NK1.1 (PK136), CD44 (IM7), CD127 (SB/199), and Bcl-2 (3F11). Rabbit antibodies against phospho-ERK1/2 (clone D13.14.4E, also used for immunoblots) and Bim (clone C34C5), rabbit IgG isotype control (clone DA1E), and Alexa-647 conjugated anti-rabbit IgG F(ab’)2 were obtained from Cell Signaling Technologies. Annexin V was obtained from BD Biosciences, and 7AAD was obtained from Sigma-Aldrich.

For immunoblots, the following primary antibodies were used: anti-p-Tyr (4G10, Upstate Biotechnology), anti-IκBα (Cell Signaling), anti-β-actin (Biolegend). Polyclonal rabbit anti-TCRζ serum 777, which recognizes unphosphorylated and phosphorylated TCRζ, was generated in our laboratory as described\textsuperscript{102}. Phosphorylated p21-TCRζ was identified from whole T cell
lysates using 4G10; the identity of this band was confirmed using rabbit anti-TCRζ serum (Figure 3.4b).

**Analyses of T cell activation**

For activation marker upregulation, ELISA, and cytokine capture assays, LLO56 and LLO118 CD4+ T cells purified by magnetic bead negative selection were cultured with T cell-depleted B6 splenocytes at a 1:4 ratio. Stimulations were performed in duplicate or triplicate wells in DMEM + 10% FCS (HyClone) at 37°C/5% CO₂. The mouse cytokine capture assay was obtained from Miltenyi Biotec and done according to their protocol, except that culture media contained bovine rather than murine serum. For this assay, αCD3+αCD28 was used for stimulation; PMA + ionomycin was not used with this assay to avoid cytokine capture *in trans* by non-secreting cells. Intracellular cytokine staining was done as previously described. Briefly, cells were treated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) plus 1 µg/mL ionomycin (Sigma-Aldrich) and incubated for 30 minutes. All cultures were then treated with 2 µg/mL Brefeldin A (Sigma-Aldrich) and incubated for an additional 4 hours. Samples were harvested and stained for surface markers and with 7AAD, fixed in 4% paraformaldehyde in PBS, and permeabilized in FACS buffer containing 0.5% saponin (Sigma-Aldrich), then stained for cytokines.

**Surface Plasmon Resonance**

Binding experiments were done with a Biacore 2000 SPR instrument essentially as described. Briefly, CM5 sensor chips (GE Healthcare) were activated by a 20 minute pulse of a 1:1 mix of
N-hydroxysuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide HCl (EDC). Soluble LLO(190-205)/I-A$^b$ was amine coupled to the chip in 20 mM sodium citrate pH 4.5 to a total response of 300 RU, after which unreacted NHS groups on the chip were blocked by a 6 minute pulse of 1M ethanolamine pH 8.5. Concentration series of soluble LLO56 and LLO118 single-chain (scTCR) in HEPES buffered saline + BSA (10 mM HEPES, 3 mM EDTA, 150 mM NaCl, 0.05% Tween-20, and 1% BSA) were injected in duplicate over the prepared flow cells at a flow rate of 30 μL/min. Specific SPR responses of LLO TCR binding to LLO(190-205)/I-A$^b$ were obtained by first subtracting the response from injections over a flow cell coupled with unexchanged soluble I-A$^b$, then correcting for bulk flow effects by subtracting the response from injections of plain buffer. Sensorgrams were fitted by Biacore software to a 1:1 Langmuir binding model to derive values for the rate constants $k_d$ and $k_a$, and the dissociation constant $K_D$ ($=k_d/k_a$).

**Soluble protein preparation**

Soluble, single-chain LLO56 and LLO118 scTCRs, designed as Vβ-linker-Vα constructs, were engineered by error prone mutagenesis and conformational selection of stable mutants by yeast display as described$^{41}$. The scTCR genes were cloned into pET28a via NheI-XhoI restriction sites, placing them in-frame with a 6x-His tag. Protein expression in *E. coli* was induced with 1mM IPTG, thus generating insoluble scTCR inclusion bodies which were harvested as described$^{23}$. Inclusion bodies were then refolded under oxidative conditions and purified by nickel bead batch purification (Qiagen) followed by S200 FPLC-size exclusion chromatography. Purified protein was concentrated using Amicon centrifugal filters and quantified by A280 using
extinction coefficients of 1.690 and 1.674 for LLO56 and LLO118, respectively. For preparation of LLO/I-A\textsuperscript{b} complexes, soluble I-A\textsuperscript{b} covalently tethered to 3R peptide via a thrombin-cleavable linker was provided by Eric Huseby. 3R/I-A\textsuperscript{b} complexes were thrombin-cleaved to release the peptide, which was then exchanged with LLO(190-205) peptide by incubation in sodium carbonate buffer pH 10.5 at 37°C for 48 hours.

**Phospho-ERK flow cytometry**

Cells were prepared in triplicate 100µL samples in serum-free IMDM and held on ice prior to activation. Upon addition of 100µL PMA at 2X concentration (200 ng/mL), each tube was briefly mixed then placed in a 37°C water bath to begin the stimulation. Afterwards, tubes were removed from the water bath and immediately fixed by adding 200µL 4% paraformaldehyde in PBS. After 20 minutes of fixation at room temperature, tubes were filled with 4mL ice-cold 100% methanol and held at 4°C overnight. The following day, cells were washed twice, incubated with rabbit anti-phospho-ERK, and then stained with Alexa 647-conjugated anti-rabbit IgG along with antibodies against relevant surface markers.

**Immunoblots**

Cells were stimulated as indicated at 37°C, then lysed immediately in ice-cold buffer containing 1% Nonidet P-40, 10 µg/ml leupeptin and Pepstatin A each, 1 mM PMSF, and 1 mM sodium orthovanadate. Lysates were cleared of insoluble material by centrifugation at 16000 x g for 10 min at 4°C, then mixed with Laemmli buffer, boiled for 5 min and resolved on a 12% SDS-PAGE gel. Following overnight transfer to nitrocellulose membranes (10V, 4°C), blots were
blocked for 1 hour with a 1:1 mix of PBS and Odyssey Blocking Buffer (LI-COR), incubated overnight at 4°C with mouse and rabbit primary antibodies, then incubated with Alexa 680-conjugated anti-rabbit IgG (Molecular Probes) and IRDye 800-conjugated anti-mouse IgG (LI-COR) secondary antibodies. All antibody incubation steps were done in 1:1 PBS + Odyssey Blocking Buffer with 0.1% Tween-20. Membranes were imaged using an Odyssey infrared scanner (LI-COR), and densitometry done using ImageJ software (NIH).

**Calcium flux experiments**

T cells purified by magnetic sorting were stained for 30 minutes at 37°C with 2 μM Indo-1 AM (Molecular Probes) in the presence of 0.02% Pluronic-F127. Cells were washed twice, resuspended in buffered saline containing 1mM CaCl₂ and 1mM MgCl₂ and rested at room temperature for 20-30 minutes. All samples were prewarmed to 37°C for 5 minutes immediately prior to analysis. After establishing a baseline with unstimulated cells, ionomycin was added to a final concentration of 5 μg/mL.

**Flow cytometry and cell sorting**

FACS analysis was conducted using a BD FACSCalibur or BD LSR II cytometer. For cell sorting, single-cell suspensions of thymus or pooled spleen and lymph nodes were stained for populations of interest using antibodies against CD4, CD8 and CD5. For sorting of B6 T cells by CD5 expression, CD4⁺ and CD8⁺ T cells were pre-enriched by magnetic sorting. CD44hi, CD25⁺ and NK1.1⁺ were removed as part of our sorting strategy in some experiments in order to generate populations of naive conventional CD4⁺ and CD8⁺ T cells. Samples were routinely co-
stained with 7AAD and antibodies against CD11b, CD11c, B220 and F4/80 to facilitate exclusion of dead and unwanted cells. All cell sorting was done using a FACSARia II sorter (BD Biosciences). Data analysis for all experiments was done using FlowJo version 8.8.6 (Treestar).

**Adoptive Transfer Experiments**

LLO56 and LLO118 CD4^+^ T cells were purified by magnetic bead negative selection. 1-3x10^6 LLO CD4^+^ T cells were transferred intravenously to B6, MHC Class II^-/-^, Cα^-/-^, or H2-M^-/-^ recipients for 1, 2 or 4 days, depending on the experiment, then harvested and magnetically enriched as described^103^. For experiments where donor cells were assayed for IL-2 production, cell enrichment was routinely done by positive selection for their unique congenic markers, with similar results if cells were enriched by negative selection. For experiments where donor cells were assayed for phospho-ERK or transferred into *Listeria*-infected mice, cells were purified exclusively by negative selection.

**Listeria infection**

Frozen stocks of *Listeria monocytogenes* strain 10403S in PBS + 20% glycerol were thawed and serially diluted to 10^4 CFU/mL in PBS. 100μL of this solution was injected retro-orbitally to give an inoculum of 10^3 CFU/mouse. Injection titers were confirmed by colony counting aliquots of injection solution plated on brain-heart infusion agar.
Statistics

Statistical testing was done as indicated in each figure legend. $P < 0.05$ was designated as the criterion for significance; p-values for experiments are as reported in each figure. Decisions to use the indicated statistical analyses were assisted by the results of the Shapiro-Wilk normality test and the F test (to compare variances). All statistical analyses were conducted using Prism 6 for Mac OS X (Graphpad).
Figure 3.1. Cell surface phenotyping of unstimulated mature LLO56 and LLO118 T cells.

(a) Differences in CD5 expression among LLO56 and LLO118 T cells. The distribution of CD5 expression on polyclonal B6 CD4+ T cells is presented for reference. (b) Expression of TCR signaling components and costimulatory molecules on LLO56 and LLO118 T cells. Data are representative of analyses from at least three mice.
Figure 3.1. Cell surface phenotyping of unstimulated mature LLO56 and LLO118 T cells.
Figure 3.2. LLO56 and LLO118 T cells diverge in their IL-2 responses to specific or nonspecific stimuli. (a and b) Upregulation of CD69 and CD25 (a), and IL-2 ELISA (b) for LLO56 and LLO118 T cells treated with LLO(190-205) peptide, 100 μM MCC(83-101) peptide, or 10 μg/mL αCD3+αCD28 mAbs. (c) Surface plasmon resonance binding analysis of LLO56 and LLO118 scTCRs to LLO(190-205)/I-A\(^{b}\). Results show a concentration series of scTCR injections beginning at 40 μM (topmost curves), with two-fold serial dilutions going from top to bottom. (d) Primary (left) and graphed (right) data for IL-2 capture assay of LLO56 and LLO118 CD4\(^{+}\) T cells stimulated with 10 μg/mL αCD3+αCD28. Numbers in plots are the %IL-2\(^{+}\) among CD4\(^{+}\) cells. (e) Primary (left) and graphed (right) data for intracellular IL-2 (top), IFN\(\gamma\) (middle), and TNF\(\alpha\) (bottom) assays of LLO56 and LLO118 CD4\(^{+}\) T cells stimulated with PMA + ionomycin. Numbers in plots are the percent of cytokine\(^{+}\) CD4\(^{+}\) cells. All data are representative of at least three experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests. P values for comparisons are as indicated in the figure.
Figure 3.2. LLO56 and LLO118 T cells diverge in their IL-2 responses to specific or nonspecific stimuli.
Figure 3.3. Stronger LLO56 IL-2 responses are linked to greater activation-induced phospho-ERK and basal phospho-TCRζ than LLO118. (a) ERK phosphorylation kinetics of LLO56 and LLO118 T cells stimulated with PMA for the indicated times. (b) IκBα degradation kinetics of PMA-stimulated LLO56 and LLO118 T cells. IκBα band densities are normalized to β-actin for quantitation. (c) Flow cytometric analysis of calcium flux in LLO56 and LLO118 T cells, with one measurement taken every second. (d) Basal p21-TCRζ phosphorylation in unstimulated LLO56 and LLO118 whole cell lysates. Densities of p21 bands are normalized to p16-TCRζ, and are reported relative to LLO56. All data are representative of at least three independent experiments. Bar graphs depict means ± SEM, with statistical analysis done using unpaired two-tailed Student’s t tests.
Figure 3.3. Stronger LLO56 IL-2 responses are linked to greater activation-induced phospho-ERK and basal phospho-TCRζ than LLO118.
Figure 3.4. Validation of LLO56 and LLO118 signaling results. (a) Immunoblot and quantitation of phospho-ERK kinetics of purified LLO56 and LLO118 CD4+ T cells, representative of two experiments. (b) Confirmation of p21 band in 4G10 blots of unstimulated CD4+ whole cell lysates as phospho-TCRζ using rabbit polyclonal anti-ζ serum 777. 4G10 and anti-ζ serum staining was performed on the same blot. LLO56 was used for this validation as it gave the most easily detectable p21 band. Data are representative of at least three experiments.
Figure 3.4. Validation of LLO56 and LLO118 signaling results.
Figure 3.5. Strength of intrinsic IL-2 responses and signaling in polyclonal B6 CD4+ and CD8+ T cells correlates with CD5 expression. (a and b) CD4+ and CD8+ T cells were stimulated as indicated and gated into four equal fractions (Q1 through Q4, from lowest to highest CD5 expression). Fractions were analyzed for IL-2 production (a) or ERK phosphorylation (b). Primary (upper panels) and graphed (lower panels) data are presented. (c) Basal TCRζ phosphorylation in whole cell lysates of unstimulated B6 CD4+ and CD8+ T cells FACS-sorted from the Q1 and Q4 CD5 fractions. Densities of p21 bands are normalized to p16-TCRζ, and are reported relative to Q4. (d-f) Analysis of IL-2 production (d), ERK phosphorylation (e) and basal TCRζ phosphorylation (f) in sorted naive conventional (CD44lo-int, CD25-, NK1.1-) CD4+ and CD8+ T cells. All data are representative of at least three independent experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests.
Figure 3.5. Strength of intrinsic IL-2 responses and signaling in polyclonal B6 CD4+ and CD8+ T cells correlates with CD5 expression.
Figure 3.6. CD5\textsuperscript{hi} B6 CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are more responsive to stimulation than their CD5\textsuperscript{lo} counterparts. (a) Primary (top) and graphed (bottom) data from IL-2 capture assay analysis of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells stimulated with αCD3+αCD28. Cells were gated into four equal fractions (Q1 through Q4, from lowest to highest CD5 expression). (b) Comparison of CD5 expression on stimulated and unstimulated CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. (c) Overlays of FACS-sorted CD5 fractions (Q1 through Q4, from lowest to highest CD5 expression) from stimulated B6 CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in intracellular IL-2 and phospho-ERK assays. Data are representative of two or three experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests.
Figure 3.6. CD5\(^{hi}\) B6 CD4\(^+\) and CD8\(^+\) T cells are more responsive to stimulation than their CD5\(^{lo}\) counterparts.
Figure 3.7. Functional attributes of LLO56 and LLO118 T cells emerge during positive selection, during which LLO56 receives a stronger signal from selecting self-pMHC. (a) Analysis and quantitation of LLO56 and LLO118 thymocyte subsets and total thymic cellularity, compiled from 12 (LLO118) or 13 (LLO56) thymi. (b) Expression of markers reflecting avidity for self-pMHC in DP (pre-selection) and CD4SP (post-selection) thymocytes, representative of at least three LLO56 and LLO118 thymi each. (c and d) Intracellular IL-2 analysis of PMA + ionomycin stimulated LLO56 and LLO118 thymocytes (c), and ERK phosphorylation of LLO56 and LLO118 thymocytes stimulated with PMA for 3 minutes (d), gated and analyzed by subset. Data are representative of at least three experiments. (e) Basal p21-TCRζ phosphorylation in FACS-sorted, unstimulated DP and CD4SP thymocytes (1.7x10^6 cells per lane) from LLO56 and LLO118 mice. Densities of p21 bands are normalized to p16-TCRζ, and are reported relative to DP thymocytes. Data are representative of five experiments. (f) Annexin V and 7AAD staining of LLO56 and LLO118 thymocyte subsets and peripheral CD4^+ T cells stimulated 24h with 1 μg/mL αCD3+αCD28, representative of four experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests.
Figure 3.7. Functional attributes of LLO56 and LLO118 T cells emerge during positive selection, during which LLO56 receives a stronger signal from selecting self-pMHC.
Figure 3.8. Detailed analysis of selection of LLO56 and LLO118 thymocytes, and the intrinsic responses of mature AND T cells developed on different MHC backgrounds.

(a) Identification of post-selection (TCR<sup>hi</sup> CD69<sup>+</sup> thymocytes) from total viable LLO56 and LLO118 thymocytes, representative of at least three experiments. (b) Frequencies of NK (CD3<sup>-</sup> NK1.1<sup>+</sup>), NKT (CD3<sup>+</sup> NK1.1<sup>+</sup>), and γδ T cells (GL3<sup>+</sup>) among DN thymocytes. Data are representative of thymi from three mice. (c) Gradual emergence of LLO56 and LLO118 IL-2 (left) and pERK (right) responses among stimulated thymocytes, occurring along the DP to CD4SP transition (cells gated in fractions labeled 1 through 5). Numbers in plots are the %IL-2<sup>+</sup> cells (left plots) or pERK MFI (right plots). DN thymocytes were gated out for clarity. Data are representative of at least three experiments. (d) IL-2 responses of AND H-2<sup>b</sup> and AND H-2<sup>k</sup> TCR transgenic CD4<sup>+</sup> T cells stimulated with PMA + ionomycin, representative of three experiments. (e) Analysis of various cell survival-associated markers among LLO56 and LLO118 thymocyte subsets and mature CD4<sup>+</sup> T cells, representative of 2 or 3 experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests.
Figure 3.8. Detailed analysis of selection of LLO56 and LLO118 thymocytes, and the intrinsic responses of mature AND T cells developed on different MHC backgrounds.
Figure 3.9. Deprivation of self-pMHC ligands compromises intrinsic IL-2 responsiveness, ERK activation, and in vivo response to Listeria. (a) IL-2 responses of LLO56 and LLO118 T cells transferred to B6 or MHC Class II−/− recipient mice for 4 days. Data are compiled from three experiments; each data point comprising the bar graphs is the frequency of IL-2+ LLO T cells from a single recipient. (b) Phospho-ERK responses of LLO56 and LLO118 T cells transferred to B6 or MHC Class II−/− recipient mice for 4 days. Data are compiled from three or four experiments. The pERK MFI of LLO T cells from each Class II−/− recipient is reported relative to the pERK MFI of LLO T cells from B6 recipients analyzed in the same experiment. (c) Experimental strategy to examine the effect of self-pMHC withdrawal on response to Listeria infection. (d) Expansion of LLO56 and LLO118 T cells either deprived or not deprived of self-pMHC 7 days post-transfer to Listeria-infected B6 mice. Data are compiled from 6 experiments (LLO56) or 4 experiments (LLO118). Bar graphs depict means ± SEM. For (a) and (b), statistical analyses done using unpaired two-tailed Student’s t tests; for (d), paired two-tailed Student’s t tests were used. The number of animals (n) analyzed in each experimental group is given in the figure.
Figure 3.9. Deprivation of self-pMHC ligands compromises intrinsic IL-2 responsiveness, ERK activation, and in vivo response to *Listeria.*
Figure 3.10. Loss of LLO56 and LLO118 IL-2 responses in adoptive transfer experiments tracks with removal of self-pMHC, independent of recipient-derived T cells. (a) Comparison of cell surface phenotype following 4-day transfer of LLO56 and LLO118 T cells to B6 or MHC Class II<sup>−/−</sup> recipients, representative of at least three recipients. (b) IL-2 responses of LLO56 and LLO118 T cells transferred to Cα<sup>−/−</sup> or H2- M<sup>−/−</sup> recipient mice for four days. Data are compilations of four experiments. (c) IL-2 responses of LLO56 T cells transferred to H2-M<sup>−/−</sup> recipients for the indicated periods of time. For (b) and (c), data are compilations of three or four experiments; each data point comprising the bar graphs is the frequency of IL-2<sup>+</sup> LLO T cells from a single recipient. The number of recipients (n) analyzed in each experimental group is presented in the figure. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests.
Figure 3.10. Loss of LLO56 and LLO118 IL-2 responses in adoptive transfer experiments tracks with removal of self-pMHC, independent of recipient-derived T cells.
Figure 3.11. CD5 antagonizes signal from self-pMHC and intrinsic IL-2 and pERK responses. (a and b) Expression of functional markers in WT and CD5<sup>−/−</sup> LLO56 (a) and LLO118 (b) thymocyte subsets and peripheral cells. (c) Intrinsic IL-2 response in WT and CD5<sup>−/−</sup> LLO56 and LLO118 T cells. (d) Phospho-ERK response in WT and CD5<sup>−/−</sup> LLO56 and LLO118 T cells. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests. Displayed data are representative of two (for LLO118) or three (for LLO56) experiments.
Figure 3.11. CD5 antagonizes signal from self-pMHC and intrinsic IL-2 and pERK responses.
4.1 Differential recognition of C-terminal extended LLO peptides

It is well established that T cells can somehow sense the presence of peptide residues not residing within the peptide binding pocket. For instance, a study examining CD4\(^+\) T cell hybridomas specific for human CLIP/I-A\(^b\) pMHC complexes demonstrated a requirement for peptide residues occupying the P10 and P11 positions\(^{104}\). Importantly, residues beyond P11 did not contribute to T cell responses. Another study of T cell hybridoma recognition of CLIP/I-A\(^b\) demonstrated a remarkable sensitivity of the TCR for residues occupying C-terminal positions, such that truncation or conservative substitutions markedly reduced or abrogated T cell activation\(^{105}\). Importantly in these studies, these flanking residues did not affect TCR recognition via altering peptide binding to I-A\(^b\), which has been shown to be a potential factor in at least one instance\(^{106}\).

In studies of the hen egg lysozyme (HEL) epitope comprised of residues 48-63 presented by I-A\(^k\), T cell hybridomas lacking CD4 critically required the presence of the flanking residues W62 and W63 while CD4-sufficient hybrids did not\(^{107}\). Immunization studies with HEL peptides demonstrated stronger responses in mice receiving peptides with HEL residues 62 and 63 present compared to mice receiving peptide lacking these residues. Mice immunized with the different length HEL peptides showed differential V region usage, a finding also made when hybridomas dependent and independent of HEL residues 62 and 63 were compared\(^{84}\).
That the T cell responses to flanking residues are peptide specific, dependent on the distance of the residues from the TCR, and are not influenced by altered MHC binding all suggest that the TCR is key player in the differential responses to C-terminally extended peptides. That peptides with and without flanking residues can expand different T cell populations in vivo further supports this point. However, the structural mechanisms mediating such recognition by the TCR are unclear. A structural study of I-A\(^b\) complexed with human CLIP peptide demonstrated that C-terminal flanking residues are solvent accessible and, thus, may be accessible by the TCR\(^{87}\). The binding footprint of TCR on pMHC would suggest that CDR1\(\beta\) or CDR3\(\beta\), with their proximity to the peptide C-terminus, would be likely candidate regions important in an putative interaction with flanking residues\(^{76, 84}\). A peculiar finding was made in the context of structural studies of an HLA-DR1 bound peptide derived from HIV Gag, whose C-terminal flanking residues formed a hairpin loop with residues P9-P13 that was required for activation of a human HIV-specific T cell\(^{108}\). How the TCR engages a peptide in such a configuration remains unclear without a TCR:pMHC cocrystal structures.

The LLO56 and LLO118 TCRs provide a unique opportunity to understand how the TCR interacts with C-terminal residues extending from the MHC Class II peptide binding pocket. The high degree of structural similarity between these TCRs, where most of the amino acid differences are concentrated at CDR3\(\beta\), will facilitate our ability to pinpoint the foci important for recognition of these extended peptide residues. While we have generated useful insights and hypotheses from our peptide mutagenesis studies, elucidation of the differences between LLO56
and LLO118 interaction with LLO peptide flanking residues will ultimately require detailed biochemical and structural studies. These approaches will prove invaluable in clarifying several issues. First, they will determine whether LLO56 and LLO118 TCRs make contacts directly with peptide flanking residues. Second, it will suggest whether charge repulsion between LLO56 CDR3β and the peptide C-terminus is a plausible mechanism for its poor response to LLO peptides lacking residues 202 and 203. Third, we can assess the extent to which the presence of flanking residues alters TCR binding kinetics and thermodynamics.

Finally, structural approaches may suggest alternative mechanisms by which the TCR senses peptide flanking residues. Of particular interest is whether the TCRs are responding to an indirect conformational effect of the flanking residues on amino acids occupying the binding groove. There is precedent for this in the interactions of I-E\(^k\) MHC with Hb(64-76) and D73 peptides, which differ by a single methylene group at the P6 MHC contact position\(^{26}\). This slight change of a side chain not accessible by the TCR slightly shifted the backbone conformation and caused the P8 Ile residue to adopt an altered rotameric configuration, which could be detected by the TCR. Mutagenesis studies showed that the 3.L2 TCR was least tolerant of changes at P8, which provides a mechanistic explanation for how such a small change could so profoundly affect TCR recognition.
4.2 Intrinsic T cell responsiveness set and sustained by avidity for self-pMHC

We extensively characterized the \textit{in vitro} and \textit{in vivo} reactivity of LLO56 and LLO118. Inasmuch as LLO118 showed superior \textit{in vivo} expansion during primary responses to LM compared to LLO56, LLO56 had an intrinsically stronger capacity to mount IL-2 responses, as revealed by nonspecific stimuli which bypass the TCR. Experiments done with T cells from B6 mice confirmed the authenticity of this phenotype for naive and bulk CD4$^+$ and CD8$^+$ T cells. Remarkably, that LLO56 and LLO118 have such different biology despite identical affinities for cognate pMHC underscores the notion that factors besides overall TCR binding strength for cognate antigen play key roles in determining T cell biology.

LLO56 and LLO118 have compensating two-fold differences in binding kinetics which underlie their similar affinities, a finding whose significance is presently unclear. Earlier studies of TCR:pMHC binding kinetics using surface plasmon resonance-based assays have correlated dissociation kinetics (“off-rate”) and T cell activity$^{9, 13}$. Recently, however, the importance of association kinetics (“on-rate”) was strikingly demonstrated by “2D $K_D$” measurements of TCR:pMHC affinity, in which binding measurements were made with TCR and pMHC in a cellular context$^{32, 33}$.

The primacy of on-rate in \textit{in situ}, cell-based binding experiments is likely due to TCR clustering and constrained ligand orientation and diffusion in the plasma membrane, all of which would encourage rebinding and rapid association. As surface plasmon resonance makes measurements
with one binding partner completely immobilized and the other free in solution, it inadequately captures essential features of the TCR:pMHC interaction as occur at a cell-cell contact. A good example of this comes from one recent study, in which the binding properties of the L\textsuperscript{d}-restricted 42F3 TCR to a peptide called p3A1 were determined\textsuperscript{109}. While 42F3 bound p3A1/L\textsuperscript{d} in SPR experiments with relatively high affinity, this peptide was completely unstimulatory. 2D K\textsubscript{D} and multimeric pMHC binding both demonstrated poor binding of 42F3 to p3A1, consistent with its poor agonist activity. Crystal structures of 42F3 TCR with p3A1/L\textsuperscript{d} revealed that the 42F3 TCR docked onto the pMHC with a different orientation from most other TCR:Class I MHC complexes. While the precise mechanism for this behavior remains speculative, it is clear that binding measurements that take cellular spatial constraints into account better associate with agonist activity. Future 2D K\textsubscript{D} studies will undoubtedly yield valuable insights into the biophysical features of the TCR:pMHC interaction associated with useful CD4\textsuperscript{+} T cell responses.

Next, we showed that the intrinsic responsiveness of the LLO56 and LLO118 T cells was set during thymic education in apparent proportion to TCR:self-pMHC avidity. There has been great interest in understanding the factors that determine the fate and function of developing T cells in the thymus. For conventional \(\alpha\beta\) T cells, iNKT cells, and Tregs\textsuperscript{110, 111, 112}, considerable evidence has demonstrated that TCR interaction with self-ligands provides essential instructive signals that drive maturation into these lineages. It is indeed a fascinating concept that what the TCR “sees” can guide an uncommitted thymocyte toward one of a variety of cell types with quite disparate effector functions. This begs the question of how TCR ligation of self-moieties induces distinct developmental signals leading to such profoundly different fates.
Recent work utilizing Nur77-GFP as a reporter of TCR signal strength\(^{46}\) demonstrated that Treg and iNKT cells receive a stronger signal from selecting ligands than conventional αβ T cells. This was corroborated by studies demonstrating that such agonist-selected T cells indeed require strong TCR signals and store-operated calcium entry for their development\(^{113}\). Our studies show that the nature of the selecting signal from the Class II self-pMHC provides instructive signals that set the functional properties of CD4\(^+\) T cells, including intrinsic IL-2 responses and proclivity to cell death.

In terms of signaling, TCR ligation of self-pMHC complexes has generally been studied by analyzing basal activation of proximal TCR signaling pathway components like TCR\(\zeta\)\(^{54, 57, 94}\). Whether this basal TCR signal from self-pMHC has downstream effects has received less attention, but might be expected given the many aforementioned functions of TCR:self-pMHC interactions\(^{47, 48, 49, 50, 51, 53, 56, 90}\). Recently, it was shown that B cells receiving a stronger signal from endogenous antigen gave stronger calcium responses upon stimulation, but similar behavior was not observed in T cells\(^{114}\). Our studies, using PMA as a tool to provoke responses far downstream of the TCR, demonstrated a link between ERK phosphorylation and TCR avidity for self-pMHC. Deprivation of self-pMHC reduced the PMA-induced ERK phosphorylation, directly implicating TCR:self-pMHC interactions in maintaining the strength of this response. Thus, TCR ligation of self-pMHC complexes impacts tonic signaling of mature T cells to a greater extent than previously appreciated, such that even PMA stimulation cannot bypass its influence.
An interesting mechanistic possibility for this draws from the demonstration that slight changes in signal strength from thymic self-pMHC could induce different selection outcomes and distinct patterns of localization of ERK. In that study, stronger, negatively selecting signals promoted plasma membrane positioning of ERK, while weaker, positively selecting signals promoted localization of ERK to the Golgi apparatus. Applying those concepts to our system, the stronger proximal signaling from self-pMHC in LLO56 T cells could better facilitate assembly of signaling components to the plasma membrane, thus concerting and strengthening ERK activation upon stimulation.

Strikingly, our results provide the first evidence to our knowledge that removal of self-pMHC compromises a CD4+ T cell response to an infection. This was in accord with all of our other self-pMHC withdrawal experiments in that LLO56, which receives stronger tonic signaling from self-pMHC, showed greater functional detriment when self-ligands were removed than did LLO118. However, given that LLO56’s heightened propensity to cell death also tracked with its stronger interactions with self-pMHC, one might expect that self-pMHC withdrawal would mitigate the amount of cell death seen, leading to improved rather than exacerbated in vivo expansion.

That LLO56 nevertheless expanded more poorly than LLO118 whether the cells were deprived of self-pMHC or not suggested that the same cell-death driven differences in expansion were manifest in our self-pMHC withdrawal model as in our standard infection model. This may be a consequence of the fact that all cells, deprived of self-pMHC or not, were ultimately transferred to B6 mice for the Listeria infection. Even if cells deprived of self-pMHC were
primed as such, they would eventually be able to encounter the self-pMHC milieu normally present in B6 mice. It is possible that even if LLO56’s proclivity to cell death phenotype were reset by the initial withdrawal of MHC Class II, it could rebound in the presence of self-pMHC over the course of the 7-day response to the infection.

Recently, it was reported that CD4\(^+\) T cell self-reactivity correlates with affinity for cognate pMHC and efficacy in responding to pathogen \(^{94}\). This study argued that because more strongly self-reactive cells are more efficiently positively selected, selection effectively enriches the mature T cell repertoire with higher-affinity clones capable of stronger anti-pathogen responses. Our studies with the LLO56 and LLO118 T cells demonstrate that selection not only preferentially admits clones with more self-reactive TCRs to the mature T cell pool, but actively imprints their intrinsic functional properties and basal signaling in apparent proportion to TCR:self-pMHC avidity.

Based on these results, one might understandably predict that the CD5\(^{hi}\) LLO56 T cells should make the most effective responders during infection. However, it is the CD5\(^{lo}\) LLO118 T cell that prevails during primary \textit{in vivo} responses to \textit{Listeria}, a finding which challenges the generality of associations made between self-reactivity, affinity for cognate pMHC, and the efficacy of responses to pathogens. It is reasonable to suggest that if selection favors TCRs able to recognize general structural features of pMHC complexes, the resulting repertoire would be enriched with TCRs that might more strongly bind any particular pMHC. However, the repertoire is nevertheless anticipatory with respect to the innumerable variety of foreign peptides.
that might be encountered; there is no direct selection dictating that the handful of TCRs able to bind a specific set of pathogen-derived epitopes must have a particular avidity for self-pMHC beyond that necessary to get the cell through selection. Thus, we would expect that T cells with a suitable affinity for foreign pMHC could be found across the spectrum of CD5 expression. LLO56 and LLO118, which lie on opposite ends of this spectrum yet have identical affinities for LLO/I-A<sup>b</sup>, provide a clear example of this. Ultimately, the discrepancy between the strong <i>in vitro</i> response and weak <i>in vivo</i> expansion of the LLO56 cells results from its propensity to undergo cell death, demonstrating a potential mechanism by which self-reactivity and <i>in vitro</i> sensitivity may be decoupled from <i>in vivo</i> response.

Finally, our results suggest why polyclonal CD4<sup>+</sup> T cells expressing the highest levels of CD5 only showed an average ~2-3 fold greater expansion <i>in vivo</i> than cells expressing the least CD5. It was suggested that since death by neglect would cull the least reactive cells, one might expect the difference between CD5<sup>hi</sup> and CD5<sup>lo</sup> <i>in vivo</i> T cell responses to be relatively modest. The existence of cells like LLO56 and LLO118 suggests an additional explanation: clones whose self-reactivity does not correlate (or correlates negatively) with the strength of their <i>in vivo</i> response would reduce the observed disparity in expansion when CD5<sup>hi</sup> and CD5<sup>lo</sup> T cells are compared. Alternatively, unappreciated phenotypic heterogeneity in the naive CD4<sup>+</sup> T cell pool could impact comparisons of CD5<sup>hi</sup> and CD5<sup>lo</sup> cells. For example, it was shown recently that Ly-6C<sup>-</sup> CD4<sup>+</sup> T cells are enriched in CD5<sup>hi</sup> cells that preferentially developed into induced Tregs, and exhibited poorer <i>in vivo</i> expansion than Ly-6C<sup>hi</sup> cells<sup>116</sup>. It will be of interest to determine whether such a functional dichotomy among naive CD4<sup>+</sup> T cells impacts the biology of the
LLO56 and LLO118 T cells, as well as the responses of polyclonal T cells, in the context of infections.

Indeed, how a given CD4\(^+\) T cell will respond to pathogen challenge involves the interplay of a number of contributing factors. Our work underscores the importance of factors other than affinity for cognate pMHC, including inherent responsiveness to stimulation as set and maintained by self-pMHC, and proclivity to cell death. Notably, thymic education was shown to be a critical inflection point about which these attributes, and thus the performance of CD4\(^+\) T cells during the response to pathogen \textit{in vivo}, were determined.

4.3 Future directions for the LLO TCR transgenic project

The LLO56 and LLO118 TCR transgenic mice have proven versatile and informative reagents, with which we have been able to interrogate a number of issues relevant to T cell activation in response to a pathogenic epitope, such as the role of TCR ligation of cognate, the influence of intrinsic sensitivity set by self-pMHC, the basis for disparate performance in primary or recall responses, and the role of CD5 in T cell signaling in response of TCR:self-pMHC interactions. These studies have opened up many avenues for further investigation.

Our motivation to undertake this project was to understand what characteristics of TCR:pMHC interactions led to the provision of better helper function during infection. While the ability of the cells to expand during infection is an important part of this, it will be important in the future to assess whether the potential of LLO56 and LLO118 to assume different helper phenotypes
differs as the cells respond to LM infection. While LM is known to be a Th1 polarizing infection, we are interested in whether development to other lineages is also affected, such as Th17 and TfH. Whether LLO56 and LLO118 differ in their ability to differentiate into induced Tregs is of particular interest given their marked difference in self-reactivity.

While CD4⁺ T cells are dispensable for primary responses to LM, they are required for mounting robust memory CD8⁺ T cell responses. Using an adoptive CD4⁺ T cell transfer model, we have attempted to test the effect of LLO56 and LLO118 T cells on the ability to provide help to CD8⁺. This type of experiment requires that recipient mice have no other CD4⁺ T cells that could provide help, but which have an intact polyclonal pool of CD8⁺ T cells and an intact ability to present MHC Class II peptides. We first attempted experiments in which 5x10⁵-10⁶ B6 CD8⁺ T cells were transferred along with either LLO56 or LLO118 cells into Cc⁻/⁻ mice, then infected and rechallenged with LM expressing ovalbumin (LM-OVA). As polyclonal CD8⁺ contain OVA(257-264)-responsive cells, one could determine the impact of CD4⁺ help by looking at the strength of the primary and recall CD8⁺ T cell responses to OVA peptide. The problem with this set up is the incredibly low frequency of OVA-reactive T cells in a naive repertoire, estimated to be approximately 1 in 150,000 CD8⁺ cells. Thus, mice were receiving on average 3-6 OVA responsive CD8⁺ T cells, but could easily have received no cells. Thus, many mice did not show CD8⁺ responses to OVA, and the ones that did showed unacceptably high variability.

We also attempted pilot CD4⁺ help experiments using ThPOK⁻/⁻ recipients, which seemed an appropriate model given their lack of CD4⁺ T cells but spared CD8⁺ compartment.
Importantly, the lack of CD4$^+$ T cells does not originate from lack of Class II MHC in these mice, and thus was suitable for assessing CD4$^+$ helper responses$^{119,120}$. However, CD4$^+$ help experiments with this model also gave inconsistent results. Also, a recent study reported that CD8$^+$ T cells deficient in ThPOK showed weaker primary and memory CD8$^+$ T cell expansion in vivo, further demonstrating that this model is not appropriate for studying CD4$^+$ help of CD8$^+$ T cell responses$^{121}$.

Provision of CD4$^+$ T cell help to B cells could be tested via a relatively uncomplicated model, with LLO T cells transferred to Cα$^{-/-}$ mice prior to immunization. However, LM does not induce a strong antibody response even after multiple rounds of infection, even though provision of anti-Listeria antibody aid resistance to the infection$^{66}$. Attenuated strains of LM, deficient in key virulence factors such as ActA and InlB, are of particular interest given their strong immunogenicity despite markedly reduced virulence. Interestingly, these attenuated strains have been shown to raise strong humoral responses after repeat vaccination$^{122}$. The greater antibody response may be due to the larger dose of bacteria that are routinely used in infections, taken together with the fact that the bacteria’s reduced infectivity forces a greater extracellular tropism where they may be more effectively recognized by B cells. Attenuated LM expressing various pathogenic epitopes, including anthrax protective antigen and HIV-Gag, have been engineered$^{123}$, allowing examination of the efficacy of these LM strains as vaccine vectors. How CD4$^+$ T cells like LLO56 and LLO118 differ in their respective abilities to impact antibody responses may have direct relevance to the use of attenuated LM as vaccine vectors.
Finally, the basis for LLO56 and LLO118’s strikingly different performance in primary and recall responses LM is another issue we do not yet fully understand, but suggests that different CD4$^+$ T cell clones may be better suited for expansion during these different responses. The poor recall response of LLO118 T cells seemed to be associated with the marked TCR downregulation observed. LLO56 shows the typical pattern of a primary response followed by a stronger recall response, but it is currently unknown to what extent this better response results from proliferation versus cell death. Moreover, though LLO56 and LLO118 show strong proliferation by day 4 of the primary response, their relative proliferation during the massive expansion taking place between days 4 and 7 has not been studied in detail. As CFSE-labeled LLO T cells had nearly completely diluted the dye by day 4, this method is unlikely to be of use in analyzing later proliferative responses. Thus, the extent of cell division will need to be determined by a different method, such as BrdU incorporation.

Cellular metabolism is another unaddressed issue with potential relevance to the in vivo responses of LLO56 and LLO118 T cells is metabolism. It has been shown for CD8$^+$ T cells that long-lived memory cells have greater mitochondrial mass and spare respiratory capacity than short-lived effectors$^{124}$. IL-15, in promoting memory CD8$^+$ differentiation, also promoted increased mitochondrial mass. Based on this, it was suggested that part of IL-15’s role in memory CD8$^+$ development was to induce metabolic changes needed for rapid recall responses. It will be interesting to determine to what extent mitochondrial metabolism impacts the responses of the LLO56 and LLO118 T cells.
Lastly, now that CD5\(^{-/-}\) LLO56 and LLO118 T cells are available, we can assess conclusively whether this molecule impacts LLO T cell responses to *Listeria*. In our previous studies, sorted CD5\(^{hi}\) LLO118 T cells seemed to show weaker expansion than sorted CD5\(^{lo}\) LLO118 cells. The CD5\(^{-/-}\) mice will clarify whether this directly involves CD5 itself or something indirectly selected for by our sorting strategy.
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