An Essential Role for Self-pMHC throughout the Life of a CD4 T Cell: Contributions in the Thymus and Periphery

Stephanie Rodriguez
Washington University in St. Louis

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An Essential Role for Self-pMHC throughout the Life of a CD4 T Cell: Contributions in the Thymus and Periphery

by

Stephanie Nicole Rodriguez

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

December 2014
St. Louis, Missouri
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APL</td>
<td>CD4 single positive</td>
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<tr>
<td>CD4SP</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CFA</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CFSE</td>
<td>Class II-associated invariant chain peptide</td>
</tr>
<tr>
<td>CLIP</td>
<td>Double negative</td>
</tr>
<tr>
<td>DN</td>
<td>Double positive</td>
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<tr>
<td>DP</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELISA</td>
<td>Extracellular signal-related kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LLO</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MACS</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>PMA/I</td>
<td>PMA + Ionomycin</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pERK</td>
<td>Peptide-bound Major histocompatibility complex</td>
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<tr>
<td>pMHC</td>
<td>Pre T cell receptor alpha</td>
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<tr>
<td>Rag</td>
<td>Recombinase activating gene</td>
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<tr>
<td>scTCR</td>
<td>Single-chain T cell receptor</td>
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<tr>
<td>SP</td>
<td>Single-positive</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>TCP</td>
<td>T cell progenitor</td>
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<td>Tg</td>
<td>Transgenic</td>
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ABSTRACT OF THE DISSERTATION

An Essential Role for Self-pMHC throughout the Life of a CD4 T Cell: Contributions in the Thymus and Periphery

by

Stephanie Nicole Rodriguez

Doctor of Philosophy in Biology and Biomedical Sciences
Immunology

Washington University in St. Louis, 2014

Professor Paul Allen, Chairperson

It is well established that self-peptide Major Histocompatibility Complexes (hereafter self-pMHC) are essential for the development of a broad repertoire of mature, self-tolerant CD4 and CD8 T cells. Despite clear knowledge that the pool of self-ligands is critical for positive and negative selection, the exact kinetics and dynamic nature of thymocyte interaction with self-pMHC class II (self-pMHCII) bearing antigen-presenting cells (APCs) during development is still largely a mystery. The enigmatic nature of selecting self-pMHC is not unique to the thymic environment; increasingly evident is the dependence of peripheral T cells on extra-thymic self-pMHC expression, specifically selecting self-pMHC, for their homeostatic maintenance and peripheral functionalities. Unequivocal resolution as to the exact nature of selecting self-pMHC engagement with thymocytes and mature peripheral T cells has been hindered by the lack of known TCR:self-pMHC pairs. Our laboratory has identified the first bona fide, naturally occurring self-pMHCII, which positively selects a known CD4 transgenic (tg) T cell. The gp250/I-E^k ligand is able to positively select the ANDtg CD4 T cell, which recognizes the
agonist ligand MCC/I-E\(^k\). Using this system, we have surveyed and quantified the endogenous presentation of a naturally occurring positive selecting ligand-MHCII complex in the thymus and periphery. The redundant presentation of gp250/I-E\(^k\) on positive selecting cortical epithelial cells as well as tolerance inducing APCs lends credence to an affinity model of thymic selection, dependent upon re-recognition of “public” self-pMHC complexes in the thymus. The peripheral expression of this public ligand is affected by the activation state of peripheral APCs, suggesting that the recent immune status can impact the homeostasis of T cell repertoires unrelated to the ongoing immune response. Importantly, the expression of functional self-pMHCII complex does not correlate with mRNA levels, indicating a need to functionally detect the presence of these important ligands in order to gain a complete understanding of how they orchestrate the critical processes of T cell development and peripheral maintenance.

In an ongoing attempt to more completely understand the early events and cellular interactions critical to thymic selection, we have developed a novel method of \textit{in situ}, intravital, two-photon imaging of the murine thymus. This groundbreaking technique allows for the visualization of thymic selection events that occur with intact vasculature and innervation, providing an advantage over current explantation methods to view intrathymic events. Indeed, published data in regards to the post-positive selection motility of thymocytes in explant preparations appear to overestimate the intrathymic cellular dynamics when compared to intravital imaging. Not only can utilization if the intravital imaging technique give a complete view of ongoing selection events, it is currently the only method to visualize the earliest thymic seeding events. Within 15 minutes of intravenous administration, fluorescently labeled bone marrow cells can be viewed within the thymic vasculature. Over the time of imaging, bone marrow cells can be viewed at
each stage of extravasation into the thymic tissue. The ability to visualize the earliest thymic seeding events will provide a novel method to address longstanding questions in the field of T cell selection.

A T cell’s dependence on recognition of self-pMHC does not end in the thymus; mature peripheral cells continue to require self-pMHC signals for survival. The exact nature of this interaction continues to be a topic of active research. Curiously, it appears that the same ligands experienced during thymic selection play a unique role in naive T cell homeostasis. Investigation of gp250/I-Ek mediated homeostatic maintenance of ANDtg CD4 T cells suggests that this confirmed endogenous selecting ligand can augment homeostatic proliferation. How this weak TCR engagement mediates homeostatic responses is unknown. Hypothesizing that the strength of self-reactivity dictates the degree of homeostatic proliferation, we turned to a different TCRtg system, LLO. We observed that despite only a few amino acid differences in their TCR and equivalent affinity for the same agonist pMHCII, the LLO118 CD4 T cell underwent reduced homeostatic proliferation compared to the LLO56 counterpart. The main difference between these two TCR is the strength of self-reactivity; LLO118 is less self-reactive as indicated by CD5 expression, suggesting that self-reactivity is correlated with homeostatic capacity.
CHAPTER I

Introduction: Self-pMHC in the development and function of mature T cells
Some of the figures presented here represent the data of Wan-Lin Lo and Scott Weber, former graduate student and postdoctoral fellow in our laboratory, respectively. They are credited for their contributions as indicated in each figure legend. That work has been published as articles in *Nature Immunology* 2009\(^1\) and *PNAS* 2012\(^2\).

Every organism faces the challenge of protecting itself from external and internal assaults. Commonalities exist within the entire animal kingdom, however among higher vertebrate species evolution of an adaptive immune response provides the capacity to remember exposure to pathogens and better prepare for future responses. Unlike the non-specific protection afforded by the innate immune system, adaptive immunity offers unique protection against specific pathogens and protects from re-infection, thus the basis of vaccination. All components of adaptive immunity, including: the myriad T cell subsets, antigen-presenting cells (APCs), B cells and antibodies, and primary and secondary lymphoid organs, work in concert to accomplish this protection. In regard to the T cell arm of adaptive immunity, protective capacity is established early in life, and the complicated process of T cell development produces a repertoire of T cells that will protect against infection for the entirety of life. At a cellular level, this is widely the result of the combinatorial diversity exhibited by T lymphocyte antigen receptors and the subsequent selection of effective mature T cells. This chapter will specifically discuss the important steps of T cell receptor generation and T cell quality control via thymic selection, as well as the critical nature in which intrathymic development shapes mature, peripheral T cell repertoire stability and function.

**T cell development is initiated upon progenitor entry into the thymus**

T cells, so named because of their development in the thymus, undergo a complex maturation process before establishing their effector capacities. This begins with the entry of bone marrow
resident progenitor cells into the thymus\(^3\). Because the thymus does not contain self-renewing T cell progenitors (TCPs) these cells must be imported from the bone marrow through the blood. Unknown thymic niche factors control the volume of TCP entry independent of the number of TCP available in the blood\(^4\). Interestingly, the thymus appears to undergo periods of receptivity to TCP entry, and the mechanisms of this gate-keeping are currently under investigation in the field\(^5\). It is clear that the entry of TCPs requires a multi-step adhesion cascade utilizing expression gradients of molecules such as P-selectin and the chemokine CCL25\(^6\). More recent work suggests that the observed waves of TCP entry may be correlated to the temporal regulations of these, and other, adhesive and attractant molecules\(^7\). Regardless the hitherto unknown mechanisms of TCP entry, upon gaining access to the thymic microenvironment TCPs undergo an intricate maturation process on the way to becoming mature, self-tolerant T cells. This developmental process is closely linked to the 3-dimensional architecture and unique stromal environments comprising the thymus. The development of a lifelong T cell pool occurs early in life, and the thymic atrophy that accompanies aging is closely linked to a reduction in thymic output over time\(^8\). An unanswered question in T cell adaptive immunity is how the thymus shape a T cell repertoire that so effectively predicts what TCR will be needed for lifelong protection.

**Thymic architecture and unique cellular composition orchestrate T cell selection**

The thymic microenvironment is an essential factor in the development of a mature T cell repertoire. As indicated above, cues from this environment are essential for the initial recruitment of T cell progenitors (TCPs). From here, the dependence grows. Before a thymocyte rearranges its antigen receptor or depends on self-peptide MHC (self-pMHC) signals for
survival, the thymic stroma provides an uncommitted TCP the Notch ligands required for T cell lineage commitment\(^9,10\). Reciprocal bone marrow (BM) transfers between nude and SCID mice were used to determine that the thymic stroma was crucial for thymocyte development. Nude mice have a defect in epithelial development while SCID mice have TCR rearrangement defects; both are T cell deficient. TCPs from nude mice develop normally in SCID BM recipients, which possess functional thymic environments. However, SCID TCPs cannot develop into mature T cells regardless which thymic environment due to intrinsic defects\(^11\). It is clear that there is a necessary interplay between thymocytes and epithelial cells, each depending on the other for fully efficient development and survival\(^12,13\).

The stages of intrathymic selection are largely segregated into distinct anatomical locations within the thymus. The TCPs enter at the cortical-medullary junction through blood vessels and undergo a concerted journey throughout the thymus. The self-pMHC independent stages of thymocyte development first occur in the outer subcapsular region of the thymus, and subsequently move toward the medulla after surviving the 3-4 day gauntlet of self-pMHC mediated positive selection. Positive selection occurs in the cortex among cortical thymic epithelial cells (cTECs) and very few bone marrow derived APCs, and will be discussed in complete detail later in this chapter. This migratory pattern is thought to depend on the specific chemokines produced by the stromal cells in each location. Tolerance inducing negative selection occurs primarily in the medulla among thymic DCs and medullary thymic epithelial cells (mTECs)\(^14,15,16\). The cortical and medullary regions of the thymus can be distinguished based on the tight packing of bone marrow derived DCs and macrophages in the medulla and their absence in the cortex. Furthermore, mTECs are morphologically distinct from their positive
selecting cTEC counterparts, and exhibit broad and sheet-like morphology as opposed to the thin reticular shape of the cTECs\textsuperscript{17}. Due to the dynamic cellular interaction occurring during thymic selection, these morphological distinctions may contribute to the unique functions of the cortical and medullary TECs. The unique steps of thymocyte selection and the critical role of self-peptide expression within the unique thymic environments will be further discussed.

**Two-Photon microscopy and the study of thymocyte selection**

The intrathymic dynamics of T cell development have been of interest for some time. Specifically, the precise kinetics and dynamics of thymocytes during a bona fide positive selection event *in vivo* is unknown. Using state of the art two-photon imaging techniques, investigators have begun to visualize the interaction of thymocytes undergoing intrathymic selection in various *in vitro* and *in vivo* systems\textsuperscript{18,19}. It is still unclear from these systems if positive selection requires a few, long duration contacts with selecting self-pMHC expressing cTECs or many short-lived interactions; some studies indicate that both types of interactions occur in a positive selecting environment followed by directed migration toward the medulla\textsuperscript{19,20}. Elucidating the cellular dynamics of thymocyte:thymic APC interaction during negative selection is likewise of interest. Thymocytes that enter the medulla after positive selection are thought to have a fast motility rate that allows them to make multiple and transient contacts with medullary DCs and mTECs; negatively selected thymocytes are then thought to slow their motility and remain in a confined zone, engaging with medullary APCs for longer periods of time before being quickly engulfed by medullary macrophages after dying\textsuperscript{21}. The frequent and abundant contact of thymocytes with medullary APCs may be key for central tolerance, allowing thymocytes to interact extensively with the thymic environment in search of self-pMHC signals.
As no in vitro system fully replicates in vivo T cell development, it is necessary to develop in vivo systems for investigation. Unequivocal determination of the kinetics and cellular dynamics involved in positive selection have been hindered by the available systems, of which CD8 selection models predominant, and in which the study of CD4 T cell selection has been limited to MHC deficient environments and artificial TCR:selecting ligand pairs \(^{20,21}\). As more confirmed endogenous selecting ligand:TCR pairs, such as the CD4 ANDtg:gp250/I-E\(^k\) positive selection system\(^1\), are identified, visual evaluation of bona fide selection events will be tremendously informative.

Advances in microscopic techniques allowing for increased time and depth of imaging have lead to optimism in regards to being able to visualize a selection event unfold. However, to date, visualization of thymocyte selection has required the ectopic growth of thymic rudiments, or explantation and slicing of whole thyi\(^{17,18}\). Visualization of T cell development in situ would be a significant advance. Using current techniques, the initial events of thymic seeding can be investigated using fluorescent imaging because they are intimately linked to the thymic vasculature. Given the known complexities of thymocyte selection and the plethora of cues required for development, it is not unlikely that removing the thymus from its native environment has wide spread effects related to changed in innervation and chemical signals\(^{22,23}\). The work herein describes a novel technique for the in vivo imaging of the mouse thymus. Careful surgical procedures are used to image the thymus with intact vasculature and innervation while reducing the movement cause by cardiac and respiratory functions.
The self-pMHC independent stages of thymocyte development

Upon entering the thymus, thymocytes express neither the CD4 nor CD8 coreceptor, and are termed double negative (DN). DN thymocytes migrate from the cortico-medullary junction toward the outer subcapsular region within the cortex of the thymus. Throughout the DN stages, thymocytes undergo RAG (recombination activating gene) mediated V(D)J recombination in attempts to generate a functional TCRβ chain. At the final DN4 stage, the β chain pairs with the invariant preTα chain and the cell commits to the αβ lineage\textsuperscript{24,25}. Nonhomologous V(D)J recombination generates billions of diverse TCR sequences throughout the random joining of TCR gene segments. Further random modification adds variability centered at the CDR3 region that contacts pMHC. Elegant studies early on showed that MHC restriction was imprinted in the thymus\textsuperscript{26}. While the large pool of unselected thymocytes develops independently of self-pMHC recognition, the final TCR repertoire is only about 5% of this starting population. The quality control mechanisms that pare down this initial TCR pool are referred to as positive and negative selection, and depend on the landscape of self-pMHC presented within the thymus\textsuperscript{27,28}.

The self-pMHC dependent stages of thymocyte development: positive and negative selection

After the self-pMHC independent generation of a TCR β:pTα pair, thymocytes begin their lifelong dependence on self-pMHC. During the transition from CD4\textsuperscript{−} CD8\textsuperscript{−} DN to CD4\textsuperscript{+} CD8\textsuperscript{+} double positive (DP) thymocyte, developing T cells rearrange their TCR β and α loci until a functional receptor is identified via interaction with self-pMHC. Crosslinking of the TCR by self-pMHC recognition results in the inactivation of RAG and subsequent termination of further α chain rearrangement, a phenomenon known as allelic exclusion\textsuperscript{29,30}. Allelic exclusion of the TCR α chain is an incomplete process, and as such there do exist mature T cells that posses two
functional TCRα chains. There is a distinct possibility that these “dual alpha” T cells can uniquely contribute to alloreactivity\(^\text{31}\) and autoimmunity\(^\text{32}\) by allowing for the maturation of a TCR that evades the rigors of thymic selection via piggybacking on a properly selected TCR on the same cell\(^\text{33}\). DN thymocytes that appropriately crosslink their TCR pass the first stage of selection and graduate to become CD4 CD8 DP thymocytes and undergo the gauntlet of positive and negative selection.

**Positive and negative selection**

The TCR diversity generated through V(D)J recombination is dramatically reduced through the processes of positive and negative selection. These processes ensure that the mature T cell pool is comprised of members that recognize pathogens with exquisite specificity while avoiding self-reactivity, and both depend on the interaction of newly generated TCR with self-pMHC molecules\(^\text{34}, \text{35}, \text{36}\). The exact mechanisms as to how a thymocyte can require constant self-pMHC engagement for survival (positive selection) yet undergo apoptosis mediated death as a consequence of the same interaction (negative selection) has been a topic of active investigation for some time. A variety of non-mutually exclusive models have been proposed to address this paradox and, despite the differences, tend to hinge on the affinity of the interaction between TCR and self-pMHC\(^\text{37}\). Without the proper engagement with self-pMHC ligands, DP thymocytes die due to neglect, however if the interaction affinity is too great, the DP thymocytes die of negative selection.
Positive selection requires the weak interaction of DP thymocytes with self-pMHC on cTECs, while negative selection of thymocytes with strong reactivity for self-pMHC is thought to occur primarily on mTECs and medullary thymic DCs\textsuperscript{34, 35, 38}. Many studies, using CD8 systems or detuned agonist peptides, have shown that the selecting self-pMHC repertoire for a given T cell is rare and of a weak affinity; interactions of positive selecting ligands with TCR are often unable to be measured by standard 3D biophysical and tetramer decay methods\textsuperscript{39}, and do not stimulate mature T cells. Our identification of only 1 out of 95 I-E\textsuperscript{k} binding peptides with the ability to positively select an I-E\textsuperscript{k} restricted transgenic T cell further indicates the relatively rare nature of selecting ligands\textsuperscript{1}. It is further suggested that positive selecting and tolerance inducing thymic APCs may display some set of private, non-overlapping peptides that provide additional tests for developing thymocytes\textsuperscript{40}. The mechanisms by which cTECs and negative selecting thymic APCs may present unique self-pMHC, and the impact of this difference on the mature T cell repertoire, will be discussed.

Regardless of the derivation of the selecting self-pMHC, the cascade of intracellular events initiated by the TCR:self-pMHC interaction either lead to survival and CD4 or CD8 lineage commitment, or death. Specifically, negative selection is accompanied by a transient and intense burst of ERK activation and calcium influx, but the survival of positively selected cells depends on the sustained maintenance of these signals\textsuperscript{36, 41, 42}. Like positive and negative selection, the decision to become a CD4 or CD8 single positive (SP) T cell is also thought to depend on the strength of recognition through the TCR, in addition to signals provided via the CD4 and CD8 coreceptors. Within the permissive range for positive selection, it is thought the highest affinity TCRs develop into natural regulatory T cells or innate-like T cells, while those within the mid
range of the selection affinity window become CD4 and the lower end CD8 SP cells\textsuperscript{43, 44}. The strength of interaction, along with the choreographed display of coreceptor during positive selection, results in a temporal discrepancy between CD4 and CD8 SP thymocyte development. No matter the final fate, DP thymocytes initially down-regulate their CD8 coreceptor. Should the thymocyte be selected on a class II self-pMHC, the strong and sustained signal leads to the rapid establishment of CD4 SP cells. However, if the DP thymocyte is receiving class I mediated self-pMHC signals, it must undergo the process of coreceptor reversal in which CD8 is re-expressed. As such, CD8 SP cells require an increased development time of up to four days\textsuperscript{45, 46}.

In addition to the expression, or lack of expression for DN thymocytes, of coreceptors, there are a variety of other surface markers that allow for the identification of discrete stages in thymocyte development. While DN thymocytes are distinguished based on CD44 and CD25 expression, DP thymocytes undergoing positive selection also exhibit characteristic surface phenotypes. Pre-selection DP thymocytes express low levels of CD5, CD69 and TCRβ. Studies indicate that CD69 is an early marker of positive selection, and increases on post-positive selection DP and SP cells, but is not expressed pre-selection DP or DN cells. Positive selection by self-pMHC is also accompanied by increases in CD5 and TCRβ levels\textsuperscript{28, 47}. The timing and subset specific expression of these markers have been utilized herein for the experimental investigation of the role self-pMHC play throughout the life of a CD4 T cell. Specifically, the CD69 expression pattern will be utilized as tool for visualizing positively selected cells within this dissertation. CD5, another marker of positive selection, is also an option for identification of positively selected thymocytes, however its functional role in T cell biology is not firmly established and its usefulness as a marker of weak TCR:self-pMHC engagement is discussed below\textsuperscript{48}. Some think
that within the affinity threshold of positive selection, CD5 expression levels may dictate a T cell’s peripheral functionality\textsuperscript{2, 49}, which is an attractive hypothesis given the tremendous consequences of thymic selection on the mature T cell functionality and repertoire\textsuperscript{50}. It is not entirely evident what biological impact positive selection has on the mature T cell, and some believe that thymic selection may enrich for TCR that better recognize foreign antigens\textsuperscript{51}. Indeed, work from our laboratory confirms the intimate relationship between a thymocyte’s reactivity to self-pMHC in the thymus and its effector capacities as a mature T cell, though perhaps not in a simply correlative manner\textsuperscript{52}.

The early discoveries of MHC self-restriction and of TCR binding peptide loaded MHC complexes illuminated many key elements of immune recognition, but did not clarify how self-tolerance was established\textsuperscript{53, 54}. Along with other laboratories, our laboratory provided some of the first clues that self-tolerance developed in the thymus. Using the Hb/I-E\textsuperscript{k} system, endogenous self-pMHC were identified on both peripheral and thymic APCs, suggesting that thymus might be the location of self-tolerance induction\textsuperscript{55}. It is now known that the immunological sense of self is defined in the thymus via positive selection and enforced via negative selection\textsuperscript{51}. The elimination of strongly self-reactive thymocytes before they escape into the periphery is one key component of self-tolerance and autoimmunity prevention. Though this primarily occurs in the distinct medullary region of the thymus, should a thymocyte interact too strongly with self-pMHC at any thymic location, it can be deleted. The molecular mechanisms that allow the same TCR to signal dramatically different cell fates, positive selection induced proliferation or negative selection induced apoptosis, in response to slightly different ligand affinities is an
ongoing area of investigation, and may depend on nonexclusive mechanisms involving changes in TCR and CD5, voltage-gated sodium channels and intracellular signaling molecules.\textsuperscript{27, 56}

**Establishing the self-pMHC landscape**

The expression of self-pMHC expressing APCs is not uniform within the thymus, and it is clear that the cortical or medullary location of a self-antigen will impact the developmental stage at which the thymocyte experiences the self-pMHC; note that DP cells can be negatively selected in the cortex if the TCR engagement is of sufficient affinity. One mechanism for the differential expression of self-pMHC throughout the thymus is the utilization of distinct processing machinery. As in the periphery, housekeeping and immunoproteasomes are both available for the generation of pMHCI complexes; the immunoproteasome is specifically expressed in mTECs and medullary thymic DCs and is independent of IFN\textgreek{g} stimulation. cTECs constitutively express a version of the housekeeping proteasome called the thymoproteasome containing the \( \beta5t \) subunit, and generate their own diverse self-peptide repertoire. Using different proteasomal processing ensures that cTECs can display disparate self-pMHCI from mTECs and DCs.\textsuperscript{57} Further processing differences are evident within the MHCII system as well. cTECs utilize thymus-specific-serine-protease (TSSP) and Cathepsin L, specifically, for degradation of proteins in the endosomal and lysosomal compartments, while mTECs and DCs utilize Cathepsin S, thus generating distinct peptide pools for MHCII expression.\textsuperscript{58} These mechanisms allow thymic APCs to present both unique, private self-pMHC ligands, as well as overlapping, public, ligands. Overall, the differences between cortical and medullary thymic APCs’ processing pathways illuminate a selection system in which the negatively selecting components most closely resemble peripheral APCs.
In addition to the antigen processing differences between the cortex and medulla, there are also important genetic differences. Though cTECs and mTECs are derived from a common Foxn1 dependent precursor\textsuperscript{59}, unique gene expression further allows for the differential expression of self-pMHC that likely dictates intrathymic selection. mTECs are particularly notorious for promiscuous gene expression; this trait is dependent on expression of AIRE, and the transcriptional mechanisms by which AIRE promotes the ectopic expression of tissue restricted antigens is being investigated. Though previous investigations as to the thymic APCs sufficient for negative selection highlighted the importance of DCs over mTECs\textsuperscript{38}, recent studies have confirmed the ability of mTECs to not only express tissue restricted antigens, but present them to thymocytes and induce negative selection\textsuperscript{60}. Prior to these studies, it had been thought that mTECs simply provide a reservoir of self-antigen for medullary DCs through cross presentation mechanisms. It is clear that DC play a critical role in central tolerance, and that they retrieve their self-pMHC cargo through a variety of mechanisms, including cross-presentation of mTEC antigens and direct membrane transfer; immigrated DCs are also known to present peripherally and blood derived self-antigens within the medulla\textsuperscript{61,62}. The differential self-pMHC expression among thymic APCs is an interesting phenomenon addressed herein. A complete understanding of the self-pMHC ligandome is essential for understanding how the mature T cell repertoire is shaped. Critically, these peptides inform the generation of T cells that anticipate all future pathogenic exposures at the beginning of life.
Subthreshold ligands: the effect of weak TCR engagement with self-pMHC

Self-pMHC are critical ligands in the life of a T cell. The interactions between the TCR of naive cells and self-pMHC, like that between thymocyte TCR and self-pMHC, are weak and do not typically activate the T cells. Though the T cell is not activated, this signal is registered through the TCR, as indicated by partial tyrosine phosphorylation of the TCRζ chain which is often used as an indication of weak TCR engagement\(^63\). Various effects have been proposed to result from TCR interaction with weak ligands. During positive selection, these weak interactions provide the essential stimuli for selection and the subsequent survival, proliferation and maturation of DP thymocytes. Careful investigation showed that despite lower levels of TCR expression, pre-selection thymocytes are more sensitive to signals through the TCR than their mature counterparts\(^64\). Our lab has recently shown that this increased sensitivity can be attributed, in part, to expression of specific voltage-gated sodium channels that allows for sustained calcium flux in response to weak interaction with positively selecting ligands\(^39\). Interestingly, the decreased sensitivity of mature T cells compared to DP thymocytes does not preclude the peripheral engagement of mature TCR with selecting self-pMHC.

The effects of peripheral recognition of self-pMHC by T cells is a topic of much debate, with data suggesting roles for self-pMHC in naive cell homeostasis, as discussed below, increased recognition of foreign antigens, and the peripheral prevention of autoimmunity. Some studies also point to a role for selecting self-pMHC in coagonism\(^1,39,65\), perhaps through sustaining the TCR sensitivity to foreign pMHC through partial tyrosine phosphorylation of TCRζ and polarization of TCR signaling components. Others suggest that TCRs that bind well to weak ligands in the thymus are simply poised for increased recognition of foreign antigens in the
periphery\textsuperscript{66}. Work from our laboratory does not confirm this finding. In the LLO TCRtg system, which is utilized herein and described in detail below, the CD5\textsuperscript{High} LLO56 CD4 T cell recognizes self-pMHC with a higher affinity than LLO118, as indicated by CD5 expression levels. A key component of this system is both TCR recognize foreign antigen with the same affinity. However, LLO118 excelled in terms of expansion during the primary response to \textit{Listeria monocytogenes}\textsuperscript{2}. The discrepancy in finding from different groups suggest that more work is required to unequivocally determine the exact mechanisms by which thymic self-recognition informs that peripheral immune response. Additionally, separate work using syngeneic skin grafts in the presence or absence of self-pMHC indicate that self-pMHC recognition is necessary for the efficient prevention of autoreactivity\textsuperscript{67}.

The recognition of weak TCR ligands is thus a critical area of investigation, with important questions remaining as to how the sensitive thymocyte reactivity allowing positive selection can be modulated to prevent autoreactivity among mature T cells using the same TCR. One molecule of interest is CD5. Aside from its role as a marker for self-reactivity, it is thought to be a negative regulator of TCR signaling\textsuperscript{68}. TCR with recognition on the high end of the positive selection spectrum seem to be associated with higher CD5 levels both within the thymus and the periphery, which some speculate detunes the TCR as a preventative measure against reactivity to self-antigens\textsuperscript{37,68,69,70}. Though CD5 KO mice do not exhibit defects in polyclonal T cell development, many key pieces of data using transgenic systems indicate that the CD5 molecule may be an important component of intrathymic selection\textsuperscript{71}. The exact biological functions of this molecule remain enigmatic.
Measuring the affinity of TCR for self-pMHC has proven difficult due to the weak nature of the interaction. Though new technology in the field of 2D kinetic measurements are improving our ability to approximate low affinities, the studies of TCR engagement with weak pMHC have relied on assumptions of affinities based on correlative marker expression and TCR phosphorylation. Furthermore, lack of known, bona fide selecting self-pMHC ligands has forced the use of detuned agonist and antagonist ligands (altered peptide ligands, APLs) to investigate models of self-peptide recognition. The rare nature of cTECs which positively select thymocytes and the weak affinity of self-pMHC for TCR have made identification of endogenous selecting ligands particularly difficult. Though these altered peptide ligands provide an indication of the TCR affinity spectrum and the functional consequences of these affinities, the few known positively selecting ligands often do not resemble their agonist counterparts and as such, peptide mimics may not be the best tool for investigating weak TCR:self-pMHC interactions. Having identified the first naturally occurring CD4 T cell positive selection system, AND:gp250/I-Ek, our laboratory can contribute to the understanding of the mechanisms at play when a bona fide positively selecting ligand interacts with its TCR. This transgenic system and our use of it to investigate the self-pMHC landscape are described below.

**Self-pMHC are required in secondary lymphoid organs**

Numerous studies have contributed to the current understanding of the intricate mechanisms of positive and negative selection, the intrathymic processes that shape the development of a mature T cell repertoire that is both MHC restricted and self-tolerant. Both of these processes are intimately dependent on the recognition of self-peptide MHC (self-pMHC), and the diversity of self-pMHC available during intrathymic selection dramatically impacts the mature T cell
repertoire\textsuperscript{27,73}. Though it is obvious why the deletion of self-reactive thymocytes during negative selection should rely on the recognition of self-peptide presenting MHC molecules, it is less clear why signals from self-pMHC are required for the survival of DP thymocytes during positive selection, especially considering that MHC-restriction, which occurs during positive selection, appears to depend on germline encoded affinity of TCR for MHC\textsuperscript{36,74}. In the late 1990’s, it became clear that some amount of naive T cell homeostasis in the periphery was dependent on self-pMHC, specifically the selecting self-pMHC\textsuperscript{75,76}, indicating a potential link between the self-pMHC mediating positive selection and those found in the periphery.

Mature T cells that have escaped the gauntlet of intrathymic selection still have to compete for survival factors when they enter the periphery. Once selected, they require a constant supply of homeostatic factors to ensure their survival and functionality. Though the thymus produces fewer T cells with age, the peripheral number of T cells remains relatively constant during and after T cell emigration from the thymus. Additionally, the number and diversity of the T cell pool must remain constant after the contraction phase of an immune response, indicating the existence of critical homeostatic mechanisms in the peripheral environment. These include cytokine, specifically IL-7, and self-pMHC:TCR signaling\textsuperscript{77,78}.

Over the course of multiple studies, it has become increasingly evident that self-pMHC provide support for naive T cells to survive in the absence of antigenic stimuli, and proliferate under lymphopenic conditions\textsuperscript{76,77,79}. Further studies indicate a requirement for the peripheral self-pMHC repertoire to reflect the selecting self-pMHC specifically; naive CD4 T cells that develop in a normal T cell environment do not proliferate or transition into CD4\textsuperscript{High} memory phenotype.
cells when transferred into a pMHCII restricted H2DM$^+$ host. However, if the naive CD4 cells first develop in an H-2DM$^+$ environment, they proliferate and convert as is expected when transferred into an H-2DM$^+$ host. Though many investigators have come to this same conclusion, others contend that, though naive CD4 T cells do engage selecting self-pMHCII in the periphery, this interaction is not required for their maintenance$^{63}$, or that is responsible for dampening the naive T cell response to autoantigens$^{67}$ and not homeostasis. Much of the controversy stems from differences in the transfer systems used to study T cell homeostasis; lymphopenic conditions create space that allows naive T cells to proliferate and transition into memory phenotype cells when they would otherwise remain mostly quiescent in a lymphoreplete environment$^{80,81}$. It is argued that the confounding factor of lymphopenia prohibits the investigation of naive cell maintenance in steady state conditions.

Additionally, there are differences in the forms of lymphopenia. Chronically lymphopenic (Rag$^{-/-}$ or SCID) mice are constitutively presenting peptides from nonpathogenic commensal bacteria. However mice that are rendered lymphopenic acutely (sublethal irradiation) have MHC that are predominately loaded with endogenous self derived antigens$^{79}$. Though “self” antigens derived from endogenous or commensal sources are both able to induce homeostatic proliferation$^{82}$, acutely lymphopenic conditions are thought to better illuminate the roles of endogenous selecting self-pMHC in naive T cell homeostasis, which is the topic of investigation in the aims below. Not only does the source of self-pMHC impact the peripheral maintenance of naive T cells, some studies indicate that the affinity of the TCR, dictated in the thymus, intimately regulates a T cell’s homeostatic ability. Not all TCRtg cell lines undergo lymphopenia induced proliferation, and some studies indicate that high affinity interaction with self-pMHC, as indicated by high
CD5 expression levels, is required for peripheral T cell maintenance and functionality\textsuperscript{66, 83}, however this has yet to be unequivocally confirmed. Interestingly, ANDtg T cells from the H-2K\textsuperscript{b} background, have high CD5 expression levels but are not thought to undergo homeostatic proliferation upon transfer into lymphopenic hosts, indicating a need to more closely evaluate the link between CD5, TCR:self-pMHC affinity and peripheral T cell maintenance. Work in this vein is described herein.

**ANDtg:gp250/I-E\textsuperscript{k}: A bona fide, naturally occurring CD4 positive selection system**

Our lab has recently identified the ANDtg CD4 T cell to be positively selected by gp250/I-E\textsuperscript{k}\textsuperscript{1}. Investigation as to the exact nature CD4 selecting self-pMHC has lagged behind similar studies among CD8 T cells due to the inability to identify a naturally occurring selecting self-pMHC\textsuperscript{84, 85}. To this end, previous researchers in our laboratory generated a panel of 95 peptides that naturally bind I-E\textsuperscript{k} on the CH27 B cell line\textsuperscript{86} and they were loaded onto I-E\textsuperscript{k} Ig dimers. DP thymocytes from four separate I-E\textsuperscript{k} reactive CD4tg lines, bred onto non-selecting MHC background to ensure the absence of CD4 SP post selection thymocytes, we analyzed for CD69 upregulation against the self-peptide I-E\textsuperscript{k} panel. Of the 380 potential TCR:self-pMHC\textsuperscript{II} pairs, only one showed CD69 upregulation, AND:gp250/I-E\textsuperscript{k} (Figure 1.1). This is in accordance with early data suggesting that ligands able to select a given TCR are quite rare among the entire self-pMHC population\textsuperscript{87, 88}. In reaggregate cultures with the ANV/I-E\textsuperscript{k} cTEC cell line (a cell line that expresses very few self-pMHC) pulsed with gp250, ANDtg DP thymocytes became CD4 SP thymocytes and upregulated standard markers of positive selection: CD5, CD69 and TCR\textbeta\textsuperscript{ (Figure 1.2)}. In further confirmation of the extreme specificity of a TCR for its selecting self-pMHC, altered peptide ligands (APLs) of gp250 at the predicted TCR contact residues were
unable to mediate the positive selection of ANDtg CD4 SP cells in reaggregate culture (Figure 1.3), despite the fact that these mutations mimicked agonist ligand residues. Of note is the fact that gp250 has no sequence homology to ANDtg CD4 T cells’ agonist peptide moth cytochrome C (MCC)\textsuperscript{1,89}. That this bona fide positive selecting ligand does not structurally mimic the agonist ligand suggests that the popular hypothesis that mimicry of agonist ligands is a predominate purpose of thymic positive selection is likely incomplete.

In addition to identifying gp250 as an endogenous positive selecting ligand and confirming that it is bona fide in its ability to generate CD4 SP cells from DP thymocytes, our laboratory further elucidated that this selecting ligand likely plays roles in the peripheral maintenance and function of mature ANDtg CD4 T cells. Using a novel \textit{in vitro} assay to modulate the ratio of agonist MCC:positive selecting gp250 peptide loaded onto I-E\textsuperscript{k} dimers, we have shown a potential role for gp250 in augmenting ANDtg CD4 T cell responses to agonist ligand, in other words, acting as a coagonist (Figure 1.4). Additionally, exogenous addition of gp250 peptide was shown to increase the recovery of ANDtg CD4 T cells after adoptive transfer into chronically lymphopenic Rag2\textsuperscript{−/−} recipients (Figure 1.5). These data are in line with hypotheses as to the role of self-pMHC in coagonism and homeostasis. Further establishing the role of selecting ligands and TCR self-reactivity in the peripheral maintenance of CD4 T cells is a predominate focus of this dissertation.

The gp250 peptide is derived from the sortilin-related LDL endocytic receptor, encoded by the \textit{LR11} gene. Its transcripts are expressed to various degrees in all known APC subsets \textsuperscript{90,91} (Figure 1.6). To more completely evaluate the selection of ANDtg CD4 T cells by gp250/I-E\textsuperscript{k},
as well as the peripheral role of this naturally occurring positive selecting ligand, we obtained the gp250KO mice from Hideaki Bujo and Yasushi Saito\textsuperscript{92}. We bred them to the ANDtg B6.K Rag1\(^{-/-}\) mice and initially observed that selection of ANDtg CD4 cells was severely diminished in the absence of gp250, with reduced numbers of mature ANDtg CD4 cells making it into the periphery (Figure 1.7). Other than defects in the development of ANDtg CD4 T cells, the gp250KO mice are immunologically normal. Using this system, our laboratory is uniquely suited to investigate longstanding questions in regards to the endogenous landscape of a confirmed positive selecting self-pMHC, in addition to the intricate relationship between an effective, predictive T cell repertoire and the ligands that select it.

**LLO TCRtg system: investigating thymocyte self-reactivity and peripheral T cell function**

As discussed, CD5 expression levels represent the strength of TCR self-reactivity, and this interaction is thought to critically inform the thymic development and peripheral functions T cells\textsuperscript{40,48,67}. Our laboratory has generated multiple TCRtg pairs that recognize the same cognate antigen, but exhibit disparate avidities for self-peptide as measured by CD5 expression. Herein, we utilize the LLO TCRtg system to further investigate how the strength of positive selection and self-peptide recognition impact the homeostatic maintenance of mature, peripheral CD4 T cells. Initially created by Scott Weber, a former postdoctoral fellow in the laboratory, the LLO56 and LLO118 TCRtg CD4 T cells recognize the same immunodominant epitope listeriolysin O (190-205) from *Listeria monocytogenes* (LLO\textsubscript{190-205}/I-A\textsuperscript{b})\textsuperscript{2}. These TCRs were chosen from LLO\textsubscript{190-205}-specific T cell hybridomas having identical V\(\alpha\) and V\(\beta\) variable gene usage, namely V\(\alpha\)2 and V\(\beta\)2. They differ in only 15 amino acids, primarily located in the CDR3 regions. Furthermore, the only discernable difference in surface marker expression was that of CD5
(Figure 1.8). LLO56 exhibits higher CD5 levels than its LLO118 counterpart. Given these findings, we have generated TCRtg CD4 T cells that recognize the same agonist ligand, but presumable have differing affinity for self-pMHCII.

In keeping with the theory that the self-reactivity of a T cell impacts its peripheral function, our laboratory observed a discrepancy in the primary response of the LLO TCRtg CD4 T cells to primary *Listeria* infection. Contrary to correlative studies suggesting that higher self-reactivity begets increased response to foreign antigen\(^{66}\), we observed that the $\text{CD5}^{\text{High}}$ LLO56 cells responded less well to *Listeria* infection compared to LLO118 in terms of the proliferative response in the primary response. This discrepancy was reversed in the recall response, with LLO56 predominating (Figure 1.9). The mechanisms and details of this interesting phenomenon are the work of a previous graduate student in the laboratory\(^ {52}\). Herein, we investigate a potential role for the difference in self-reactivity in the homeostatic maintenance of these LLO TCRtg CD4 T cells.
**Figure 1.1. The screening for positive selecting ligands.** A panel of 95 unique endogenous I-E\(^k\) self-peptides previously identified from a CH27 B cell line\(^{29}\) were individually loaded onto I-E\(^k\)-immunoglobulin (I-E\(^k\)-Ig) dimers, and immobilized on plates along with anti-CD28. Thymocytes from four TCR transgenic mice (AND.\(Rag1^{+/−}\)H-2d, N3L2.\(Rag1^{+}\)H-2b, 2.102.\(Rag1^{+}\)H-2b and A1.\(Rag1^{+}\)H-2b) were incubated overnight in plate and then analyzed by flow cytometry. The initial screens utilized crude peptides (> 80% pure), and those inducing CD69 expression on greater than 1% of thymocytes were regarded as candidate peptides. Those potential candidates identified in the initial screenings were further confirmation with HPLC purified peptides (> 99% pure). Those candidate peptides shown in grey were not confirmed by HPLC-purified peptides, and thus were considered negative. The candidate peptide shown in red was confirmed by HPLC-purified peptide in multiple syntheses. Data are representative of two independent experiments for each TCR transgenic mouse strain. *Lo et al. 2009, Nature Immunology.*
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| Figure 1.1. The screening for positive selecting ligands. Lo et al. 2009, Nature Immunology.
**Figure 1.2. Positive selection of AND TCR–transgenic thymocytes selected by gp250 in reaggregate cultures are phenotypically mature.** (a) Flow cytometry analysis of the expression of CD4 and CD8 by DP thymocytes from newborn AND*Rag1*–*H-2* mice, cultured for 96 h with the I-E-transfected cortical thymic epithelial cell line ANV41.2 (I-E), along with 30 µM gp250 peptide or the control peptide Hb (GKKVITAFNEGLK). Numbers above outlined areas indicate percent positive selected CD4SP cells. Data are representative of five independent experiments. (b) CD4SP differentiation induced by various doses of gp250 peptide (horizontal axis) or 30 µM Hb in cells cultured as described in a. (c) Expression of markers of positive selection on CD4SP and DP cells in reaggregate cultures pulsed with 30 µM gp250 or 30 µM Hb (control peptide). *P < 0.05 (two-tailed Mann-Whitney test). Data represent three-five independent experiments (mean and s.e.m.). Lo et al. 2009, *Nature Immunology.*
Figure 1.2. Positive selection of AND TCR–transgenic thymocytes selected by gp250 in reaggregate cultures are phenotypically mature. Lo et al. 2009, Nature Immunology.
Figure 1.3. Positive selection by gp250 has a high degree of specificity. (a) Aligned amino acid sequences of gp250 and MCC. Residues P2, P3, P5 and P8 are predicted TCR contact residues (red, upward arrows). (b) Frequency of CD4 SP positively selected in reaggregate culture by Hb (negative control peptide), the peptides with single-residue substitutions reflecting agonist residues, or gp250 (30 µM). Results are presented as percent CD4 SP cells among total thymocytes. *$P < 0.05$ (two-tailed Mann-Whitney test). Data represent four independent experiments (mean and s.e.m.). Adapted from Lo et al. 2009, Nature Immunology.
Figure 1.3. Positive selection by gp250 has a high degree of specificity. Adapted from Lo et al. 2009, Nature Immunology.
**Figure 1.4. The gp250 peptide acts as a coagonist for peripheral ANDtg CD4 T cells.**

Coagonist ability of Hb (control peptide), gp250 (a) or gp250 peptides with single substitutions (described in Figure 1.3), tested with I-Eκ–Ig heterodimers loaded with various ratios of peptides (horizontal axis (a) or 1:100 (b)) and coated onto triplicate wells of 96-well tissue culture plates; CD4 peripheral T cells isolated from AND TCR–transgenic (H-2b) spleen cells were cultured on the plates for 3 d and pulsed with [3H]thymidine for the final 18–24 h of culture. Data are representative of five (a) or three (b) independent experiments (mean and s.d. of triplicate determinations (a) or mean and s.e.m. of triplicate wells (b)). *Lo et al. 2009, Nature Immunology.*
Figure 1.4. The gp250 peptide acts as a coagonist for peripheral ANDtg CD4 T cells. Lo et al. 2009, Nature Immunology.
Figure 1.5. Administration of 0.1mg of gp250 enhances survival of homeostatically proliferating ANDtg CD4 T cells. CD4+ peripheral T cells were isolated from AND.Rag1⁻/⁻H-2k spleen cells, CFSE labeled, and transferred to B6.K.Rag1⁻/⁻ mice by retro-orbital injection on day 0. The B6.K.Rag1⁻/⁻ mice were injected intraperitoneally with 0.1 mg gp250 or control peptide (Hb) on days -1 and +1. CD4 and CFSE were analyzed by flow cytometry. (a) The representative dot plots and (b) histogram are representative of eight individual mice from a total of three separate experiments. (c) The total number of T cells recovered from pooled inguinal and auxiliary lymph nodes (mean ± s.e.m.) from three separate experiments from mice receiving either control (Hb, n = 4) or gp250 (n = 4) peptide. *P > 0.05 by two-tailed Mann-Whitney test. Lo et al. 2009, Nature Immunology.
Figure 1.5. Administration of 0.1mg of gp250 enhances survival of homeostatically proliferating ANDtg CD4 T cells. Lo et al. 2009, Nature Immunology.
Figure 1.6. Positive selecting ligand gp250 is derived from an endocytic receptor whose transcripts are variably expressed. (a) Illustration (by Wan-Lin Lo) depicting the transmembrane protein from which the gp250 peptide is derived. (b) NCBI Geo repository of gene expression data shows LR11 expression in various tissues (top) thymic APCs (bottom left) splenic DCs (bottom middle) and naive or activated splenic B cells (bottom right).
Figure 1.6. Positive selecting ligand gp250 is derived from an endocytic receptor whose transcripts are variably expressed.
Figure 1.7. ANDtg CD4 T cells are positively selected by gp250/I-Ek in vivo. The development of CD4 SP cells in the thymus and their presence in the periphery are severely diminished in ANDtg/ gp250−/−/Ragk/k mice (a). Intrathymic injection (courtesy of Wan-Lin Lo) of gp250 peptide can restore selection of ANDtg CD4 SP cells while irrelevant Hb peptide does not; strong agonist peptide, MCC, results in deletion of ANDtg cells (b).
Figure 1.7. ANDtg CD4 T cells are positively selected by gp250/I-E^k in vivo.
Figure 1.8. LLO118 and LLO56 differ only in few TCR amino acids and CD5 expression.

(a) Primary sequence differences in the CDR3α and β as well as CDR2β V-regions of the TCR. Not shown are the CDR regions of perfect sequence identity between LLO56 and LLO118 TCR.

(b) Flow cytometry analysis of common T-cell surface markers. T cells were isolated from the spleen and cell surface markers were directly analyzed without any stimulation. Data are representative of three independent experiments with at least three mice per group each experiment. Adapted from Weber et al. 2012, PNAS
Figure 1.8. LLO118 and LLO56 differ only in few TCR amino acids and CD5 expression.

Adapted from Weber et al. 2012, PNAS
Figure 1.9. LLO118 T cells have a better primary response, whereas LLO56 T cells have a better secondary response to L. monocytogenes infection. (a) CD4+ T cells from LLO118-Ly5.1 or LLO56-Thy1.1 mice were purified by negative selection and 3x10^3 cells were transferred to C57BL/6 recipient mice. The mice were subsequently infected with 1x10^4 CFU of L. monocytogenes and cell numbers were measured 7 d later by flow cytometry. (b) On day 35 after the primary infection recipient mice were infected with 1x10^5 CFU of L. monocytogenes. Four days after the secondary infection cell numbers were determined by flow cytometry. Data are representative of three independent experiments with at least three mice per group each experiment. **P < 0.01 (Student t test). Weber et al. 2012, PNAS
Figure 1.9. LLO118 T cells have a better primary response, whereas LLO56 T cells have a better secondary response to *L. monocytogenes* infection. *Weber et al. 2012, PNAS*
CHAPTER II

Self-pMHCII complexes are variably expressed in the thymus and periphery independent of mRNA expression but dependent on the activation state of the APCs
This chapter has been drawn almost entirely from a manuscript currently in press with *Molecular Immunology*.

**Introduction**

The development and maintenance of a functional and self-tolerant T cell repertoire depends on the continued interaction between TCR: self-pMHC \(^{36}\). It is well established that these self-peptides are constitutively processed and presented \(^{55,93,94}\). Much progress has been made toward elucidating the key mechanisms of thymic selection that result in the production of a mature T cell pool that not only effectively detects self-peptides presented on MHC molecules (positive selection), but also avoids autoreactivity to self-peptides (negative selection). The undisputed requirement for recognition of self-pMHC at both the positive selection and self-tolerizing stages of thymocyte development appears paradoxical, and a variety of non-mutually exclusive models have been proposed to explain how the recognition of self-peptides can be both essential for survival but also potentially deadly to developing thymocytes \(^{27,95}\). Decades of investigation have suggested myriad models hypothesizing how this might be. Early work suggested that positive selecting cortical thymic epithelial cells (cTECs) display unique peptides for positive selection that are not duplicated among APCs involved with tolerance induction in the thymus or periphery. However subsequent work showing overlap between cTEC and splenic eluted peptide \(^{96}\), and *in vitro* assays suggesting the ability of low amounts of agonist ligand or antagonist and partial agonist ligands to induce positive selection have led to more current models emphasizing the avidity and affinity of TCR: selecting self-pMHC interaction \(^{96,97,98}\).

Currently, intrathymic selection models are evolving with our understanding of the thymic microenvironment, and not only account for the affinity of the TCR: selecting self-pMHC
interaction, but the quantity, quality, spatial and temporal characteristics of the selecting ligands and the APCs that present them \(^{35}\). Interestingly, a revival of the discrete peptide model is ongoing as it becomes clear that the APCs vital for positive selection and central tolerance are differentially equipped to process and present unique peptides \(^{91}\). Though elucidation of the exact mechanisms that generate self-pMHC able to mediate thymic selection is ongoing, it is clear they are complicated in nature and challenge our current understanding of antigen processing.

Involvement of antigen processing machinery outside of the “classical” pathways may prove to play a vital role in establishing the selecting ligandome that leads to functional and self-tolerant CD4 and CD8 T cell repertoires \(^{99}\). Unequivocal determination of which model, or more likely, combination of models, most accurately reflects \textit{in vivo} thymocyte selection depends intimately upon gaining an understanding of the landscape of selecting self-pMHC complex expression. Specifically, a clear understanding of if and how the ligand complexes mediating positive selection differ from those orchestrating negative selection will bring clarity to the debate \(^{58,100}\).

Elucidation of the selecting self-pMHC ligandome has proven difficult for a variety of technical reasons \(^{40}\). First, very few naturally occurring TCR: selecting self-pMHCII pairs have been identified and validated \(^{85}\). Given the clear differences between CD4 and CD8 T cell development and maintenance \(^{101,102}\) it is critical to evaluate these two systems independently. Second, the rare abundance of individual thymic APC subsets compounded with the low concentration of any one unique selecting self-pMHC complex makes \textit{ex vivo} biochemical analysis of these complexes challenging, and skewed toward the few most abundant self-peptides \(^{103}\). Alternative routes to investigate the self-peptide repertoire have focused on evaluating mRNA levels \(^{104}\). Unfortunately, surveillance of eluted peptides and message levels do not
provide any insight as to the spatial and temporal nature of the functional unit of selecting self-peptide in complex with MHC. Recent discoveries of TCR: selecting self-pMHCII pairs *in vitro* have opened the field for investigation of naturally occurring selecting ligands, however to date no studies have shown directly the presence of these ligand complexes on the selecting thymic APCs.

It is clear that a T cell’s dependence on self-pMHC recognition does not end in the thymus. In addition to the clear intrathymic requirements for self-pMHC expression, continued engagement of TCR by self-pMHCII is required for the homeostatic maintenance of mature T cells, the mechanisms of which are not yet completely understood. Studies suggest that the same ligands present in the thymus are utilized in the periphery for maintaining the T cell repertoire. Given that the entire life of a T cell depends on continued recognition of (selecting) self-pMHC, it is critical to understand the natural landscape of these complexes in the thymus and periphery. The technical hurdles involved with ex vivo analysis of the peptide repertoire indicate a need to functionally detect these selecting self-pMHC complexes in their natural environments.

Our laboratory has recently identified a naturally occurring positive selecting self-pMHC for the ANDtg T cell, called gp250/I-E\(^k\). The gp250 self-peptide was isolated from self-peptides bound to I-E\(^k\) on the B cell line CH27. In the present report, we have generated a T cell hybridoma specific for gp250/I-E\(^k\). Using this hybridoma, we have functionally analyzed the expression of naturally occurring selecting self-pMHCII complexes on thymic and peripheral APCs. Because T cell hybridoma responses rely solely on pMHC engagement and are not affected by co-stimulatory molecules, this tool will only reflect the presence of gp250/I-E\(^k\)
complexes on surveyed APCs. The results indicate that naturally occurring selecting ligand gp250/I-E\textsuperscript{k} is ubiquitously presented by positive selecting cTECs, negatively selecting mTECs and thymic DCs, as well as peripheral MHCII expressing APCs. These data show for the first time the presence of a naturally occurring selecting self-pMHCII on both cTEC and mTECs, suggesting that despite unique processing capabilities, positive and negative selecting thymic APCs do functionally present overlapping pMHC complexes. Additionally, we observed discrepancies between gene expression and functional complex presentation. Furthermore, the peripheral presentation of this selecting complex changes with recent immune status, suggesting the existence of a dynamic self-pMHC landscape with potential consequences to the existing mature T cell repertoire. These findings support a thymic selection model that merges, not excludes, currently proposed systems, and suggests that investigation of the self-pMHC landscape must include careful functional evaluation of pMHC complexes in addition to broadly visualizing the gene expression and eluted peptide profiles.

**Results**

*Generation of a sensitive T cell hybridoma specific for the positive selecting ligand gp250/I-E\textsuperscript{k}*  
We generated a sensitive T cell hybridoma to investigate the constitutive expression of the naturally occurring gp250/I-E\textsuperscript{k} positive selecting protein complex by various APCs. Attempts to analyze the self-peptide landscape throughout various tissues have previously been performed on a transcriptional level. However, mRNA expression does not always correlate with protein expression, and this correlation is further complicated when focusing on functional pMHC complexes. We sought to use a T cell hybridoma as a tool to functionally detect selecting self-pMHCII complexes. The inability of co-stimulation to affect a hybridoma response means that
this tool will solely reflect the presence of self-pMHC presented by the surveyed APCs. A panel of hybridomas from H-2^k gp250 deficient mice, primed with gp250 peptide in CFA, were tested for activity against the CH27 B cell line from which the gp250/I-E^k selecting complexes were identified biochemically. Reactive hybridomas, identified by IL-2 production after co-culture with the CH27 APC line, were subcloned by limiting dilution three times to achieve clonality (Figure 2.1). Hybrid specificity was tested using gp250 peptide pulsed CHO-E^k APCs. Because this I-E^k restricted cell line very poorly presents endogenously processed peptides, we are able to confirm that hybridoma reactivity is specific to our peptide of interest, and not an unknown peptide entity. The chosen gp250/I-E^k specific T cell hybridoma (out of ~100), herein termed Ga2.6, expresses the Vα4 (TRAV4), Vβ1 (TRBV1) TCR with CDR3 regions that are 10 and 12aa long, respectively (data not shown).

Despite the low concentration of a given self-pMHC on an APC, this small amount of complex is biologically relevant, providing essential stimuli to both developing thymocytes and mature T cells 39,63,64. Because levels of most endogenously presented self-pMHC are very low, it was important that the chosen gp250/I-E^k specific T cell hybridoma could sensitively and specifically detect physiological levels of the selecting self-pMHCI complex gp250/I-E^k. The Ga2.6 hybridoma proved extremely sensitive, producing detectable amounts of IL-2 when activated by as few as 1,000 cells from the CH27 B cell line, and 0.05µM peptide on pulsed CHO-E^k cells (Figure 2.2 A, B). Ga2.6 was tested for broad recognition of gp250 altered peptide ligands (APLs) at the TCR contact residues, and was shown to be completely intolerant to alterations at three out of four contact locations, producing no IL-2 when stimulated with respective APL pulsed CHO-E^k cells. Ga2.6 exhibited flexibility in peptide recognition exclusively at the P8
position, thus exhibiting exquisitely specific pMHCII recognition, as was described for the primary ANDtg CD4 T cell selected by gp250/I-E\(^k\) \(^1\) (Figure 2.2 C). It is known that T cell hybridomas do not depend on adhesion molecules or co-stimulation to become activated \(^{105}\). To verify that Ga2.6 works independently from these factors, and to survey the presentation of selecting self-pMHCII in the absence of adhesion or co-stimulatory molecules, we stimulated Ga2.6 with a soluble gp250/I-E\(^k\) monomer and noted a sensitive response to this stimulus (Figure 2.2 D). To confirm that recognition by Ga2.6 is I-E\(^k\) restricted, we used anti-I-E\(^k\) monoclonal antibodies to block Ga2.6 stimulation. The ability of anti I-E\(^k\) 14.4.4 antibody to block Ga2.6 activation indicates that recognition by Ga2.6 is MHCII I-E\(^k\) restricted (Figure 2.2 E). Together, these characteristics show that the Ga2.6 T cell hybridoma is an ideal functional probe for the detection of endogenously processed and presented gp250/I-E\(^k\).

**Characterization of a sensitive T cell hybridoma specific for the positive selecting ligand gp250/I-E\(^k\)**

Detection of endogenous gp250/I-E\(^k\) was closely investigated using primary splenic or thymic single cell preparations from either wild type H-2\(^k\) restricted or gp250 deficient H-2\(^k\) restricted mice. Using Ga2.6, we were able to functionally detect expression of gp250/I-E\(^k\) protein complexes in both whole thymus and spleen from low numbers of primary cells. As expected based on its role in the selection of the ANDtg T cell, selecting self-pMHCII complexes were detected within whole thymic preparations from gp250 sufficient mice (Figure 2.3 A). Though peripheral expression of self-pMHC is a well-described phenomenon, the purpose of this peripheral expression is hitherto unknown. Using Ga2.6 as a functional tool, we sought to survey the peripheral expression of selecting ligand gp250/I-E\(^k\) within splenic preparations. Ga2.6 can
detect gp250/I-E\(^{k}\) complexes presented by as few as 5,000 primary splenic APCs (Figure 2.3 B). Importantly, single cell preparations from gp250KO/H-2\(^{k}\) mice were unable to promote IL-2 production by Ga2.6. Thymic and splenic preparations from B6 (I-E-negative) mice failed to activate Ga2.6, confirming the I-E\(^{k}\) specific recognition of this hybridoma (Figure 2.3). The intermediary response produced upon stimulation with gp250 heterozygote splenic and thymic preparations, compared to B6.K mice, indicates that Ga2.6 can detect two fold differences in gp250/I-E\(^{k}\) expression (Figure 2.4). Together, these data show that endogenously processed and presented selecting ligand gp250/I-E\(^{k}\) is present in both the thymus and periphery, and can be detected by the T cell hybridoma Ga2.6 in a dose dependent manner.

**Transcript analysis predicts variable expression of the selecting ligand gp250 among APC subsets**

Data repositories such as NCBI Geoprobe\(^{106}\) and ImmGen\(^{107}\) are excellent resources for gaining an understanding of organism-wide gene expression. Due to the technical limitations in peptide analysis from small numbers of cells, transcriptome analysis is often the best available indicator of potential self-peptide expression. Databases indicate that LR11 mRNA, from which gp250 is derived, can be detected in various APC populations, including cortical and medullary thymic epithelial cells; notably, LR11 message is minuscule in dendritic cells from both the thymus and spleen (GEO: GDS4303\(^{90}\), GEO: GDS1655\(^{91}\), GEO: GDS297). In our hands, quantitative PCR analysis of LR11 expression in sorted thymic and splenic APC populations (Figure 2.5) confirms the expression profile described in available databases. Specifically, positive selecting cTECs contain abundant levels of LR11 message; negatively selecting thymic DCs express very low amounts while expression in mTECs is intermediary (Figure 2.6 A). LR11
mRNA was also detected in sorted peripheral APCs. While high expression of LR11 was detected in B cells, much lower levels were observed in splenic DCs (Figure 2.6 B). LR11 message was undetected in gp250 deficient spleen and thymus.

The positive selecting gp250/I-\(E^k\) complex is constitutively and efficiently presented by thymic APCs

Using the Ga2.6 hybridoma, we surveyed FACS sorted thymic APCs for functional expression of the positive selecting self-pMHCII, gp250/I-\(E^k\) protein complexes. We detected gp250/I-\(E^k\) on all three thymic APC populations, cTECs, mTECs and DCs, providing a picture of the naturally occurring selecting self-pMHCII expression landscape (Figure 2.7 A). To our knowledge, this is the first demonstration of a validated and endogenous positive selecting ligand being expressed on both the positive selecting cTECs as well as tolerance inducing mTECs and thymic DCs. This close investigation of selecting ligand presentation within the thymus showed that the hierarchy of LR11 transcript abundance among the cells responsible for intrathymic selection (Figure 2.6) is not reflected in gp250/I-\(E^k\) protein complex expression, as detected by Ga2.6. Despite LR11 transcript expression being most abundant in cTECs, significantly diminished in mTECs, and nearly undetectable in thymic CD11c+ DCs, the Ga2.6 hybridoma detected the most efficient presentation of selecting ligand gp250/I-\(E^k\) on the thymic APCs with the lowest LR11 gene expression. Specifically, CD11c+ DCs exhibited more potent and sensitive stimulation of Ga2.6 than did mTECs. Meanwhile, the expression of gp250/I-\(E^k\) complexes on positive selecting cTECs was present, but low, as detected by the Ga2.6 hybridoma. To elucidate if differences in Ga2.6 stimulation by thymic APCs were due to inherent differences in peptide processing or simply a result of differing pMHCII expression, exogenous gp250 peptide was added to each cell...
type, such that differences in processing would be overridden by direct loading of gp250 peptide onto MHCII I-E\(^k\) molecules. Ga2.6 responds similarly to peptide pulsed primary cTECs, mTEC and DCs, with the DCs tending to be insignificantly yet reproducibly elevated in their self-pMHCII expression (Figure 2.7 B). This equivalent presentation of exogenous peptide indicates that differences in gp250/I-E\(^k\) complex expression likely arise during the endogenous processing of this naturally occurring ligand, and are not due to differences in pMHCII abundance. The hybridoma’s co-stimulation independent functionality further ensures that the differences we see are not due to the variable co-stimulatory capacity of the APC subsets. Of note, all three main thymic APC subsets express similar levels of MHCII (data not shown). These results suggest that the unique endogenous processing machinery of each thymic APC subset dictates the efficiency with which positive selecting ligand gp250/I-E\(^k\) is expressed. Overall, Ga2.6 was able to functionally detect selecting self-pMHCII gp250/I-E\(^k\) complexes on all thymic APCs involved in intrathymic selection. Notably, RNA expression did not correlate with the functional expression of pMHCII complexes as evidenced by Ga2.6.

Constitutive presentation of the positive selecting ligand gp250/I-E\(^k\) in the periphery is affected by the activation state of the splenic APCs.

Self-peptides, including positive selecting ligands, are known to be expressed in the peripheral lymphoid tissues and play essential roles in the survival and activation of peripheral T cells. The precise roles of positive selecting self-pMHCII in the periphery have yet to be unequivocally determined. Understanding the peripheral APC network that expresses these selecting ligands will be critical in elucidating their peripheral function. Immune responses occur in the periphery and lead to explicit changes in the APC network toward the elimination of pathogens. Of interest
is how the landscape of selecting self-pMHCII expression changes in response to unrelated antigenic stimulation. Having already established the detection of gp250/I-E\(^k\) within whole splenic preparations (Figure 2.3), we used the Ga2.6 hybridoma to assess the peripheral expression of positive selecting ligand gp250/I-E\(^k\) on specific APC subsets. B cell and DC populations were enriched from whole spleen by magnetic bead enrichment and FACS (Figure 2.5). gp250/I-E\(^k\) selecting complexes were detected on both B cell and DC enriched and sorted populations (Figure 2.8). Similar to the thymic APC populations, the hierarchy of gp250/I-E\(^k\) protein complex expression did not reflect the LR11 gene expression hierarchy (Figure 2.6). CD11c+ DCs more efficiently present the selecting ligand, despite CD11c+ cells having dramatically less expression of the LR11 gene. This confirms the benefit of a system that functionally detects naturally occurring selecting self-pMHCII protein complexes. Because the proposed roles of self-pMHC in the periphery include augmenting an immune response to foreign antigen, we sought to investigate any changes in the expression of selecting ligand gp250/I-E\(^k\) that occur as the activation state of the APCs change. A close look at the expression of the selecting ligand on peripheral B cells shows that \textit{ex vivo} activation leads to decreased presentation of gp250/I-E\(^k\). Activating B cells using a regimen that mimics immune complex activation, anti-CD40/anti-IgM, reduces the efficiency of self-pMHCII expression. This efficiency is even more dramatically reduced upon activation through TLR 4 using LPS. (Figure 2.9 A) We next sought to investigate any changes in the self-pMHCII landscape during the course of an \textit{in vivo} infection. Three days post infection with \textit{Listeria monocytogenes} B cells (Figure 2.9 A) and DCs (Figure 2.9 B) were enriched from the spleen and surveyed for gp250/I-E\(^k\) expression. In both B cell and DC compartments, activation of Ga2.6 was reduced during bacterial infection. Despite dramatic reductions in the maximal IL-2 production generated in
response to stimulation with *Listeria* infected APCs, the half-maximal cell dose was similar to that of steady-state APCs. Given the well-established role for dendritic cells during the course of a *Listeria* infection, it was not surprising to see changes in the landscape of presented peptides within this APC subset. However, the reduced presentation of gp250/I-E\(^k\) by B cells was curious, as these cells are not known to play a role in the direct presentation of *Listeria* antigen on MHCII. To investigate the extent of *Listeria* pMHCII expression by B cell and DC subsets, *Listeria* specific TCR transgenic CD4\(^+\) T cells (LLO56) were co-cultured with APCs enriched as above. CD69 upregulation was assessed 15-20 hours later as a measure of T cell activation (Figure 2.9 C). As expected, DC enriched populations were efficiently presenting *Listeria* antigens as indicated by CD69 upregulation. Meanwhile, B cell enriched populations appeared to be presenting suboptimal levels of *Listeria* pMHCII. These results suggest that the landscape of selecting self-pMHC is a dynamic entity, experiencing antigen non-specific changes in expression patterns throughout the course of immune responses.

**Discussion**

There are several technical hurdles involved in evaluating the endogenous self-peptide landscape. To date, the most common insight into self-ligand expression is gained through transcriptional analysis. The discrepancy viewed herein between endogenous mRNA and self-pMHCII complex expression is just one clear example of the caution required when evaluating the functional self-pMHC repertoire. The mechanisms behind this modulation may have a direct impact on the overall peptide landscape. Only recently has the presence and impact of post-translational modifications (PTMs) on presented pMHC complexes become clear\(^\text{108}\). Some of the PTMs identified are regulated by immune response factors, such as inducible nitric oxide.
synthase (iNOS)\textsuperscript{109} and have direct impacts on the immunogenicity of presented peptide epitopes. As such, the link between self-pMHC modifications and autoimmunity has been previously described\textsuperscript{110}, suggesting that PTMs may explain the differences between the expression of mRNA and self-pMHCII complexes observed here. It is also possible that the variation between APC subsets can be attributed to the their unique processing machinery. Increasingly clear is that PTMs and MHC processing are not mutually exclusive, and that PTMs can directly and indirectly influence the pMHC landscape.

We were able to functionally detect differences in the peripheral presentation of a single natural positive selecting ligand in response to a bacterial infection. Given the intricate regulation of the pMHC-ligandome, particularly as a result of an immune response, this is not entirely surprising. This suggests that the peripheral self-peptide landscape is dynamic, and has implications for the maintenance of the mature T cell pool. Assuming the widely held paradigm that self-pMHC, in particular selecting ligands, are essential for the peripheral maintenance of T cells holds true, dramatic reductions in the expression of a given self-pMHC might be expected to result in the loss of T cells dependent on that homeostatic signal. In the case of an immune response, there exists the potential to lose T cell pools completely unrelated to the ongoing reactions. In practice, this would likely not be a completely detrimental consequence. Studies with single peptide and selecting peptide deficient mice suggest that a single peptide or peptide family can support multiple T cell clones, and that non-dominant self-pMHC exist that can select a single T cell clone in the absence of the dominant selecting ligand\textsuperscript{111}. This would imply that peripheral T cells ought to receive homeostatic signals from more that one specific self-pMHC. Further work is needed to determine how the self-pMHC repertoire contributes to the maintenance of the
mature T cell pool and fits into the models of immune response driven T cell repertoire fluctuations\textsuperscript{112}.

To our knowledge, this is the first description of the presence of a known MHCII positive selecting ligand that is naturally occurring on both positive and negative selecting thymic APCs. These data support the affinity model for how a CD4\textsuperscript{+} T cell can be positively selected on a "public" ligand, but not be negatively selected by the same ligand\textsuperscript{40}. The non-mutually exclusive possibility that some CD4\textsuperscript{+} T cells are selected by “private” class II bound peptides, uniquely generated by Cathepsin L or TSSP mechanisms, is not excluded by these data. How our findings relate to the positive selection of CD8\textsuperscript{+} T cells was not established in this study. The thymoproteasome, containing the β5t subunit, has been shown to generate "private" peptides in cTECs in addition to the “public” peptides concurrently generated. The currently growing list of proteases being associated with antigen processing suggests we are only beginning to understand the intricate mechanisms and regulation of pMHC generation\textsuperscript{113}. Given the inefficiency of positive selection it is likely that both "public" and "private" peptides are involved in the positive selection of a broad T cell repertoire. It remains to be determined if there are functional differences between such T cells selected on "private" or "public" peptides. "Public" peptides may have a specific role in the peripheral maintenance of T cells, which requires re-recognition of thymic self-pMHC in the periphery. What is clear from this study is that the naturally occurring self-pMHC landscape is intricately modulated. Complete understanding of the self-ligandome will be critical in elucidating the exact mechanisms of thymic development and how this process and the ligands involved impact the T cell for life.
Conclusion

We have generated a sensitive T cell hybridoma that is specific for the bona fide, naturally occurring gp250/I-E\textsuperscript{k} positive selecting ligand. This hybridoma is I-E\textsuperscript{k} MHCII restricted and specific for the gp250 epitope identified as the selecting ligand for the ANDtg CD4 T cell. Using this tool, we were able to assess the expression of gp250/I-E\textsuperscript{k} protein complexes in thymus and periphery. The efficiency of selecting self-pMHCII expression did not correlate with the levels of LR11 gene expression in either location. Despite nearly absent gene expression in the CD11c compartments of the thymus and spleen, Ga2.6 detected the most efficient presentation of gp250/I-E\textsuperscript{k} within these populations. To our knowledge, the detection of this naturally occurring selecting ligand on cTECs as well as mTECs and thymic DCs is the first unequivocal proof that the same self-ligand is present on both positive selecting and tolerance inducing thymic APCs. In the periphery, the extent of gp250/I-E\textsuperscript{k} presentation was dependent on the activation state of the CD19\textsuperscript{+} B cells and CD11c\textsuperscript{+} DCs, with B cells predominating in the naive setting and the DCs playing a bigger role in self-pMHCII presentation while in the mature state. These findings suggest that despite the unique processing capabilities of the thymic APCs, they do present overlapping functional self-pMHCII complexes. In regards to the peripheral presentation of these complexes, the landscape is dynamically regulated, and depends on the recent immune status. In either location, evaluation of the self-ligandome should include functional detection self-pMHC complexes in addition to transcriptional analysis.
**Materials and Methods**

**Mice**

gp250 deficient mice \(^92\) were bred to the B6.K (H-2\(^k\) restricted) line (JAX 001148). The mice were extensively backcrossed to B6.K, and were monitored by the analysis of microsatellite markers at the Rheumatic Disease Core Center, Washington University School of Medicine (St. Louis, MO). The gp250 deficient mice are immunologically normal. gp250 is derived from the sortillin-related LDL endocytic receptor LR11, and while primary expression is in the cortical brain, its transcripts are expressed to varying degrees in all known APC subsets. gp250 has no sequence homology to ANDtg CD4 T cells’ agonist peptide MCC \(^1,89\). The MCC–I-E\(^k\) specific AND TCRtg\(^2\), B6.K (H-2\(^k\)), C57BL/6 (B6, H-2\(^b\)) (JAX 000664) and B6.\(Rag1^{−/−}\) (JAX 002096) mice were purchased from The Jackson Laboratory. The AND TCRtg line was bred onto \(Rag1^{−/−}\) background: AND.\(Rag1^{−/−}\)H-2\(^k\). The LLO TCRtg mice were generated in our laboratory as previously described \(^2,24\). All mice were bred and housed in specific pathogen–free conditions of the animal facility at the Washington University Medical Center. The use of all laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

**Development of gp250-Specific T cell hybridomas**

gp250 specific hybridomas were generated by using a standard fusion protocol \(^55\). Briefly, popliteal lymph nodes were taken 7 to 10 days after immunization of gp250 deficient mice in the hind footpads with 40 nMole of gp250 peptide emulsified in complete Freund's adjuvant H37Ra (BD Difco). A single-cell suspension was prepared and these bulk cultures were fused with the BW5147αβ fusion partner. Successful fusions were enriched by hypoxanthine-aminopterin-
thymidine (HAT) selection. The Ga2.6 hybridoma was subcloned three times by limiting dilution analysis to ensure clonality of the line.

**Flow cytometry and APC isolation**

For the enrichment and purification of thymic APC populations, 5-30 thymi were digested as previously described \(^{114,115}\). Briefly, thymi were isolated into CO\(_2\) independent media (Life Technologies) supplemented with 10% FCS, thymocytes were released and thymic remnants were digested by either stirring at 1200 rpm for 30 minutes in 0.125% w/v Collagenase D (Roche) at 37°C or with 1.8mg/mL Liberase TH using the gentleMACS dissociator Spleen_01 program, followed by a 15 minute rotation at 37°C and final dissociation with program Spleen_02 (Miltenyi). Each digest was supplemented with 0.025% w/v DNaseI (Roche). For the enrichment and purification of splenic B cell and DC populations, 5-10 spleens were digested as with the Collagenase D digested thymi above. CD45, CD11c or CD19 positive and negative populations were separated by magnetic bead positive selection using the respective microbeads (Miltenyi). CD45- and CD45+ thymic populations were stained for cTECs, mTECs and CD11c+ DCs using the following antibodies: UEA-1 FITC (Vector Laboratories), EPCAM (G8.8) PE, MHCII (M5) PECy7, Ly51 (6C3) APC, CD45.1 (A20) or CD45.2 (104) APCCy7, and CD11c (N418) PacBlue (eBioscience or BioLegend). Splenic populations were stained for B cell and DC populations using CD19 (6D5), B220 (RA3-6B2), CD11c and MHCII. APCs were sorted on the FACSARiaII into serum and plated at the indicated concentrations in 96-well flat bottom plates.
**Cell lines, peptides and monoclonal antibodies**

The CH27 (H-2\(^a\), I-E\(^k\)) B cell line, the MHCII I-E\(^k\) transfected Chinese hamster ovary (CHO-E\(^k\)) cell line, and the BW5147αβ cell line, have been described\(^{116,117}\). The CHO-E\(^k\) cells were cultured in MEM-\(\alpha\) containing 5% (vol/vol) bovine growth serum (Hyclone) and 0.5 mg/ml of G418, and 50\(\mu\)M 2-mercaptoethanol. BW5147αβ cells and the Ga2.6 hybrid generated here were grown in Iscove's medium containing 20% (vol/vol) FCS. Hybrids were subcloned and maintained in IMDM medium containing 10% (vol/vol) FCS, as were the CH27 cells. CTLL cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 5% (vol/vol) FCS. Media were supplemented with 2 mM Glutamax (Invitrogen) and 50\(\mu\)g/ml of gentamicin.

Monoclonal antibody 14.4.4S, specific for the I-E\(\alpha\)-chain, was obtained from the American Type Culture Collection and was purified from ascites fluid and used at the indicated concentrations\(^{86}\). All peptides were synthesized, purified, and analyzed as previously described\(^{86}\). gp250 (SAPGLIATGSGVK) and CLiP (PVSKMRMATPLLMA) I-E\(^k\) biotinylated monomers were obtained from the NIH tetramer facility.

**Hybridoma stimulation assays**

This assay system examines the *in vivo* presentation of gp250 by APCs to the gp250-specific T-cell hybridoma Ga2.6. APCs used were the CH27 B cell line, CHO-E\(^k\) cell line (10\(^5\)/well), or primary APCs isolated from enzymatically digested spleen or thymus. In the case of plate-bound monomer stimulations, 96-well round bottom plates were first coated overnight at 4°C with 1\(\mu\)g/well streptavidin. Plates were then washed and coated with biotinylated monomer at the indicated concentrations, overnight at 4°C. To these was added the T-cell hybridoma Ga2.6 (10\(^5\) cells/well) in the presence or absence of exogenous gp250 peptide. APCs and hybridoma cells
were incubated for 15-20 hours at 37˚C in 96-well flat-bottom plate. T-cell stimulation was measured by quantifying the release of interleukin 2 (IL-2) by the T-cell hybridoma. IL-2 in the supernatant was measured by IL-2 ELISA, or by measuring [3H]thymidine incorporation by the IL-2-dependent CTLL line\textsuperscript{118,119}. ELISA was performed using 100 µg/well capture anti-IL-2 mAb (JES6-1A12; Biolegend), 50 µg/well biotinylated-anti-IL-2 detection mAb (JES6-5H4; Biolegend), 100 µl/well 1/10,000 dilution streptavidin-HRP (Southern Biotech), and developed using 100 µl/well 1-Step-Ultra tetramethylbenzidine substrate (Thermo Scientific). The reaction stopped at 15 min by the addition of 100 µl/well 2 M sulfuric acid, and assessed using a Victor3 plate reader (PerkinElmer). Regression analysis of IL-2 standards using GraphPad Prism 6 (GraphPad Software) was used to convert absorbance into amount of IL-2. Alternatively, CTLL cells (10^4 in 100µl) were added to the 100µl of supernatant and were cultured for 24-32 hours. Each well was pulsed with 0.2µCi of [3H]thymidine during the final 6 hr of culture, and plates were harvested and [3H]thymidine incorporation was measured.

\textit{T cell receptor sequencing and quantitative PCR}

RNA was isolated with an RNeasy kit (Qiagen) from cell lines and splenic or thymic APCs enriched through the use of magnetic-activated cell sorting beads and purified by flow cytometric sorting. cDNA was synthesized using random hexamer primers (SuperScript II kit; Invitrogen). TCR were amplified as previously described\textsuperscript{120}. Briefly, nested PCR was used to amplify TCR α and β transcripts\textsuperscript{121}, which were bulk ligated into pCR2.1 TOPO sequencing vector, sequenced, and analyzed using Lasergene 8 (Invitrogen, Genewiz, DNASTAR). For quantitative PCR analysis, SYBR Green PCR master mix and ABI7000 machinery (Applied Biosystems) were used as previously described\textsuperscript{122}. Cycling conditions were 50 °C for 2 min,
followed by 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and a dissociation stage (95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s); β-actin was included as internal control for all samples. Changes were calculated by the ΔΔCT method with CH27 B cell line samples as the baseline sample and Actb as the reference gene. The following primers (5′ to 3′; forward and then reverse) were used: LR11, GGGAATTTCTTCAGGCCCCA and TCCGAAGTTGGAGTGTAGC; β-actin, CTAAGGCCAACCGTAAAG and ACCAGAGGCATACAGGGACA.

**Bacterial infections and in vitro activation**

The *Listeria monocytogenes* strain 10403S was used in this study. Frozen stocks of *L. monocytogenes* in PBS plus 20% glycerol were thawed and serially diluted to a density of 1x10⁴ colony-forming units (CFU) per ml in PBS. Mice were infected three days prior to APC isolation retro-orbitally with 10³ CFU L. monocytogenes 52. For *ex vivo* activation, enriched splenic B cells were stimulated in 6-well plates with 10µg/mL anti-CD40 and 10µg/mL anti-IgM F(ab)2, or 20µg/mL LPS for 48 hours at 37°C 123.

**Analysis of Listeria specific response**

For analysis of Listeria specific response, LLO56 CD4⁺ T cells purified by magnetic bead positive selection were cultured with varying amounts of sorted CD11c⁺ DCs or CD19⁺ B cells from the spleens of naive or Listeria infected mice in triplicate, in 96 well plates. After 15-20 hours at 37°C in 5% CO₂ in Iscoves medium containing 10% FCS, CD4⁺ T Cells were analyzed for upregulation of CD69 by flow cytometry.
Figure 2.1. Isolation and subcloning of Ga2.6 hybridoma. (A) Greater than 85 hybridomas were tested for reactivity against the CH27 B cell line as measured by IL-2 production; 12 were selected for further investigation. (B) Sensitive hybridomas were subcloned and tested for reactivity to high doses endogenously processed gp250/I-E<sup>k</sup> from whole spleen.
Figure 2.1. Isolation and subcloning of Ga2.6 hybridoma.
Figure 2.2. Ga2.6 hybridoma response is specific for gp250/I-E\textsuperscript{k}. Enzyme-linked immunosorbent assay (ELISA) of IL-2 produced by Ga2.6 detecting gp250/I-E\textsuperscript{k} in overnight culture with the indicated number of (A) CH27 B cell lymphoma line (B) exogenous gp250 peptide presented by $10^5$ CHO-E\textsuperscript{k} cells or (C) gp250 altered peptide ligands with proline mutations at TCR contacts presented by CHO-E\textsuperscript{k}. (D) IL-2 production by Ga2.6 stimulated overnight with plate-bound gp250/I-E\textsuperscript{k} monomer. (E) $5\times 10^5$ whole splenocyte or thymic cells cultured with (black bars) or without (white bars) anti-MHCII clone 14.4.4; Ga2.6 activation was measured by IL-2 response. N.D. = not detected. Data are representative of at least 3 experiments and depict mean ± SEM.
Figure 2.2. Ga2.6 hybridoma response is specific for gp250/I-E^k.
Figure 2.3. Ga2.6 sensitively detects endogenous gp250/I-E^k complexes. Detection of gp250/I-E^k complexes from titrated doses of single cell (A) thymic or (B) splenic preparations by IL-2 ELISA of Ga2.6 culture supernatant. Mice expressed gp250/I-E^k (B6.K-black circles), I-E^k without gp250 (gp250 KO-triangles) or gp250/I-A^b (B6-crosses). Data are representative of at least 3 experiments and depict mean ± SEM.
Figure 2.3. Ga2.6 sensitively detects endogenous gp250/I-E\(^k\) complexes.
Figure 2.4. Ga2.6 recognizes endogenous differences in gp250/I-E^k doses. IL-2 production, as measured by ELISA, indicates intermediary detection of gp250/I-E^k complexes from the indicated number of (A) spleen and (B) thymus single cells from gp250 heterozygote mice, compared to homozygote and deficient littermate controls. Data are representative of at least three experiments and depict mean ± SEM.
Figure 2.4. Ga2.6 recognizes endogenous differences in gp250/I-E^k doses.
Figure 2.5. Thymic and splenic APC sorting strategy. (A) cTECs, mTECs were gated as CD45-, EPCAM and MHCII+, then CDR1+ or UEA-1+ respectively. Thymic DCs (tDC) were gated as CD45+, CD11c and MHCII+. (B) Splenic B cells were gated as CD19+, then B220 and MHCII+, while splenic DCs (sDC) were gated as CD19-, then CD11c and MHCII+. 
Figure 2.5. Thymic and splenic APC sorting strategy.
Figure 2.6. Variable LR11 gene (encoding gp250) expression in thymic and splenic APCs.
Quantitative RT-PCR analysis of LR11 mRNA expression in various FACS sorted (A) thymic and (B) splenic APC populations; results are presented as ΔΔC_T fold change relative to Actb (encoding β-actin) with CH27 as the baseline sample. tDC = thymic CD11c+ DC, sDC = splenic CD11c+ DC. Data are representative of two-three experiments and depict mean ± SEM.
Figure 2.6. Variable LR11 gene (encoding gp250) expression in thymic and splenic APCs.
Figure 2.7. Ga2.6 detects endogenous gp250/I-Ek complexes on positive and negative selecting thymic APCs. IL-2 production by Ga2.6 after overnight culture with FACS sorted thymic APCs (equivalent numbers of DC, mTEC, or cTEC) in the (A) absence or (B) presence of exogenous gp250 peptide. gp250/I-Ek detection is measured via [3H]thymidine incorporation during the IL-2 dependent proliferation of CTLL cells. To accommodate for experimental variation in maximal cTEC recovery and IL-2 production, equal numbers of all thymic APCs, ranging from 4,000-15,000, were utilized within each assay. Data were normalized to the maximal [3H]thymidine incorporation in each experiment. Data are a compilation of five experiments and depict mean ± SEM. One-way Anova, **P<0.01, ***P<0.0001.
Figure 2.7. Ga2.6 detects endogenous gp250/I-E<sup>k</sup> complexes on positive and negative selecting thymic APCs.
Figure 2.8. Ga2.6 detects gp250/I-E\(^k\) complexes on peripheral splenic APCs. (A) IL-2 production by Ga2.6 measured after overnight culture with FACS sorted splenic B cells and DCs. (B) EC\(_{50}\) indicates the number of APCs required to produce a half-maximal IL-2 response. Data are representative of three experiments and depict mean ± SEM. Mann-Whitney test, **p<0.01.
Figure 2.8. Ga2.6 detects gp250/I-E<sup>k</sup> complexes on peripheral splenic APCs.
Figure 2.9. Expression of gp250/I-E\(^k\) is affected by the recent immune status. IL-2 production by Ga2.6 in response to (A) B cells activated \textit{ex vivo} using anti-CD40 and anti-IgM or LPS for 48 hours prior to co-culture with Ga2.6. Alternatively, \textit{in vivo} activated B cells and (B) DCs were harvested from the spleens of B6.K mice infected with \textit{Listeria monocytogenes} three days prior to co-culture with Ga2.6. (C) Presentation of Listeria-pMHCII as measured by CD69 upregulation (mean fluorescence intensity-MFI) on LLO specific CD4 TCRtg T cells stimulated with B cells or DCs from Listeria mice. Data are representative of at least three experiments and depict mean ± SEM.
Figure 2.9. Expression of gp250/I-E<sup>k</sup> is affected by the recent immune status.
CHAPTER III

Intravital, *in situ* imaging of the murine thymus
This chapter represents work completed in collaboration with the laboratory of Mark J. Miller. The surgical technique described herein is performed by Yizheng Tu.

**Introduction**

Migration and cell-cell interactions are a vital component of both adaptive and innate immunity. Whether in the earliest stages of development and tissue seeding of hematopoietic cells, the critical circulation of these effector cells when surveying the body for pathogens, or the cellular dynamics that occur during an active immune response, the choreography of immune cells is critical to their function. Two-photon microscopy has ushered a transition from the static evaluation of cellular interactions to the real-time visualization of live cells\textsuperscript{124,125,126,127}. This is particularly valuable for the study of T cell development, a process that depends intimately on the interaction of developing cells with the thymic microenvironment. The stages of thymocyte development occur in distinct locations within the thymus\textsuperscript{14,15,16}. Studies of explanted thymi suggest that the stromal network act as roads guiding developing thymocytes as well as scaffolds for thymic dendritic cells to adhere\textsuperscript{128}. Indeed, disruptions of thymic three-dimensional architecture are directly linked to inefficient development of the T cell repertoire. In addition to three-dimensional architecture, \textit{in vitro} organ cultures require the supplementation of various soluble factors to support the development of mature T cells\textsuperscript{129}. As such, unequivocal elucidation of intrathythic dynamics requires investigating these processes in the native thymic environment, complete with known and unknown endogenous cues.

Early studies, predating the use of two-photon microscopy in the study of immunology, showed that the thymus was composed of an intricate three-dimensional epithelial network\textsuperscript{130,131}. Using standard histological, immunohistochemical and electron microscopy techniques, it was
appreciated that the thymic epithelial cells were distinct from the typical flat, two-dimensional, basement membrane anchored epithelial networks observed in other organs\textsuperscript{132}. Within these intricate sponge-like networks exist clusters of developing thymocytes, and the cross talk between these developing T cells and the thymic stroma is well documented. Specifically, observations of cortical and medullary thymic structure before and after hematopoietic reconstitution of immunodeficient mice indicated dynamic restructuring of the epithelia compartments with the addition of bone marrow derived cells\textsuperscript{133}. The plasticity of cortical and medullary epithelia cells depends on thymocytes of specific developmental stages to coordinate the unique three-dimensional environment supporting efficient T cell development; the reversion from three-dimensional to two-dimensional stromal architecture was observed in conjunction with early blockage in thymocyte development\textsuperscript{134,135,136,137}. It is clear that the interdependence of thymocytes and the thymic stroma to generate a three-dimensional microenvironment dictates efficient T cell development, however the exact mechanism of this regulation are still largely a mystery.

Despite the lingering questions regarding intrathymic cross talk, progress has been made toward elucidating the endogenous cues that coordinate T cell development within the thymic microenvironment. Immunohistochemical analysis suggests that the spatial relationship of medullary thymic APCs around intermediate, but not large thymic vessels or capillaries, suggest that unique vascular derived cues may contribute to intrathymic dynamics\textsuperscript{138}. Aside from vascular clues, there is also a known interaction between the neuroendocrine and immune systems\textsuperscript{139}. Specifically, it is thought that growth hormone increases both the thymic migration of developing thymocytes as well as the output of mature T cells\textsuperscript{23,140}. Other than a handful of
chemokines, the soluble factors that orchestrate intrathymic dynamics are poorly understood and difficult to investigate *ex vivo*.

Studying the contributions of endogenous cues to the development of mature T cells using current imaging approaches is inadequate due to the explanted or ectopically expressed nature of the thymus being visualized. In valiant attempts to visualize the specific interactions that mediate the effective development of a mature T cell repertoire, a number of creative techniques have been employed in conjunction with two-photon imaging. Explantation and imaging of whole and sliced thymi have offered tremendous insight into the thymic architecture and likely migratory behavior of developing thymocytes\textsuperscript{19,21,141,142}. Though valuable, systems such as the thymic slices, which require slicing explanted thymi with a Vibratome and the addition of exogenous, sorted donor thymocytes to oxygen perfused slices, involve dramatic changes to the thymic architecture and supply of endogenous cues. In attempts to generate more physiologic conditions, some groups have used two-photon microscopy to image ectopically transplanted thymi in the kidney capsule to study thymic development\textsuperscript{143}. Though all of these techniques have led to new insights into intrathymic cellular dynamics, none are able to completely reproduce the intact thymic microenvironment, and it is known that *in vitro* T cell selection systems do not perfectly mimic the endogenous process. Technical difficulties have also hindered the intravital, *in situ* visualization of thymic development thus far. The location of the thymus, within the mediastinum, makes it particularly difficult to image while avoiding cardiac and respiratory movement that impedes high-resolution time-lapse imaging\textsuperscript{144}. Herein, we describe a novel technique that will allow for live imaging of a mouse thymus with complete, *in situ* vasculature and innervation while minimizing motion artifacts. Outlined below is a detailed description of
the surgical procedure used to access the thymus with intact vasculature while minimizing cardiac and respiratory movement for the purpose of two-photon intravital imaging.

**Detailed technical protocol**

All of these imaging experiments are terminal procedures, and the animals are euthanized immediately following the end of image acquisition.

1. Anesthetize animals with Avertin (tribromoethanol) 150mg/kg intraperitoneally.
2. Shave the fur from the chest and place mice on the imaging stage with temperature control capabilities.
3. Endotracheally intubate and mechanically ventilate the animals with a rodent ventilator (120 breaths/min and with a tidal volume of 0.5 mL).
4. Perform a thoracotomy using standard procedures to expose the heart and thymus.
5. In order to prevent respiratory and cardiac function from creating movement artifacts during imaging, insert a thin piece of surgical cotton above the pericardial sac and below the thymus to act as a "tissue sling". Cautiously avoid thymic vessels and internal thoracic artery as well as neighboring cardiac vessels that line the thymus.
6. Attach the spring loaded imaging slide and secure it near the thymus capsule.
7. Ensure the tissue sling is situated without disruption of the thymic vasculature and the imaging slide is proximal to the thymus, and then gently lift at both ends to elevate the thymus above the heart (*Figure 3.1 A, B*).
8. Adjust the surgical cotton such that the maximal amount of thymic tissue is in contact with the imaging slide.
9. To maintain the thymus’ position above the pericardial sac and in contact with the imaging slide, attach each end of the tissue sling to the imaging slide using super glue.

10. Administer 655 nm non-targeted quantum dots 20µl in 100µl PBS (Invitrogen) intravenously after the surgical preparation to confirm that healthy blood flow is intact.

11. Add the animal stage to the microscope (Figure 3.1 C).

12. Turn on heaters and ensure that the temperature is maintained at 37°C.

13. Continually monitor the animal for sustained anesthesia. Administer additional doses of anesthesia as needed.

14. After image acquisition, euthanize animals in accordance with approved laboratory protocols.

**Technical considerations**

Animal viability must be closely monitored for effective intravital imaging. Over the course of imaging anesthesia will wear off; care must be taken to ensure the animal remains properly anesthetized. In addition, it is important to monitor the animal’s body temperature. Automated regulation of the imaging stage temperature should be used to maintain body temperature during imaging and the mouse given saline subcutaneously to prevent dehydration.

The key technical advance in this approach is the use of the tissue sling that minimizes cardiac and respiratory movement artifacts on the thymus and permits the *in vivo* visualization of intrathymic single-cell dynamics. The amount of pressure used when securing the imaging slide will directly impact both the blood flow to the thymus and well as the motion artifacts. If blood flow is impaired, as assessed by bright field observation or the flow of quantum dots in vessels...
using two-photon, then the pressure being exerted on the thymus must be reduced by releasing slightly the spring loaded imaging slide until robust tissue perfusion is restored. Contact between the thymus by the upper imaging slide is important for dampening undesired thymic movement due to heart and lung functions, so a balance must be struck between stability and good tissue perfusion. Within stable preparations, tissues depths of over 300µm have been evaluated (Figure 3.2).

Results

Direct comparison of intravital in situ and explant thymic preparations

Common methods of studying intrathymic cellular dynamics require continued perfusion of thymic preparations with media saturated with oxygen. Given that cell motility in ex vivo thymic preparations may be affected by non-physiological oxygen concentration, temperature and tissue manipulation, it is important to compare results between explant and in vivo imaging approaches. In the case of T cell dynamics in peripheral lymph node, intravital and explanted data are in close agreement. To investigate how this novel technique compares with common thymic explant procedures, we have employed a previously unpublished reporter mouse, B6.CD69YPET.

Thymocyte dynamics upon positive selection have been extensively studied in explanted systems. Given that thymocyte CD69 expression is an early indicator of positive selection by self-pMHC, we sought to use a CD69 reporter mouse, expressing YPET from the CD69 promoter, to compare the intrathymic dynamics of post-positive selection thymocytes in explanted and intravital, in situ thymic imaging preparations. We first confirmed by flow
cytometry that YPET expression correlated with CD69 expression in these thymocytes (Figure 3.3). As expected, post-positive selection DP and SP thymocytes expressed YPET in correlation with CD69. There was a low level of YPET expression in the DN compartment, and this was correlated with the CD25+ DN2 and DN3 populations. Given the low abundance of these cells within the thymus and the low intensity of YPET expressed, they do not contribute significantly to the YPET+ thymocytes observed in the thymus.

In vitro investigation of the timing of YPET induction on reporter thymocytes after non-specific stimulation was performed using CD69YPET/ANDtg/Ragd/d reporter thymocytes. In these mice, the H-2d non-selecting background forces ANDtg thymocytes to arrest at the pre-positive selection, CD69 negative DP stage (Figure 3.4 A). These assays indicated that YPET surface expression occurs as early as one hour post stimulation, reaching a maximum level by 3.5 hours as detected by flow cytometry. YPET expression accurately traces CD69 expression as measured by flow cytometry, represents roughly 50% of total surface CD69 expression. This is useful for two-photon analysis as it will reduce the total number of fluorescent cells observed within the thymus, allowing for the identification of unique cellular events. A single-cell suspension of these cells visualized by confocal microscopy showed maximal YPET expression after roughly 6 hours of stimulation (Figure 3.4 B, C). Having confirmed that the B6.CD69YPET reporter reflects the known expression pattern of CD69 in thymocytes and that endogenous YPET expression can be observed using confocal microscopy, we used this tool to compare explanted and intravital, in situ intrathymic cellular dynamics after positive selection using two-photon microscopy.
Post-positive selection YPET+ thymocyte dynamics can be visualized using standard two-photon imaging of the novel thymic preparation described above. These cells are tracked as previously described using Imaris analysis software (Figure 3.5 A). Immediately following intravital, *in situ* image acquisition, we excised the contralateral thymic lobe and perfused it with warm oxygenated media in accordance with common thymic explant procedures. Imaging and tracking of post-positive selection thymocytes within these explanted lobes was performed as with the intravital preparation (Figure 3.5 B). The cellular dynamics of both preparations were quantified using two-photon analysis software developed by the Miller laboratory (in collaboration with Johannes Textor, University of Utrecht). Unlike published data showing similar T cells dynamics between intravital and explanted peripheral lymph nodes, we observed reduced migratory dynamics of post-positive selection thymocytes in the intravital, *in situ* thymus preparation compared to the explanted lobe (Figure 3.6). While explanted thymi showed mean velocities of 6µm/min, intravital imaging revealed a slower average velocity for 4µm/min along with a reduction in track straightness. These data suggest that current explant and ectopic techniques may overestimate the migratory behavior of developing thymocytes, and highlight the usefulness of this novel surgical preparation and imaging technique.

*Visualization of thymic entry*

To date, studying the entry of thymic seeding cells by two-photon microscopy has not occurred because *in situ* thymic preparations with intact vasculature have not been possible. The novel intravital, *in situ* thymus preparation described herein allows for the real-time visualization of thymic entry and seeding. Bone marrow was isolated cyan fluorescent protein (CFP) transgenic mice (Jackson 004218) and adoptively transferred by retro orbital injection into recipient mice.
that have been prepared for thymus imaging as described above. Within 15 minutes, CFP+ bone marrow derived cells are observed within the thymus vasculature, visualized by the injection of quantum dots (Figure 3.7 A). Over time, CFP+ cells are also found outside of vessels and within the thymic stroma, indicating that these cells have undergone extravasation. Tissue resident cells have been identified as early as two hours after intravenous administration. To our knowledge, this is the first report in which thymic seeding cells have been observed entering the thymus via endogenous vasculature in vivo. Unlike ex vivo analysis, where the circulation is lost, intravital imaging of the thymus allows the earliest steps of thymocyte trafficking to be studied for the first time. Moreover, in vivo imaging will permit thymocyte development to be studied in the native context of important autocrine and paracrine regulators of selection, as well as distal endocrine cues coming from the circulation.

Cells that are entering the thymus can be observed and tracked at various stages of extravasation (Figure 3.7 A,B). We inquired whether trafficking of bone marrow derived cells via the blood and into the thymus resembled the behaviors observed in earlier studies of leukocyte homing to peripheral tissues. Within the blood vessels, some cells were moving at rapid speeds, and appear only as flashes during time-lapse imaging and as a sequence of spots within a 15 frame averaged z-step (Figure 3.7 A, right). Taking advantage of the known frame rate of our system (~25f/sec), these velocities were measured; each spot corresponds to a single video-rate frame and the distance between them equals the distance traveled in 1/25th of a second. Using this method, intravascular cells were found to move between 8-90 µm/s. Though this velocity is much quicker than the average velocities observed of lymphocytes in tissues, it is slower than the reported circulating velocity of neutrophils. In addition to rapidly circulating bone marrow
derived cells, CFP+ cells were observed rolling and adhering within the vessels and perivascular space, and these dynamics could be quantified (Figure 3.7 C). As such, we have described a novel technique in which the earliest events of thymic entry can visualized and quantified. This methodology will open the door to future investigation into the mechanisms and control of T cell precursor thymic entry.

Conclusions
We have generated a method to perform intravital live imaging of the intact thymus. We used a novel "tissue sling" surgical preparation that minimized respiratory and cardiac induced movement artifacts that hinder thymus imaging and have performed successfully the first intravital, in situ analysis of intrathymic thymocyte dynamics. To compare the migratory behavior of thymocytes in explanted thymi and the thymi of live mice, we used a previously unpublished reporter mouse, B6.CD69YPET. This reporter faithfully represents CD69 expression on developing thymocytes. Our data suggest that explanted and ectopic thymic preparations may overestimate the velocity and migratory behavior of developing thymocytes, perhaps due to endogenous factors that are missing or altered during tissue explanation. Using this novel thymic preparation, it is now also possible to visualize the initial steps of cell entry into the thymus. Because the endogenous cues that mediate thymocyte development in vivo are incompletely understood, and in vitro selection models do not fully replicate in vivo thymic selection, intravital in situ imaging approach, such as the one described here, will be important in understanding the role of thymic vasculature, innervation and tissue environment on thymocyte trafficking and development.
Discussion

Published data indicate that thymocytes exhibit slow and random movement before positive selection. Explanted and slice preparations estimate mean speeds of 3-8µm/min and varied direction before positive selection. After dwelling while in contact with positive selecting cTECs, post-positive selection thymocytes are then thought to move rapidly, greater than 13µm/min, and in a straight and directed fashion toward the medulla\textsuperscript{18, 19, 153}. Our intravital data indicate post selection, YPET+ thymocytes moving akin to published data for pre-selection cells, roughly 3µm/min in a random manner. Though our explanted preparations showed increased thymocyte motility compared to intravitaly imaged tissues, around 6µm/min, speeds were still lower than expected from published data. However, similarly to published data, post-selection YPET+ thymocyte tracks were straight.

Discrepancies in observed migration dynamics between laboratories could be attributed to different acquisition rates and time resolutions. Additionally, perfusion, oxygenation, and temperature have been reported to variably affect lymphocyte dynamics in lymph nodes; differences of only a few degrees can have a dramatic impact on cell behavior. Future work in visualizing thymocyte development should address these components and their affect on cell motility directly. A caveat to the approach is that it wasn't possible to verify that the thymus temperature was at the core body temperature. However, we do not expect the thymus temperature to be far outside the physiological range as the water-dipping objective uses a highly insulating ceramic face to minimize heat loss, blood flow is robust and warming the upper imaging plate had no noticeable effect on cell motility. The temperature of explanted tissues can be more tightly regulated, yet even when tissues were at 37 degrees C, cell migration speeds
were substantially less than published values using different imaging preparations, suggesting that although temperature is an important factor, it cannot completely explain the decreased motility we observed. The addition of heating elements to the top imaging slide, as well as measuring mouse body temperature during imaging using a rectal thermometer, would help verify that physiological temperature is maintained during intravital imaging.

Elevating the thymus above the pericardial sac to avoid cardiac and respiratory movement from interfering with imaging is a significant component of this technique, as even under anesthesia, these movements can impede image acquisition. Slight adjustments of the pressure used to stabilize the spring-loaded imaging slide can dramatically change any undesired movement that remains after thymus elevation. Being able to eliminate cardiac and respiratory motion while keeping the blood flow intact allows the visualization if thymic seeding cells entering from the blood. While this technique maintains the endogenous thymic environment and cues, it is not without its caveats. It should be noted that the surgical process itself and mode of anesthesia may affect lymphocyte motility, and as previously discussed, temperature regulation must still be carefully monitored as with previous thymus imaging techniques\textsuperscript{124, 154}.

In a short time, two-photon microscopy has led to incredible advancements in how the immune system is thought to function. As more and more critical questions are being addressed with immunoimaging, the technology is continuing to advance, making strides to overcome previous limitations such as the depth, length, and time resolution at which real time imaging can occur\textsuperscript{142, 155}. Generation of fluorescent reporter systems that unambiguously mark specific cells and can reflect intracellular dynamics are also expanding the scope of questions being investigated by
two-photon microscopy\textsuperscript{156, 157}. Access to and stability of organs to be imaged has also been a limitation. We developed an intravital, \textit{in situ} technique to overcome this hurdle in regards to the thymus, which will allow for important advances in studying intrathymic cellular events and their regulation.

**Methods**

**Mice**

B6.129 (ICR)-Tg (CAG-ECFP) CK6Nagy/J Actin\textsuperscript{CFP} mice (Jackson 004218) and Gt(ROSA)26Sor\textsuperscript{tm4(ACTB-tdTomato,-EGFP)}Luo/J (Jackson 007576) were bred to the B6.K (H-2\textsuperscript{k} restricted) line (Jackson 001148) to generate what we refer to as Actin\textsuperscript{CFP} and Rosa\textsuperscript{tdTom} mice respectively. B6.CD69\textsuperscript{YPET} transgenic reporter mice were generated in Andrey Shaw’s laboratory by using a BAC transgene in which YPET was inserted at the initiating methionine of the CD69 gene. CD69\textsuperscript{YPET} reporter mice have also been bred to ANDtg/Rag\textsuperscript{d/d} mice for use in \textit{in vitro} assays, and are referred to as CD69\textsuperscript{YPET}/ANDtg/Rag\textsuperscript{d/d}; note that these thymocytes are blocked at the pre-selection DP stage of development. All mice were bred and housed in specific pathogen–free conditions of the animal facility at the Washington University Medical Center. The use of all laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

**Bone marrow isolation**

Femurs and tibias of Actin\textsuperscript{CFP} reporter mice were cut at the ends and flushed with PBS using a 21G needle and syringe to generate a single cell suspension. Cells were centrifuged at 1200 RPM
for 5 minutes and resuspended in PBS. 1-5x10^7 unfractionated bone marrow cells were injected intravenously into recipient B6.K mice for intravital imaging.

**In vitro CD69 upregulation assay**

1x10^6 CD69<sup>YPET</sup>/ANDtg/Rag<sup>d/d</sup> DP thymocytes were plated in 96-well round bottom plates and stimulated with 100 ng/ml PMA (Sigma-Aldrich) plus 1 µg/ml ionomycin (Sigma-Aldrich) for the indicated times. Placing the cells on ice stopped the stimulation, and staining was performed to analyze the timing of CD69 expression by flow cytometry.

**Antibodies**

The following antibodies were used for flow cytometry: Live/Dead UV-Violet (Invitrogen), anti-CD4 (RM4-4, RM4-5 and GK1.5), anti-CD8 (53-7.8), anti-CD69 (H1.2F3), anti-CD25 (PC61), CD44 (IM7) anti-CD5 (53-7.3) from eBioscience or Biolegend.

**Confocal Microscopy**

For confocal analysis, 2x10^7 CD69<sup>YPET</sup>/ANDtg/Rag<sup>d/d</sup> DP thymocytes were plated in 35mm glass microwell dishes (MatTek). Images were acquired every minute in 4 fields of focus for 3 hours. CD69<sup>YPET</sup>/ANDtg/Rag<sup>d/d</sup> DP thymocytes were stimulated with PMA/I as above to visualize the initiation, rise and maintenance of YPET expression in CD69<sup>YPET</sup>/ANDtg/Rag<sup>d/d</sup> DP thymocytes. The baseline CD69<sup>YPET</sup> expression level was established prior to addition of PMA/I.
Two-Photon microscopy data acquisition and analysis

Time-lapse imaging was performed with a custom-built dual-laser video-rate two-photon microscope as previously described\textsuperscript{99,150}. Quantum dots (Invitrogen) as well as tdTomato, YPET and CFP labeled cells were excited with a Chameleon XR Ti:sapphire laser (Coherent) and visualized with an Olympus XLUMPlanFI 20\times objective (water immersed, numerical aperture, 0.95). YPET positive cells were excited by a laser tuned to 920 nm, CFP and tdTomato and fluorescence were excited using a laser tuned to 820 and 1000 nm respectively. Fluorescence emission was separated passing through 510- and 560-nm dichroic mirrors placed in series and detected as red (560–650 nm), green (490–560 nm), and blue (second harmonic) signal (<458 nm) channels by three head-on Bialkali photomultiplier tubes (PMTs). A customized version of ImageWarp (A&B Software) was used during real-time acquisition to process and archive the image data. To create time-lapse sequences, we scanned volumes of tissue in which each plane consists of an image of 100 × 120 \mu m (x and y = 2 pixel/\mu m). Z-stacks were acquired by taking 31 or 121 sequential steps at 2.5\mu m spacing at 27–35-s intervals for up to 20 min. To increase signal contrast, we averaged 15 video frames for each z-slice. Multidimensional rendering and cell tracking was performed with Imaris (Bitplane). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software) and 2Ptrack.net (Miller laboratory and Johannes Textor).
Figure 3.1. Intravital, *in situ* thymus imaging setup.

(A) Diagram and (B) image depicting how exposure of the thymus is achieved for intravital, *in situ* imaging. After opening the thoracic cage, surgical tissues is used to gently lift the thymus away from the pericardium. Care is taken to avoid the local vasculature. (C) Complete imaging setup showing (from left to right, red asterisks) ventilator mask, spring-loaded imaging slide over thymus, objective and temperature regulation probe(s).
Figure 3.1. Intravital, *in situ* thymus imaging setup.
Figure 3.2. Quality of intravital thymus imaging.

Live imaging of Rosa^tdTom^ reporter thymus at 1000nm shows (A) an imaging depth of over 300μm (121 z-steps) (B) z merged rendering (ez3D, Imaris). (C) Intravital imaging of B6.K thymi immediately after intravenous injection of quantum dot 655 reveals maintenance of blood flow within thymic tissue and the absence of detrimental cardiac or respiratory movement.
Figure 3.2. Quality of intravital thymus imaging.
Figure 3.3. Evaluation of B6.CD69<sup>YPET</sup> reporter representation of CD69 surface expression.

Flow cytometry with a panel of standard thymocyte development markers was used to assess the endogenous expression of YPET as an indicator of CD69 expression. (A) Standard gating of single positive (left) double negative (middle) and pre- or post-positive selection double positive (right) thymocytes is shown. (B) YPET (right) and surface antibody stained (left) levels of CD69 expression in the indicated thymocyte populations. (C) Quantification of frequency (left) and absolute number (right) of the indicated thymocyte populations.
Figure 3.3. Evaluation of B6.CD69<sup>YPET</sup> reporter representation of CD69 surface expression.
Figure 3.4. *In vitro* analysis of CD69<sup>YPET</sup> expression after non-specific stimulation.

(A) Thymocytes (right) are arrested at the DP stage of development compared to B6.K thymocytes (left). (B) Time course of surface (red and black) and YPET (yellow and grey) CD69 expression in reporter positive (Rptr+) or control (Ctrl) DP thymocytes stimulated with PMA/I for the indicated time. (C) Intensity of YPET expression detected by confocal microscopy after stimulation with PMA/I. Note that YPET intensity decrease dramatically upon cell death. Data are representative of 2-5 experiments.
Figure 3.4. *In vitro* analysis of CD69<sup>YPET</sup> expression after non-specific stimulation.
**Figure 3.5. Intravital, in situ imaging and analysis of post-positive selection thymocytes.**

Still representations of live two-photon imaging of the B6.CD69YPET reporter thymus after the intravenous administration of quantum dot 655. Full view (left) of endogenous thymocytes from (A) intravital or (B) explanted thymi are shown. For analysis purposes, a representative quarter of the entire video (middle) was fully analyzed using Imaris spot tracking. Representative track data are shown (right). Bars indicate 50µm.
Figure 3.5. Intravital, *in situ* imaging and analysis of post-positive selection thymocytes.
Figure 3.6. Comparison of standard migratory measurements between intravital and explanted preparations.

X, Y and Z positional data as well as YPET intensity data gathered using Imaris analysis were used in combination with 2Ptrack.net (Miller laboratory and Johannes Textor) and GraphPad Prism to compare thymocyte behavior within the live and explanted thymic preparations. (A) Mean speed, (B) track straightness, (C) motility coefficient (D) mean square displacement (E) the correlation between mean YPET intensity and speed were evaluated.
Figure 3.6. Comparison of standard migratory measurements between intravital and explanted thymic preparations.
Figure 3.7. *Intravital, in situ* imaging allows for the visualization and quantification of thymic seeding events.

Actin$^{\text{CFP}}$ reporter bone marrow is administered intravenously into B6.K mice after the post-surgical addition of quantum dot 655. Within 15 minutes after transfer, bone marrow derived cells can be (A) visualized at various stages of extravasation (left), including rolling (middle) and rapid circulation in the blood vessels (right). These dynamics were tracked (B) in Imaris (left) and represented using 2Ptrack.net software (right). These dynamics can be quantified using Imaris and GraphPad (C) highlighting the motility circulating cells and tracks (left and middle left) as well as extravasating cells and tracks (middle right and right).
Figure 3.7. Intravital, *in situ* imaging allows for the visualization and quantification of thymic seeding events.
CHAPTER IV

Self-pMHCII and TCR self-reactivity impact the peripheral homeostasis of mature CD4 T cells
**Introduction**

T cell immunity is a tightly regulated process in which a naive pool of T cells capable of responding to pathogens must be maintained in the steady state. Pathogen specific T cells then undergo massive expansion during an immune response, followed by dramatic contraction to the steady state level while maintaining a small population of memory cells for future protection against said pathogens. The mechanisms of steady state homeostasis of naive and memory T cells are being worked out, and it is widely thought that though naive and memory CD4 and CD8 T cells are regulated by a unique synergy of mechanisms, they all rely to some degree on cytokine and self-pMHC derived signals. The study of exactly how or if self-pMHCII impact CD4 T cell homeostasis has been mired with debate, with most of these studies relying on MHCII ablation to study the role of TCR:pMHC in steady state homeostasis.

Some of the controversy surrounding the role for self-pMHC in CD4 T cell homeostasis stems from differences in the systems utilized. In normal lymphoreplete hosts with complete immune systems, polyclonal CD4 cells undergo minimal proliferation. However when rendered lymphopenic, either chronically (Rag\(^{-/-}\)) or acutely (sublethally irradiated), CD4 cells undergo proliferation and conversion into memory phenotype cells. It has been proposed that the source of homeostatic self-pMHCII signals differ depending on the lymphopenic environment, and some argue that lacking MHCII results in enough lymphopenia that interpretation of these results are confounded by space-induced proliferation, and do not solely reflect the role of pMHCII in naive CD4 T cell homeostatic proliferation. Well regarded studies have suggested that since Rag\(^{-/-}\) mice are chronically infected with commensal bacteria, and “self” proteins are predominately derived from this source during chronic lymphopenia, the homeostatic
proliferation observed is largely commensal organism driven. Under acutely lymphopenic conditions however, low affinity self-peptides predominately drive proliferation of naive CD4 T cells\textsuperscript{79, 82}. A closer look at the T cells that undergo homeostatic proliferation after being adoptively transferred into lymphopenic recipients shows that some of have acquired a CD44\textsuperscript{high} memory phenotype. In this study, we have focused on the sublethally irradiated, acutely lymphopenic homeostatic system.

Despite the ongoing debate, overwhelming evidence points to some role for self-peptides in maintaining the mature T cells pool, even suggesting that it is the same ligands that mediate intrathymic selection that are required for peripheral T cell homeostasis\textsuperscript{73, 75, 161}. The fact the selected T cells have a small range of affinity for self-peptide as a result of the selection process has prompted the question of whether the self-reactivity of a given T cell impacts it peripheral homeostasis. The sensitivity of mature T cells for self-pMHC is established during thymic development; the strength of this spMHC:TCR interaction is represented by CD5 expression levels\textsuperscript{48, 52}. The few studies that have began to investigate the role of self-reactivity in T cell homeostasis suggest that T cells with a high affinity for self-pMHC have a competitive advantage in homeostatic survival and proliferation\textsuperscript{83, 162}.

**Results**

As discussed above, addition of exogenous gp250 positive selecting peptide to chronically lymphopenic B6.K Rag1\textsuperscript{−/−} recipients of adoptively transferred ANDtg CD4 T cells significantly increased the survival of these transgenic T cells (**Figure 1.5**). This is in keeping with the notion that the same ligands mediating intrathymic positive selection also provide essential cues for the
homeostatic maintenance of naive CD4 T cells in the periphery. To our knowledge, this is the first explicit example of a bona fide selecting ligand impacting peripheral maintenance. We sought to further confirm that the naturally occurring positive selecting self-pMHCII, gp250/I-E\(^k\), supported homeostatic maintenance of ANDtg CD4 T cells by utilizing the gp250 deficient animals as recipients, compared to gp250\(^{+/+}\) B6.K recipients. During routine analysis of our newly generated polyclonal gp250 KO mice, we uncovered a significant reduction in polyclonal V\(\beta\)3 expressing CD4 T cells (Figure 4.1). Though the ANDtg CD4 T cell expresses a V\(\beta\)3 TCR, it is unlikely that one ligand is responsible for the selection of a majority of V\(\beta\)3 expressing polyclonal CD4 T cells. Indeed, the V\(\beta\)3 TCRs are known to be highly amenable to endogenous retroviral (mouse mammary tumor virus) super-antigen mediated deletion\(^{163}\). As such, we closely investigated the genomic heterogeneity of the gp250 KO mice; heterogeneity within the genome was revealed, though not at loci known for super-antigen mediated V\(\beta\)3 deletion\(^{164,165}\). With the help of the Rheumatic Disease Core Center, we used speed congenics to further backcross the gp250 KO mice to B6 background resulting in heterogeneity confined solely to chromosome 9 on which the LR11 gene, from which gp250 is derived, is located (Figure 4.2). The ANDtg/Rag1\(^{+/−}/\)gp250\(^{−/−}\) mice were re-derived by breeding ANDtg/Rag\(^{kk}\) animals with these fully backcrossed gp250 KO mice, and evaluated for the production of V\(\beta\)3\(^+\)V\(\alpha\)11\(^+\) ANDtg CD4 T cells. As opposed to the dramatic loss of ANDtg CD4 T cells initially observed (Figure 1.7), the new litters showed only modest reductions in ANDtg CD4 T cell development (Figure 4.3). Though work in our laboratory and others has indicated a high degree of specificity among the self-pMHC that can select a given TCR, it is not surprising to find that, though sufficient for positive selection, gp250 is not the sole ligand capable of mediating the intrathymic development of the ANDtg CD4 T cell. Despite the revelation that gp250/I-E\(^k\) is not a necessary self-pMHCII
ligand responsible for the selection of ANDtg CD4 T cells, it has proven to be a sufficient and dominant selecting ligand and useful tool in the evaluation of endogenous, physiological self-pMHCII characteristics and functions.

Having identified one of only a few bona fide CD4 T cell positive selection systems, we sought to closely evaluate the contribution of gp250/I-Ek self-pMHCII to the maintenance and proliferation of naive CD4 T cells. Given that acutely lymphopenic, sublethally irradiated recipients are widely thought to support predominately self-pMHC mediated homeostatic proliferation, we focused our attention on the role of selecting ligand gp250 in this setting. One week after adoptive transfer of ANDtg CD4 to cells into acutely irradiated B6.K or gp250 KO recipients, we observed no significant differences in the survival, as measured by recovery, or proliferation of the transferred ANDtg CD4 T cells (Figure 4.4). Given our recent discovery that gp250/I-Ek was a sufficient but not necessary selecting ligand for the ANDtg CD4 T cell, it is possible that unknown ANDtg selecting ligands are providing the necessary homeostatic cues in both the B6.K and gp250 KO recipients.

We next investigated whether exogenously increasing the amount of the naturally occurring positive selecting ligand would impact the homeostatic characteristics of the ANDtg CD4 T cells in the acutely lymphopenic setting. We added gp250 peptide intraperitoneally one day before and after the adoptive transfer of ANDtg CD4 T cells into acutely lymphopenic B6.K recipients. After 7 days, we noticed no significant difference in total ANDtg CD4 T cell survival or division with the addition of exogenous gp250 peptide (Figure 4.5 A-C). However, upon closer inspection we identified a consistent pool of rapidly dividing ANDtg CD4 T cells upon addition
of exogenous gp250 peptide; without exogenous peptide, no rapidly dividing population existed. The ANDtg CD4 T cells that underwent rapid proliferation also exhibited a CD44<sup>high</sup> memory phenotype (Figure 4.5 D-F). Thus, it appears that increasing the amount of positive selecting ligand may prompt homeostatically proliferating CD4 T cells to divide more rapidly.

Having a novel, naturally occurring CD4 T cell selection system in which to investigate the role of selecting ligands in peripheral T cell homeostasis is an incredible advancement, however as with other known selecting ligands we do not have an affinity measurement for the weak ANDtg CD4 TCR:gp250/I-E<sup>k</sup> interaction. To further investigate the characteristics of self-pMHC that mediate homeostatic maintenance, we employed the LLO TCRtg system generated in our laboratory. Using this system, we can investigate how two T cells which recognize the same cognate antigen with equivalent affinities, yet differ in their reactivity to self-pMHC, are maintained in the periphery. As described in the introduction LLO56 TCRtg CD4 T cells express higher levels of CD5 than LLO118 TCRtg CD4 T cells (Figure 1.8), a finding we have interpreted to indicate that the LLO56 cells have an increased reactivity to self-pMHC than do LLO118 cells. One week after adoptive transfer of LLO TCRtg CD4 T cells into acutely irradiated B6 mice, we found that the CD5<sup>high</sup> LLO56 cells underwent increased proliferation when compared to the LLO118 TCRtg CD4 T cells. Despite this increased proliferative capacity, the LLO56 TCRtg CD4 T cells survive less well as indicated by the frequency and number of recovered cells (Figure 4.6). The same was true of a disparate TCRtg pair; both recognize hemoglobin (Hb<sup>d(64-76)/I-E<sup>k</sup></sup>) as their cognate antigen but differ in CD5 expression<sup>166, 167</sup>. After adoptive transfer into acutely lymphopenic hosts, we noted that the CD5<sup>high</sup> 2.102 TCR exhibited poorer survival but increased proliferation in comparison to the CD5<sup>low</sup> N3L2 TCRtg CD4 T
cells (Figure 4.7). These data would suggest that, though CD5\textsuperscript{high} expression results in an increased proliferative capacity in response to homeostatic cues, presumably self-pMHC, these highly self reactive cells are not well maintained.

We next investigated if exogenous addition of a pool of I-A\textsuperscript{b} binding self-peptides could increase the homeostatic proliferation of LLO118tg CD4 T cells, similarly to as was seen with the naturally occurring ANDtg CD4 T cell selecting self-pMHCII gp250/I-E\textsuperscript{k}. Though none of these ligands are confirmed LLO TCRtg positive selecting ligands, they are naturally occurring self-peptides identified from activated B cells and macrophages\textsuperscript{168}. One week after adoptive transfer and intraperitoneal administration of a 10 I-A\textsuperscript{b} self-peptide pool we observed no significant increase in the survival or proliferation of LLO118tg CD4 T cells (Figure 4.8). We sought to determine if altered peptide ligands (APLs), which bind LLO56 or LLO118 TCR but do not stimulate the T cell directly, can impact the homeostatic characteristics of the LLOtg CD4 T cells. Using a panel of LLO APLs previously identified in the laboratory we performed adoptive transfer assays as described above. We observed that the addition of antagonist peptides increased the amount of rapid proliferation that occurred over the 7 day period for both LLO56 and LLO118. This effect was considerably more dramatic for the CD5\textsuperscript{low} LLO118tg than the CD\textsuperscript{high} LLO56tg CD4 T cell (Figure 4.9). This suggests that antagonist peptides can augment homeostatic proliferation, and that less self-reactive CD4 T cells are more susceptible to changes induced by these ligands.

We were curious if CD5 was playing a direct role, either positive or negative, in the homeostasis of LLOtg CD4 T cells. Though often utilized simply as a surrogate for self-pMHC affinity, CD5
has also been attributed with the negative regulation of TCR signaling. Indeed, work from our laboratory has shown that LLO TCRtg CD4 T cells lacking CD5 exhibit increased IL-2 production and ERK phosphorylation when stimulated. We utilized the LLO56 and LLO118 CD5KO mice as donors in our acute lymphopenia homeostatic assay. With both LLO56tg and LLO118tg CD4 T cells, the absence of CD5 elevated their homeostatic proliferation and survival. No cells were covered from the negative control H-2DM KO recipients (Figure 4.10). These increases were most marked for the less self reactive LLO118tg CD4 T cells. This suggests that CD5 may function to dampen the steady state response to self-pMHC. However, given that LLO56tg CD4 T cells did not exhibit a vast increase in homeostatic proliferation and survival, there are likely compensatory mechanisms for maintaining the homeostatic expansion of highly self reactive TCRs at an appropriate level.

**Conclusion**

In this study we have shown that self-peptides and the degree of self-reactivity can impact the homeostatic proliferation of CD4 T cells. Specifically, the exogenous addition of the bona fide selecting ligand gp250/I-Eκ to acutely lymphopenic hosts can increase the prevalence of rapidly divided ANDtg CD4 T cells. Investigating how the TCR affinity for self-pMHCII impacted peripheral homeostasis employed two TCRtg systems, LLO and HbD. In each system it was revealed that the less self-reactive CD5high TCRtg pair, LLO56 and 2.102 respectively, underwent increased homeostatic proliferation. Despite the increased proliferative capacity of the CD5high CD4 cells they survived less well compared to their CD5low counterparts. Antagonist peptides were able to induce a rapid proliferation of LLO TCRtg CD4 T cells similar to that of gp250 and the ANDtg CD4 T cell. This effect was more pronounced when investigating
LLO118tg CD4 T cell antagonists, suggesting that CD5\textsuperscript{low} cells with reduced affinity for self-pMHCII are more responsive to changes in the peptide landscape.

**Discussion**

We have investigated the interplay between self-pMHC, strength of self-reactivity and naive CD4 T cell homeostasis. Initially, we had hypothesized that if positive selecting self-pMHC were in fact required for the homeostatic proliferation and maintenance of naive CD4 T cells, then eliminating the dominant selecting ligand for ANDtg CD4 T cells in the gp250KO mice would eliminate the proliferation and survival of these cells. However, given that gp250/I-E\textsuperscript{k} is not the sole ligand capable of mediating ANDtg CD4 T cell selection, it is not surprising that elimination of this ligand alone did not preclude the proliferation and survival of the ANDtg T cells. This does not negate the importance of (selecting) self-peptides in the homeostatic maintenance of CD4 T cells. Rather, it suggests that other self-ligands, besides the positive selecting ones, may compensate for the homeostatic cues provided by gp250/I-E\textsuperscript{k}.

Because we do not have an affinity measurement for the weak interaction of ANDtg CD4 TCR with the positive selecting gp250/I-E\textsuperscript{k} ligand, we sought to investigate how the strength of self-reactivity impacted homeostasis. In two separate TCRtg systems, we found that increased self-reactivity resulted in increased homeostatic proliferation. Despite the increased presence of dividing cells, these highly self-reactive TCR were less prone to survival compared to their TCRtg counterparts exhibiting lower self-reactivity. It has been proposed in other studies that homeostatic proliferation and survival are not necessarily regulated by the same cues. Indeed, it
appears that the while strong self-reactivity may augment proliferation, this trait does not lend itself to increased survival.

The studies wherein exogenous peptide was added before and after adoptive transfer of TCRtg cells suggest that the abundance self-peptide can impact the homeostatic characteristics of a naive CD4 T cell. Specifically, they increase the frequency of rapid “burst-like” divisions. As has been proposed, we observe that these cells have a memory phenotype, expressing high levels of CD44 compared to the slowly dividing transferred cells. The purpose of these cells is of interest; unlike traditional memory CD4 T cells, these CD44^{high} cells have not experienced cognate antigen. Extensive “burst-like” proliferation was also observed when adding antagonist peptide to the LLO systems. Though not bona fide positive selecting ligands, antagonist peptides have long been used to mimic positive selection due to their inability to classically activate T cells while still interacting with the TCR. Interestingly, the antagonists had a greater effect on the homeostatic proliferation of the CD5^{low} LLO118tg CD4 T cells compared to LLO56tg. This suggests that peptide abundance may play a larger role in the homeostasis of T cells that are less self-reactive, as measured by CD5 expression levels.

The exact functional role of CD5 is debated. It is an accepted readout of a TCR’s affinity for self-peptides, and this has proven a useful tool in these studies. However, we were interested in whether CD5 played a functional role in regulated T cell characteristics. In regards to homeostasis, it appears that CD5 does in fact reduce the homeostatic proliferation and survival of naive CD4 T cells, as CD5KO LLOtg cells showed increases in these parameters compared to the CD5 sufficient LLOtg CD4 T cells. We had anticipated that any direct effect of CD5 would
be exacerbated in the CD5\textsuperscript{high} LLO56tg CD4 T cells compared to CD5\textsuperscript{low} LLO118. However, we observed the opposite. We suggest that highly self-reactive T cells are endowed with more abundant compensatory negative regulatory components than cells that are less prone to self-reactivity. As such, removing CD5 from the equation would augment proliferation to a greater capacity in the less self-reactive CD4 compartment which posses less stringent negative regulatory elements.

There appears to be a complicated interplay between the landscape of self-ligands, the abundance of these ligands and a T cell’s inherent affinity for self-peptides. The experiments herein did not address the important role of cytokines and competition for homeostatic cues that will also influence the steady state maintenance of a T cell pool. Taken together these data suggest that self-peptides and a TCR’s affinity for such ligands impact their peripheral maintenance in a complex manner.

**Materials and methods**

**Mice**

The gp250 deficient mice \textsuperscript{92} were bred to the B6.K (H-2\textsuperscript{k} restricted) line (JAX 001148). The mice were extensively backcrossed to B6.K, and were monitored by the analysis of microsatellite markers at the Rheumatic Disease Core Center, Washington University School of Medicine (St. Louis, MO) as described above. The MCC–I-E\textsuperscript{k} specific AND TCRtg\textsuperscript{89,170}, B6.K (H-2\textsuperscript{k}), C57BL/6 (B6, H-2\textsuperscript{b}) (JAX 000664) and B6.\textit{Rag}1\textsuperscript{−/−} (JAX 002096) mice were purchased from The Jackson Laboratory. The AND TCRtg line was bred onto \textit{Rag}1\textsuperscript{−/−} background: AND.\textit{Rag}1\textsuperscript{−/−}H-2\textsuperscript{k}. AND.\textit{Rag}1\textsuperscript{−/−}H-2\textsuperscript{k} mice were bred to fully backcrossed gp250 KO mice to
generate ANDtg/Rag1−/−gp250−/− mice. The LLO TCRtg mice were generated in our laboratory as previously described. The LLO56 and LLO118 TCRtg CD4 T cells recognize the same immunodominant epitope listeriolysin O (190-205) from Listeria monocytogenes (LLO190-205/IA)2,24. CD5−/− mice were obtained as part of the NIAID Exchange Program from the transgenic mouse repository maintained by Taconic. These mice were bred to the LLO TCRtg mice to generate LLO56 CD5KO and LLO118 CD5 KO mice. The n3.L2/B6.K and 2.102/B6.K mice were previously generated in our laboratory and were crossed to the Rag1−/− line166.

H-2DM−/− 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 H-2DM KO mice used in this study. All mice were between the ages of 4-12 weeks and were bred and housed in specific pathogen–free conditions of the animal facility at the Washington University Medical Center. The use of all laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

**Adoptive transfer assays**

For acute lymphopenia homeostatic assays, recipient mice were sublethally irradiated with a dose of 600 rads on day -2. On day 0, spleen and lymph node are harvested from donor mice, made into a single cell suspension and counted. CD4 T cells were isolated by magnetic bead positive selection (Miltenyi). CD4 purified cells are CFSE labeled, and 1x10^6-2x10^6 are intravenously transferred to recipient mice by tail vein injection. Spleen and axial, brachial and inguinal lymph nodes from individual mice are harvested on day 7. Flow cytometric analysis of donor cell recovery and division follows. “Rapid” divisions are representative of the final 2-3
peaks of CFSE dilution, while “slow” division includes the first 2-3 peaks of CFSE dilution.

Data in this study show lymph node populations. In assays where exogenous peptides are added to the homeostatic systems, 100µg of peptide is administered intraperitoneally on day -1 and day +1; non-peptide controls receive and equal volume of PBS intraperitoneally. Statistical analysis is performed using GraphPad Prism 6 (GraphPad Software).

**Peptides**

All peptides were synthesized, purified, and analyzed in house as previously described using a Symphony automated solid-phase peptide synthesizer via Fmoc chemistry and high performance liquid chromatography. gp250 peptide is described above.

**I-Ab binding self-peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
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<tr>
<td>FcgR(231-245)</td>
<td>EVGEYRQPSGGSVPV</td>
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<tr>
<td>DEC205(562-579)</td>
<td>DPDSRGEYSWAVAQGVKQ</td>
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<td>Int B1(778-794)</td>
<td>GENPIYKSAVTTVVNPK</td>
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<tr>
<td>CD98(207-223)</td>
<td>GQNAWFLPAQADIVATK</td>
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<tr>
<td>CD22(25-39)</td>
<td>DWTVDHPQTLFAWEG</td>
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<tr>
<td>IgM(376-391)</td>
<td>EKYVTSPMPEPAG</td>
</tr>
<tr>
<td>LDLR(486-501)</td>
<td>RNIYWTDSVPGSVSVA</td>
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<tr>
<td>I-Ab(50-64)</td>
<td>RPDAYWNSQPEILE</td>
</tr>
<tr>
<td>Actin(163-177)</td>
<td>VPIYEGYALPHAILR</td>
</tr>
<tr>
<td>GAPDH(227-242)</td>
<td>TGMAFRVPTPNSVVVD</td>
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**LLO altered peptide ligands**

<table>
<thead>
<tr>
<th>Peptide</th>
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<tbody>
<tr>
<td>LLO(190-205)K197</td>
<td>NEKYAQAKPVNSAKID</td>
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<tr>
<td>LLO(190-205)F197</td>
<td>NEKYAQAFPNVSAKID</td>
</tr>
<tr>
<td>LLO(190-205)G194</td>
<td>NEKYQGYAPNVSIAKID</td>
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**Flow cytometry**

The following antibodies were used for flow cytometry: Live/Dead UV-Violet (Invitrogen), anti-CD4 (RM4-4, RM4-5 and GK1.5), anti-CD44 (IM7) anti-CD5 (53-7.3), anti CD45.1 (A20), anti-Thy1.1 (OX-7), anti-Vβ3 (8F10), and anti-Vα11 (RR8-1) from eBioscience or Biolegend as well as CFSE (Invitrogen).
Figure 4.1. gp250 deficient mice have polyclonal V\(\beta\)3 TCR deletion. Flow cytometric analysis of polyclonal gp250 KO mice exhibited reduced V\(\beta\)3 TCR expression on CD4 SP thymocytes (A) as well as mature CD4 cells in the lymph node and spleen (B) compared to B6.K (WT) cells. Data are representative of at least 3 experiments and depict mean ± SEM.
Figure 4.1. gp250 deficient mice have polyclonal Vβ3 TCR deletion.
Figure 4.2. Speed congenic backcrossing of gp250 deficient mice. Chromosomal map of gp250 KO mouse compared to B6 before (A) and after (B) backcrossing via speed congenics. Regions of heterogeneity are indicated in green; chromosomal locations of known Vβ3 TCR deleting endogenous retroviruses are indicated in red; the orange box indicates the approximate chromosomal location of LR11, also named SorLA, at 41.983-41.985 cM.
Figure 4.2. Speed congenic backcrossing of gp250 deficient mice.
Figure 4.3. ANDtg CD4 T cells continue to be selected in the absence of gp250. Flow cytometric analysis of (A) thymus and (B) spleen from the newly backcrossed ANDtg/gp250KO/Rag\(^{kk}\) mice shows no difference in the production of V\(\beta\)3\(^+\) V\(\alpha\)11\(^+\) ANDtg CD4 T cells between gp250 deficient (KO) and sufficient (WT) mice. Data are representative of at least 3 experiments.
Figure 4.3. ANDtg CD4 T cells continue to be selected in the absence of gp250.
Figure 4.4. ANDtg CD4 T cells maintain their homeostatic characteristics in the absence of gp250/I-E<sup>k</sup> positive selecting ligand. (A) CFSE dilution profile of ANDtg CD4 T cells adoptively transferred into acutely lymphopenic gp250 deficient (KO) and B6.K (WT) recipients. Quantification of (B) absolute number recovered (C) percent of transferred cells recovered and (D) percent divided.
Figure 4.4. ANDtg CD4 T cells maintain their homeostatic characteristics in the absence of gp250/I-Ek positive selecting ligand.
Figure 4.5. Addition of exogenous gp250 positive selecting ligand promotes the rapid division of ANDtg CD4 T cells. Quantification of (A) absolute number recovered (B) percent of transferred cells recovered and (C) percent of cells divided after intraperitoneal injection of gp250 peptide before and after adoptive transfer of ANDtg CD4 T cells into acutely lymphopenic B6.K mice that did (+gp250) and did not (no peptide) receive exogenous gp250 peptide. (D) CFSE dilution profile of ANDtg CD4 T cells. “Rapid” division indicates 7 or more divisions, while “slow” division represents 2-3 rounds of CFSE dilution. (E) Quantification of the frequency of rapid and slowly dividing cells among total ANDtg CD4 T cells undergoing division, with or without exogenous peptide. (F) CD44 expression differences between rapidly and slowly dividing ANDtg CD4 T cells in the presence of exogenous gp250 peptide.
Figure 4.5. Addition of exogenous gp250 positive selecting ligand promotes the rapid division of ANDtg CD4 T cells.
Figure 4.6. CD5\textsuperscript{high} LLO56 TCRtg cells exhibit increased proliferation and decreased survival compared to CD5\textsuperscript{low} LLO118 TCRtg counterpart. (A) CFSE dilution profile of donor CD5\textsuperscript{high} LLO56 and CD5\textsuperscript{low} LLO118 TCRtg CD4 T cells one week after adoptive transfer into acutely (600 rads) lymphopenic B6 hosts. Quantification of (B) absolute number (C) recovery rate and (D) frequency of division among LLO56 and LLO118 donor CD4 T cells.
Figure 4.6. CD5^{high} LLO56 TCRtg cells exhibit increased proliferation and decreased survival compared to CD5^{low} LLO118 TCRtg counterpart.
Figure 4.7. CD5<sup>high</sup> 2.102 TCRtg cells exhibit increased proliferation and decreased survival compared to CD5<sup>low</sup> n3L2 TCRtg counterpart. (A) Flow cytometric analysis of CD5 expression levels between Hb<sup>d</sup> specific CD4tg T cells 2.102 and n3L2. Quantification of (B) absolute number (C) recovery rate and (D) frequency of division among 2.102 and n3L2 donor CD4 T cells.
Figure 4.7. CD5$^{\text{high}}$ 2.102 TCRtg cells exhibit increased proliferation and decreased survival compared to CD5$^{\text{low}}$ n3L2 TCRtg counterpart.
Figure 4.8. Addition of exogenous I-A<sup>b</sup> self-peptide pool does not significantly the homeostatic characteristics of LLO118 TCRtg CD4 T cells. (A) CFSE dilution profile one week after intraperitoneal injection of an I-A<sup>b</sup> self-peptide pool before and after adoptive transfer of LLO118tg CD4 T cells into acutely lymphopenic B6 mice that did (118 + peptide) and did not (56 or 118) receive exogenous I-A<sup>b</sup> self-peptide. Quantification of (B) absolute number (C) recovery rate and (D) frequency of division among LLO56tg, LLO118tg and LLO118tg with exogenous peptide.
Figure 4.8. Addition of exogenous I-A<sup>b</sup> self-peptide pool does not significantly the homeostatic characteristics of LLO118 TCRtg CD4 T cells.
Figure 4.9. LLO TCRtg antagonist peptides promote the rapid proliferation of LLO56tg and LLO118tg CD4 T cells. (A) Panel of LLO altered peptide ligands with differing TCR binding characteristics. Red highlights the antagonist peptides for LLO118tg (F197) and LLO56tg (G194 and K197) CD4 T cells. CFSE dilution profiles of (B) LLO118tg and (C) LLO56tg one week after intraperitoneal injection of antagonist peptides before and after adoptive transfer of LLOtg CD4 T cells into acutely lymphopenic B6 mice that did and did not (LLO56 or LLO118) receive exogenous antagonist peptide. (D) Quantification of the rapidly divided LLOtg CD4 T cells in the presence of absence of exogenous antagonist peptide.
Figure 4.9. LLO TCRtg antagonist peptides promote the rapid proliferation of LLO56tg and LLO118tg CD4 T cells.
Figure 4.10. CD5 deficient LLOtg CD4 T cells exhibit increased homeostatic characteristics. (A) CD5 expression levels of CD5KO and CD5 sufficient LLOtg CD4 T cells. (B) CFSE dilution profile of donor CD5 KO LLO56tg (left) and CD5 KO LLO118tg (right) CD4 T cells one week after adoptive transfer into acutely (600 rads) lymphopenic B6 hosts. Quantification of (C) absolute number (D) recovery rate and (E) frequency of division among CD5KO LLO56 and LLO118 donor CD4 T cells. Black indicated CD5 sufficient LLOtg CD4 T cells, while red indicates CD5 KO LLOtg T cells as donors into acutely lymphopenic B6 recipients. Blue indicates homeostasis negative control H-2 DM deficient recipients of CD5 sufficient LLOtg donor CD4 cells.
Figure 4.10. CD5 deficient LLOtg CD4 T cells exhibit increased homeostatic characteristics.
CHAPTER V

Discussion and future directions
The studies performed in this dissertation have made novel advances in the fields of CD4 T cell development, including live, in situ visualization of intrathymic dynamics and characterizations of a physiological selecting self-pMHCII landscape, and homeostatic maintenance of peripheral CD4 T cells. We can further take advantage of the systems described in the following ways.

**Future investigation of the endogenous self-pMHCII landscape**

We have successfully surveyed the expression of a bona fide positive selecting ligand in the thymus and periphery. We suspect that post-translational modification (PTMs) may impact this landscape. In future work, we will perform western blot analysis to elucidate if PTMs, such as glycosylation, phosphorylation and ubiquitination, are causing the observed discrepancy between mRNA expression and functional expression of gp250/I-E\(^k\) complexes. Given that immune response factors are known the impact the PTM of pMHC and this has been suggested to play a role in autoimmunity\(^{110}\), it will be of particular interest to look at PTMs in steady state and activated APCs. Aside from Listeria infections, we investigate if parasitic or viral pathogens alter the self-pMHCII landscape. Many environmental factors have been suggested to trigger autoimmunity\(^{171}\), and future studies may investigate if changes to the self-pMHC landscape and subsequent alterations to the T cell pool may be a potential mechanism of such triggers.

Advances in sequencing technology and reductions in cost allow to potential to investigate the TCR repertoire in response to self-pMHC landscape alterations.

Additionally, having unequivocally shown, for what we believe to be the first time, that a physiological self-pMHCII can be presented on both positive selecting and tolerance induce thymic APCs, we are in a position to further investigate the role of public, widely expressed, and private, uniquely expressed, ligands in T cell development and peripheral maintenance. We
know that ANDtg CD4 T cells can be selected by more than one self-pMHCII. Though herculean, it may be of interested to identify an additional self-ligand for comparison to the public gp250/I-E\(^k\) ligand.

**Intravital characterization of thymus seeding events and intrathymic selection**

One of the biggest advantages of our novel intravital, *in situ* thymus imaging preparation is the ability to visualize the entry of T cell progenitors into thymus from the blood. Future studies will replicate these initial experiments wherein whole bone marrow cells were intravenously administered and observed entering the thymus. Because mature T cells are not known to recirculate through the thymus to any significant degree, we suspect that the thymus is a stringent gate keeper and the bone marrow cells we observe entering the thymus are common lymphoid progenitors. However, we can FACS sort common lymphoid progenitor prior to administration for two-photon analysis to purify the population of thymic seeding cells. We will also investigate the role of soluble molecules suggested to regulate thymus entry and intrathymic development in this system.

Additionally, we can generate a reporter system to in which we can observe a bona fide positive selection event. Intrathymic administration of exogenous gp250 peptide was previously shown in our laboratory to enhance the selection of ANDtg CD4 T cells. Thus, we have the ability to initiate a positive selection event by adding exogenous gp250 peptide to our transgenic CD4 T cell system. We propose to generate reporter mice in which the components of the ANDtg:gp250/I-E\(^k\) positive selection system can be identified, and use these reagents to determine the kinetics of a positive selecting event. Combining our ANDtg system with the
B6.CD69\textsuperscript{YPET} reporter mice described herein, we can start to investigate the early events involved in positive selection using tow photon microscopy\textsuperscript{28,47}. By including carefully selected reporters for the thymic APCs into these imaging assays the cellular interplay between thymic APCs and the ANDtg thymocytes in the presence of exogenous gp250/I-E\textsuperscript{k} can be evaluated. We will thus be able to analyze the migratory behavior and cellular interactions of a known CD4 TCR with selecting thymic APCs.

Finally, we will closely investigate how temperature is impacting both the intravital and explant imaging preparations. As has been described elsewhere, even small changes in temperature can impact cellular motility\textsuperscript{142}. We will perform the imaging preparations as described herein, while carefully controlling the temperature of the automated temperature regulation unit and perfusion media. We will also monitor the animal’s core body temperature in relation to the mechanical temperature to confirm that the heating elements are accurately maintaining the animal’s internal body heat.

**Clarification of the interplay between homeostatic cues**

Differences between the experimental systems used to study naive T cell homeostasis have made it hard to reconcile the work done across many groups, and indeed our studies suggest that the interplay between self-reactivity, (selecting) self-pMHCII peripheral landscape and homeostasis requires more investigation. The role of lymphopenia is a major source of discrepancy. Naive TCRtg and polyclonal cells have been shown to undergo increased proliferation and CD44\textsuperscript{high} conversion upon adoptive transfer into chronically lymphopenic compared to acutely lymphopenic recipients\textsuperscript{79}. The source of pMHC is also shown to differ in those setting, with
acute radiation induces lymphopenia being self-pMHC mediate, as no foreign antigens were involved. However in an MHCII monoclonal antibody mediated depletion of self-pMHCII interaction, BrdU remained to be incorporated in a population of CD44\textsuperscript{high} cells, indicating a self-pMHCII independent meditated proliferation\textsuperscript{172}. They show a role for cytokine-driven homeostatic proliferation in supplying the memory phenotype pool; cytokine involvement in CD4 T cell homeostasis is an important component we will investigate in the future. Our experiments adding self and antagonist self-peptide mimics to acute homeostasis transfer systems suggest that self-pMHCII do play a role in homeostatic proliferation. However the many differences in the systems make these difficult to compare, specifically the mode of lymphopenia or pMHC inhibition, the transfer versus endogenous source of CD44\textsuperscript{high} cells and the tracking of division be CFSE dilution or BrdU incorporation.

Having identified these antigen inexperienced CD44\textsuperscript{high} memory phenotype cells, questions regarding their function have naturally arisen. Naive animals have been estimated to have 10-20\% CD44\textsuperscript{high} T cells in the stead state\textsuperscript{173}. Induction of acute, radiation-induced lymphopenia increases the frequency, however few of these memory phenotype cells were shown to make TNF\textalpha or IFN\gamma\textsuperscript{79}. Some have speculated that they supply a pool of cross-reactive memory cells or play some kind of role when antigen experienced memory cells are not available\textsuperscript{67,174}. In the future, our laboratory can utilize the ANDtg system to investigate the role of CD44\textsuperscript{high} memory phenotype ANDtg CD4 T cells in response to cognate MCC antigen. The identification of gp250 as the a bona fide selecting ligand will allow for investigation of how selecting self-pMHCII impacts the response to foreign antigens.
We have made a connection between the strength of self-reactivity, self-pMHCII, and rapid proliferation and CD4^high memory phenotype conversion. While other laboratories have noted a concurrent increase in both homeostatic proliferation and survival as measured by recovery, for CD5^high self-reactive T cells, we observed in two separate systems that increased homeostatic proliferation was in opposition to survival. We push this observation further by showing that the CD5^low CD4 T cells are more receptive to increases in self-MHCII. In the future, we will investigate whether the antagonist LLO peptide utilized herein are capable of selecting LLOtg CD4 T cells in *in vitro* fetal thymic organ culture, as some antagonists have been shown to do. This will confirm that these antagonists are effective selecting self-peptide mimics. We will also attempt to establish where within the narrow affinity window of allowable self-reactivity the mature ANDtg CD4 T cells lie, to further investigate the interplay between self-reactivity and peripheral self-pMHCII, using a bona fide physiological self-pMHCII.

Comparing the CD5 levels of polyclonal B6.K and ANDtg CD4 T cells will be an initial step. We can then utilize the previously identified I-E^k self-pMHC to see if any naturally occurring self-ligands impact polyclonal and ANDtg CD4 T cell homeostasis in the periphery. These same I-E^k self-peptides can be used in assays with the Hb^d TCRtg system. Though it is known that these peptides do not positively select the TCRs in question, these assays will show if any non-selecting self-peptides augment the homeostatic traits of the CD4 T cells. It should be noted that peripheral CD5 expression levels have been reported to fluctuate in the periphery. Given that our laboratory has shown that CD5 itself can augment homeostatic proliferation within these experiments, as well as antigen responses in published data, it will be important to further elucidate what impact CD5 has on peripheral T cells outside of reporting self-reactivity.
References


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77. Takada K, Jameson SC. Naive T cell homeostasis: from awareness of space to a sense of place. *Nature reviews Immunology* 2009, **9**(12): 823-832.


171. Temajo NO, Howard N. The mosaic of environment involvement in autoimmunity: the abrogation of viral latency by stress, a non-infectious environmental agent, is an intrinsic
prerequisite prelude before viruses can rank as infectious environmental agents that trigger autoimmune diseases. *Autoimmunity reviews* 2014, 13(6): 635-640.


