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How CD4+ T Cells Recognize Allostimulatory Peptide-MHC

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LIST OF ABBREVIATIONS

APC	antigen-presenting cell
APL	altered peptide ligands
CFA	complete Freund's adjuvant
CHO	Chinese hamster ovary
CTL	cytotoxic T lymphocyte
DN	double-negative
DP	double-positive
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
GP	glycoprotein
GVHD	graft versus host disease
Hb	hemoglobin
HEL	hen egg-white lysozyme
i.p.	intra-peritoneal
i.v.	intra-venous
LCMV	lymphocytic choriomeningitis virus
LLO	Listeriolysin O
MCMV	murine cytomegalovirus
MHC	Major Histocompatibility Complex
MLR	mixed lymphocyte reaction
MOG	myelin oligodendrocyte glycoprotein
nTreg	natural Treg
PMA	phorbol 12-myristate 13-acetate
pMHC	peptide-MHC
PPD	purified protein derivative
sc	single chain
s.c.	sub-cutaneous
SP	single-positive
TCR	T cell receptor
Treg	regulatory T cell

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ABSTRACT OF THE DISSERTATION

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Critical in determining transplantation outcome, whether tolerance is achieved or not, are $CD4^+$ T cells that can recognize peptides presented on allogeneic (non-self) Major Histocompatibility Complex (MHC) molecules in addition to their conventional ligands, peptides presented on self MHC. It is an enigma as to how these alloreactive T cells can bind to allogeneic MHC given that T cells undergo stringent positive selection during development to bind to peptides presented on self MHC. We hypothesize that T cells bind to peptides on non-self MHC using the same properties involved in binding their conventional ligands. We identified allostimulatory peptide-MHC (pMHC) ligands (I-E^k as the allogeneic MHC) for two LLO/I-A^b-specific $CD4^+$ T cells, LLO118 and 1G5.1. Both T cells recognized their allostimulatory ligand with a high degree of specificity and sensitivity for the allopeptide, similar to how they recognized their cognate ligand. Allopeptide recognition was also shown to not merely reflect mimicry of the cognate peptide. The integral role of the peptide in alloreactivity was further confirmed by the ability to

convert previously non-alloreactive T cell hybrids into becoming responsive with the addition of peptide pools. In addition, the binding affinity and kinetics of LLO118 to its allostimulatory and cognate pMHC ligands were compared using surface plasmon resonance (Biacore system). LLO118 bound its alloligand using similar affinity and kinetics compared to its cognate ligand. In comparing alloreactivity with conventional recognition, which has not been done before for $CD4^+$ T cells, we have found that similar peptide specificity and binding affinity are used to recognize both ligands, shedding light on the fundamental binding properties of the T cell receptor (TCR) for pMHC.

Within the population of alloreactive T cells, a significant percentage is comprised of dual TCR T cells. This occurs from incomplete allelic exclusion of the TCRα loci during thymic development, allowing for simultaneous rearrangement of TCRα on both alleles until positively selecting signals are received through the TCR. In dual TCR T cells, only one TCR needs to mediate positive selection, and an autoreactive TCR can be masked from negative selection through decreased surface expression. This generates a repertoire of T cells containing secondary TCRs unconstrained by thymic selection. We set out to investigate the impact of secondary TCRα rearrangement to determine what benefit this has on thymic development and further define its contribution to peripheral T cell responses. Our hypothesis is that secondary TCRα rearrangement positively impacts the development of T cells, but atypical TCR properties that arise contribute to alloreactivity and autoimmunity. We examined mice heterozygous for the T cell receptor α chain constant region ($TCR\alpha^{+/-}$), which have only one functional TCRα rearrangement. The mice had a defect in generating mature T cells attributable to decreased positive selection. Elimination of secondary TCRs did not broadly alter the peripheral T cell

compartment, though deep sequencing of the TCRα repertoire demonstrated unique TCRs resulting from secondary rearrangements. The functional consequence of these unique TCRs was evidenced by the significantly reduced frequencies of TCRα$^{+/-}$ T cell binding to autoantigen and alloantigen pMHC tetramers as well as decreased *in vivo* alloreactivity. Analysis of responses to altered peptide ligands (APLs) revealed that dual TCR T cells had increased flexibility in their recognition of allogeneic ligands, indicating a mechanism for their importance in alloreactivity. Our results show that the role of secondary TCRs in alloreactivity appears to be more significant than what has been assumed.

Another factor we wanted to investigate in alloreactivity - as well as in autoimmunity and conventional T cell responses - is the effect of MHC composition, specifically the impact of increasing the types of MHC molecules expressed. It is perplexing why vertebrates express a limited number of MHC molecules when theoretically, having a greater repertoire of MHC molecules would increase the number of epitopes presented, thereby enhancing thymic selection and T cell response to pathogens. It is possible that any positive effects would either be neutralized or outweighed by negative selection restricting the T cell repertoire. We hypothesize that the limit on MHC number is due to negative consequences arising from expressing additional MHC. We compared T cell responses between B6 mice (I-A^{+}) and B6.E^{+} mice (I-A^{+}, I-E^{+}), the latter expressing a second class II MHC molecule, I-E^{b}. The naive TCR Vβ repertoire was altered in B6.E^{+} thymi and spleens, suggesting a potential for mediating different outcomes in T cell reactivity. In alloreactivity, the B6.E^{+} T cell response was significantly dampened. We wondered if similar effects would be seen in other types of immune responses. The B6 and B6.E^{+} responses to hen egg-white lysozyme (HEL) protein immunization remained similar, but the

quality of the T cell response was subtly altered in viral infection and there was markedly enhanced susceptibility to experimental autoimmune encephalomyelitis (EAE) in B6.E^+ mice. The EAE phenotype could be explained by decreased percentage of natural regulatory T cells (nTregs) in the B6.E^+ mice. Our data suggest that expressing an additional class II MHC can produce both positive and negative effects on a wide range of T cell responses. In conclusion, new insight into $CD4^+$ T cell alloreactivity has been gained, with our research indicating that specificity of peptide binding, weak affinity, flexibility in recognition by dual TCR T cells, and MHC composition all contribute significantly to allorecognition.

CHAPTER I

Introduction

The ability of T cell receptors (TCRs) to recognize peptides presented on allogeneic (non-self) Major Histocompatibility Complex (MHC) molecules presents a fascinating puzzle to immunologists. TCRs are generated randomly through gene rearrangement and are then subjected to strict selection processes during T cell development to bind self MHC, but not so strongly that they would cause autoimmunity in the periphery. Knowing this, why can a significant portion of TCRs recognize allogeneic MHC, and what are the factors driving the participation of T cells in altered reactivities? In this chapter, I will review TCR generation and selection, discuss what we know about the characteristics of T cell binding to allogeneic pMHC, and introduce factors in selection – namely secondary TCRα rearrangement and MHC composition – that may unduly influence T cell alloreactivity as well as autoimmunity, another type of immune response that eludes thymic selection processes. Here, alloreactivity is not merely an interesting enigma to ponder, but an area of study that could shed light on fundamental T cell binding properties and factors leading to unwanted immune responses.

I. T cell development and ligand recognition

I.I. Positive and negative selection in the thymus lead to the development of self MHC-restricted yet non-autoreactive T cells

T cells are a critical component of the adaptive immune system, with antigen recognition by TCRs initiating a cascade of events including cytokine production, activation of other cells, and elimination of cells infected with pathogens, amongst other things. The TCR is comprised of TCRα and TCRβ chains generated by gene segment recombination during thymocyte

development. The process of gene rearrangement is important for generating a functional T cell repertoire. First, the TCRβ chain rearranges in CD4 and CD8 double-negative (DN) thymocytes under stringent allelic exclusion and ceases when an in-frame product is made and expressed (1, 2). CD4 and CD8 co-receptors become upregulated, and in these double-positive (DP) cells TCRα chain recombination occurs until halted by positively selecting signals through the TCR (3, 4). Unlike the TCRβ locus, there is a lack of allelic exclusion for TCRα, evidenced by thymocytes and peripheral T cells with 2 in-frame rearrangements of TCRα (2, 5) as well as mature T cells with dual TCR expression on the surface (6-8). As it is a difficult process for thymocytes to successfully undergo positive selection, with approximately 90% of thymocytes dying (9-13), having simultaneous and iterative TCRα rearrangement on both chromosomes would presumably increase the chances of generating a TCR that can bind peptide-MHC (pMHC) (14). At the end of thymic development, a positively selected T cell downregulates one co-receptor and matures into a CD4 or CD8 single-positive (SP) cell, depending on whether its TCR is specific to MHC class II or class I molecules, respectively (10).

Two selection processes in thymic development act to generate T cells that can recognize self MHC yet remain tolerant to self. Positive selection leads to the survival of T cells that can recognize self-pMHC ligands on cortical thymic epithelial cells, allowing peripheral T cells to become activated by self MHC molecules presenting cognate ligands (10, 15-17). Negative selection is induced by medullary thymic epithelial cells and bone marrow derived antigen presenting cells (APCs) to delete T cells containing TCRs with high affinity to self-pMHC, preserving self-tolerance (11, 18). Approximately 5% of thymocytes are eliminated through negative selection mediated by a single MHC molecule (19).

I.II. T cells that recognize allogeneic MHC or autoligands can result despite stringent thymic selection processes

Although during thymic development T cells are selected to be responsive to peptides on self MHC, an astounding percentage of T cells in the periphery are capable of reacting to peptides on allogeneic, or non-self, MHC. In fact, it is estimated that 1-10% of T cells are alloreactive, approximately 1,000-fold higher than the percentage of T cells that can bind their cognate ligands on self MHC (20-22). An explanation for this phenomenon, discussed in greater detail in a subsequent section, is that there is germline affinity of the TCR for MHC molecules, leading to an ability of T cells to bind MHC molecules they have not encountered in the thymus.

Additionally, negative selection is not a perfect process, with low avidity, self-reactive T cells escaping negative selection and migrating to the periphery. And, not all peripheral antigens are expressed or presented sufficiently in the thymus to allow for elimination of autoreactive T cells. Though peripheral tolerance mechanisms are in place, autoimmunity may still ensue when it is broken by the release of self antigens through tissue damage or by infection with a pathogen containing epitopes similar to self peptides (23).

I.III. T cells exhibit distinct binding properties to peptide-MHC ligands

The TCR recognizes antigen comprised of peptide bound to MHC molecules expressed on the surface of other cells. How the TCR interacts with pMHC is one of the most distinctive features

of T cell biology. MHC class I-restricted TCRs bind to peptides of approximately 8-10 residues that can bulge outwards from the MHC peptide-binding groove. The ligand for MHC class II-restricted TCRs consists of longer peptides that can extend past the open grooves of the MHC (24). TCRs interact with pMHC using characteristically weak affinity (approximately 1 - 100μM), slow on-rates, and fast off-rates (25, 26). Interestingly, despite this weak affinity interaction, ligand recognition by TCRs occurs with exquisite specificity as well as plasticity. The specificity of recognition is seen with varying T cell responses to subtly different APLs and drastically divergent T cell fates – death by neglect, positive selection, and negative selection – upon encountering pMHC ligands in the thymus. TCR plasticity is the fundamental ability of TCRs selected on endogenous peptides presented by self MHC to react to pathogen-derived peptides in the periphery (27, 28). Another characteristic feature of TCRs is that they generally sit over the peptide-MHC in a similar, diagonal orientation relative to the long axis of the peptide-binding groove, with the TCR Vα domain contacting the N-terminus of the peptide and the TCR Vβ domain at the C-terminus end. Contacts with the MHC are primarily made by the germline-encoded CDR1 and CDR2 loops of the TCR, whereas the somatically rearranged CDR3 loops bind to the highly variable peptide portion (24).

II. $CD4^+$ T cells in alloreactivity

II.I. Germline-encoded affinity of TCR for MHC provides an explanation for alloreactivity

It has also been proposed that the phenomenon of alloreactivity is a manifestation of the propensity of TCRs to bind MHC. Whether this propensity results from an intrinsic affinity of

TCR for MHC or from thymic selection restricting the T cell repertoire to TCRs that can bind to MHC has been a fiercely debated topic. In support of the latter possibility, it was found in a structural analysis of 8 TCRs co-complexed with MHC class I that the structures could be split into 2 groups – 4 TCRs in each group – based on the location on the peptide-MHC surface where the TCR Vα domains contacted. Interestingly, 1 group consisted of CD8-dependent TCRs, while the other group had the CD8-independent TCRs (29). This observation lends support to the idea that extrinsic factors, such as the co-receptor, contribute to how TCRs bind to their ligands. Additionally, the Lck sequestration model of thymic selection proposes that the association of the signaling protein tyrosine kinase, Lck, with the CD4 and CD8 co-receptors only allows thymocytes with MHC-restricted TCRs able to bind pMHC along with their co-receptor to transduce positive selection signals and mature. That the expression of co-receptor-free Lck instead of co-receptor-associated Lck was sufficient to select out an MHC-independent T cell repertoire supports the idea of Lck association with co-receptors mediating the generation of MHC-restricted T cells (30).

On the other hand, an elegant set of experiments has been performed to demonstrate that the TCR repertoire prior to MHC selection has an inherent affinity for MHC molecules. In this study, thymi from MHC-deficient mice were matured in organ cultures by treatment with antibodies to TCR and CD4. Upon stimulation with allogeneic cells, the frequency of responding, pre-selection $CD4^+$ T cells was comparable to the alloreactive frequency of MHC-selected $CD4^+$ T cells derived from thymic organ cultures from MHC class II sufficient mice (31). Another study probed the effects of thymic negative selection on TCR-MHC interactions. $CD4^+$ T cells from wild-type mice were compared to cells derived from mice expressing I-A^b

with a single peptide, in which positive selection can still occur but negative selection is severely limited. This study noted that with decreased negative selection, T cells overall were much more reactive to allogeneic MHC (32). Thus, it appears there is an intrinsic ability of TCRs to broadly recognize MHC, but this becomes constrained by negative selection.

Building upon this idea of inherent TCR affinity for MHC, recent studies have reported convincing data documenting germline-encoded interactions between TCR and MHC. A structural analysis of 4 Vβ 8.2 TCRs complexed with their pMHC ligands revealed that they bind nearly identically despite differences in the TCRβ D and J segments, TCR Vα chains, and the pMHC recognized. In particular, 3 amino acids in the Vβ CDR2 loop – Tyr48, Tyr50, and Glu56 – made remarkably conserved interactions with residues on the MHC (33). These TCR Vβ amino acids were demonstrated to be required for efficient thymic selection, as mice with a modified TCR Vβ repertoire, expressing only a TCR Vβ8 chain containing an alanine substitution at any of the 3 residues (paired with the full TCRα repertoire), yielded decreased numbers of mature T cells. That the same result was seen when one of these amino acids was mutated in a different β chain family – TCRβ6 – is convincing evidence for conserved interactions between TCRs and MHC, lending credence to the idea of an inherent TCR affinity for MHC (34).

II.II. There is increasing evidence that the peptide is highly involved in alloreactivity

Whether or not interactions between TCRs and the peptides bound to MHC also contribute to allorecognition is an important question for understanding the molecular basis behind T cell alloreactivity. A convincing study demonstrating the importance of the peptide utilized H2-DMα$^-$

$^{/-}$ mice as donor organs in a transplantation experiment. Without H2-DMα, MHC class II molecules are primarily associated with an invariant chain peptide (clip), significantly decreasing the repertoire of peptides presented to $CD4^+$ T cells. Interestingly, H2-DM$\alpha^{-/-}$ (H-2^b) cardiac grafts survived much longer than B6 wild-type grafts in H-2^d recipient mice, accompanied by a diminished host T cell response, demonstrating the considerable impact of the peptide in mounting an alloreactive response (35). The importance of allopeptides for MHC class I-restricted T cells has also been documented. Analysis of the 2C TCR binding to an alloligand, dEV8 peptide presented by H-$2K^{bm3}$, revealed that an MHC residue responsible for allorecognition repositions the presented peptide, and this peptide alteration dramatically changes how the TCR recognizes the pMHC surface to allow for allo-pMHC binding (36).

Additionally, the specificity of the T cell alloresponse to peptides has been shown. Cytotoxic T lymphocyte (CTL) clones generated to be alloreactive to murine cytomegalovirus (MCMV) antigen presented by H-$2L^d$ showed exquisite peptide specificity, as they were unable to recognize other viral or endogenous L^d peptides (37). Three T cells alloreactive to I-E^k were each identified by our lab to respond to multiple I-E^k peptides. We demonstrated that allopeptide mutants attenuated T cell responses, so peptide specificity rather than degeneracy explained the ability of the T cells to react to multiple allostimulatory pMHC ligands. The TCRs also bound differently to each allopeptide-I-E^k surface, suggesting that the presented peptide makes important contributions to how alloreactive TCRs approach and bind their ligands (38). Furthermore, another study from our lab showed that the self-peptide repertoire limits the ability of T cells to be alloreactive. A panel of T cell hybrids not alloreactive to H-2^k or H-2^p could be converted to becoming alloreactive upon addition of peptide mimotope pools with H-2^k or H-2^p

MHC binding anchors and all 20 amino acid options at TCR contact positions, demonstrating that the endogenous peptide repertoire is a limiter on alloreactivity (39). This result allows us to reconcile the idea that TCRs have inherent affinity for MHC and the observation that not all T cells are alloreactive.

II.III. It is unknown how $CD4^+$ T cell alloreactivity compares to conventional recognition

All of the observations about T cell alloreactivity suggest that alloreactive T cells would require important contacts with both the allopeptide and MHC to bind, but whether this binding looks similar or different compared to recognition of cognate pMHC is still an unresolved issue. An understanding of this would yield insight into the fundamental properties of TCR-pMHC binding. Assessing crystal structures of TCRs co-complexed with both allostimulatory and cognate pMHC ligands is the best way to compare the detailed chemical interactions between alloreactive and conventional T cell recognition. To date, there are only 2 known studies comparing TCRs co-complexed with distinct allostimulatory and cognate ligands, and both involve MHC class I-restricted TCRs. One study compared structures of the 2C TCR in complex with its allostimulatory ligand, the QL9 peptide presented on H-$2L^d$, and in complex with its cognate ligand, dEV8 peptide on H-$2K^b$. Results from this investigation showed that the 2C TCR binds to its two ligands fairly dissimilarly. For instance, 2C binds more perpendicularly to QL9-L^d relative to the long axis of the MHC groove compared to dEV8-K^b. As a result, there are many pairwise interactions that are unique between the two co-complexes. 4 TCR-MHC contacts are identical between the two, but given the 80% sequence identity of the K^b and L^d helices, a few preserved contacts would be predicted (40).

A study of the LC13 TCR, however, suggested a different mechanism for how a TCR binds to its allostimulatory pMHC ligand. Here, crystal structures were compared between LC13 in complex with an allopeptide on HLA-B*4405 and in complex with its cognate ligand, a viral peptide presented on HLA-B*0801. Though the surface-exposed residues of the 2 MHC molecules are not highly similar – 5 amino acids are different between the 2 – the footprints of LC13 on the MHC surfaces revealed striking similarity in binding. Additionally, 3 C-terminal residues of the viral peptide known to be critical for contact (P6-Ala, P7-Tyr, P8-Gly) are not identical to the corresponding residues of the allopeptide (P6-Ala, P7-Phe, P8-Thr), yet the LC13 TCR binds to both peptide ends with highly similar interactions, making conserved hydrogen bonds to the peptide backbone despite the different amino acid side chains. Thus, the authors concluded that molecular mimicry is the mechanism for how alloreactive TCRs bind to their ligands (41). Whether $CD4^+$ T cell alloreactivity is different from or similar to conventional recognition, whether it follows the conclusions drawn from 2C or LC13, remains to be determined. Work comparing $CD4^+$ T cell recognition of an autoantigen and foreign antigen showed that reactivity to the two ligands involves conserved peptide recognition and only a small number of amino acid differences on the MHC surface (42). It is unknown whether this observation of $CD4^+$ T cell mimicry would also be seen when comparing $CD4^+$ T cell alloantigen and foreign antigen recognition.

II.IV. Dual TCR T cells contribute disproportionately to alloreactivity

The lack of allelic exclusion for TCRα results in 1-10% of peripheral T cells in mice and humans expressing 2 functional TCRs on the cell surface (7). In these cells, each TCRα chain pairs with the same β chain, giving the cell 2 distinct pMHC ligand specificities (43-45). Not all the T cells with two rearranged alpha chains express two TCRs on the cell surface, due primarily to preferential pairing of α and β and the degradation of unpaired α chains (2, 8, 46). There has been some debate on whether the secondary TCR, which most likely is not involved in positive selection, actually exerts an important role in peripheral immune responses. In particular, it is intriguing to wonder whether dual TCR T cells play a role in atypical immune reactions such as alloreactivity and autoimmunity. T cells with 2 TCRs may not follow stringent selection requirements during thymic development. One TCR can successfully mediate positive selection, freeing the secondary TCR from participating in positive selection and needing to bind self pMHC (43, 44). Additionally, the expression of secondary TCRs can mask autoreactive TCRs from deletion, likely due to TCRα chain competition for the single TCRβ chain decreasing the surface expression of the pathogenic TCR (47).

There are reports that the secondary TCR is not required for autoimmune diseases such as arthritis, experimental autoimmune encephalomyelitis (EAE), and lupus (48, 49). On the other hand, the contribution of naturally-arising dual TCR T cells to autoimmunity has been shown for diabetes using NOD mice (49). The impact of dual TCR T cells on alloreactivity has also been investigated. Using TCR$\alpha^{+/-}$ mice which are heterozygous for a mutation in the T cell receptor α chain constant region that disrupts the formation of a functional TCRα chain, resulting in the lack of dual TCR T cells, our lab demonstrated that secondary TCRs were responsible for over 40% of the alloreactive response even though they constitute only about 10% of the peripheral T

cell repertoire (44). Additionally, we demonstrated the impact of secondary TCRs on alloreactivity in patients with acute graft versus host disease (GVHD) after allogeneic hematopoietic stem cell transplantation. In these patients, dual TCR T cells were expanded, activated, and responded preferentially to mismatched alloantigens (50). In addition to having an impact in autoimmunity and alloreactivity, secondary TCRs were also shown to contribute to the response to a foreign ligand (43).

III. The effect on T cell responses from expressing additional MHC molecules in mice

III.I. MHC composition affects alloreactivity

There is evidence that the composition of the positively and negatively selecting MHC molecules can shape the alloreactive T cell repertoire. In studying the repertoire of T cells resulting from selection with a single peptide-MHC class II molecule – a system that can positively select a full repertoire of T cells but exhibits limited negative selection – it was revealed that positive selection results in T cells that can bind to many different allogeneic MHC molecules, with negative selection establishing MHC restriction (32). Germ-line affinity of T cells for MHC can also impact the formation of the alloreactive T cell repertoire; $CD4^+$ TCRs that contain mutations of Vβ amino acids identified to mediate MHC binding could no longer respond to the majority of allogeneic MHC haplotypes tested (34). In studying the relationship between the selecting MHC and allostimulatory MHC, it was discovered that the avidity of the 2C T cell for its alloantigen is affected by what MHC is present during selection (51). Along the same line, allopeptide recognition can be influenced by the homology of the stimulatory MHC to the self MHC

molecule (52). Altogether, it would appear that the MHC present during selection affects the alloreactive T cell population.

III.II. Adding MHC molecules to the repertoire has conflicting results on T cell immunity

It is an interesting question as to why more types of MHC molecules are not expressed by vertebrates. As the MHC plays an integral role in selecting mature T cells and presenting pathogenic peptides in the periphery (53), having more MHC would theoretically be beneficial, resulting in more T cells that make it past positive selection and an increased ability to respond to pathogens. Indeed, mathematical modeling suggests that the number of MHC molecules can be increased significantly, up to as many as 1,500 MHC molecules, without suffering adverse effects on the ability to mount a response to pathogens (54, 55). The question of what impact expressing additional MHC molecules has on the immune response has intrigued immunologists over the years yet has not been answered thoroughly.

Currently in the field, there are conflicting reports on whether increasing the repertoire of MHC molecules expressed would be beneficial or detrimental to immune responses. MHC heterozygotes – obtained from crossing the F1 progeny of MHC-congenic mice – were shown to have greater pathogen resistance compared to their littermate MHC homozygote mice (56, 57). On the other hand, studies looking at the F1 progeny of parents with disparate MHC haplotypes revealed a dampening of T cell reactivity in the context of viral infections. Both naive T cell precursor numbers and T cell cytokine production during infection with influenza and vaccinia viruses were shown to be reduced (58, 59). The approach of looking at MHC heterozygote mice,

however, is a complicated system that may result in heterogeneous responses in offspring within the same cross due to complex contributions from the varied MHC molecules (60).

An alternative, simpler method is to investigate the effects of adding just one more MHC molecule. Studies employing this method focused on autoimmunity, with most concluding that expressing more MHC is beneficial. Adding I-E expression to the non-obese diabetic (NOD) background protected from diabetes, with I-E-mediated thymic deletion of pathogenic Vβ5 TCR-expressing T cells proposed to be a mechanism (61-64). Expression of I-E on other backgrounds including H-2^b and H-2^q likewise resulted in protection from lupus, experimental autoimmune myasthenia gravis, and collagen-induced arthritis (65-67). Yet there is a report that the addition of I-E^b actually resulted in detrimental effects, enhancing susceptibility to disease in autoimmune thyroiditis (68). Having negative effects from the addition of MHC was also corroborated in a different model (viral infection) with a different MHC being investigated (I-A) (69). What effect expressing additional MHC molecules exerts on alloreactivity has not been tested. And, the previous studies summarized here have each focused only on one disease model. It remains unknown whether adding a particular MHC exerts distinct effects on different T cell responses.

Conclusion

The studies on T cell alloreactivity have revealed incredible insight into the factors contributing to alloreactivity. Germline-affinity of TCRs for MHC molecules and recognition of the presented allopeptide have been shown to be important in allorecognition. Compared to conventional recognition, $CD8^+$ T cell alloreactivity can mimic conventional recognition as well as involve disparate binding mechanisms, though how $CD4^+$ T cell alloreactivity compares is unknown.

Additionally, it was discovered that secondary TCRα rearrangement, which generates dual TCR T cells, contributes disproportionately to alloreactivity as well as to some types of autoimmunity. What benefit this process has in thymic development remains to be seen. And, MHC composition, specifically how many types of MHC molecules are expressed, has been shown to affect autoimmunity with its role in alloreactivity suggested but not yet determined.

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CHAPTER II

Identification of alloligands for $CD4^+$ T cells and comparison of allorecognition with conventional recognition reveal similarities

Introduction

An enigma in T cell biology is how T cells are able to recognize allogeneic MHC after undergoing stringent selection processes during thymic development to bind self MHC. It is particularly puzzling that such a high frequency of T cells are alloreactive, between 1-10% (1, 2). Germline affinity of TCRs for MHC, in which conserved amino acids in the TCR CDR1 and CDR2 loops consistently bind to similar regions on the MHC, has been proposed to explain this phenomenon (3). As recognition of allogeneic MHC has not been limited by negative selection, it is conceivable that a high percentage of T cells recognize allogeneic MHC, an idea supported by the finding that limited negative selection yields a T cell repertoire that is more alloreactive (4). Convincing experimental evidence in support of germline affinity as a basis for alloreactivity includes decreased alloreactivity after mutating the conserved TCR CDR1 and CDR2 amino acids as well as dependence on these residues for allorecognition after altering the peptide-contacting CDR3 loop (5, 6).

A longstanding question of T cell alloreactivity is how it compares to conventional recognition of foreign peptides presented by self MHC. Studies thus far have started to show that alloreactivity utilizes fundamental properties seen in conventional T cell recognition of pMHC. A study of CTL clones generated to be alloreactive to MCMV peptide presented by H-2L^d revealed that alloreactivity exhibits peptide- and MHC-specificity (7). Additionally, comparable MHC binding was found for CTLs recognizing H-2L^d as an alloligand compared to CTLs binding it as its syngneic MHC (8). Polyspecificity in alloreactivity has been documented, with alloreactive CD4$^+$ T cell hybrids recognizing each of their distinct allopeptide ligands with a high

degree of peptide specificity and utilizing different MHC contacts when bound to each peptide (9). Regarding binding affinity and mechanisms for T cell alloreactivity, differences between alloreactivity and conventional recognition were noted. Using the 2C TCR system, it was found that binding to the allogeneic MHC H-2L^{d} is an order of magnitude stronger than binding to the syngeneic MHC H-2K^{b} (10) with additional differences in enthalpy- and entropy-driven binding (11). The most direct way to understand how alloreactivity relates to conventional recognition is to compare how one T cell binds to its alloligand versus its cognate ligand. To date, T cells with identified paired ligands (distinct from each other) that have been used for this analysis have been restricted to CD8^{+} T cells. These studies found that CD8^{+} T cell alloreactivity can reflect molecular mimicry (12) as well as bind with divergent mechanisms compared to conventional recognition (11).

Ligand recognition by CD4^{+} T cells is of a dissimilar nature compared to CD8^{+} T cells; not only does the pMHC surface look different, but the CD4 co-receptor itself also binds less strongly to MHC and is associated with more Lck, affecting signaling downstream of TCR activation (13-15). Thus, the conclusions drawn from CD8^{+} T cell alloreactivity cannot be applied to understanding whether CD4^{+} T cell alloreactivity is similar or different compared to conventional recognition. We hypothesize that CD4^{+} T cell alloreactivity is similar to conventional recognition and exhibits the same binding properties. To address this, we identified paired ligands (allo and cognate pMHC ligands) for multiple CD4^{+} T cells and compared how they recognize their alloligands and cognate ligands, assessing the specificity of peptide recognition and binding kinetics. For one T cell responsive to Listeriolysin O (LLO)/I-A^{b} as its cognate ligand, LLO118, we found that it is naturally alloreactive to I-E^{k}. Subsequent screening

of a 95 I-E^k peptide library identified one allopeptide for LLO118. Another T cell also responsive to LLO/I-A^b as its cognate ligand, 1G5.1, was not naturally alloreactive to H-2^k. However, we were able to identify an I-E^k allopeptide mimic for the T cell using peptide pools with a hemoglobin (Hb) epitope as the peptide backbone and multiple amino acid options at the TCR contact sites. Studies in the field have successfully used allopeptide mimics to examine alloreactivity (16), even resulting in conclusions identical to those obtained from studying a related, naturally occurring allopeptide (12). Additionally, we found that T cell alloreactivity is limited by the endogenous peptide repertoire, necessitating the use of peptide mimics to study the alloreactive potential of T cells. Comparison of LLO118 and 1G5.1 peptide binding for cognate recognition versus alloreactivity revealed that mimicry did not explain the ability of the T cells to be alloreactive as well as demonstrated a high degree of specificity in binding. Additionally, binding affinity and kinetics studies using surface plasmon resonance revealed that LLO118 binds with similar affinity and kinetics to both its cognate and allostimulatory ligands. Our data therefore suggest that $CD4^+$ T cell alloreactivity is of a similar nature compared to conventional recognition, providing insight into universal properties of T cell binding.

Results

The LLO/I-A^b-specific T cell, LLO118, is naturally alloreactive to kidins220/I-E^k

LLO118 is a $CD4^+$ Vα2, Vβ2 T cell hybrid that was originally generated in the Unanue lab (unpublished data). With antibodies available to both TCRα and TCRβ chains as well as LLO118 TCR transgenic mice on the RAG-/- background made, this T cell has many useful reagents for probing immunologic questions. Specific to $LLO_{190\text{-}201}$ presented by I-A^b as measured by enzyme-linked immunosorbent assay (ELISA) for IL-2 production (**Fig. 2.1A**), LLO118 was also determined to be alloreactive, with significantly increased IL-2 production to irradiated B6.K (H-2^k) APCs compared to medium. The alloresponse was directed to I-E^k, as blocking antibodies to I-E^k abrogated the IL-2 response to H-2^k APCs (**Fig. 2.1B**). We sought to identify its I-E^k-restricted allopeptide in order to have two defined ligands and directly compare alloreactive and conventional recognition. To do this, we utilized a library of 95 self-peptides eluted from I-E^k, previously used by our lab to successfully identify allopeptides for 9 out of 32 alloreactive T cells (9). We used this library to screen for an allopeptide able to stimulate LLO118 IL-2 production upon addition of peptide to Chinese hamster ovary (CHO) cells stably transfected with I-E^k (CHO-E^k), which have limited endogenous peptides and do not stimulate LLO118 on their own. One peptide (#75) from the library was able to stimulate IL-2 production from LLO118 in a dose-dependent manner. The lack of stimulation from peptide #10 is representative of the results obtained from the rest of the peptides in the library (**Fig. 2.1C**). Peptide #75 is derived from a predicted kinase D-interacting substrate of 220 kDal (kidins220). Thus, we now have two defined ligands for LLO118: its cognate ligand LLO/I-A^b and its

alloligand kidins220/I-E^k.

An I-E^k allopeptide mimic can be identified for the LLO/I-A^b-specific T cell, 1G5.1

1G5.1, a Vα1, Vβ1 $CD4^+$ T cell hybridoma, was generated by immunizing B6 TCR $C\alpha^{+/-}$ mice containing a fixed 2.102β chain with the $LLO_{190-201}$ peptide (that is presented by I-A^b). Heterozygosity at TCR Cα prevents the expression of dual TCRs in the hybrids, and the β chain is from the well-defined 2.102 T cell specific for Hb/I-E^k (17). Reactivity to LLO is shown by IL-2 production measured by ELISA (**Fig. 2.2A**). Although 1G5.1 was not naturally alloreactive to H-2^k APCs (**Fig. 2.2B**), we sought to identify an I-E^k allopeptide mimic for it because a second T cell with defined, paired pMHC ligands in addition to LLO118 would enhance our investigations into how alloreactivity compares to conventional recognition as well as broaden the applicability of the conclusions drawn from our comparisons. To identify an allostimulatory mimotope for 1G5.1, we screened pools of peptides consisting of the I-E^k-binding Hb epitope as the backbone, reasoning that the 2.102 TCRβ chain may predispose the TCR to recognize Hb and facilitate our screen. The pools contained different amino acid groups substituted at TCR contact P3 in addition to all 20 amino acid possibilities (denoted as "X") at P5. Conversely, we also screened peptide pools consisting of all 20 amino acids at P3 with more limited amino acid groups at P5. Three peptide pools each containing groups of 4 amino acids at P3 and all 20 options at P5 elicited T cell responses. One pool with all 20 amino acids at P3 and D,E,N,Q at P5 also provoked T cell production of IL-2 (**Fig. 2.3A**). These pools were deconvoluted into the Hb peptide with single amino acid substitutions based on the amino acids present in the stimulatory pools. A phenylalanine (F) at P3 resulted in the most IL-2 production (**Fig. 2.3B**). This

allopeptide mimic, Hb F70, was purified, analyzed by mass spectrometry, and tested for its ability to stimulate 1G5.1. The T cell response to Hb F70 was dose-dependent and highly sensitive (**Fig. 2.3C**).

Allopeptide recognition by $CD4^+$ T cells is specific

Reports of how $CD8^+$ T cell alloreactivity compares to conventional recognition have revealed that it involves mimicry of cognate pMHC binding (12) or encompasses divergent binding mechanisms (11). Upon initial characterization, the peptide sequences of cognate and allostimulatory peptides for 1G5.1 and LLO118 are not similar at the TCR contact sites (**Fig. 2.4A**). To test if mimicry can explain LLO118 and 1G5.1 allopeptide binding, APLs were generated and tested to see if alloresponses could still occur when amino acids at the TCR contact positions P2, P3, or P5 of the allopeptides are replaced with the corresponding amino acids from the cognate peptides. As measured by IL-2 ELISA, LLO118 and 1G5.1 did not respond or responded minimally to these APLs (**Fig. 2.4B**). Likewise, APLs of the cognate peptide, LLO, containing amino acids from the allopeptides at the TCR contact positions did not stimulate significant responses from LLO118 and 1G5.1 (**Fig. 2.4C**). Therefore, peptide mimicry cannot explain LLO118 and 1G5.1 alloreactivity. Thus far, with the APLs tested, it appears that alloreactivity is peptide-specific. Knowing that a fundamental property of conventional T cell recognition is a high degree of peptide specificity, we sought to rigorously determine whether $CD4^+$ T cells exhibit similar allopeptide specificity. Our results will contribute to understanding whether $CD4^+$ T cell alloreactivity is inherently different or strikingly similar to conventional recognition. A comprehensive series of APLs containing single amino acid substitutions at TCR

contact sites P2, P3, or P5 were generated for both the allopeptides and cognate ligands. Substitutions with both conserved and disparate residues were made. LLO118 and 1G5.1 responses to the APLs were severely attenuated in comparison to wild-type peptides, suggesting peptide specificity is involved in allorecognition (**Fig. 2.5**).

That the allopeptide is recognized as a distinct surface from the cognate peptide and with a high degree of specificity suggest that recognition of a particular allopeptide is required for $CD4^+$ T cell allorecognition. Seeing that not all T cells are naturally alloreactive, we wondered if providing T cells with an increased repertoire of allopeptides would be sufficient to enable alloreactivity. We took a panel of I-A^b-restricted $CD4^+$ T cell hybrids not naturally alloreactive to H-2^k or H-2^p APCs and investigated whether adding peptide mimotope pools to the APCs would result in reactivity. Using IL-2 ELISA as a read-out, we saw that the addition of peptide pools comprised of I-E^k or I-E^p MHC anchor residues plus all 20 amino acid options at the TCR contact sites P2, P3, P5, and P8 yielded responses from the T cell hybrids (**Fig 2.6A**). Deconvolution of the peptide pools was performed by substituting more limited pools of 4 amino acid options at one TCR contact site in conjunction with unlimited amino acids at the other 3 contact sites, then further deconvoluting into single peptide species depending on the patterns of reactivity to yield individual allopeptide mimotopes for each T cell hybrid. We saw that recognition of the allopeptide mimotopes was highly specific, as APLs with mutated residues at P3 or P5 strongly inhibited alloreactivity (**Fig. 2.6B**). This is consistent with our LLO118 and 1G5.1 data and what others noticed in previous studies of allopeptide specificity (7, 9). Put together, our results indicate that recognition of the allopeptide is not merely mimicry of the cognate peptide, allopeptide response is highly specific, and expanding the peptide repertoire is

sufficient for enabling allorecognition of otherwise non-alloreactive T cells.

LLO118 binds its alloligand and cognate ligand with similar binding affinity and kinetics

Weak affinity, slow association, and fast dissociation are all characteristic properties of T cell recognition of conventional ligands (18, 19). We used surface plasmon resonance (Biacore system) to measure kinetics and determine whether $CD4^+$ T cells use these same properties to bind to their allostimulatory ligands or whether the properties of allorecognition are inherently different. LLO/I-A^b and kidins220/I-E^k were coupled to a Biacore CM5 chip. Soluble single chain (sc) LLO118, with the TCR Vα and Vβ chains expressed as a single-chain construct, was injected and flowed over the chip until equilibrium was reached. We saw that the on-rate, off-rate, and binding affinities were similar between scLLO118 binding to its cognate ligand and its alloligand (**Fig. 2.7, Table 2.1**). Additionally, binding affinities were weak and within the 1-100μM range typically reported for TCR-pMHC interactions, confirming the values obtained were reasonable.

Discussion

Much insight has been gained in understanding T cell alloreactivity by our lab and others. This includes the remarkable discovery of germline-encoded contacts between TCR and MHC and recent findings that suggest an importance for the allopeptide in alloreactivity. However, there are still questions about how $CD4^+$ T cells bind to allostimulatory pMHC ligands that remain unanswered and are integral for gaining a full picture of how alloreactivity occurs. Namely, how does $CD4^+$ T cell alloreactivity compare to recognition of cognate ligands? Finding an answer to this question is important because we still do not know whether $CD4^+$ T cell alloreactivity is fundamentally similar to conventional recognition or whether it embodies divergent binding mechanisms, with either scenario having important implications for understanding fundamental T cell binding properties. To address this issue, the identification of paired ligands, allostimulatory and cognate, was performed for two $CD4^+$ T cells, LLO118 and 1G5.1. These T cells were then used to directly compare alloreactive and conventional recognition, revealing a high degree of peptide specificity in both types of recognition. Additionally, we found that molecular mimicry of the cognate peptide could not explain the ability of the T cells to respond to the allopeptide. Further emphasizing the importance of the peptide in alloreactivity, we determined that naturally non-alloreactive $CD4^+$ T cells could be converted to becoming alloreactive by the addition of allopeptide mimotopes, suggesting that the endogenous repertoire of peptides is a limiting factor for T cell alloreactivity. Finally, biochemical studies of LLO118 suggested very similar binding kinetics and affinity values between alloreactivity and cognate recognition. Thus far, these studies of $CD4^+$ T cells favor the interpretation that alloreactivity exhibits highly similar binding properties compared to cognate pMHC recognition.

We currently do not know how LLO118 and 1G5.1 bind their allogeneic MHC compared to their syngeneic MHC. Crystal structures of these TCRs co-complexed with their allostimulatory and cognate pMHC ligands would best elucidate which MHC residues are contacted and how. Our prediction is that the TCRs would bind their ligands distinctly. Structural comparison of the MHC class I restricted TCR 2C complexed with its allo- and cognate-pMHC ligands revealed highly divergent binding footprints of the TCR on the two surfaces, with a more perpendicular TCR binding orientation on the alloligand relative to the long axis of the MHC groove (11). Though no comparable crystal structure comparison for MHC class II restricted TCRs has been done, analysis of $CD4^+$ T cell recognition of multiple allo-pMHC ligands (dissimilar peptides presented by $I\text{-}E^k$) has been performed. Using a panel of MHC mutants each with a different surface residue disrupted, it was found that these $CD4^+$ T cells recognize their $I\text{-}E^k$ alloligands distinctly (9). The allopeptides and cognate peptides for LLO118 and 1G5.1 are likewise different, and distinct MHC binding would be predicted. Although greatly similar TCR contacts with MHC were seen in LC13 binding to its cognate ligand and alloligands, in this case the peptide binding mechanisms were identical, with the TCR CDR3 loops contacting conserved C-terminus features in both the cognate and allostimulatory peptides (12). No such peptide mimicry was seen in LLO118 and 1G5.1, so we would not anticipate similar MHC contacts between the allo- and cognate-MHC molecules.

A fundamental understanding of how $CD4^+$ T cells contact their allo-pMHC ligands has clinical implications, especially in the area of allopeptide-induced tolerance strategies. Both direct and indirect allorecognition play an integral role in graft rejection, with the former dominating acute

rejection and the latter mediating chronic rejection. Direct allorecognition involves T cells contacting allogeneic MHC molecules, while indirect recognition is a response to syngeneic MHC molecules presenting allostimulatory peptides such as those derived from the allogeneic MHC (20). Strategies to achieve graft tolerance target both allorecognition pathways. Intrathymic injection of allostimulatory antigens - including MHC-derived peptides, soluble antigen, and cells - prevented rejection, possibly due to elimination of alloreactive T cells in the thymus and/or an increase of regulatory T cells (Tregs) (21). Extrathymic inoculation strategies also may be promising. For instance, intratracheal injection of donor splenocytes or an MHC-derived allopeptide promoted the survival of cardiac allografts (22). With the knowledge that $CD4^+$ T cell alloreactivity can exhibit the same binding affinity and kinetics as conventional recognition, it is likely that allostimulatory antigen injection is a practical way to achieve tolerance since excessive amounts of antigen or unconventional injection strategies will not be needed to achieve tolerance. And, viral- and bacterial-specific T cells may not be stimulated since our work with LLO118 and 1G5.1 shows peptide allorecognition is not molecular mimicry of cognate peptides, allowing for a tolerance strategy that is specific. Additionally, the use of allo APLs to induce tolerance has been probed. Two APLs of a peptide derived from the HLA molecule DR1 were generated and shown to antagonize the T cell response to wild-type peptide presented by DR11, an example of a way to modulate indirect alloreactivity (23). The highly allopeptide-specific $CD4^+$ T cell response shown by us and by other labs strengthens the idea that allo APLs represent a promising therapeutic strategy.

The ability to recognize allogeneic pMHC ligands, which T cells do not encounter during thymic selection, is fascinating because it may represent fundamental binding properties normally not

apparent when selection restricts the T cell repertoire. A related concept supporting this idea is that although negative selection is crucial for maintaining self-tolerance, it can hide evolutionarily conserved TCR-MHC binding properties (24). Indeed, an analysis of the T cell repertoire resulting from fairly normal positive selection but greatly limited negative selection revealed that the T cells are significantly more alloreactive (4). As such, our data on $CD4^+$ T cell alloreactivity support the idea that peptide specificity and low binding affinity of TCR-pMHC interactions are fundamental, conserved characteristics of T cells.

Figure 2.1 Identification of an I-E^k-restricted allopeptide for the LLO/I-A^b-specific T cell, LLO118

LLO118 T cells were stimulated overnight with (**A**) varying concentrations of $LLO_{190-201}$ plus irradiated B6 splenocytes; (**B**) irradiated B6.K splenocytes with or without 10μg/mL αE^k antibody (clone 14-4-4S); (**C**) CHO-E^k cells with or without E^k peptides (left) and varying concentrations of E^k #75 peptide plus CHO-E^k cells (right). Supernatant was taken and analyzed for IL-2 production by ELISA. Data are compiled from 3 independent experiments each performed in triplicate wells (**A, C** left) or are representative of 2 independent experiments each in triplicate (**B, C** right), shown as mean ± sem.

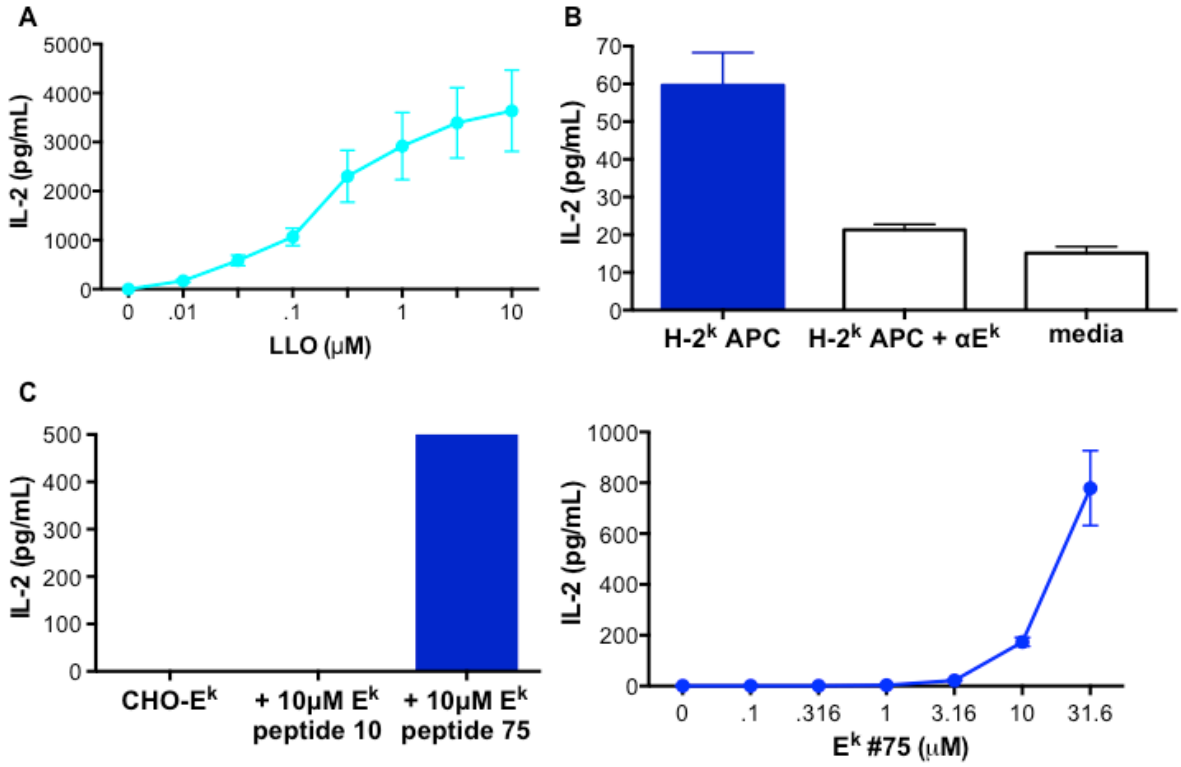

Figure 2.1 Identification of an I-E^k-restricted allopeptide for the LLO/I-A^b-specific T cell, LLO118

Figure 2.2 The T cell 1G5.1, also specific to LLO/I-A^b, is not naturally alloreactive

1G5.1 T cells were stimulated overnight with (**A**) varying concentrations of $LLO_{190-201}$ plus irradiated B6 splenocytes and (**B**) either irradiated B6 splenocytes with or without LLO or CH27 (H-2^k) APCs. Supernatant was taken and assessed for IL-2 production by ELISA. Data are representative of 3 independent experiments each done in triplicate wells, mean ± sem.

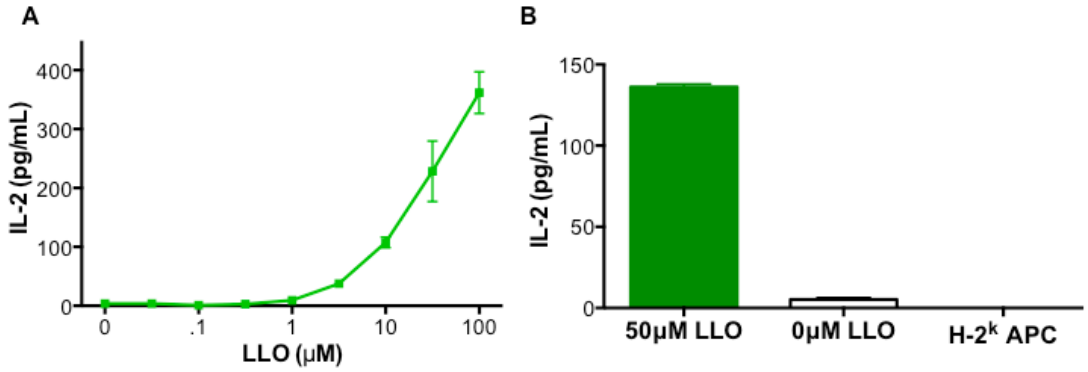

Figure 2.2 The T cell 1G5.1, also specific to LLO/I-A^{b}, is not naturally alloreactive

Figure 2.3 Identification of an allopeptide mimic for 1G5.1

1G5.1 T cells were stimulated overnight with CH27 APCs plus (**A**) peptide pools containing the Hb_{64-76} epitope as the backbone with groups of 4 amino acids at the P3 position and all 20 amino acid options (denoted as "X") at P5 (top) or X at P3 and groups of 4 amino acids at P5 (bottom); (**B**) peptides containing the Hb_{64-76} epitope with single amino acid substitutions at P3 or P5; and (**C**) the indicated concentrations of Hb F70. Supernatant was taken and assessed for IL-2 production by ELISA. Data are representative of 2 independent experiments done in triplicate wells, shown as mean ± sem.

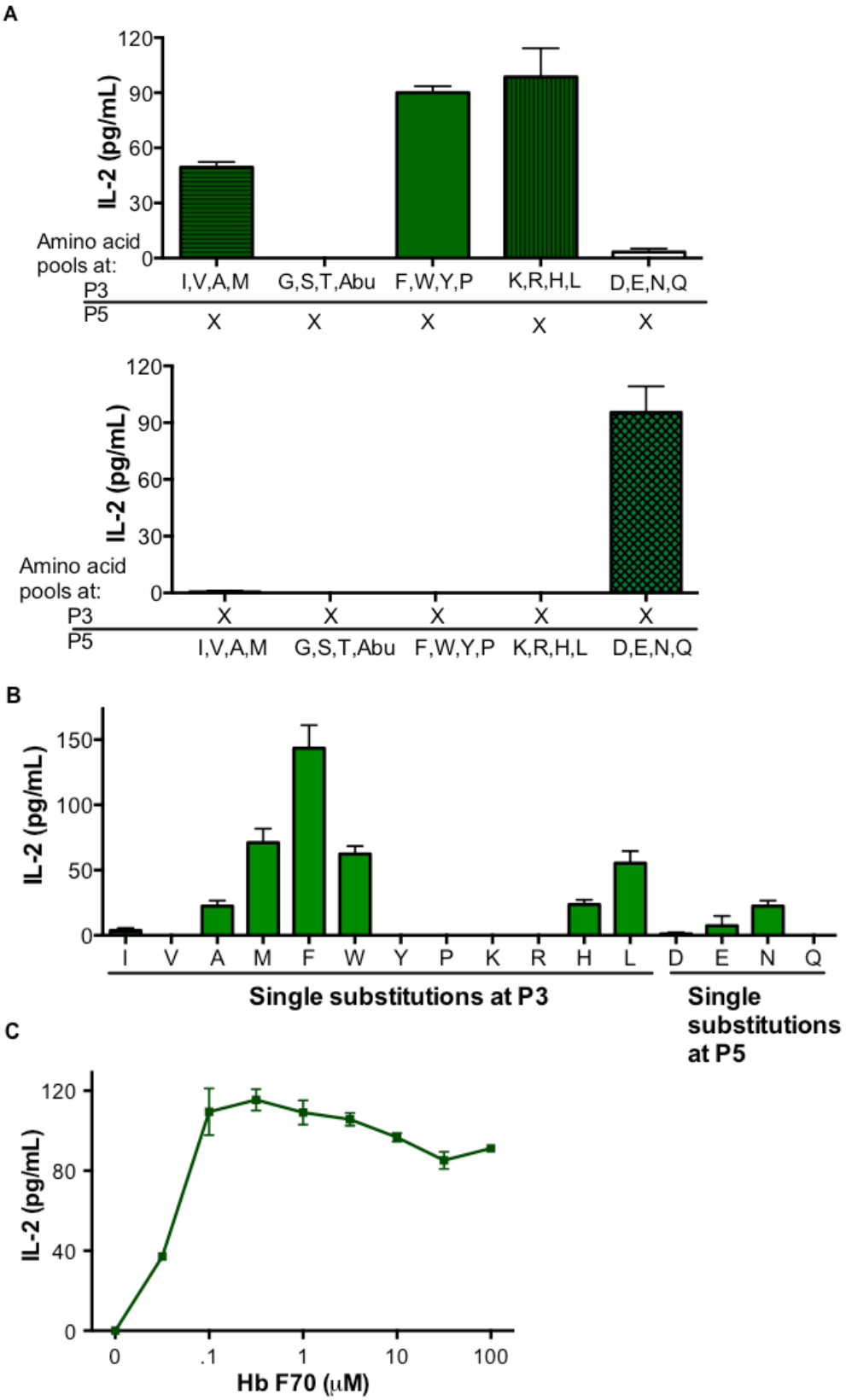

Figure 2.3 Identification of an allopeptide mimic for 1G5.1

Figure 2.4 Molecular mimicry cannot explain allopeptide recognition by LLO118 and 1G5.1

(**A**) Comparison of TCR contact sites P2, P3, and P5 between $LLO_{190-201}$, the cognate peptide of both LLO118 and 1G5.1, and $kidins220_{47-62}$ and Hb $F70_{64-76}$, the allopeptides of LLO118 and 1G5.1, respectively. (**B**) LLO118 and 1G5.1 T cells were stimulated over night with CHO-E^k and CH27 APCs, respectively, along with allopeptide APLs with residues at P2, P3, or P5 mutated to the corresponding amino acids of LLO. For LLO118, peptides were added at 31.6μM and for 1G5.1, peptides were added at 1μM. Supernatant was taken and analyzed for IL-2 production by ELISA. (**C**) LLO118 and 1G5.1 T cells were stimulated overnight with irradiated B6 splenocytes plus LLO APLs with the P2, P3, or P5 residues mutated to the corresponding amino acids of kidins220 (for LLO118 stimulation, 0.1μM) or Hb F70 (for 1G5.1 stimulation, 31.6μM). Supernatant was taken and analyzed for IL-2 production by ELISA. Data are representative of 2 independent experiments done in triplicate wells and depicted as mean ± sem.

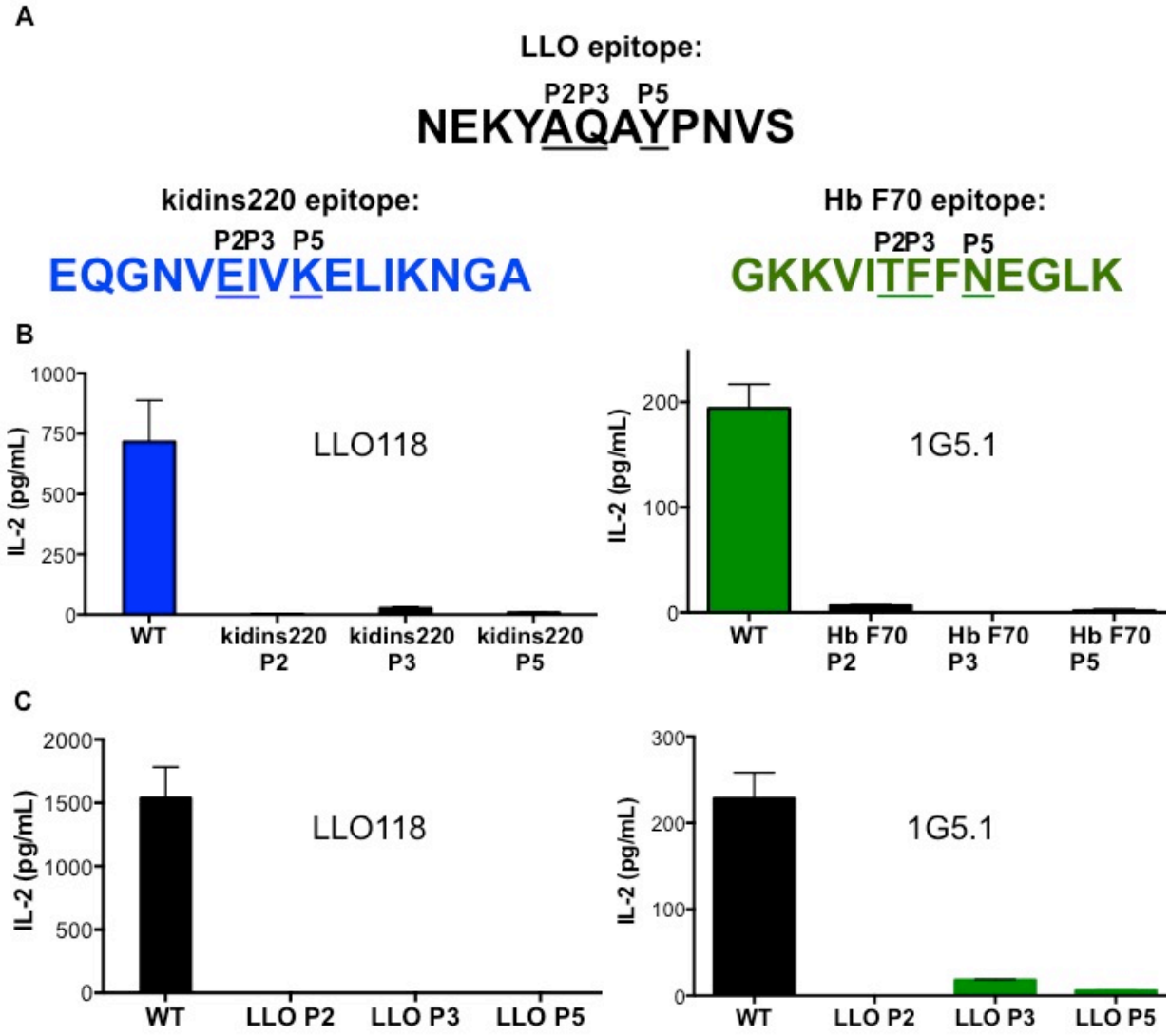

Figure 2.4 Molecular mimicry cannot explain allopeptide recognition by LLO118 and 1G5.1

Figure 2.5 LLO118 and 1G5.1 exhibit a high degree of peptide specificity for both alloreactivity and conventional recognition

LLO118 and 1G5.1 T cells were stimulated overnight with CHO-E^k cells + kidins220 APLs and CH27 cells plus Hb F70 APLs, respectively, at the indicated concentrations (top). LLO118 and 1G5.1 T cells were also stimulated overnight with irradiated B6 splenocytes plus LLO APLs at the indicated concentrations (bottom). The specific amino acid substitutions at P2, P3, or P5 for the peptide APLs are depicted. Supernatant was taken and assayed for IL-2 by ELISA. Data are representative of 2 independent experiments and presented as mean ± sem.

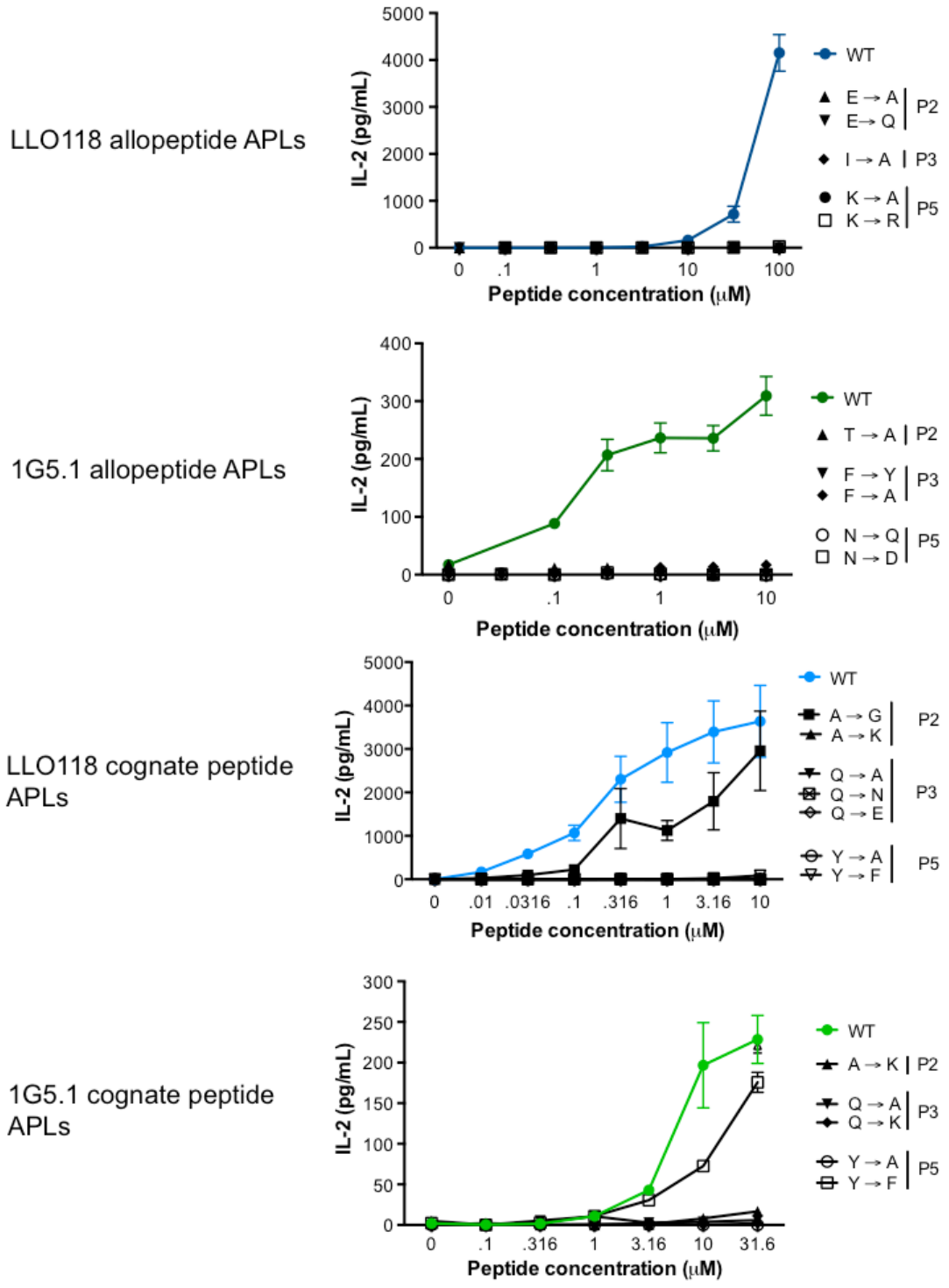

Figure 2.5 LLO118 and 1G5.1 exhibit a high degree of peptide specificity for both alloreactivity and conventional recognition

Figure 2.6 The alloreactive T cell repertoire is expanded by the addition of peptide mimotope pools and exhibits a high degree of specificity in peptide mimotope recognition (**A**) A panel of non-alloreactive, $CD4^+$ T cell hybrids was cultured for 24 h with irradiated B6.K ($H\text{-}2^k$) or B6.P ($H\text{-}2^p$) splenocytes with or without 200μM of the EKX or EPX peptide pool, respectively. The EKX pool consists of canonical $I\text{-}E^k$ MHC binding residues along with all 20 amino acid options at the TCR contact positions P2, P3, P5, and P8 (amino acid sequence of GKKVIXXFXEGXK, with "X" denoting all 20 amino acid options), while the EPX pool has peptides with canonical $I\text{-}E^p$ MHC anchor residues and unrestricted amino acid usage at P2, P3, P5, and P8 (GKKVIXXSXGSXS). Supernatant was taken and assessed for IL-2 by ELISA. Results are depicted as means from 3 independent experiments each done in triplicate wells. (**B**) T cell hybrids 4C9 and 1A10 were analyzed for allopeptide specificity. T cells were stimulated overnight with CHO-E^k APCs with different concentrations of $I\text{-}E^k$ peptide mimotopes (black line) or mimotope APLs (grey lines) containing single amino acid substitutions at P3 or P5. Supernatant was taken and assessed for IL-2 production by ELISA. Data are representative of 3 independent experiments each done in triplicate wells and shown as mean ± sem.

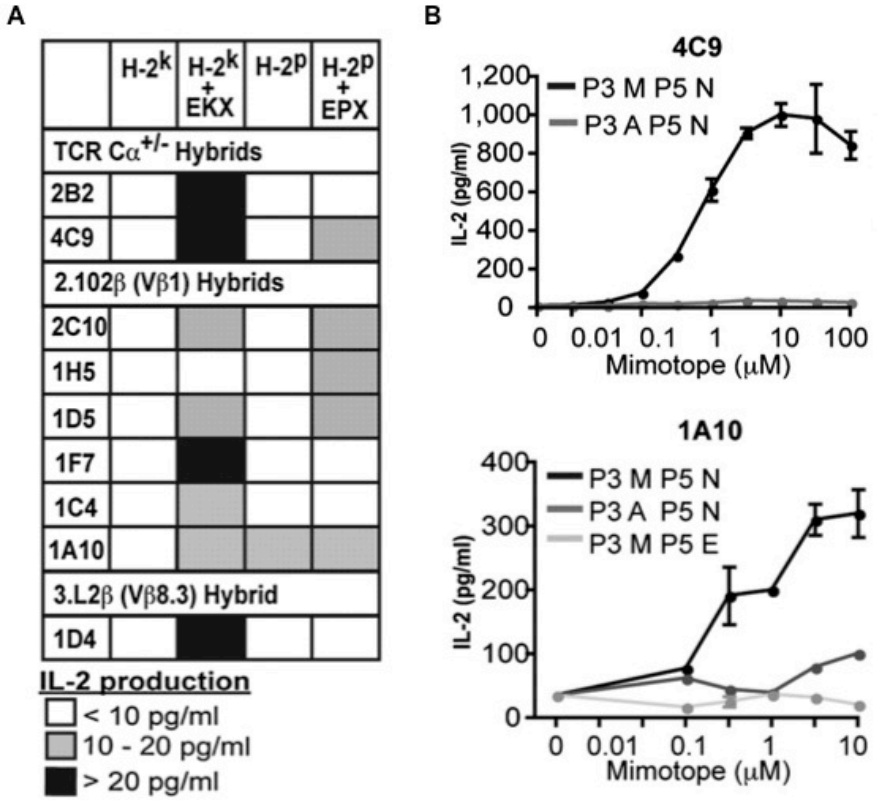

Figure 2.6 The alloreactive T cell repertoire is expanded by the addition of peptide mimotope pools and exhibits a high degree of specificity in peptide mimotope recognition

Figure 2.7 LLO118 binds its alloligand and cognate ligand with similar kinetics and affinity (**A**) Sample sensorgrams of scLLO118 binding to LLO/I-A^b (cognate ligand) and kidins220/I-E^k (alloligand). A concentration series of scLLO118 (40, 16, 8, 4, 2 μM for cognate and 40, 10, 2.5 μM for allo) was flown over pMHC bound to a Biacore CM5 chip. (**B**) Equilibrium binding analysis of scLLO118 to its cognate (left) and allo (right) ligands was performed with linear Scatchard plots shown in the insets that confirm a 1:1 binding. Graphs shown are representative of 3-5 experiments.

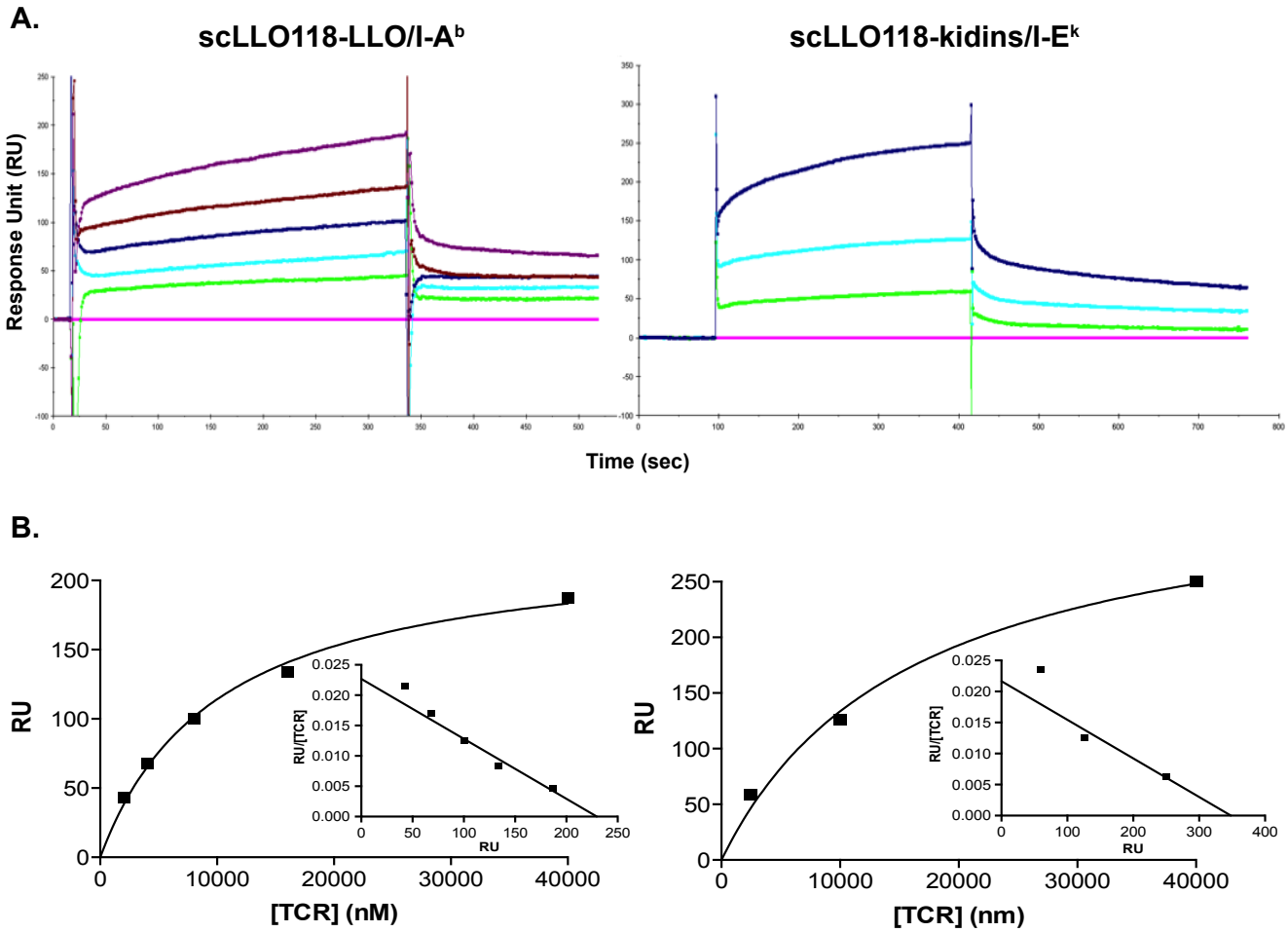

Figure 2.7 LLO118 binds its alloligand and cognate ligand with similar kinetics and affinity

Table 2.1 Kinetic values obtained by surface plasmon resonance for LLO118 binding to its cognate and allostimulatory ligands

On-rate, off-rate, and binding affinities obtained from surface plasmon resonance are presented as means ± SEM combined from 3-5 experiments.

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (k_{off}/k_{on}) nM	Equilibrium K_D (nM)
Cognate ligand (LLO/I-A^b)	**265.0** ± 66.39	**0.003** ± 0.0007	**14,704** ± 4,839.9	**16,117** ± 3,989.16
Alloligand (kidins220/I-E^k)	**277.8** ± 106.0	**0.014** ± 0.0098	**36,360** ± 13,788	**24,260** ± 4,450.24

Table 2.1 Kinetic values obtained by surface plasmon resonance for LLO118 binding to its cognate and allostimulatory ligands

Methods

Generation of $CD4^+$ T cell hybrids

B6 TCR $C\alpha^{+/-}$ mice and B6 TCR fixed β $C\alpha^{+/-}$ mice (β chain from 2.102 or 3.L2 TCR) were immunized in the footpads with 10 μM $LLO_{190-201}$ emulsified with complete Freund's adjuvant (CFA). 7 days later, popliteal lymph node cells were taken and fused with BW5147 TCRα-β- cells. Subcloning was performed by limiting dilution. TCR of the T cell hybrids was identified by DNA sequencing, with only one TCR expressed in each hybrid due to heterozygosity of the TCRα constant region loci.

IL-2 ELISA

1 x 10^5 T cells were cultured in 96-well plates with 1 x 10^5 CH27 APCs, 1 x 10^5 CHO APCs transfected with I-E^k (25) or 1 x 10^6 irradiated splenocytes along with any indicated peptides for 24 h. Supernatant was transferred to Immulon 2 HB plates (Thermo Scientific) pre-incubated with anti-IL-2 antibody (Bio X Cell). Biotinylated anti-IL-2 antibody (BioLegend) was used to detect IL-2, followed by streptavidin-conjugated horseradish peroxidase (Southern Biotech) and 1-step Ultra TMB-ELISA substrate (Thermo Scientific). 2M sulfuric acid was used to stop the reaction, with absorbance values read at 450 nm.

Peptide pools

Peptides were synthesized by Fmoc chemistry on a Rainin Symphony/Multiplex multiple peptide synthesizer (Protein Technologies). Peptide pools were generated by the addition of multiple amino acids at the P2, P3, P5, and/or P8 positions during synthesis. Individual peptides were

purified by reverse phase HPLC on a C18 column (Vydac), and peptide composition and purity were confirmed by MALDI mass spectrometry (Washington University Mass Spectrometry Facility). All peptides were sterilized through .22 μM filtration.

Surface plasmon resonance

The scLLO118 TCR construct was cloned and provided by Steve Persaud (26). scLLO118 protein was produced in *Escherichia coli* inclusion bodies, combined and refolded in a guanidine hydrochloride based buffer, and purified by size exclusion chromatography. LLO covalently linked to I-A^b was produced in S2 cells and purified from the supernatant using the histidine tag on the MHC and nickel affinity chromatography (27). I-E^k dimer was produced in S2 cells and purified with protein A, with peptide exchanged conducted to add the peptide to the MHC (9). pMHC complexes were amine-coupled, either directionally attached using neutravidin-biotin interactions (LLO/I-A^b and control gp250/I-E^k) or not (kidins220/I-E^k dimer and control Hb/I-E^k dimer), to a Biacore CM5 chip. A concentration series of scLLO118 was flowed over the chip until equilibrium was reached, using a Biacore 2000 surface plasmon resonance instrument. Control sensorgrams were subtracted from experimental sensorgrams to eliminate non-specific binding and fitted to a 1:1 Langmuir binding model using BiaEval version 4.1 (Biacore AB) to obtain measurements of association and dissociation as well as affinity.

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CHAPTER III

The ability to rearrange dual TCR T cells contributes to efficient positive selection of T cells and to alloreactivity through increased flexibility in peptide recognition

Introduction

Gene rearrangement of the TCRα and TCRβ chains generates T cells with receptors that specifically and sensitively recognize self and foreign pMHC ligands to appropriately navigate development and mediate immune responses (1). Although the TCRβ chain rearranges under tight allelic exclusion and ceases when an in-frame product is made and expressed, TCRα chain recombination does not exhibit allelic exclusion, rearranging simultaneously and iteratively on both alleles until positively selecting signals are received through the TCR (2-5). The majority of thymocytes die from an inability to rearrange a TCR that can mediate positive selection (6-9). Iterative revision of TCRα has been demonstrated to be important for efficient positive selection of T cells (10). However, the effect of simultaneous TCRα rearrangement on thymocyte selection is unclear. The lack of allelic exclusion for TCRα results in thymocytes and peripheral T cells with 2 in-frame rearrangements of TCRα (3, 11), and 1-10% mature T cells with dual TCR expression on the surface (12-14). In dual TCR T cells, each TCRα chain pairs with the same β chain, giving the cell 2 distinct pMHC ligand specificities (15-17). Many questions remain regarding the impact of these cells in the periphery.

Dual TCRs in developing thymocytes provide an unusual lessening of the stringent requirements for thymic selection. One TCR can successfully mediate positive selection, enabling the presence of a secondary TCR that does not participate in positive selection (15, 16, 18). Expression of secondary TCRs can also factor importantly during negative selection, masking autoreactive TCRs from deletion (19). The contribution of naturally-arising dual TCR T cells to the autoreactive T cell repertoire has been suggested by studies of diabetes in NOD mice (20). The

potential for TCRs to be exempted from the stringent requirements of thymic selection prompted our lab in previous studies to investigate dual TCR T cells in alloreactivity. Alloreactive responses were examined in mice genetically lacking dual TCR T cells ($TCR\alpha^{+/-}$, heterozygous for a mutation in the T cell receptor α chain constant region that disrupts formation of a functional TCRα chain). Our lab found that dual TCR T cells constitute over 40% of the response to allogeneic stimuli, a disproportionate contribution since they make up about 10% of the T cell repertoire (16). The impact of secondary TCRs in pathologic alloreactivity was also demonstrated in patients developing acute GVHD following allogeneic hematopoietic stem cell transplantation. In these patients, dual TCR T cells were expanded, activated, and responded preferentially to mismatched alloantigens (21).

The observed potential for atypical or unwanted TCR specificities harbored by dual TCR T cells suggests that simultaneous rearrangement of TCRα loci must present a significant benefit in thymocyte development to counteract the detrimental effects. We hypothesize that secondary TCRα rearrangements improve efficiency of DP thymocyte positive selection but result in an increase in the naive autoreactive and alloreactive T cell repertoire. To address this, we examined thymocyte development and antigen reactivity in $TCR\alpha^{+/-}$ mice. Mice unable to produce secondary TCRs demonstrated decreased positive selection, providing a novel demonstration of their importance to effective thymocyte development. While this did not broadly impact peripheral T cell numbers or TCRVα and TCRJα gene segment use, $TCR\alpha^{+/-}$ mice had diminished binding to autopeptide-MHC and allopeptide-MHC tetramers but not to foreign antigen. Decreased *in vivo* alloreactivity was also seen with $TCR\alpha^{+/-}$ T cells. Analysis of binding to APLs of an allostimulatory peptide showed that the presence of secondary TCRs enabled

flexibility in recognition of pMHC ligands, providing a mechanism for the significant contribution of secondary TCRs to the responses to atypical ligands such as allogeneic or autologous pMHC.

Results

Secondary TCRα rearrangements enable efficient positive selection

The effects of simultaneous TCRα rearrangement on thymocyte development were evaluated by analyzing thymus cellularity and populations in TCRα$^{+/-}$ mice, heterozygous for a mutation in the TCRα constant region that disrupts formation of a functional TCRα chain (4). Thymi from B6 and TCRα$^{+/-}$ mice had similar total number of cells (**Fig. 3.1A**). There were comparable percentages of immature DN (8.8±1.5% vs. 7.6±2.0%) and DP (70.1±3.0% vs. 76.4±3.6%) cells, though TCRα$^{+/-}$ thymi had significantly decreased percentages of mature SP cells (14.7+2.2%) compared to B6 (19.7+2.5%) (**Fig. 3.1B, C**). Further examination revealed that TCRα$^{+/-}$ mice had significantly reduced percentages of post-selection CD3^{high} thymocytes (8.7±0.6%) compared to B6 (13.3±0.7%) (**Fig. 3.1D, E**). This specific decrease of post-positive selection thymocytes implies that secondary TCRα rearrangements are important for efficient thymic selection. Co-transfer studies of congenically marked B6 and TCRα$^{+/-}$ cells were performed to attribute the deficiency of generating mature SP thymocytes to a cell-intrinsic defect. Pre-selection CD53^{-} cells from B6.Ly5.1 and TCRα$^{+/-}$.Thy1.1 thymocytes were mixed at a 1:1 ratio and intra-thymically injected into recipient B6 mice. Transferred cells were analyzed 7 days post-injection by flow cytometry. The ratio of B6.Ly5.1/TCRα$^{+/-}$.Thy1.1 among total thymocytes was 1.36±0.11, similar to the pre-injection ratio. However, examination of post-selection CD3^{high} thymocytes revealed a significant skewing toward B6.Ly5.1 cells (1.86±0.15 ratio), suggesting that the inability to perform simultaneous TCRα rearrangements impaired the ability of

thymocytes to mature. Comparison of B6.Ly5.1 and TCRα$^{+/-}$.Thy1.1 thymocyte development within individual thymi underscores this observation (**Fig. 3.2A-C**).

To determine whether the deficiency in generating mature, post-positive selection thymocytes in B6.TCRα$^{+/-}$ mice as described above could be explained by a survival defect, we analyzed thymocyte viability. B6 and B6.TCRα$^{+/-}$ thymocytes were cultured *in vitro* for 6 days and stained for Annexin V and 7-AAD. Percentages of live cells in culture were similar between B6 and TCRα$^{+/-}$ (**Fig. 3.3A**). In addition, staining of thymocytes *ex vivo* produced the same conclusion (**Fig. 3.3B**). Our results indicate that the competitive advantage of secondary TCR-sufficient thymocytes was not due to a difference in viability.

We reasoned that positive selection would be the most critical window for the influence of secondary TCRα chains, as it is estimated that pre-selection DP thymocytes have an average lifespan of only approximately 60 h during which they must form a functional TCR to mediate positive selection and continue development (9). To examine positive selection kinetics, we injected B6 and TCRα$^{+/-}$ mice with a pulse dose of BrdU and evaluated subsequent development of labeled thymocytes. There was no difference in the percentage of thymocytes (predominantly DN thymocytes) that incorporated BrdU (**Fig. 3.4A**). However, over the following 96 h, pulsed TCRα$^{+/-}$ thymocytes demonstrated a deficient progression to a post-selection CD3^{high} phenotype (18.7±2.2%) compared to B6 (27.1±1.8%) (**Fig. 3.4B**).

Absence of secondary TCRα rearrangements eliminates certain TCR specificities

The contribution of secondary TCRα chains in mediating thymocyte development suggests that their absence could affect the peripheral T cell repertoire. The lack of secondary TCRα rearrangements did not affect peripheral T cell numbers in the spleen (**Fig. 3.5A**) or CD4 and CD8 subsets (**Fig. 3.5B**). To examine potential skewing of the TCR repertoire, *TRAV* and *TRAJ* gene segment use was analyzed by TCRα cDNA sequence analysis of peripheral T cells from 3 B6 and 3 TCRα$^{+/-}$ mice. Analysis of 123,655 B6 and 104,434 TCRα$^{+/-}$ TCRα transcripts revealed similar *TRAV* (**Fig. 3.5C**) and *TRAJ* (**Fig. 3.5D**) gene segment use, indicating that the absence of secondary TCRs does not broadly affect the T cell repertoire. For a more focused examination, we compared TCRα sequences of TCRVα2^{+} (*TRAV14*) T cells from TCRα$^{+/-}$ mice with *TRAV14*$^{+}$ TCRα transcripts of dual TCR T cells (TCRVα2^{+} and TCRVα3/8/11^{+}) from B6 mice. This enabled direct comparison of TCRα repertoire composition in the presence or absence of secondary TCRs. From 3 independent *TRAV14*$^{+}$ TCRα transcript libraries for each mouse strain, we analyzed 141,353 B6 *TRAV14*$^{+}$ TCRα sequences and 148,228 TCRα$^{+/-}$ *TRAV14*$^{+}$ TCRα sequences, yielding 13,646 and 14,002 unique CDR3α sequences respectively. There was no difference between TCRα$^{+/-}$ and dual TCR B6 *TRAV14*$^{+}$ TCRα sequences in CDR3 length (**Fig. 3.6A**), amino acid composition (**Fig. 3.6B**), and *TRAJ* gene segment use (**Fig. 3.6C**).

Despite the similarities seen thus far, the majority of unique *TRAV14*$^{+}$ CDR3α sequences were different between B6 and TCRα$^{+/-}$ (66.2% of B6 repertoire), indicating a potential for significant differences between the 2 repertoires (**Fig. 3.6D**). It is possible that the impact of secondary TCRα rearrangements would be more subtle and reflected in the presence or absence of specific TCRα sequences that might normally be negatively selected against if present as the only TCR. Interestingly, CDR3 sequences unique to the TCRα$^{+/-}$ *TRAV14*$^{+}$ repertoire were all low

abundance species each representing less than 0.1% of the *TRAV14*$^{+}$ repertoire, suggesting that their absence in dual TCR B6 T cells is stochastic. In contrast, sequences unique to the B6 dual TCR T cells were of relatively high abundance (> 0.1% of the *TRAV14*$^{+}$ repertoire) (**Fig. 3.6E**). We think this implies that their exclusion from the TCR$\alpha^{+/-}$ repertoire is active, likely owing to a difference in the stringency of thymic selection.

Secondary TCRs contribute to naive alloreactive and autoreactive repertoires

Findings from TCRα repertoire analysis suggest that exclusion of distinct sequences in TCR$\alpha^{+/-}$ mice could manifest as a differential ability of the naive T cell repertoire to respond to antigens. We measured the reactivity of naive B6 and TCR$\alpha^{+/-}$ T cells using class II pMHC tetramers to estimate the frequency of antigen-specific T cells (22). We generated I-E^{k} tetramers loaded with either $CD22_{654\text{-}666}$ or transferrin receptor $(TFR)_{231\text{-}244}$ peptides. These peptides were previously identified by mass spectrometry of endogenous peptides eluted from I-E^{k} molecules and known to stimulate I-E^{k}-alloreactive T cells generated from B6 mice (23). T cells were labeled with alloantigen pMHC tetramers, enriched by magnetic bead selection, and identified by flow cytometry (**Fig. 3.7A**). CD22/I-E^{k} and TFR/I-E^{k} tetramer positive and negative cells from B6 mice were sorted by flow cytometry and assessed for response to CD22 and TFR peptides. Tetramer positive populations responded to their peptide, whereas the tetramer negative cells did not (**Fig. 3.7B**), demonstrating the efficiency of our pMHC tetramer labeling. TCR$\alpha^{+/-}$ mice had a consistently decreased frequency of response to individual allogeneic pMHC complexes. The frequency of TCR$\alpha^{+/-}$ T cells recognizing the TFR/I-E^{k} tetramer was significantly decreased compared to that of B6 T cells (8.6$\pm$0.9 vs.19.9$\pm$4.7 cells/10^{5} T cells, respectively) (**Fig. 3.8A**).

CD22/I-E^k tetramer binding yielded a similar trend, though not significant (72.6±8.6 $TCR\alpha^{+/-}$ vs. 115.9±18.0 B6 cells/10^5 T cells) (**Fig. 3.8B**). There was no significant difference between B6 and $TCR\alpha^{+/-}$ T cell response to a minor histocompatibility antigen, $E\alpha_{52-68}$ presented by self-MHC I-A^b (4.3±1.0 vs. 3.1±0.9 cells/10^5 T cells, respectively) (**Fig. 3.8C**).

Consistent with our hypothesis proposing that secondary TCRα rearrangements contribute specifically to the recognition of atypical ligands, $TCR\alpha^{+/-}$ mice also had reduced frequencies of naive $CD4^+$ T cells recognizing the autoantigen myelin oligodendrocyte glycoprotein $(MOG)_{38-49}$ presented by I-A^b (8.6±2.5 cells/10^5 T cells) as compared to B6 (28.3±9.3 cells/10^5 T cells) (**Fig. 3.9A**). Conversely, recognition of I-A^b tetramer presenting a foreign antigen, lymphocytic choriomeningitis virus $(LCMV)_{66-77}$ was comparable between B6 and $TCR\alpha^{+/-}$ $CD4^+$ T cells (4.7±2.3 vs. 5.8±2.5 cells/10^5 T cells, respectively) (**Fig. 3.9B**). These results demonstrate specific contribution of secondary TCRα rearrangements to the alloreactive and autoreactive T cell repertoires.

Secondary TCRs increase early during *in vivo* alloreactivity

To evaluate the contribution of secondary TCRs during in vivo alloreactivity, we used an MHC-mismatched model of GVHD. B6.Ly5.1 and $TCR\alpha^{+/-}$.Thy1.1 T cells were mixed 1:1 (**Fig. 3.10A**), pulsed with CFSE, and injected into lethally irradiated B6.K recipient mice. Expansion of transferred T cells was assessed by flow cytometry 24 h after transfer. While the 2 cell populations were transferred at equal numbers (ratio $TCR\alpha^{+/-}$/B6 T cells = 0.99±0.07%), recovery of $TCR\alpha^{+/-}$ cells was decreased after 24 h (ratio $TCR\alpha^{+/-}$/B6 T cells = 0.41±0.09%)

(**Fig. 3.10B**). The difference was due to a decreased proliferative response by the TCRα$^{+/-}$ cells as evidenced by CFSE dilution (**Fig. 3.10C, D**). The diminished response of TCRα$^{+/-}$ T cells early after allogeneic stimulation *in vivo* illustrates a functional consequence of the decreased frequency of alloreactive T cells in the absence of secondary TCRα rearrangements and underscores the potential importance of dual TCR T cells in driving early pathologic alloreactive responses.

The presence of secondary TCRs enables flexibility in recognition of allogeneic ligands

The contribution of secondary TCRs to the alloreactive and autoreactive T cell repertoire has multiple possible mechanistic explanations. One is that elimination of secondary TCRα rearrangements results in a decreased number of TCRs, which reduces the probability of recognizing a specific antigen. However, secondary TCRs comprise less than 10% of the T cell repertoire, and our data indicate that they encompass 40-50% of the alloreactive repertoire and possibly as much as 70% of the autoreactive repertoire, but do not affect recognition of conventional foreign antigens. This specific and disproportionate effect suggests that it is not simple stochastic addition of antigen specificities to the repertoire by secondary TCRs, but rather a unique functional property of secondary TCRs.

To investigate this, we compared functional responses of CD22/I-E^{k} tetramer binding T cells from B6 and TCRα$^{+/-}$ mice to APLs. Tetramer positive cells from naive mice were sorted by flow cytometry and stimulated with the wild-type CD22 peptide or CD22 APLs containing single amino acid substitutions at TCR contact sites (P2 and P5). Responses were assessed by

measurement of IFNγ production after 24 h culture. IFNγ production to either non-specific stimulation, phorbol 12-myristate 13-acetate (PMA), or wild-type CD22 peptide was similar between the 2 cell types (**Fig. 3.11A**). However, B6 cells responded to APLs with mutations at either the P2 or P5 positions while TCR$\alpha^{+/-}$ cells did not (**Fig. 3.11A, B**). These data indicate that the contribution of dual TCR T cells to recognition of atypical ligands results from an unusual flexibility in ligand recognition.

Discussion

The existence of a minority of T cells expressing 2 TCRs in mice and humans has been recognized for some time (13), though the biological significance of these cells has not been well understood. Studies of transgenic TCR systems demonstrated that secondary TCRα recombination enables TCRs with unwanted reactivities to escape thymic selection and emigrate to the periphery (17, 19). We wondered if there are any potential benefits of simultaneous TCRα chain rearrangement in T cell development that would offset its detrimental impacts on the peripheral T cell repertoire. To address this, we used mice genetically deficient for secondary TCRα rearrangements to define their role in thymocyte development and in the generation of the T cell repertoire.

Analysis of thymocyte development in $TCR\alpha^{+/-}$ mice demonstrated defective production of mature post-positive selection $CD3^{high}$ SP cells, supporting the notion that simultaneous TCRα rearrangements occur to maximize the efficiency of mature T cell production from thymocytes that had successfully mediated β-selection. Our results demonstrate that secondary TCRα rearrangements are quantitatively important for thymocyte development, though we cannot ascribe the relative importance of their effects on mediating positive selection versus masking thymocytes with autoreactive TCRs from negative selection. This will require further investigation in systems where these questions can be separated. The elimination of secondary TCRα rearrangements did not broadly affect the peripheral T cell repertoire, either in the number of T cells, or in the general use of TCRVα and TCRJα gene segments. A more focused comparison of *TRAV14* TCR transcripts between T cells lacking secondary TCRα

rearrangements and T cells with dual TCR expression similarly revealed no differences in general TCR properties. However, dual TCR T cells had numerous high abundance CDR3 sequences that were absent among cells lacking secondary TCRα chains. The reciprocal was not true, indicating that the presence of these unique TCRα sequences depended on secondary TCRα rearrangements. These data support our hypothesis that secondary TCRs have a unique contribution to the peripheral T cell repertoire.

We hypothesize that secondary TCRα rearrangements increase the T cell repertoire for atypical self and allogeneic pMHC ligands due to the effects of dual TCR expression on the requirements for thymic selection. Indeed, our tetramer binding data revealed that secondary TCRs contribute significantly and specifically in responses to peptides involved in autoimmunity and alloreactivity, but not to cognate ligands. Mechanistically, the presence of secondary TCRs may prevent cells from becoming negatively selected when exposed to autoantigens during T cell development as well as provides TCRs that do not undergo positive selection to self MHC and may have more flexibility in binding non-self MHC. The decreased response of $CD4^+$ T cells from $TCR\alpha^{+/-}$ mice to MOG/I-A^b tetramers was somewhat unexpected given a previous study reporting no impact on EAE disease outcome in mice lacking secondary TCRs (20). In that study, the effect of secondary TCRs was measured following immunization with antigen and adjuvant as compared to our interrogation of the naive T cell repertoire. Given that the NOD diabetes model, which does not rely on adjuvants, also indicated a role for dual TCR T cells (20), it seems that secondary TCRs may be important contributors to autoimmunity under physiological conditions. In addition to pathogenic autoreactive T cells, Tregs also function by recognizing self. Interestingly, a report indicating that a majority of Tregs in humans express 2

TCRs (24) highlights the potential for dual TCR T cells to recognize self-pMHC in a high affinity capacity. It is unclear whether these dual TCR Tregs are thymically derived or result from peripheral conversion. Further studies are required to understand how recognition of self-pMHC may direct Treg lineage commitment or conversion. An additional unexpected finding is the lack of a difference between T cells from B6 and TCR$\alpha^{+/-}$ mice in recognition of the Eα/IA^{b}, which we consider a minor histocompatibility antigen between B6 and B6.K mice. This differs from previous findings from our lab using the human HA-1/HLA-A*02:01 minor histocompatibility antigen model (21). It is possible that this is a result of a nuanced but important difference between the systems - while the HA-1 antigen is a difference of a single amino acid between the 2 polymorphisms, B6 mice do not express the I-Eα molecule at all, which may make the response more similar to a foreign antigen rather than an alloantigen.

The potential for secondary TCRs to participate in pathogenic alloresponses has been demonstrated by previous studies examining dual TCR T cells in mouse models and patients with GVHD (16, 21). Our data here demonstrating decreased proliferative potential of TCR$\alpha^{+/-}$ T cells during *in vivo* alloreactivity provide insight into the mechanism by which dual TCR T cells contribute to alloresponses. Furthermore, we hypothesize that secondary TCRs are specifically important in alloreactivity as well as autoimmunity due to differential properties for their interaction with pMHC ligands derived from their uniquely relaxed constraints of thymic selection. It has been demonstrated that negative selection is important for eliminating crossreactive TCRs (25), and thus secondary TCRs masked from negative selection may be more crossreactive. This is supported by our data demonstrating the strikingly reduced stringency of recognition of an allopeptide by secondary TCRs (**Fig. 3.11**). While our data do not question the

importance of T cell recognition of peptides in alloreactivity, shown by numerous studies demonstrating integral TCR interactions with the allopeptide (23, 26) as well as our data in Chapter II, it does indicate a potential mechanism for the increased propensity for dual TCR T cells to respond to specific pMHC ligands.

In summary, we show that efficient thymocyte development requires simultaneous rearrangement of both TCRα loci. However, this comes at a cost, with resulting secondary TCRs having an increased ability to respond to self and allogeneic pMHC. Our results highlight a hitherto underappreciated role of dual TCR-expressing T cells in the development of the T cell repertoire and suggest that therapeutic strategies for combatting autoimmune diseases or transplant rejection and GVHD take into account the significant contribution of these uniquely powerful T cells.

Figure 3.1 Thymic generation of mature T cells is deficient in the absence of secondary TCRα rearrangements

(**A**) Comparison of numbers of thymocytes in 6 week old B6 and TCRα$^{+/-}$ mice. Each point represents a single mouse, *n* = 17, 4 independent experiments, mean ± sem, Mann-Whitney test. (**B - E**) The effect of secondary TCRα rearrangements on T cell development was examined by comparing thymi of B6 and TCRα$^{+/-}$ mice by flow cytometry, *n* = 10, 3 independent experiments. (**B**) Representative plots of CD4 and CD8 labeling of thymocytes from individual mice. (**C**) Percentages of mature CD4^{+} or CD8^{+} SP, CD4^{+}CD8^{+} DP, or CD4^{-}CD8^{-} DN thymocytes. Each point represents a single thymus, mean ± sem, Mann-Whitney test. (**D**) Representative plots of CD3 and CD69 labeling of thymocytes from individual mice. (**E**) Percentages of post-positive selection CD3^{high} thymocytes. Each point represents a single thymus, mean ± sem, Mann-Whitney test.

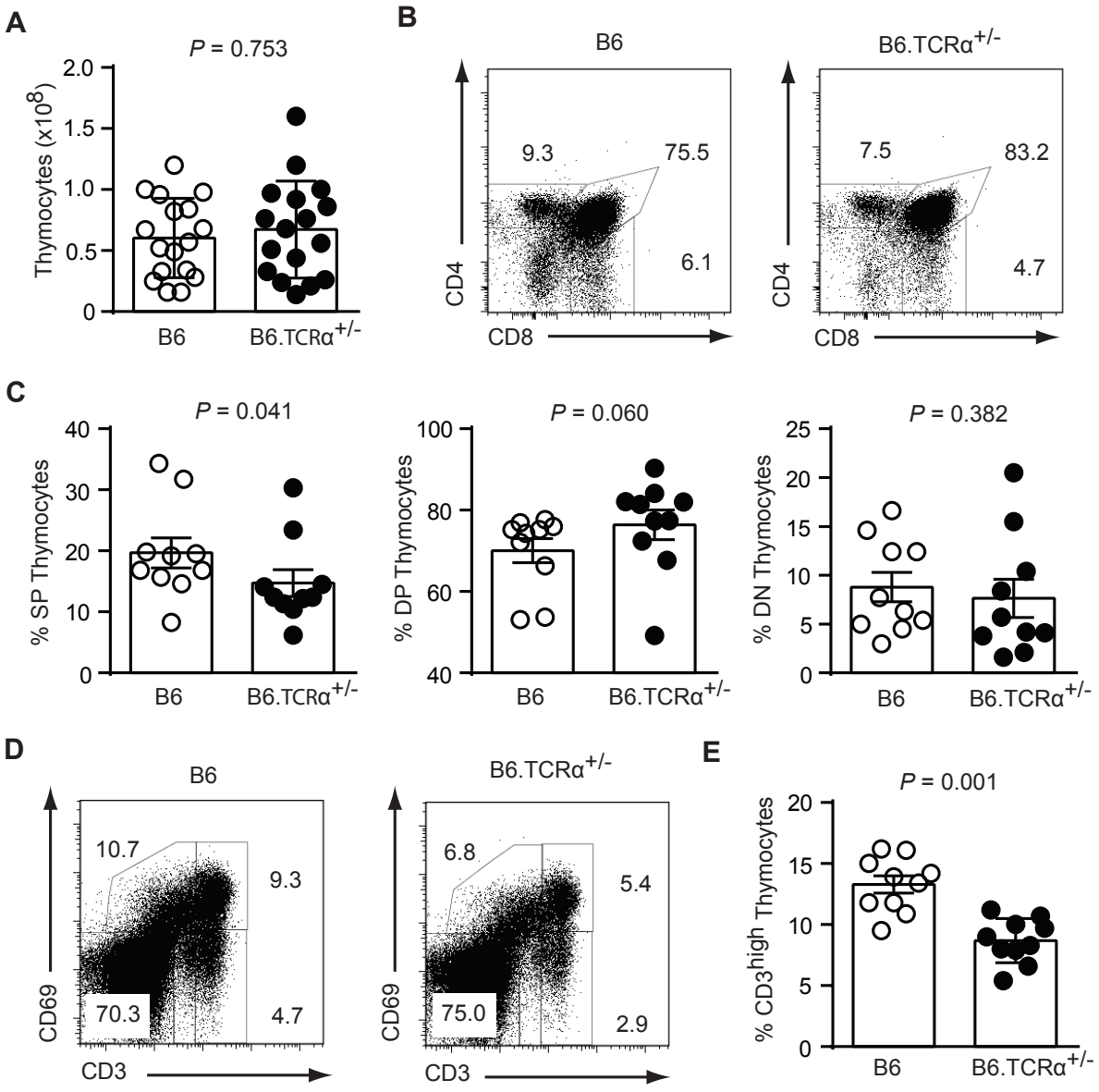


Figure 3.1 Thymic generation of mature T cells is deficient in the absence of secondary TCRα rearrangements

Figure 3.2 The deficiency in generating mature SP thymocytes is a cell-intrinsic defect of secondary TCRα rearrangements

Pre-selection thymocytes from congenically-marked B6.Ly5.1 and TCRα$^{+/-}$.Thy1.1 mice were enriched by magnetic bead selection, combined at a 1:1 ratio, and 10^7 cells transferred by intrathymic injection into B6 mice to examine development (n = 8, 3 independent experiments). Transferred cells were detected 7 d after injection by flow cytometric analysis of thymocytes. (**A**) Representative plot with congenically-marked transferred cells expressed as percentage of total thymocytes. (**B**) Ratio of B6.Ly5.1 to B6.TCRα$^{+/-}$.Thy1.1 cells among total thymocytes and post-selection CD3^{high} thymocytes. Mean ± sem, paired ratio t test. (**C**) Development of post-selection CD3^{high} thymocytes was compared between B6.Ly5.1 and TCRα$^{+/-}$.Thy1.1 thymocytes in individual mice. Transferred populations in a single mouse are linked by the line between points, paired ratio t test.

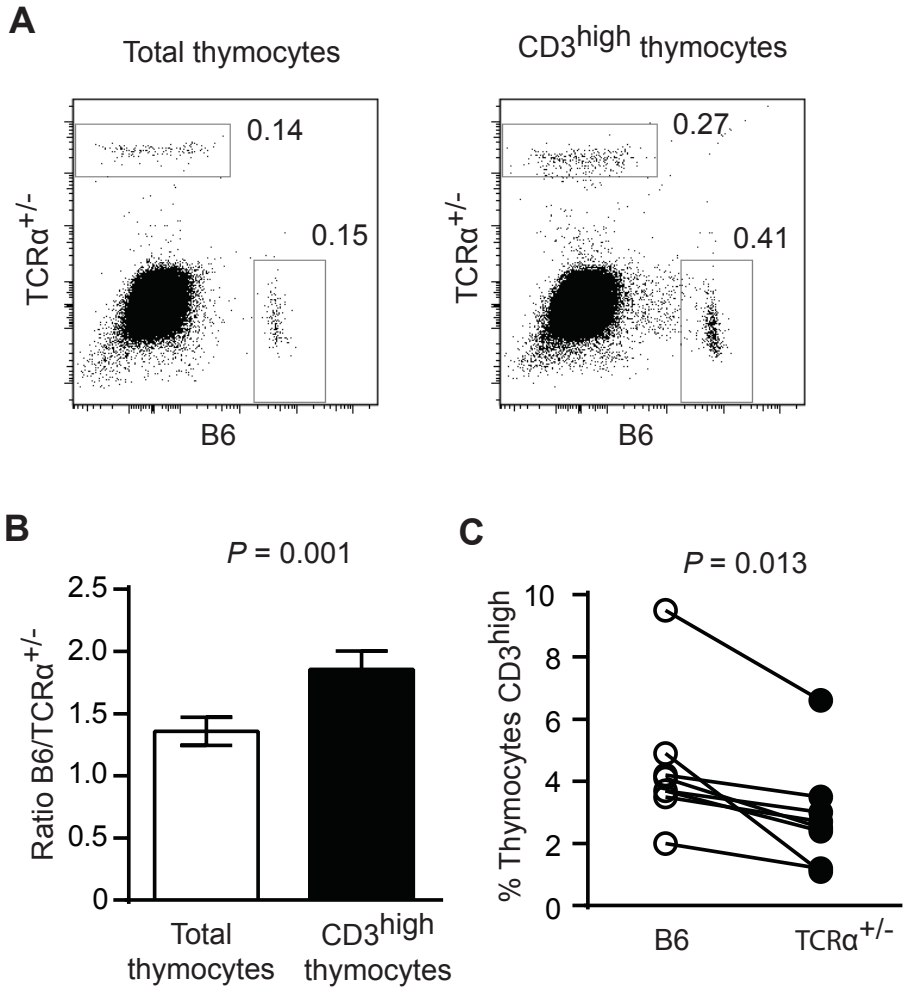


Figure 3.2 The deficiency in generating mature SP thymocytes is a cell-intrinsic defect of secondary TCRα rearrangements

Figure 3.3 The deficiency of generating mature T cells in mice lacking secondary TCRα rearrangements is not attributable to decreased cell survival

Thymocyte survival was compared between thymocytes from 6 wk old B6 and TCR$\alpha^{+/-}$ mice by labeling for CD4, CD8, Annexin V and staining with 7-AAD to detect live (Annexin V and 7-AAD negative) thymocyte populations. (**A**) Thymocytes were cultured *in vitro* at 10^7 thymocytes/ml and sampled at the indicated time points. Points are mean $\pm$ sem of 6 thymic cultures from 3 independent experiments, data analyzed by ANOVA. (**B**) Thymocytes were taken from mice and analyzed directly *ex vivo*. Each point represents one mouse from three independent experiments. Data are shown as mean $\pm$ sem, analyzed by Mann-Whitney test.

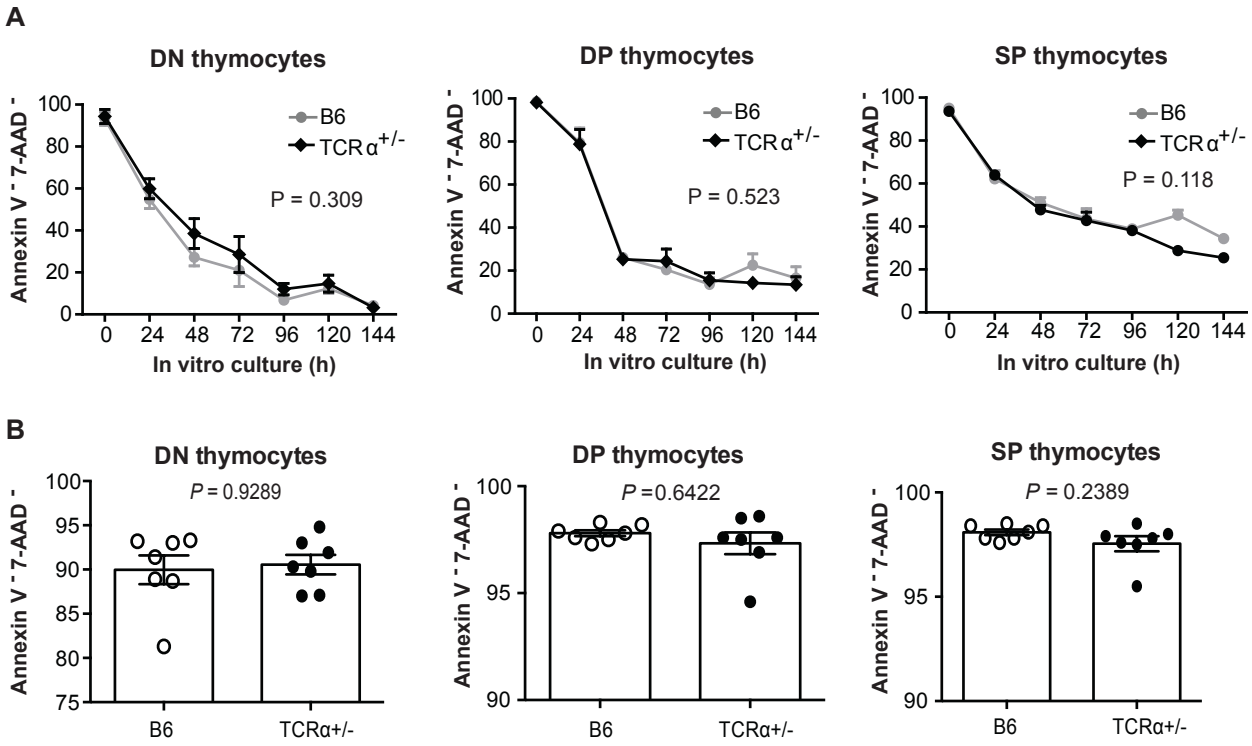


Figure 3.3 The deficiency of generating mature T cells in mice lacking secondary TCRα rearrangements is not attributable to decreased cell survival

Figure 3.4 Lacking secondary TCRα rearrangements impairs efficient positive selection

Intra-peritoneal (i.p.) administration of BrdU (1.6 mg) was done on d 0. (**A**) Thymocyte BrdU incorporation was evaluated at 24 h post-injection (n = 6, 3 independent experiments, mean ± sem, Mann-Whitney test). (**B**) Thymocyte development in BrdU-pulsed B6 and B6.TCR$\alpha^{+/-}$ mice was compared by analyzing development of $CD3^{high}$ post-positive selection thymocytes by flow cytometry at 24 h, 48 h, and 96 h post-BrdU injection. Mean ± sem of 6 mice per group in 3 independent experiments, Mann-Whitney test.

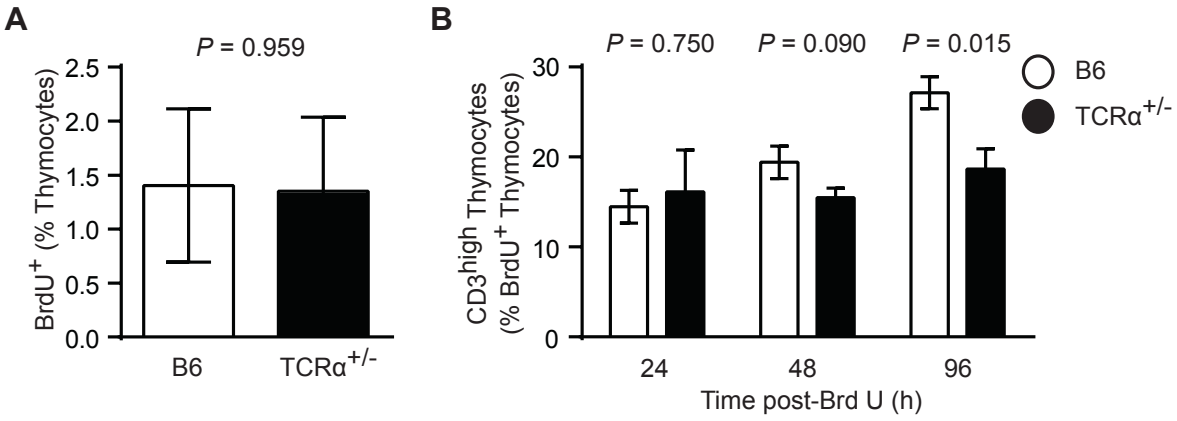


Figure 3.4 Lacking secondary TCRα rearrangements impairs efficient positive selection

Figure 3.5 Elimination of secondary TCRα rearrangements does not broadly alter the peripheral T cell repertoire

(**A**) Comparison of numbers of T cells in the spleens of 6 week old B6 and TCR$\alpha^{+/-}$ mice. Each point represents a single mouse, $n = 6$, 3 independent experiments, mean ± sem, Mann-Whitney test. (**B**) Comparison of $CD4^+$ and $CD8^+$ T cell composition, measured by flow cytometry of splenocytes. Mean ± sem of 6 mice from 3 independent experiments, Mann-Whitney test. Comparison of (**C**) *TRAV* and (**D**) *TRAJ* gene segment use by DNA sequence analysis of splenic T cells from 6 week old B6 and TCR$\alpha^{+/-}$ mice (3 mice each, 23,877 - 34,995 TCRα sequence reads/mouse). Mean ± sd for each group, data analyzed by ANOVA.

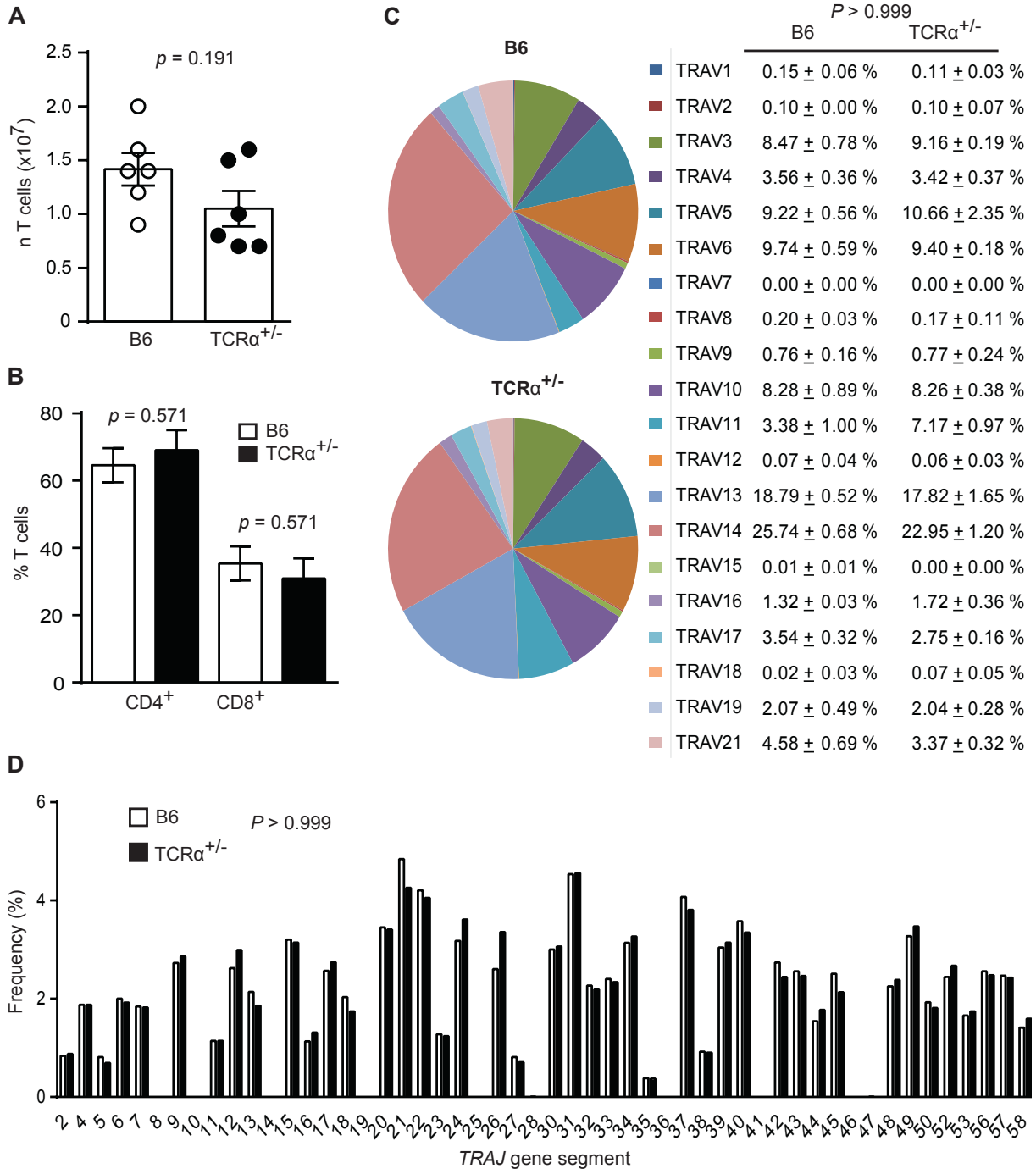
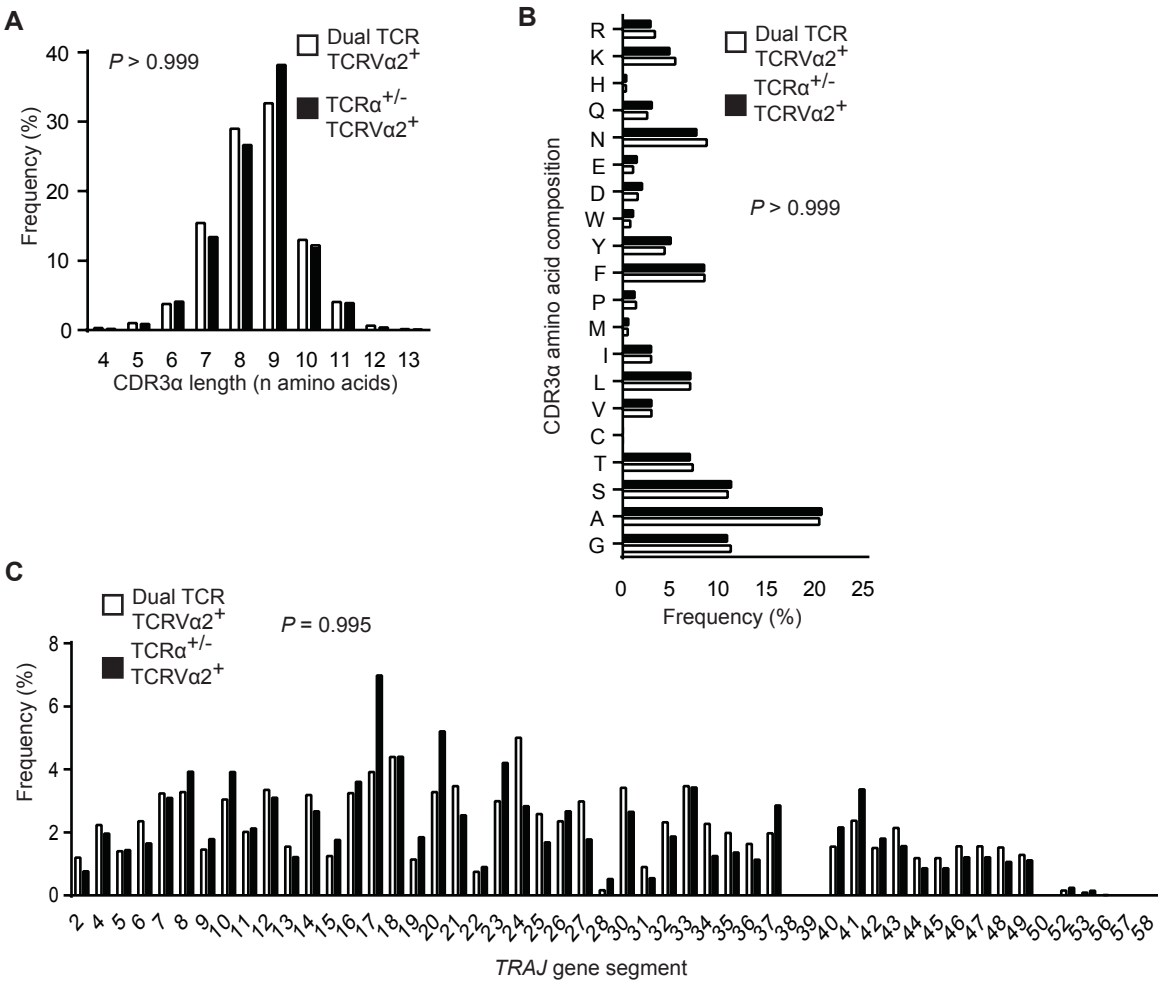
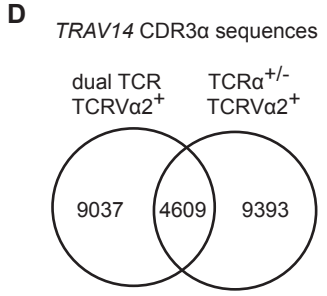


Figure 3.5 Elimination of secondary TCRα rearrangements does not broadly alter the peripheral T cell repertoire

Figure 3.6 Lacking secondary TCRα rearrangements results in elimination of specific TCRs from the naive T cell repertoire

TRAV14 T cell receptor libraries were generated from TCRα$^{+/-}$ TCRVα2^{+} T cells and B6 TCRVα2^{+} dual TCR T cells (identified by co-expression of TCRVα3, TCRVα8, and TCRVα11) sorted by flow cytometry. Three independent libraries per group were sequenced and generated 141,353 B6 *TRAV14*$^{+}$ TCRα sequences and 148,228 TCRα$^{+/-}$ *TRAV14*$^{+}$ TCRα sequences. Analysis of (**A**) CDR3 length, (**B**) CDR3 amino acid composition, and (**C**) *TRAJ* gene segment use was compared between the 2 groups. (**D**) 23,039 total unique sequences were obtained and divided among the groups as indicated. (**E**) The 5 most abundant *TRAV14* CDR3 sequences unique to each population are shown with corresponding frequencies.





E

Most abundant population-unique *TRAV14* CDR3α sequences

Dual TCR TCRVα2+ T cells		
CDR3α	Dual TCR	TCRα+/-
FCAGRSNYNVLYFG	0.36%	0
FCAATNSNNRIFFG	0.32%	0
FCAAISNYNVLYFG	0.20%	0
FCAARRETQVVGQLTFG	0.19%	0
FCAASAPPGGNYKPTFG	0.17%	0
TCRα+/- TCRVα2+ T cells		
CDR3α	Dual TCR	TCRα+/-
FCAAHDSGYNKLTFG	0	0.08%
FCAASASGSFNKLTFG	0	0.06%
FCAASEDSGTYQRFG	0	0.05%
FCAALNNYAQGLTFG	0	0.05%
FCAASEAGFASALTFG	0	0.04%

Figure 3.6 Lacking secondary TCRα rearrangements results in elimination of specific TCRs from the naive T cell repertoire

Figure 3.7 Allopeptide-MHC tetramers specifically detect alloreactive T cell populations

Splenocytes, axillary, inguinal, and popliteal lymph nodes were collected from B6 and TCR$\alpha^{+/-}$ mice, incubated with PE-labeled allogeneic pMHC tetramers, enriched by magnetic selection using anti-PE beads, and singlet T cells were analyzed by flow cytometry. (**A**) Representative B6 mouse sample labeled with TFR/I-E^k is shown. (**B**) Reactivity of pMHC tetramer-labeled cells was assessed by sorting tetramer-positive and tetramer-negative T cells by flow cytometry and measuring IFN-γ production following 18 h culture with CHO-E^k cells and 10 μM CD22 or 50 μM TFR peptide. IFN-γ production was measured by intracellular cytokine staining, with percent of IFN-γ^+ cells indicated. Data are representative examples of 3 independent experiments.

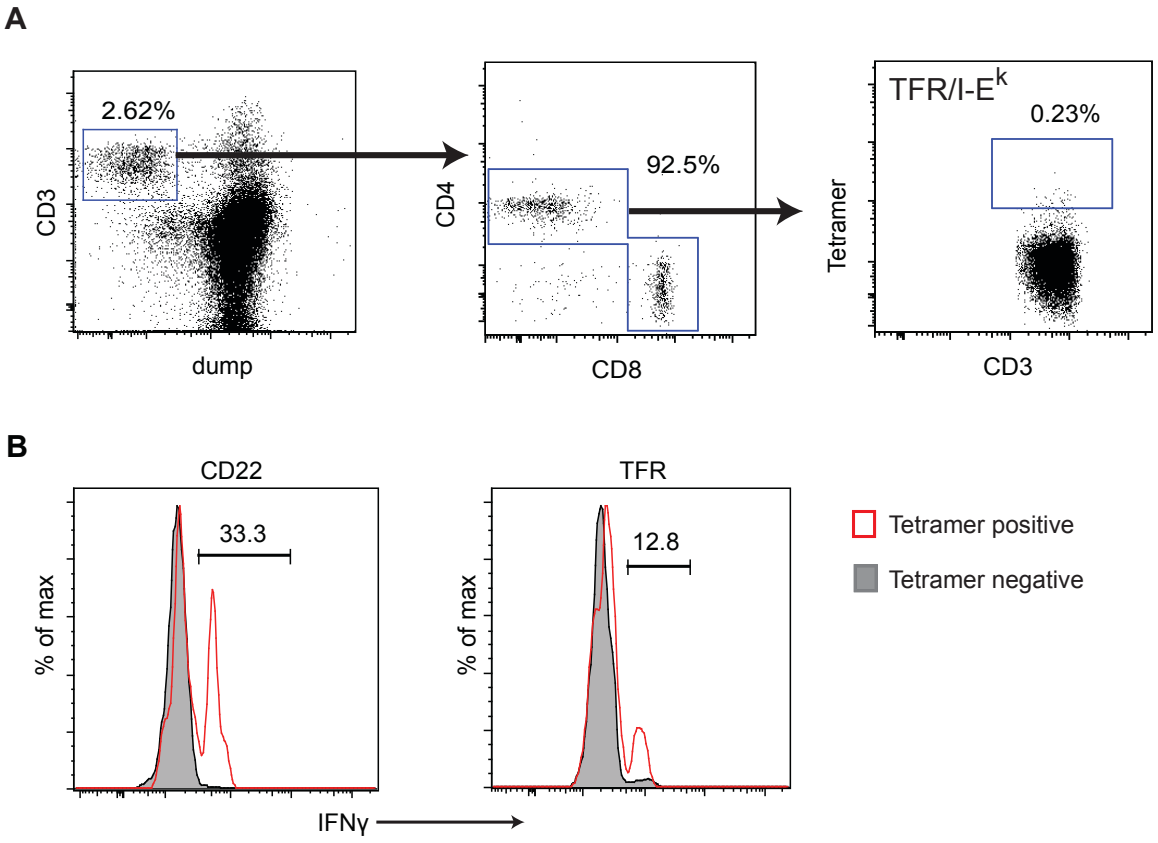


Figure 3.7 Allopeptide-MHC tetramers specifically detect alloreactive T cell populations

Figure 3.8 Secondary TCRα rearrangements increase the alloreactive T cell repertoire as measured by tetramer staining

Splenocytes, axillary, inguinal, and popliteal lymph nodes were collected from B6 and TCR$\alpha^{+/-}$ mice, incubated with APC- or PE-labeled allogeneic pMHC tetramers, enriched by magnetic selection using anti-APC or anti-PE beads, and singlet T cells were analyzed by flow cytometry. The frequency of alloantigen-reactive T cells bound to the (**A**) TFR/I-E^k, (**B**) CD22/I-E^k, and (**C**) Eα/I-A^b tetramers was calculated as the number of tetramer$^+$ cells/10^5 T cells collected from each mouse. Data points are individual mice (n = 8-11) from 4 independent experiments, mean $\pm$ sem, Mann-Whitney test.

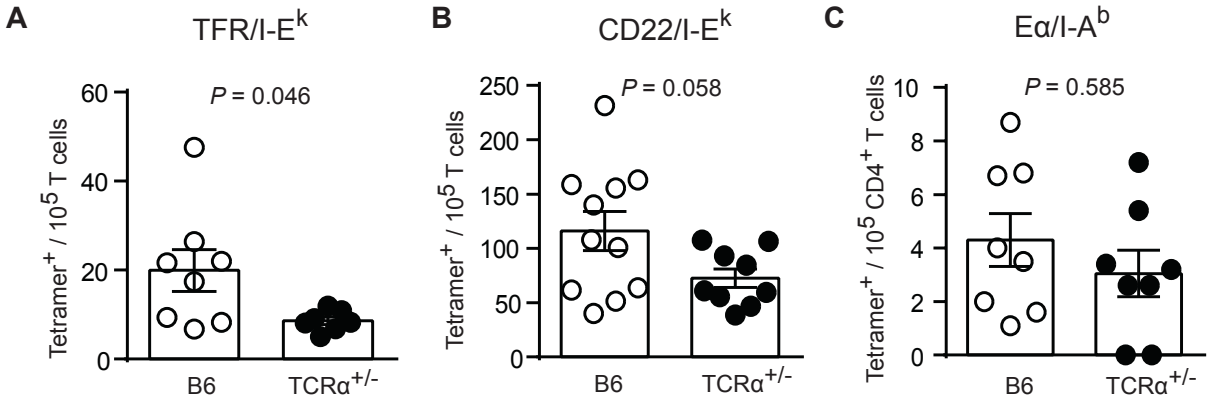


Figure 3.8 Secondary TCRα rearrangements increase the alloreactive T cell repertoire as measured by tetramer staining

Figure 3.9 Secondary TCRα rearrangements increase the frequency of T cells responsive to an autoantigen but not to cognate pMHC

Splenocytes, axillary, inguinal, and popliteal lymph nodes were collected from B6 and TCR$\alpha^{+/-}$ mice, incubated with APC- or PE-labeled allogeneic pMHC tetramers, enriched by magnetic selection using anti-APC or anti-PE beads, and singlet T cells were analyzed by flow cytometry. The frequency of (**A**) autoantigen- and (**B**) cognate peptide-reactive T cells was calculated as the number of tetramer$^+$ cells/10^5 CD4$^+$ T cells collected from each mouse. Data points are individual mice (n = 5-6) from 3 independent experiments, mean $\pm$ sem, Mann-Whitney test.

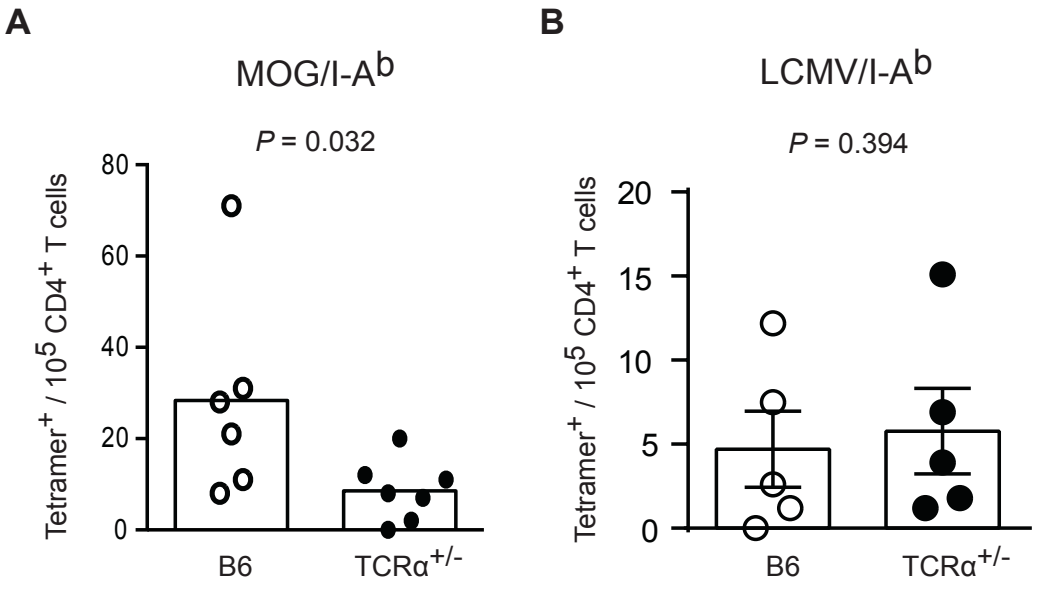


Figure 3.9 Secondary TCRα rearrangements increase the frequency of T cells responsive to an autoantigen but not to cognate pMHC

Figure 3.10 Elimination of secondary TCRα rearrangements reduced *in vivo* T cell alloreactivity

Congenically-marked B6.Ly5.1 and TCR$\alpha^{+/-}$.Thy1.1 T cells (both H-2^b) were mixed at a 1:1 ratio, pulsed with CFSE, and injected into lethally-irradiated MHC mismatched B6.K (H-2^k) recipients. (**A**) Pre-injection cells were labeled for congenic markers to confirm ratio of cells. Representative example from 3 independent experiments. (**B**) Transferred cells were analyzed at 24 h by flow cytometry of splenocytes. Cells were labeled for congenic markers to examine ratio of TCR$\alpha^{+/-}$.Thy1.1 and B6.Ly5.1 T cells pre-transfer and 24 h post-transfer. Results shown are mean $\pm$ sem of 12 recipient mice from 3 independent experiments, Mann-Whitney test. (**C, D**) Proliferation of transferred T cells was assessed by measuring CFSE dilution at 24 h post-transfer. (**C**) Representative CFSE plot of 1 recipient mouse. (**D**) Percentages of $CFSE^{low}$, divided B6.Ly5.1 and TCR$\alpha^{+/-}$.Thy1.1 T cells from 12 mice, 3 independent experiments. Data shown are mean ± sem, paired *t* test.

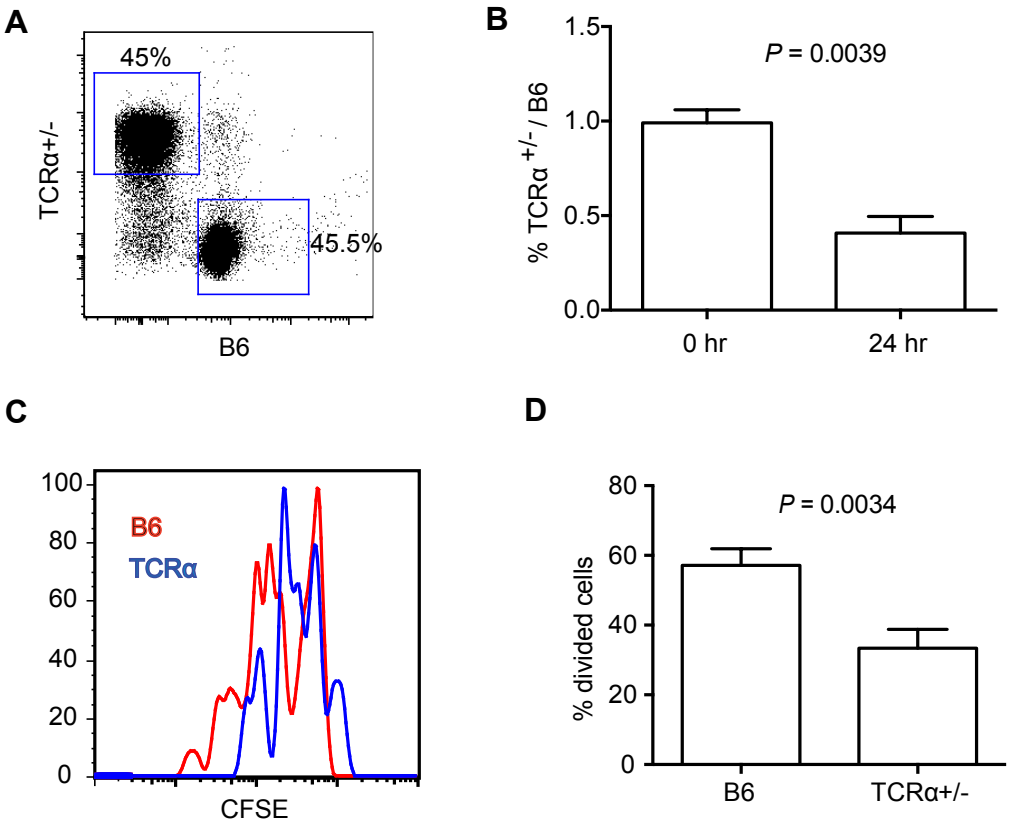


Figure 3.10 Elimination of secondary TCRα rearrangements reduced *in vivo* T cell alloreactivity

Figure 3.11 The presence of T cells with secondary TCRα rearrangements enables more flexibility in allopeptide recognition

CD22/I-E^k alloantigen-reactive T cells were collected from B6 and TCR$\alpha^{+/-}$ mice by tetramer-labeling, magnetic enrichment, and flow cytometry sorting. T cells were cultured with CHO-E^k cells for 24 h, either in the presence of the CD22 peptide or CD22 peptide APLs P2A (H to A change at TCR contact P2) and P5S (G to S change at TCR contact P5). PMA (20 ng/ml) and ionomycin (1 μM) was used as a positive control. T cell responses were measured by flow cytometry with intracellular labeling for IFN-γ production. (**A**) Representative experiment shown. (**B**) Aggregate data from 3 independent experiments with APL response normalized to the response against wild-type CD22 peptide, mean ± sem, ANOVA.

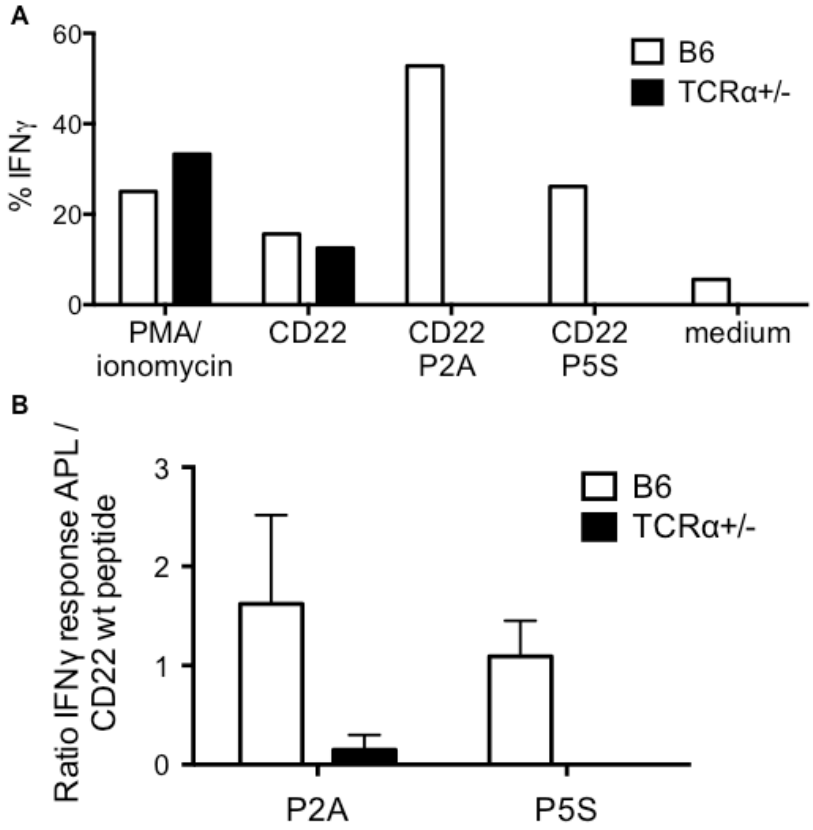


Figure 3.11 The presence of T cells with secondary TCRα rearrangements enables more flexibility in allopeptide recognition

Methods

Mice

B6, B6.Ly5.1, B6.Thy1.1 and B6.K mice were originally purchased from The Jackson Laboratory. TCR$\alpha^{+/-}$ mice, incapable of expressing two TCRα chains due to a targeted disruption at the 5' end of one copy of the *TRAC* gene, were derived by crossing *TRAC*$^{-/-}$ B6 mice (4) with B6 or B6.Thy.1.1 mice. Mice were bred and housed in specific pathogen-free conditions at Washington University Medical Center (St. Louis, MO). All use of laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

Flow cytometry

Thymocyte and T cell analyses were performed using anti-CD3 (145-2C11)-PE-Cy7, anti-CD45.2 (104)-PerCP-Cy5.5, anti-CD90.1 (HIS51)-eFluor 450 (eBioscience), anti-CD69 (H1.2F3)-PE-Cy7, anti-CD4 (RM4-5)-PerCP, anti-CD8α (53-6.7)-APC-Cy7 (Biolegend), and anti-CD45.1 (A20)-APC (BD Biosciences). Non-T cells were excluded by labeling with Pacific Blue-labeled anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), and anti-F4/80 (BM8) (Biolegend). TCRVα2^{+} and dual TCR T cells were identified among CD3^{+}B220^{-}CD11b^{-} CD11c^{-}F4/80^{-} splenocytes using anti-TCRVα2 (B20.1)-PE and anti-TCRVα3 (RR3-16)-FITC, anti-TCRVα8 (KT50)-FITC, and anti-TCRVα11 (RR8-1)-FITC (BD Biosciences). Samples were analyzed using LSR II or LSR Fortessa cytometers (BD Biosciences) with calculated compensation, and data were analyzed with FlowJo software (Tree Star). FACSAria II (BD Biosciences) was used for sorting.

Thymocyte culture

Survival was assessed by culturing 10^7 thymocytes/mL in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (PAA), 2 mM GlutaMAX, and 50 mg/ml gentamycin for 6 days. *In vitro* cultures or freshly isolated thymocytes were labeled for CD4, CD8, Annexin V, and 7-AAD (Biolegend), and analyzed by flow cytometry.

Thymocyte transfers

B6.Ly5.1 and TCR$\alpha^{+/-}$.Thy1.1 pre-selection thymocyte populations were bead-enriched for CD53$^-$ cells using anti-CD53 mAb (OX-79, Biolegend) and anti-IgM paramagnetic beads (Miltenyi Biotec). Enriched cell populations were mixed at a 1:1 ratio and injected intrathymically into sublethally irradiated (5 Gy) B6 recipient mice. Thymi from recipient mice were analyzed 7 days post-injection by flow cyomtetry.

BrdU labeling

Mice were injected intraperitoneally with 1.2 mg BrdU (Sigma) and thymi were harvested at 24, 48, and 96 h timepoints. Thymocytes were labeled for surface markers, fixed with BD CytoFix (BD Biosciences), permeabilized with Permeabilization Wash Buffer (Biolegend), treated with 1 mL DNase I (Sigma) at 50 U/mL, intracellularly labeled with anti-BrdU (BU20A)-FITC (eBioscience), and analyzed by flow cytometry.

TCR repertoire analysis

TCRVα2^{+} T cells and dual TCR T cells were sorted from TCRα$^{+/-}$ and B6 splenocytes, respectively, by flow cytometry. TCRα cDNA libraries were generated by PCR (27, 28) and sequenced by 250 cycle paired-end sequencing using an Illumina MiSeq at the Washington University Genome Sequencing Center. *TRAV* and *TRAJ* gene segment use was determined by sequence analysis using the International ImMunoGeneTics Information System nucleotide sequence database (29).

Tetramer enrichment

LCMV glycoprotein $(GP)_{66-77}$ (DIYKGVYQFKSV) / I-A^b (LCMV-A^b tetramer)-APC, mouse MHC class II antigen $E\alpha_{52-68}$ (ASFEAQGALANIAVDKA) / I-A^b (Eα-A^b tetramer)-APC, and MOG_{38-49} (GWYRSPFSRVVH) / I-A^b (MOG-A^b tetramer)-PE were obtained from the NIH Tetramer Core Facility at Emory University (Atlanta, GA). Murine $CD22_{654-666}$ (SGQDLHSSGQKLR) / I-E^k (CD22-E^k tetramer)-PE and murine $TFR_{231-244}$ (SGKLVHANFGTKKD) / I-E^k (TFR-E^k tetramer)-PE tetramers were generated using soluble I-E^k produced in *E. coli* inclusion bodies and refolded with peptide (30). Tetramer enrichment was performed according to published protocols (22). Briefly, cells were incubated with tetramer for 1 h at room temperature, washed, incubated with anti-PE and/or anti-APC conjugated microbeads for 30 min at 4°C, and passed over magnetized LS columns for positive selection (Miltenyi Biotec). Enriched populations were labeled for surface markers for 20 min. at 4°C and analyzed or sorted by flow cytometry.

Intracellular cytokine staining

T cell antigen-specific responses were assessed by 24 h culture with 10^5 CHO-E^k cells and stimulated with 20 ng/ml PMA and 1 μM ionomycin or 10–50 μM peptide in round-bottom 96-well plates at 37°C. During the last 4 h of culture, 10 μg/mL Brefeldin A (Sigma-Aldrich) was added. Live cells were stained using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Molecular Probes), labeled for surface markers, and intracellularly labeled with anti-IFNγ (XMG1.2)-FITC or isotype control (Biolegend).

***In vivo* alloreactive response**

T cells were enriched from B6.Ly5.1 and TCR$\alpha^{+/-}$.Thy1.1 spleens using anti-CD4 and anti-CD8 paramagnetic beads and LS columns (Miltenyi Biotec), mixed 1:1, pulsed with 5 mM CFSE (Sigma), and 5–10 x 10^6 cells were injected through an intra-venous (i.v.) route into lethally irradiated (10 Gy) B6.K mice. Splenocytes were harvested at 8 h and 24 h after injection, labeled for surface markers, and analyzed by flow cytometry for CFSE dilution.

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CHAPTER IV

Both positive and negative effects on $CD4^+$ T cell responses result from the addition of a second class II MHC molecule, $I\text{-}E^b$

Introduction

Humans express three types of class I (HLA-A, HLA-B, and HLA-C) and three class II (HLA-DP, HLA-DQ, and HLA-DR) MHC molecules, while mice have up to three types of class I (H2-K, H2-D, and H2-L) and only one or two class II (I-A and I-E in some) MHC molecules (1-3). The number of MHC molecules expressed can be viewed as quite low considering the important function of the MHC in presenting peptides both in the thymus and in the periphery for T cell selection and activation against pathogens, respectively (4). It is a difficult process for thymocytes to make it past selection, and positive selection is particularly stringent with approximately 90% of thymocytes dying by neglect due to an inability to sufficiently bind (pMHC) complexes (5-9). Therefore, it is curious why vertebrates do not express more MHC molecules, which would theoretically result in an increase in T cells that successfully make it past positive selection. Mathematical modeling suggests that the number of MHC molecules can be increased significantly without suffering adverse effects on the ability to mount a response to pathogens, with estimates even as high as 1,500 MHC molecules (10, 11). Understanding what impact expressing additional MHC molecules would have on immunity is important for increasing our knowledge of the factors influencing the nature of the T cell repertoire, which ultimately impacts a wide spectrum of immune responses, including the ability to tolerate transplantation in addition to reactivity to pathogens and susceptibility to autoimmune disease.

MHC molecules on medullary thymic epithelial cells and bone marrow derived antigen presenting cells APCs induce negative selection, deleting T cells containing high affinity TCRs to preserve self-tolerance (12). This role implies that increasing the number of MHC molecules

may overly restrict the T cell repertoire. Studies that support the substantial effect of negative selection on the T cell repertoire include the elimination of approximately 5% of thymocytes by a single MHC molecule (13), deletion of the TCR Vβ17-expressing T cell population by I-E (14), and the ability of 1-10% of T cells to recognize allogeneic MHC (15-17) which suggests a significant portion of T cells would bind to a new MHC encountered in the thymus, possibly too strongly. Knowing whether the introduction of additional MHC molecules results in either a net gain of positive selection or negative selection effects is integral in understanding the ultimate implications on immune responses.

Thus far, there are conflicting reports on whether the expression of additional MHC molecules would be beneficial or detrimental to immune responses. MHC heterozygotes were shown to have greater pathogen resistance compared to their littermate MHC homozygote mice (18, 19). On the other hand, F1 progeny of parents with disparate MHC haplotypes were shown to have dampened T cell reactivity in the context of viral infections (20, 21). Studies have also approached this issue by adding just one more MHC molecule to the repertoire. The majority of reports focused on autoimmunity, with most concluding that expressing more MHC is beneficial. Adding I-E expression to mice of different MHC haplotypes protected from diabetes, lupus, experimental autoimmune myasthenia gravis, and collagen-induced arthritis (22-28). Yet, it was also shown that the addition of I-E^b was detrimental, resulting in enhanced susceptibility to autoimmune thyroiditis (29). A viral infection model lent support for negative effects resulting from adding a second MHC molecule. Here, the $CD4^+$ T cell response to influenza peptides was diminished in mice expressing two I-A molecules compared to one (30).

The I-E MHC, which has been a useful tool to add back to the mouse MHC repertoire, is puzzling for its disappearance from a substantial population of mouse strains. H-2^b, H-2^s, H-2^f, and H-2^q haplotype mouse strains and approximately 20% of wild mice do not express I-E (31). Studies support the idea that there exists some advantage for losing I-E MHC expression. First, it was shown that three distinct mechanisms contribute to the loss of I-E expression. H-2^b and H-2^s haplotype mice have a deletion in the *Eα* gene, H-2^f mice synthesize Eα mRNA of an aberrant size, and H-2^q mice exhibit a defect in Eα RNA processing and/or mRNA stability (32). Second, the deletion in the *Eα* gene was found to be identical between wild mice and H-2^b plus H-2^s haplotype mice, suggesting the mutation occurred early and disseminated widely throughout the mouse species (33-35). However, it does not make sense why some mice still express both I-A and I-E class II MHC molecules and are capable of surviving normally if I-E is that harmful. Thus, we sought to resolve the important question of what impact does I-E have on immune responses. Previous studies have not tested what effect expressing additional MHC would have on alloreactivity, a T cell response in which we are particularly interested. And, prior work examined the effects of adding MHC on only one disease model (ie. only lupus was studied when an I-E molecule comprised of $E\alpha^dE\beta^b$ was expressed, or in mice additionally expressing $E\alpha^kE\beta^d$, only collagen-induced arthritis was probed). It is unknown whether adding a particular MHC exerts distinct effects on different T cell responses.

In our studies, we used B6.E^+ mice, which have the monomorphic *Eα*^{k} transgene thus enabling I-E^b expression, to address the impact of having one additional class II MHC on a range of immune responses. We hypothesized that there would be negative consequences to immune responses from adding I-E^b, providing a reason for the limit on the number of MHC molecules

expressed. We saw that in alloreactivity, the B6.E$^+$ T cell response was significantly dampened. Interestingly, diverse effects of I-E^b were seen when we probed its impact on other types of immune responses. In hen egg-white lysozyme (HEL) protein immunization, we found similar B6 and B6.E$^+$ T cell responses. In viral infection with LCMV, I-E^b elicited subtle differences in CD4$^+$ T cell cytokine production. Finally, in an autoimmune disease model, EAE, expression of I-E^b resulted in detrimental consequences. There was significantly enhanced disease in B6.E$^+$ mice compared to B6. This was not attributable to greater numbers of pathogenic T cells or increased effector cytokine production. Instead, B6.E$^+$ mice had decreased Treg cell percentages during EAE, a deficiency related to altered selection mediated by I-E^b. Altogether, the data suggest that the limitation on the number of MHC molecules we express may be to prevent autoimmunity.

Results

The expression of a second class II MHC molecule, I-E^b, alters the naive T cell repertoire

To understand the impact that increasing the number of MHC molecules has on the naive T cell repertoire, the thymus populations of B6 and B6.E$^+$ mice were compared. B6 mice express only one class II MHC molecule, I-A^b, and do not express I-E^b due to a deletion in the *Eα* gene, while B6.E$^+$ mice have both I-A^b and I-E^b resulting from transgenic expression of the monomorphic *Eα*^{k} that pairs with the endogenous I-Eβ^b chain. B6 and B6.E$^+$ mice had similar numbers of total thymocytes ($1.79x10^8$±$2.45x10^7$ cells vs $1.72x10^8$ ± $1.65x10^7$ cells, respectively). Broad analysis of thymocyte populations revealed similar percentages of B6 and B6.E$^+$ CD4$^+$ and CD8$^+$ DN cells (10.1±1.3% vs. 8.7±0.4%, respectively), DP cells (65.9±3.1% vs. 65.9±2.7%, respectively), CD4$^+$ SP cells (15.2±1.7% vs. 16.5±2.5%, respectively), and CD8$^+$ SP cells (5.1±0.4 vs. 5.3±0.3%, respectively) (**Fig. 4.1A**). However, more in-depth examination of the expression patterns of the TCR Vβ family members revealed differences. Certain Vβ family members, Vβ5, Vβ11, and Vβ12, were found to be decreased in both the B6.E$^+$ CD4$^+$ SP and CD8$^+$ SP thymus populations (**Fig. 4.1B**). These results are in line with previous work attributing the I-E molecule to the thymic deletion of these exact Vβ family members, postulated to be mediated by superantigens (23, 36, 37). What was more interesting and novel is that B6.E$^+$ CD4$^+$ SP thymocytes had increased percentages of Vβ6 and Vβ10 compared to B6 (9.3±0.4% vs. 6.6±0.3% for Vβ6 and 6.3±0.3% vs. 3.5±0.2% for Vβ10). These differences were not seen with CD8$^+$ SP thymocytes, indicating they resulted directly from adding a class II MHC molecule (**Fig. 4.1B**). Additionally, analysis of DP thymocytes revealed none of the changes in Vβ

staining, suggesting that the altered repertoire in B6.E$^+$ mice was due to selection-mediated effects (**Fig. 4.1C**). Further, splenic B6.E$^+$ CD4$^+$ T cells showed increases in the Vβ6 and Vβ10 TCR-expressing populations compared to B6 (11.4±0.5% vs. 8.8±0.4% for Vβ6 and 7.3±0.1% vs. 4.1±0.2% for Vβ10) (**Fig. 4.1D**). This demonstrates that an altered TCR repertoire is maintained in the periphery and present to potentially change the pathogenesis of immune diseases. We predict that I-E^b expression would have a negative impact.

Against allogeneic stimuli, expression of a second MHC molecule decreases the CD4$^+$ T cell response

To determine what impact MHC composition has on alloreactivity, a mixed lymphocyte reaction (MLR) was performed. 1.5 x 10^6 B6 and B6.E$^+$ splenic CD4$^+$ T cells were cultured with equal numbers of irradiated Balb/c (H-2^d) or SJL (H-2^s) APCs for 48 h, and IFNγ enzyme-linked immunospot (ELISPOT) was carried out to determine the frequency of alloreactive T cells. The B6.E$^+$ alloreactive T cell frequencies were significantly decreased upon stimulation with both Balb/c and SJL APCs compared to B6 (232.6±72.8 vs. 518±87.8 for Balb/c and 97.9±16 vs. 176.1±30.5 IFNγ spots/well for SJL). B6 and B6.E$^+$ samples had similar responses to the nonspecific stimulation PMA + ionomycin (122.8±29.1 vs. 76.3±17.1 IFNγ spots/well, respectively) as well as to no stimulation (20±4.5 vs. 21±4.4 IFNγ spots/well, respectively) (**Fig. 4.2**).

Expression of I-E^b does not change the T cell response to HEL protein immunization

We were interesting in analyzing the B6.E$^+$ T cell response in other types of immune models to determine if similar dampened T cell reactivity as in allorecognition would be seen or if the addition of I-E^b elicits different effects. Analysis of the T cell response to a single protein immunization was performed. We reasoned that should any changes be detected, this model would allow us to feasibly identify the individual epitope(s) responsible for the phenotype. The HEL protein was chosen because B6 mice exhibit minimal reactivity to it. Out of 11 HEL peptides spanning the entire 129 amino acid sequence of the protein, CD4$^+$ and CD8$^+$ T cells from B6 mice responded to only 3 epitopes (38, 39). We thought any alterations to the T cell response would be most discernible in a system where the antigen reactivity is moderate to begin with. B6 and B6.E$^+$ mice were immunized in the hind footpads with HEL emulsified in CFA, and 7 days later draining lymph node cells were taken and stimulated *in vitro* with HEL for 4 days. Samples were pulsed with [^{3}H]TdR during the last 24 h and assessed for proliferation. We found that B6 and B6.E$^+$ responses to multiple concentrations of HEL were similar, demonstrating that the addition of a second MHC molecule does not affect overall immune reactivity in this situation (**Fig. 4.3A**). Furthermore, B6 and B6.E$^+$ T cell responses to purified protein derivative (PPD), containing *Mycobacterium tuberculosis* present in CFA, was compared. The similar proliferative responses to a more complex mixture of antigens underscored the lack of I-E^b-mediated differences in protein immunization (**Fig. 4.3B**).

Addition of I-E^b impacts the quality of the CD4$^+$ T cell response to LCMV

We next investigated the CD4$^+$ T cell response to viral infection to see the addition of a second MHC II molecule elicits differences in a disease model. B6 and B6.E$^+$ mice were infected i.p.

with LCMV Armstrong, and 8 days later spleen cells were analyzed for frequency of LCMV-specific T cells by tetramer stain. To narrow our focus, we chose to compare the B6 and B6.E$^+$ T cell population specific to a defined I-A^b-restricted LCMV epitope, GP$_{66-77}$, dominant in the CD4$^+$ T cell response (40). Upon staining with the LCMV GP$_{66-77}$/I-A^b tetramer, we found that B6.E$^+$ mice exhibited similar frequencies of LCMV-specific T cells compared to B6 (900.5±177.7 vs. 624.2±78.3 cells/10^5 CD4$^+$ T cells, respectively) (**Fig. 4.4A**). T cell cytokine production was also assessed. To do this, splenic cells 8 d post-infection were stimulated *ex vivo* for 24 h with the I-A^b-restricted LCMV$_{61-80}$ peptide containing no predicted I-E^b epitopes according to an I-E^b binding motif previously used for I-E^b peptide identification (41, 42) and then analyzed by flow cytometry. The production of individual cytokines such as TNFα by itself was comparable between B6 and B6.E$^+$ CD4$^+$ T cells (4.4±0.7% vs. 3.8±0.5%, respectively). But interestingly, the percentage of CD4$^+$ T cells co-producing IFNγ and TNFα was reduced in B6.E$^+$ mice compared to B6 (0.66±0.06% vs. 0.88±0.09%, respectively) (**Fig. 4.4B**).

We wondered if the difference in B6.E$^+$ CD4$^+$ T cell production of IFNγ and TNFα was present only in an acute infection or if the cytokine decrease would persist throughout a chronic infection. Therefore, we performed chronic infection with LCMV CL13 by i.v. injection of 2x10^6 pfu of virus. Our results corroborated the dampened T cell cytokine production seen in the B6.E$^+$ mice compared to B6 (0.16±0.02% vs. 0.24±0.02%, respectively) at 28 d post-infection (**Fig. 4.4C**), demonstrating that the difference is not limited to acute LCMV infection. Altogether, expression of an additional MHC molecule resulted in a subtle impact on T cell immunity by keeping the frequency of antigen-specific CD4$^+$ T cells the same but altering the

quality of the cytokine-producing T cell repertoire, diminishing the percentage of T cells producing multiple cytokines.

I-E^b speeds the onset and increases the severity of EAE due to altered selection of the natural Treg (nTreg) population

We were interested in examining B6.E$^+$ mice in autoimmunity because there are opposing conclusions about what impact adding I-E has on autoimmune diseases. Though the majority of reports have demonstrated a protective role for I-E in a number of autoimmune models, a thorough study on autoimmune thyroiditis showed increased susceptibility imparted by I-E expression (22, 24-29). Our study provides a unique opportunity to relate the impact of I-E on autoimmunity to its effects on other types of immune responses, a comparison that has not been done before. Here, I-E^b has produced different effects on distinct immune models: it does not change the response to HEL protein immunization but alters the quality of the anti-viral response and diminishes alloreactivity. We wondered what result we would see in autoimmunity and whether it would be completely different or similar to any of our other models. We chose to investigate EAE because the impact of I-E on this autoimmune model has not been examined before and could provide further support for either a protective or detrimental role for I-E. B6 and B6.E$^+$ mice were immunized with MOG$_{35-55}$ peptide emulsified in CFA and injected with pertussis toxin. Using similar reasoning as in our LCMV infection study, we chose to immunize with an I-A^b-restricted MOG peptide rather than whole protein so that we could perform a direct and focused comparison between the B6 and B6.E$^+$ T cell response to a defined antigen.

After immunization, clinical scores were recorded throughout the course of disease. Unexpectedly, results revealed that B6.E$^+$ mice had enhanced disease compared to B6, exhibited by earlier days of onset (11.2±0.43 vs. 14±0.9, respectively) as well as higher peak clinical scores (3.5±0.25 vs. 2±0.24, respectively) (**Fig. 4.5** and **Table 4.1**). A possible explanation for increased autoimmunity would be an increased pathogenic T cell number or response. We therefore assessed T cell binding to MOG_{38-49}/I-A^b tetramer and cytokine production. Due to the difference in day of onset, we chose a time-point early in disease when we reasoned differences in lymphoid organs would be most evident. At 13 d post-EAE induction, the frequencies of MOG/I-A^b-specific CD4$^+$ T cells extracted from spleen and draining lymph nodes were similar between B6 and B6.E$^+$ mice (221.3±41.1 vs. 203.4±24.3 cells/10^5 CD4+ T cells, respectively) (**Fig. 4.6A**). To look at cytokine production, spleen and draining lymph node cells were stimulated *ex vivo* with MOG_{35-55}, and staining for cytokines relevant to EAE pathogenesis – IFNγ, IL-17, and TNFα – was performed and assessed by flow cytometry (43, 44). Surprisingly, B6 and B6.E$^+$ CD4$^+$ T cells had similar production of all three cytokines (1.2±0.3% vs. 0.9±0.1%, respectively, for IFNγ; 0.18±0.03% vs. 0.14±0.02% for IL-17; and 1.2±0.2% vs. 1.1±0.1% for TNFα) (**Fig. 4.6B**).

An alternative explanation for enhanced EAE disease is that it resulted not from increased T cell pathogenicity, but from decreased Treg cells. To test this, the percentage of Foxp3$^+$ CD4$^+$ Treg cells was assessed 13 days after EAE induction. Interestingly, a significantly decreased percentage of Treg cells was seen in B6.E$^+$ mice compared to B6 (8.7±0.8% vs. 12.7±1.2%, respectively) (**Fig. 4.7A**). To determine whether nTreg cells – which are generated in the thymus – are the subset affected, we stained the cells for Helios, a marker of nTreg cells (45). A

significant reduction in the percentage of nTreg cells during EAE was observed in B6.E^+ mice compared to B6 (63.9±2.2% vs. 72.4±2.5%, respectively), implicating a thymic selection-mediated effect of I-E^b on the Treg cell population (**Fig. 4.7B**). Analysis of Foxp3 staining in spleen and lymph node cells from naive mice revealed that the decrease in Treg cells was not present in B6.E^+ mice prior to disease (9±0.6% B6 vs. 8.1±0.9% B6.E^+) (**Fig. 4.7C**). It appears that perturbation of the immune system through autoimmunity was necessary to expose the defect of the B6.E^+ Treg cell repertoire. Put together, these EAE results provide interesting and novel insight into the differential susceptibility of self-reactive, Treg cells to increased negative selection imparted by the expression of a second class II MHC molecule in autoimmunity.

Discussion

Throughout the years, immunologists have been intrigued by the biological implications for the limit on the number of MHC molecules expressed in an individual. Knowing that the role of the MHC is to present peptides to T cells, it is reasonable to think that having additional MHC molecules would result in significantly beneficial consequences; expansion of the T cell repertoire during positive selection as well as an increase in the presented peptide repertoire in the periphery should both enhance T cell responses to pathogens. However, MHC molecules also participate in negative selection, and it is possible this would balance out or even overcome the effects from positive selection. We compared B6 mice, which have only one class II MHC molecule, I-A^b, to B6.E^+ mice, which have both I-A^b and I-E^b, in order to see what would result from expressing a second class II MHC molecule. Specifically, we were interested in determining the effect on $CD4^+$ T cell alloreactivity and if the differences seen, if any, would be observed across a wide variety of T cell responses.

Examination of naive B6 and B6.E^+ mice revealed distinct TCR usage. B6.E^+ mice exhibited higher percentages of $CD4^+$ T cells expressing Vβ6 and Vβ10 as well as decreased percentages of Vβ5, Vβ11, and Vβ12 $CD4^+$ and $CD8^+$ T cells. Though the changes in Vβ5, Vβ11, and Vβ12 expression have been shown before to be mediated by I-E and superantigen (23, 36, 37), the finding that there is increased Vβ6- and Vβ10-expressing $CD4^+$ T cells upon addition of I-E^b is novel. We discovered that the addition of a second MHC molecule resulted in diverse effects, including significantly decreased alloreactivity, no net difference in the T cell response to HEL protein immunization, a subtle alteration to the LCMV-reactive T cell repertoire, and enhanced

autoimmunity accompanied by diminished nTreg percentages. Based on a reported I-E^b binding motif (41, 42), there is one putative I-E^b peptide epitope in HEL. It is possible that though both I-A^b and I-E^b contribute to the response to HEL in the B6.E^+ mice, there was no net change in reactivity due to the altered HEL-specific I-A^b and I-E^b T cell repertoires balancing out. Or, another explanation is that there is no I-E^b-restricted response to HEL in the B6.E^+ mice. We saw that having additional antigens present and more complex immune disease models resulted in changes to the T cell response. Most intriguingly, the significantly enhanced autoimmunity in B6.E^+ mice (**Fig. 4.5**) supports our hypothesis that negative consequences on immunity limit the number of MHC molecules expressed.

We think our results can be reasonably explained by enhanced MHC-mediated negative selection dominating the effects from any increase in positive selection. For alloreactivity, thymic selection is known to play a significant role in shaping the alloreactive T cell repertoire. Positive selection results in T cells that can bind to many different allogeneic MHC molecules, while negative selection establishes MHC restriction and peptide specificity (46). We show here that the effects of negative selection outweigh those of positive selection, resulting in decreased alloreactive T cells in B6.E^+ mice. This decrease may have both beneficial and detrimental clinical consequences, theoretically leading to less GVHD or transplant rejection but diminishing the graft-versus-leukemia effect that eliminates tumors (47). Since H-2^d APCs express both I-A and I-E while the H-2^s APCs have only I-A, we can compare the I-E- and I-A-specific alloresponse to the I-A-only alloresponse. Not unexpectedly, there was a greater magnitude of alloreactivity to the H-2^d APCs compared to H-2^s, demonstrating the contribution of two allogeneic MHC molecules rather than one for stimulation. Compared to B6, B6.E^+ $CD4^+$ T cells

exhibited an approximately 50% decrease in alloresponse to both H-2^d and H-2^s APCs. This indicates that I-E^b in B6.E^+ mice can alter the T cell repertoire to affect the alloresponse to a single allogeneic MHC, I-A^s, and that having two allogeneic MHC molecules, I-A^d plus I-E^d, does not provide enough alloligands to enhance the level of B6.E^+ alloreactivity to equal B6. Whether there are similar reductions in the number of B6.E^+ T cells specific to individual allopeptides could be established in the future using H-2^d or H-2^s pMHC tetramers.

For the LCMV data, the decreased percentage of B6.E^+ $CD4^+$ T cells producing IFNγ and TNFα in response to LCMV stimulation suggests that I-E^b increases the deletion of polyfunctional LCMV-specific T cells. Sequence analysis of naturally occurring peptides eluted from I-A^b and I-E^b revealed distinct sequence motifs between the two MHC molecules (48). This lends support for the idea of I-E^b introducing a different set of pMHC ligands during thymic selection and altering the resulting T cell repertoire. Polyfunctional T cells that produce multiple cytokines have been associated with better protection from parasitic infection and more effective control of viral infection (49, 50). Thus, though the difference is subtle, it is possible that the altered T cell repertoire in B6.E^+ mice can impact viral load or disease course in subsequent challenges.

For EAE, unlike the two previous models, we did not detect a change in the B6.E^+ effector T cell population that would explain the enhanced autoimmunity. In this case, the Treg population was diminished. Further investigation identified nTreg cells as being affected, lending support for I-E^b impacting thymic development. It is possible that adding I-E^b during selection increases the chance that high affinity T cells differentiating into the Treg lineage – often characterized as experiencing a near-death encounter – are pushed into being negatively selected (51). The fact

that MOG is expressed in medullary thymic epithelial cells (52) supports this idea. Taking into account work demonstrating a saturable Treg cell selecting niche, in which the number of Tregs in the ensuing T cell repertoire plateaus when clonal frequency is increased (53), it may be that Tregs selected on I-E^b compete with and limit Tregs selected on I-A^b. This would lead to a decreased ability for the I-A^b-selected Tregs to expand and function in a MOG_{35-55}/I-A^b-induced EAE model. It is interesting that effector T cells in EAE were not impacted considering the increased T cell cytokine production seen upon depletion of Treg cells in multiple peptide immunization-induced EAE models (54, 55). This likely reflects a difference in degree of immune suppression when Tregs are depleted in other models vs. decreased in our case. Moreover, epitope spreading is known to play a role in EAE, in which T cell reactivity extends to antigens other than what was used to induce disease, which can become accessible through processes such as tissue destruction (56). Perhaps in B6.E^+ mice, effector T cells specific to I-A^b- or I-E^b-bound self peptides other than MOG_{35-55} are impacted, which we did not test in our study.

Another issue relevant to all of our immune models is the possibility that $E\alpha_{52-68}$, a peptide derived from the *Eα* gene product, binds to I-A^b and plays a role in dampening immunity. As the peptide is capable of occupying approximately 10-15% of all I-A^b molecules (29, 57), it can prevent the selection of certain I-A^b-restricted cells in the thymus or compete with the presentation of pathogenic peptides in the periphery. The Eα peptide along with the I-E^b MHC could both contribute to the altered T cell repertoire and T cell responses in the B6.E^+ mice. Intrathymic injection of the peptide into B6 mice and addition of the peptide to *in vitro* stimulations for competition with LCMV or MOG would aid in understanding what extent $E\alpha_{52-68}$/I-A^b plays in B6.E^+ T cell selection and peripheral responses, respectively.

In contemplating the evolution of the immune system, though adaptive immunity has given vertebrates advantages such as memory, it has also led to significant drawbacks of immunopathology, hypersensitivity, and autoimmunity that result from heightened responses (58). Similar to this phenomenon, our work indicates that a more complex immune system with the addition of a second class II MHC molecule elicits enhanced autoimmunity, a decidedly negative consequence. Though the results from the other immune models elicited more benign effects – no difference in HEL protein immunization, subtle alteration of the LCMV response, and decreased alloreactivity that may be both positive and negative – the detrimental impact on autoimmunity stands out as a clear deterrent to the expression of more MHC molecules. Interestingly, chickens have a minimal essential set of MHC genes within a region approximately 20-fold smaller than the human MHC locus, yet this simple and compact MHC still allows for sufficient immune responses (59). Humans, on the other hand, express a more diverse set of MHC molecules than our laboratory mouse strains. Conclusions from our work would imply that the more complex human MHC may lead to less ideal immune responses. It is in fact true that humans experience autoimmune diseases, many of which are highly associated with the MHC. This is consistent with our result of enhanced autoimmunity when adding an extra MHC molecule to the system. Perhaps humans require a certain high number of MHC molecules in order to combat the diverse repertoire of pathogens we have faced whereas the complexity of pathogens other vertebrates encounter could be sufficiently resisted with lower numbers of MHC. Knowing how different the environment is for each species, our model of expressing one additional MHC molecule, I-E, in mice is essential to experimentally address and then better understand what impact MHC number has on immune responses.

Figure 4.1 The expression of a second class II MHC molecule, I-E^b, alters the naive thymic and peripheral T cell repertoire as a result of thymic selection-mediated effects

(**A**) B6 and B6.E$^+$ thymocytes were analyzed by flow cytometry for total number of thymocytes as well as percentage of CD4$^+$ and CD8$^+$ DN, DP, and SP cells. Each point represents a single mouse, n = 7-8 from 3 independent experiments, mean ± sem. Mann-Whitney statistical test was performed. (**B**) The TCR repertoire of B6 and B6.E$^+$ thymic CD4$^+$ and CD8$^+$ T cells was examined by staining for a panel of TCR Vβ family members, n = 8 mice from 3 independent experiments, mean ± sem. 2-way ANOVA test was conducted. (**C**) The percentage of CD4$^+$ CD8$^+$ DP thymocytes positive for each Vβ is depicted for B6 and B6.E$^+$ samples, n = 8 mice from 3 independent experiments, mean ± sem, 2-way ANOVA test. (**D**) TCR Vβ staining was also performed on splenic CD4$^+$ T cells, n = 5 mice from 2 independent experiments. Data are shown as mean ± sem, with 2-way ANOVA test.

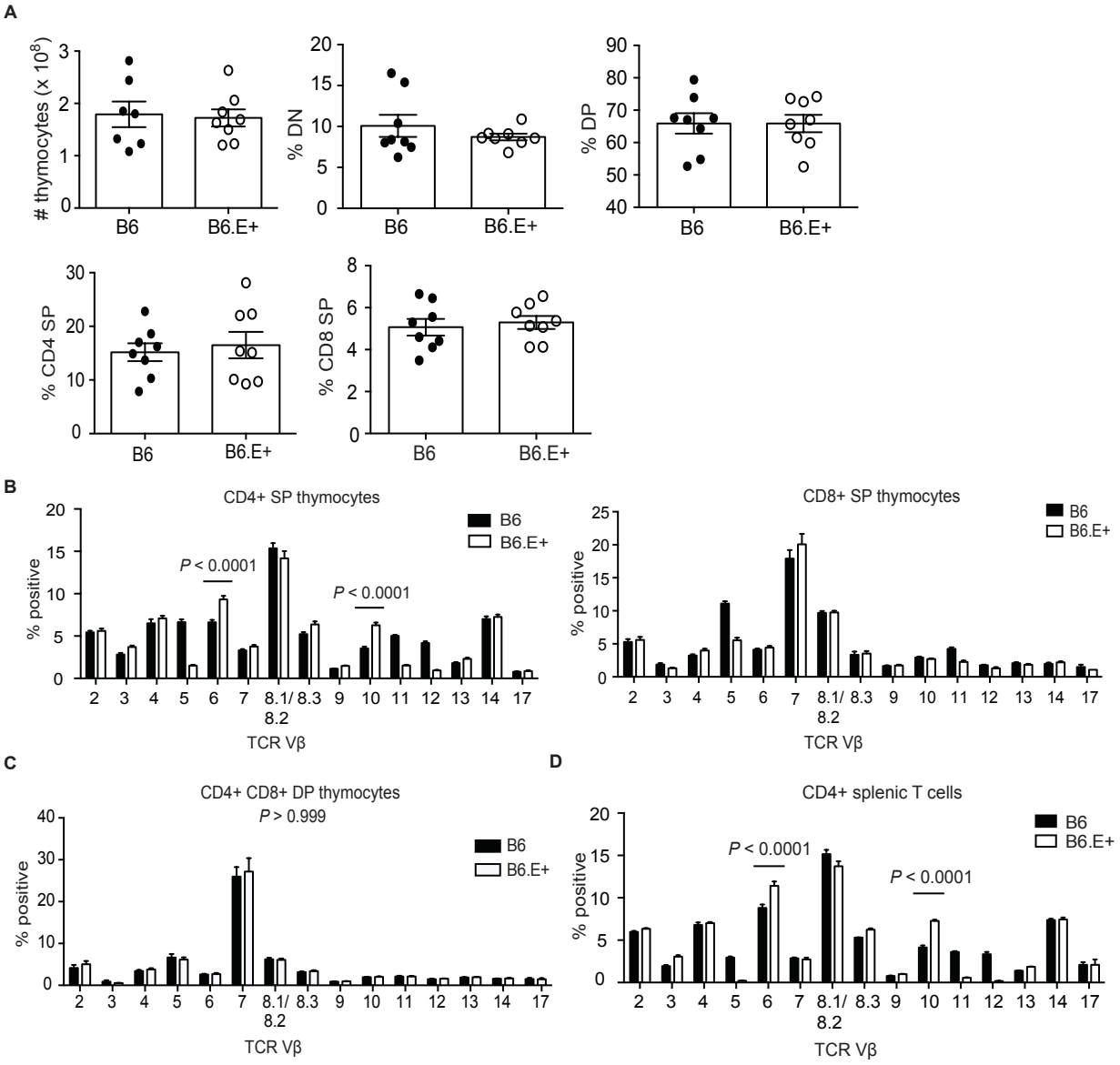

Figure 4.1 The expression of a second class II MHC molecule, I-E^b, alters the naive thymic and peripheral T cell repertoire as a result of thymic selection-mediated effects

Figure 4.2 Against allogeneic stimuli, expression of I-E^b decreases the $CD4^+$ T cell response

1.5 x 10^6 B6 and B6.E^+ splenic $CD4^+$ T cells were cultured at a 1:1 ratio with Balb/c (H-2^d) and SJL (H-2^s) APCs. Stimulation with PMA (20ng/mL) + ionomycin (1μM) and medium were used as positive and negative controls, respectively. Data are shown as mean ± sem, n = 6-9 samples compiled from 2-3 independent experiments, unpaired t test.

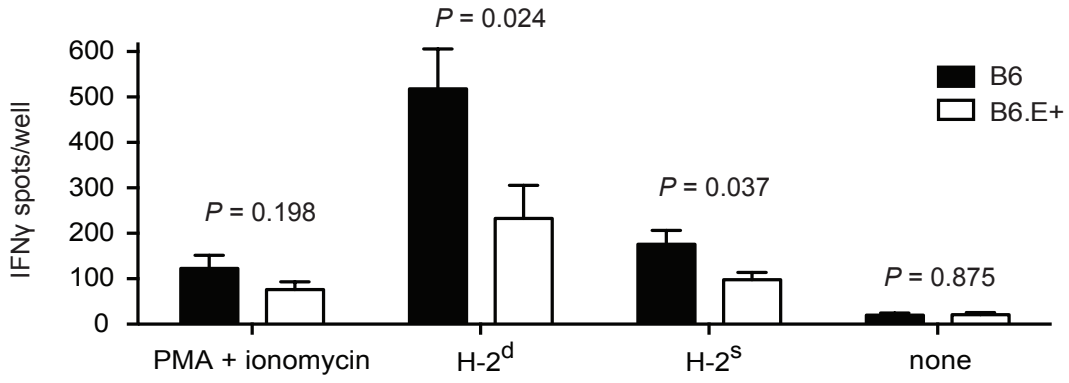

Figure 4.2 Against allogeneic stimuli, expression of I-E^b decreases the CD4$^+$ T cell response

Figure 4.3 Expression of I-E^b does not change the T cell response to HEL protein immunization

T cells from B6 and B6.E$^+$ mice immunized with 20 nmol HEL protein emulsified in CFA were stimulated in culture for 4 d with varying concentrations of (**A**) HEL and (**B**) a dilution series of PPD. [^{3}H]TdR was added during the final 24 h of culture, and proliferation was measured. No stimulation (0μM) data points are depicted to show background CPM. Data are shown as mean ± SD and are representative of three experiments.

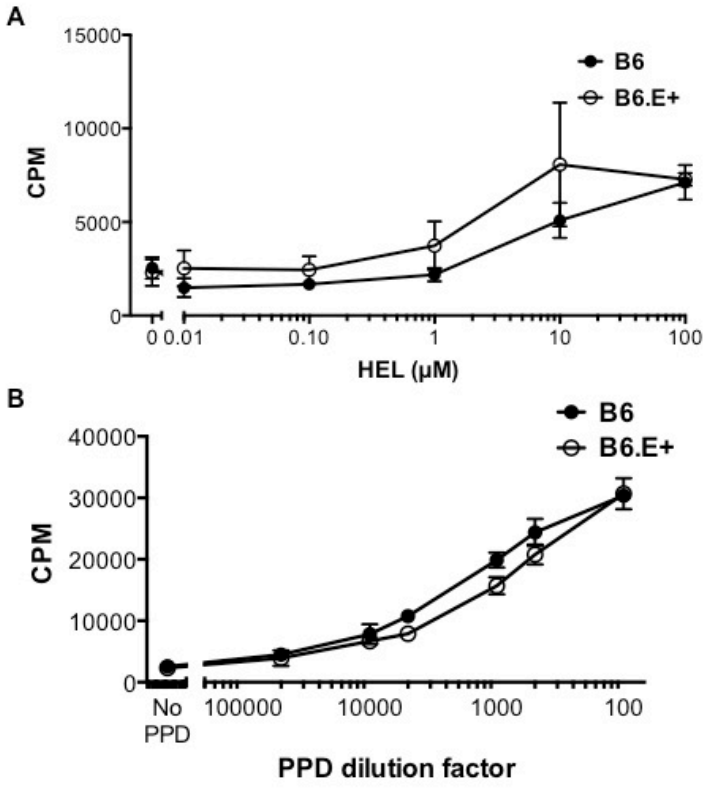

Figure 4.3 Expression of I-E^b does not change the T cell response to HEL protein immunization

Figure 4.4 Addition of I-E^b impacts the quality of the CD4$^+$ T cell response in LCMV infection

(**A, B**) Mice were infected i.p. with 4 x 10^5 pfu LCMV Armstrong, and 8 d post-infection splenic T cells were analyzed. (**A**) Cells were stained with LCMV/I-A^b APC-labeled tetramer, enriched by magnetic selection using anti-APC beads, stained for surface markers, and assessed by flow cytometry. Representative plots of CD4$^+$ and CD8$^+$ T cell tetramer-positive populations from B6 and B6.E$^+$ mice are shown (left) as well as combined data of the number of tetramer-positive CD4$^+$ T cells with each point depicting one mouse from two independent experiments, mean ± sem, Mann-Whitney test (right). (**B**) Cells were also stimulated *ex vivo* with 10μg/mL LCMV GP_{61-80} peptide for 24 h followed by intracellular cytokine staining for TNFα and IFNγ. Representative plots are shown depicting cytokine-positive populations from B6 and B6.E$^+$ CD4$^+$ T cells (left) along with combined data for TNFα positive CD4$^+$ T cells and TNFα and IFNγ double positive CD4$^+$ T cells, each point representing one mouse from two independent experiments, mean ± sem, Mann-Whitney test (right). (**C**) Mice were chronically infected i.v. with 2 x 10^6 pfu of LCMV CL13. At 28 d post-infection, spleen cells were stimulated ex vivo with 10μg/mL LCMV GP_{61-80} for 24 h, and cytokine production was assessed by flow cytometry. Representative B6 and B6.E$^+$ plots of TNFα and IFNγ positive populations are shown (left) along with combined data, each point representing one mouse from two independent experiments, mean ± sem, Mann-Whitney test (right).

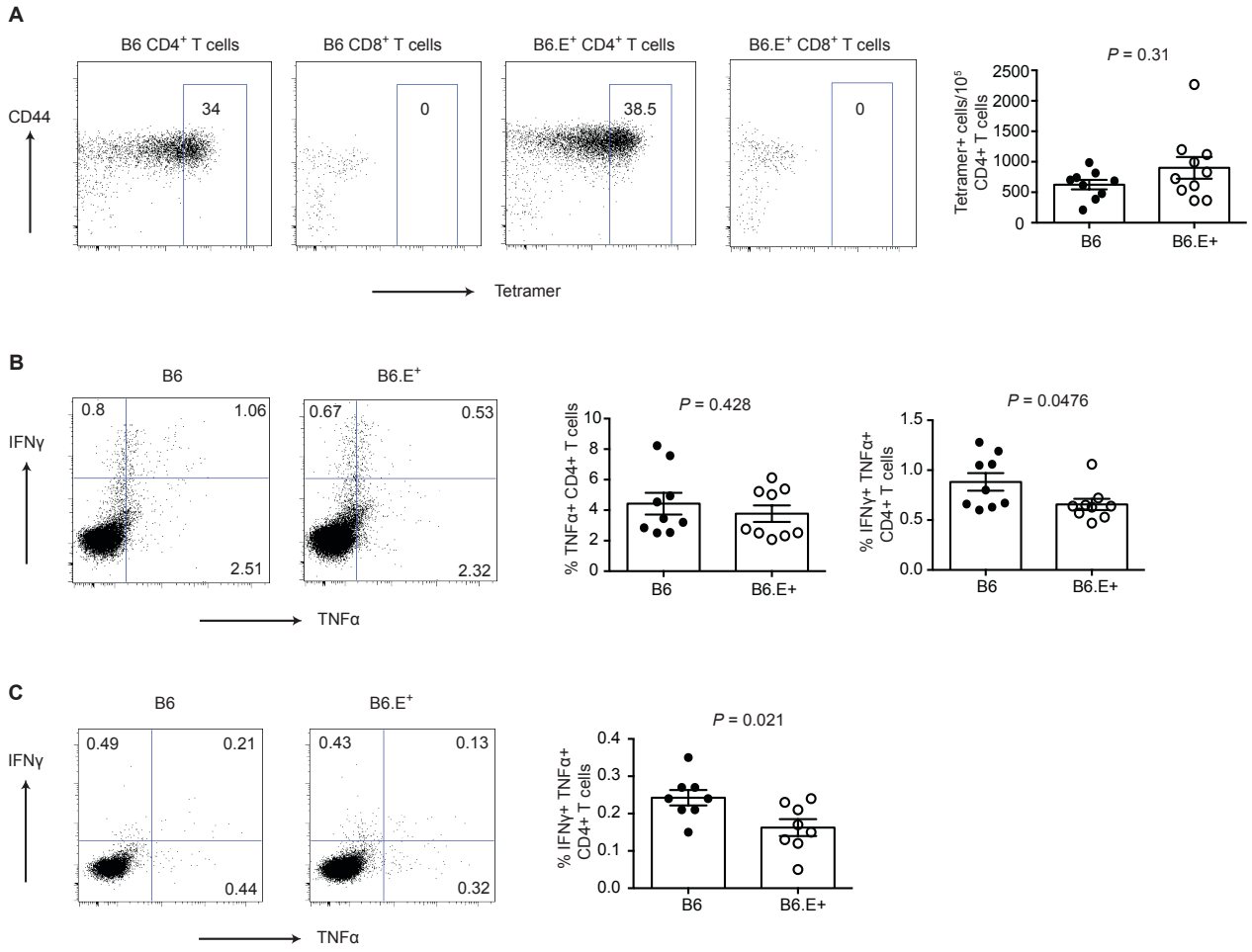

Figure 4.4 Addition of I-E^{b} impacts the quality of the CD4^{+} T cell response in LCMV infection

Figure 4.5 I-E^b speeds the onset and increases the severity of EAE

EAE was induced with sub-cutaneous (s.c.) immunization of MOG_{35-55} peptide emulsified in CFA plus i.p. injection of pertussis toxin and scored up to 28 d post-immunization on a five point scale: 0 = no weakness, 1 = limp tail, 2 = mild hindlimb paresis, 3 = moderate to severe hindlimb paresis, 4 = complete hindlimb paresis, 5 = moribund or dead. Results are mean ± sem at each timepoint and representative of three independent experiments.

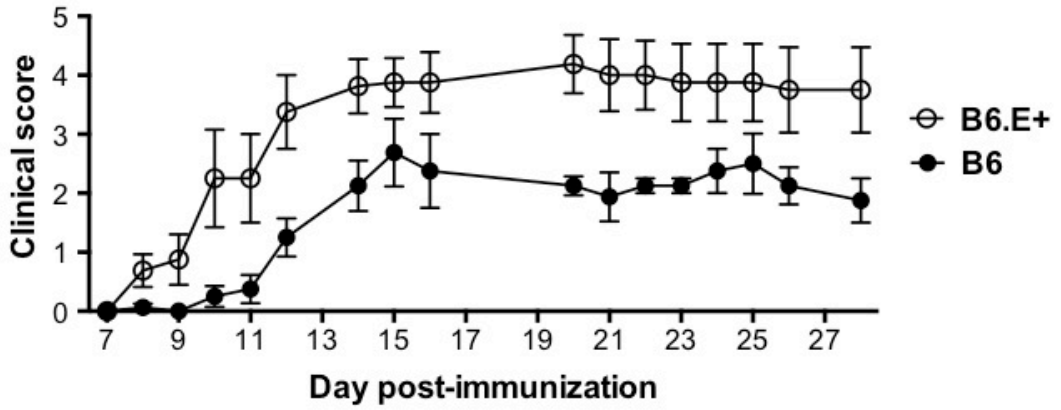

Figure 4.5 I-E^b speeds the onset and increases the severity of EAE

Figure 4.6 Enhanced EAE in mice expressing I-E^b is not associated with increased pathogenic T cell number or response

EAE was induced with s.c. immunization of MOG_{35-55} peptide emulsified in CFA plus i.p. injection of pertussis toxin. At 13 d post-immunization, spleen and draining lymph node cells were taken for analysis. (**A**) Cells were incubated with MOG/I-A^b or human clip/I-A^b (negative control) PE-labeled tetramer, enriched with anti-PE magnetic beads, and stained with surface markers for flow cytometry. Representative plots of B6 and B6.E$^+$ CD4$^+$ T cell tetramer positive populations are shown (left) as well as combined data depicting the number of MOG/I-A^b tetramer positive CD4$^+$ T cells with each point representing one mouse from four independent experiments, mean ± sem, Mann-Whitney test (right). (**B**) Cells were stimulated with 10-20μg/mL MOG_{35-55} for 24 h, stained for intracellular cytokines, and analyzed by flow cytometry. Representative plots of cytokine positive CD4$^+$ T cells are shown (left) as well as combined data from 10 mice over three independent experiments, mean ± sem, Mann-Whitney test (right).

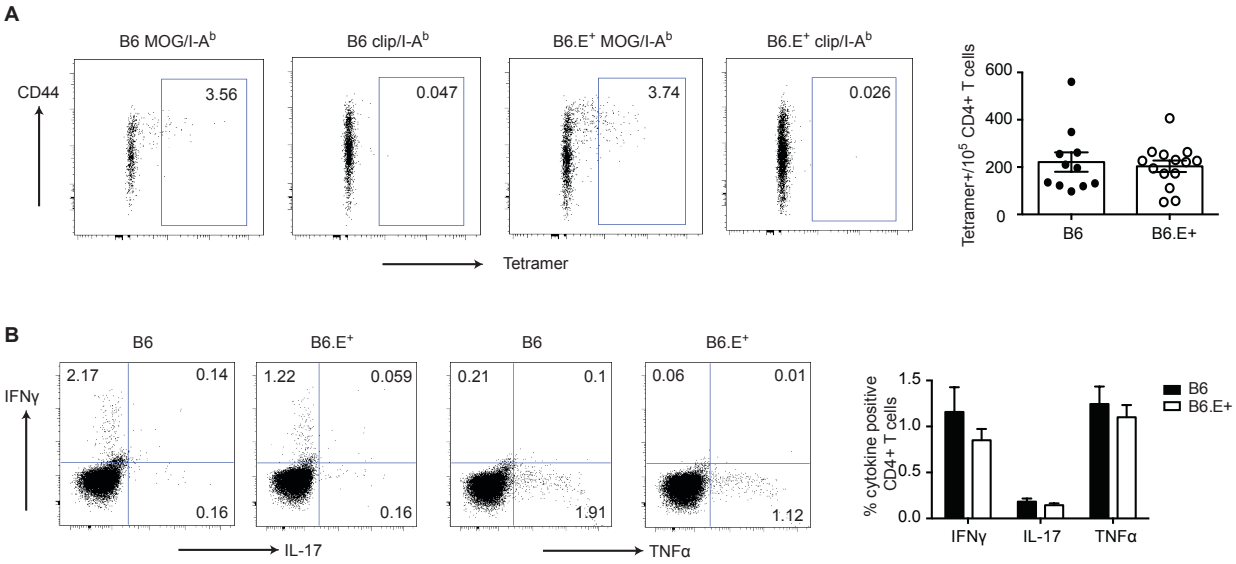

Figure 4.6 Enhanced EAE in mice expressing I-E^b is not associated with increased pathogenic T cell number or response

Figure 4.7 I-E^b-mediated enhanced EAE is associated with decreased nTreg cells

EAE was induced with s.c. immunization of MOG_{35-55} peptide emulsified in CFA plus i.p. injection of pertussis toxin. (**A, B**) At 13 d post-immunization, spleen and draining lymph node cells were taken for analysis. (**A**) Cells were stained for intracellular Foxp3 expression. Representative flow cytometry plots of Foxp3 positive B6 and B6.E^+ $CD4^+$ T cell populations are shown (left) as well as combined data (right). (**B**) Intracellular Helios staining was also performed, representative plots of $CD4^+$ T cells (left) and combined data (right) are depicted. (**C**) Intracellular staining of Foxp3 in naive splenocytes and lymph node cells was done, with representative plots (left) and combined data (right) of $CD4^+$ T cells shown. The combined data are from three independent experiments with each point on the graphs representing one mouse and are shown as mean ± sem, Mann-Whitney test.

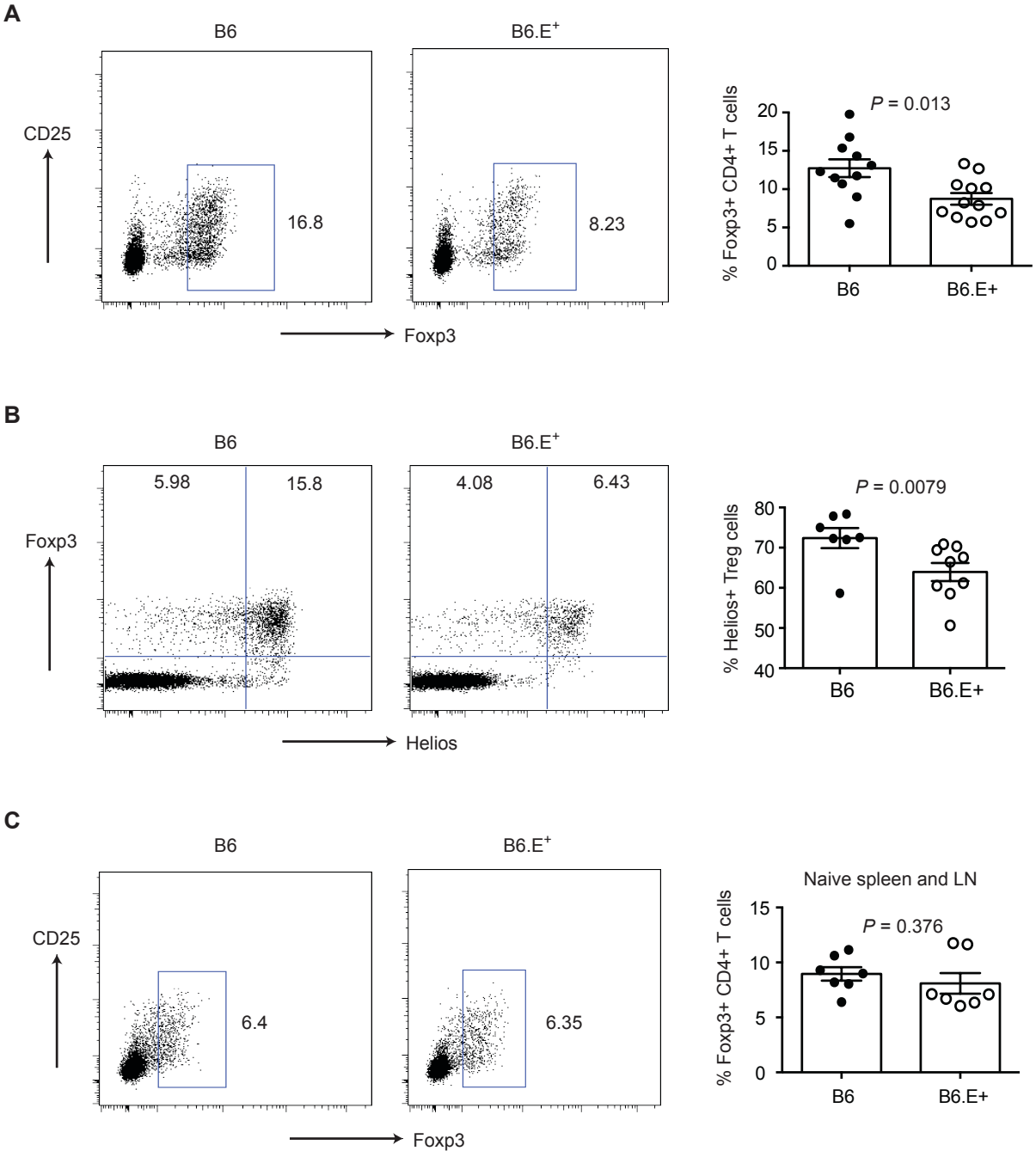

Figure 4.7 I-E^b-mediated enhanced EAE is associated with decreased nTreg cells

Table 4.1 Compilation of EAE results from four independent experiments

EAE was induced with s.c. immunization of MOG_{35-55} peptide emulsified in CFA plus i.p. injection of pertussis toxin and scored up to 28 d post-immunization on a five point scale: 0 = no weakness, 1 = limp tail, 2 = mild hindlimb paresis, 3 = moderate to severe hindlimb paresis, 4 = complete hindlimb paresis, 5 = moribund or dead.

Mouse Genotype	B6	B6.E^{+}	*P* value (Mann-Whitney test)
Incidence of disease	20/20	18/18	
Day of onset (mean ± sem)	14 ± 0.9	11.2 ± 0.43	0.015
Peak clinical score (mean ± sem)	2 ± 0.24	3.5 ± 0.25	0.0001

Table 4.1 Compilation of EAE results from four independent experiments

Methods

Mice

Balb/c, B6, and SJL mice were purchased from The Jackson Laboratory. B6.E^+ mice were the kind gift of Chella David (Mayo Clinic), and were derived from insertion of the monomorphic $E\alpha^k$ transgene into C57BL/6 x SJL embryos (60), which pairs with the endogenous I-Eβ^b chain thus allowing the expression of I-E^b. The mice were extensively backcrossed with C57BL/6 mice onto the H-2^b background, and this was confirmed by analysis of microsatellite markers at the Rheumatic Disease Core Center, Washington University School of Medicine (St. Louis, MO). Mice were bred and housed in specific pathogen-free conditions at the animal facility at the Washington University Medical Center (St. Louis, MO). All use of laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

Flow cytometry

Analysis of thymocytes and peripheral T cell populations was performed using anti-CD3 (145-2C11)-PE-Cy7, anti-CD25 (eBio3C7)-eFluor 450, anti-Foxp3 (FJK-16s)-PE (eBioscience), anti-Helios (22F6)-Alexa Fluor 647, anti-CD44 (IM7)-Alexa Fluor 700, anti-CD4 (RM4-5)-PerCP, anti-CD8a (53-6.7)-APC-Cy7 (Biolegend), and anti-Vβ TCR-FITC screening panel (BD Biosciences). All samples were analyzed using LSR II or LSR Fortessa cytometers (BD Biosciences) with calculated compensation, and the data were analyzed with FlowJo software (Tree Star).

Proliferation assay

Mice were immunized s.c. with 20 nmol HEL emulsified in CFA (Difco). After 7 days, draining lymph nodes cells were harvested, and 0.5-1 x 10^6 cells were plated in triplicate with medium only, varying concentrations of HEL, or 50μg/mL PPD of *Mycobacterium tuberculosis* for 4 days in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (PAA), $5x10^{-5}$ M 2-ME (Sigma-Aldrich), 2mM GlutaMAX (Life Technologies), 1mM sodium pyruvate (Cellgro), 0.1mM non-essential amino acids (Lonza), 25mM HEPES buffer (Cellgro), and 50μg/mL gentamicin (Life Technologies), with 0.4μCi tritiated thymidine ([^{3}H]TdR) added for the last 24 h of culture. Assays were harvested on a Skatron cell harvester and counted using a LKB (Pharmacia/LKB) β plate counter.

MLR and ELISPOT

B6 and B6.E$^+$ splenic CD4$^+$ T cells were obtained by incubating cells with anti-CD4 microbeads for 30 min. at 4°C and flowing samples through magnetized LS positive selection columns (Miltenyi Biotec). 1.5 x 10^6 T cells were cultured for 48 h with equal numbers of irradiated Balb/c or SJL splenocytes, 20ng/mL phorbol 12-myristate 13-acetate (PMA) + 1μg/mL ionomycin, or medium only in 96-well MultiScreen-IP plates (Millipore) coated with purified anti-IFNγ (RA-6A2; eBioscience). Plates were then incubated with biotinylated anti-IFNγ (XMG1.2; eBioscience) and streptavidin-conjugated HRP (Southern Biotech), developed with 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich), read on a CTL ImmunoSpot reader, and analyzed using CTL ImmunoSpot 4.0 (Cellular Technology Limited).

LCMV infection

Acute infection was performed by i.p. infection with 4 x 10^5 pfu LCMV Armstrong, and spleen cells were taken at 8 d post-infection for tetramer and intracellular cytokine stains. Chronic infection was done with i.v. infection of 2 x 10^6 pfu LCMV CL13, and at 28 d post-infection spleen cells were taken for intracellular cytokine analysis.

Tetramer enrichment

LCMV GP_{66-77} (DIYKGVYQFKSV) bound to I-A^b (LCMV/I-A^b)-APC and MOG_{38-49} (GWYRSPFSRVVH) bound to I-A^b (MOG/I-A^b)-PE tetramer were used to analyze antigen-specific T cells. Tetramers were obtained from the NIH Tetramer Core Facility at Emory University (Atlanta, GA). Enrichment was conducted according to published protocols (61). In brief, cells were stained with tetramer for 1 h at room temperature, washed, incubated with anti-PE or anti-APC conjugated microbeads for 30 min. at 4°C, and passed over magnetized LS columns for positive selection (Miltenyi Biotec). The enriched cell populations were labeled with antibodies to cell surface markers for 20 min. at 4°C and analyzed by flow cytometry.

Intracellular cytokine stain

Cells were cultured with the indicated peptide – 10μg/mL for $LCMV_{61-80}$ (GLNGPDIYKGVYQFKSVEFD) and 10-20μg/mL for MOG_{35-55} – for 24 h with 10μg/mL Brefeldin A (Sigma-Aldrich) added during the last 4 h. Live cells were stained using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Molecular Probes), labeled with surface marker mAbs, fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.5% saponin

buffer, and intracellularly stained with anti-IFNγ (XMG1.2)-FITC, IL-17 (TC11-18H10.1)-PE, and TNFα (MP6-XT22)-PE (Biolegend).

EAE induction and scoring

EAE was induced by s.c. immunization over four flanks with 200μL total volume of 200μg MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) emulsified in 500μg CFA (diluted from 10mg/mL *M. tuberculosis* H37Ra, Difco, in Incomplete Freund's Adjuvant, Sigma) and i.p. injection of 200ng pertussis toxin (List Biological Laboratories, Inc.). Spleen and draining lymph node cells at 13 d post-immunization were taken and stained for flow cytometry analysis. Mice were clinically scored on a five point scale for signs of disease. 0 = no weakness, 1 = limp tail, 2 = mild hindlimb paresis, 3 = moderate to severe hindlimb paresis, 4 = complete hindlimb paresis, 5 = moribund or dead.

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CHAPTER V

Discussion and future directions

Much insight has been gained in understanding the contributing factors to T cell alloreactivity by our lab and others. This includes the remarkable discovery of germline-encoded contacts between TCR and MHC (1-3); findings that suggest an importance for the allopeptide in alloreactivity (4-6); as well as an appreciation for the contribution of secondary TCRs, which do not mediate positive selection but arise from simultaneous and continuous TCRα chain rearrangement during the selection process (7). However, there are still questions about how $CD4^+$ T cells recognize allostimulatory pMHC ligands that remain unanswered and are integral for gaining a full picture of how alloreactivity occurs. There are two main questions: 1. How does $CD4^+$ T cell alloreactivity compare to recognition of cognate ligands? and 2. How do factors involved in thymic selection, namely secondary TCRs and the composition of the MHC, impact the alloreactive T cell population that ensues?

$CD4^+$ T cells recognize allogeneic pMHC ligands with the same properties used in conventional recognition

A comparison of $CD4^+$ T cell alloreactivity and conventional recognition is key to understanding alloreactivity because it has not been performed before, and results from $CD8^+$ T cells conflictingly suggest that alloreactivity can be different from and highly similar to binding cognate ligands (8, 9). To address this issue, allostimulatory peptides were identified for two $CD4^+$ T cells, LLO118 and 1G5.1, both responsive to LLO/I-A^b as their cognate ligand. LLO118, naturally alloreactive to I-E^k APCs, was found to alloreact to a kidins220 peptide presented by I-E^k. Though not naturally alloreactive to I-E^k, 1G5.1 was shown to respond to an allopeptide mimic, Hb F70. A direct comparison of LLO118 and 1G5.1 alloreactive and

conventional recognition revealed a high degree of peptide specificity in both types of responses. Additionally, biochemical studies of LLO118 suggested very similar binding kinetics and affinity values between alloreactivity and cognate recognition. Thus, these studies of $CD4^+$ T cells favor the interpretation that alloreactivity exhibits highly similar binding properties compared to cognate pMHC recognition.

The ultimate goal in understanding how the TCR binds pMHC is to generate a crystal structure of the TCR co-complexed with its ligand. For future investigations, it would be informative to have crystal structures for LLO118 and 1G5.1 bound to both cognate and allostimulatory pMHC to structurally compare conventional vs. alloreactive recognition as well as compare how two T cells bind the same cognate pMHC, LLO/I-A^b. From the solved crystal structures of TCRs complexed to their alloligands available in the field, it is interesting that binding to the allopeptide plays an integral role in allorecognition. For instance, upon binding allogeneic MHC molecules presenting the same peptide seen in cognate recognition, the TCR (examples from both class I and class II MHC-restricted T cells) focused on recognizing peptide conformational changes induced by the allogeneic MHC (10, 11). That TCR CDR3 loop flexibility explained the ability of a TCR to bind two different H-$2K^b$ alloligands further supports the importance of allopeptide recognition (12). Knowing this, we predict that in binding their alloligands, LLO118 and 1G5.1 will retain canonical TCR binding properties - including the diagonal orientation of the TCR over the pMHC surface - but the TCRs will use different contact sites and types of bonds given how different the allostimulatory and cognate peptides are. We think the binding would be like how the 2C TCR binds its allostimulatory and cognate ligands, with divergent mechanisms and dissimilar interactions between its paired peptides (8). Additionally, it will be

interesting to see what insight the comparison of LLO118 and 1G5.1 binding to LLO/I-A^b would give us. Other structural studies assessing multiple T cells binding the same ligand showed superimposable interactions and pinpointed amino acids that mediate germline affinity (13, 14). The T cells used in the studies were more similar to each other than LLO118 and 1G5.1 are (all were of the TCR Vβ8 family), but we think it may be possible certain peptide or MHC residues may be similarly bound by two distinct TCRs. Furthermore, how any similarities impact LLO118 and 1G5.1 allorecognition would be very fascinating to analyze.

Rearrangement of dual TCRs enhances positive selection and leads to increased alloreactive and autoreactive T cell repertoires

Understanding how thymic selection impacts alloreactivity is critical for gaining insight into the formation of alloreactive T cells. Previous work on dual TCR T cells in humans revealed their increased numbers and activation in patients with acute GVHD (15). In mice, the genetic elimination of dual TCR T cells resulted in a nearly 50% decrease in T cell alloresponse to bulk APCs *in vitro* despite the fact that only 10% of peripheral T cells express dual TCRs, demonstrating a disproportionate contribution of dual TCR T cells to alloreactivity (7). We sought to extend the studies on $TCR\alpha^{+/-}$ mice, which only rearrange one TCRα locus and thus cannot develop dual TCRs, to examine the role of simultaneous TCRα rearrangement on T cell development as well as the contribution of dual TCR T cells to recognition of individual allopeptides and *in vivo* alloreactivity. We reasoned that simultaneous TCRα rearrangement enhances T cell selection but results in dual TCR T cells that participate in atypical immune responses. We hypothesized that the secondary TCR resulting from simultaneous TCRα

rearrangement exhibits altered binding properties that allow dual TCR T cells to contribute so significantly to alloreactivity.

Our results showed deficient generation of SP thymocytes attributable to a decrease in the efficiency of positive selection in TCRα$^{+/-}$ mice. Additionally, our work further established the contribution of dual TCR T cells to alloreactivity, revealing significantly decreased binding to allo- and auto-pMHC tetramers as well as alloreactivity *in vivo* of TCRα$^{+/-}$ T cells. Interestingly, TCR sequence analysis revealed a non-random elimination of certain TCRs from the repertoire when secondary TCRα rearrangement was disabled. We found that dual TCR T cells exhibited a great deal of flexibility in allopeptide recognition and could respond to multiple peptide APLs, suggesting that secondary TCRα rearrangement enables TCRs with altered binding properties to make it past thymic selection and contribute disproportionately to alloreactivity. What is fascinating is that altered binding of T cells has been noted for another atypical immune response, autoimmunity. The crystal structure of a human TCR involved in multiple sclerosis complexed with self pMHC revealed an unconventional binding mode, with the TCR footprint shifted from a canonical, central orientation to the N-terminus of the peptide and the CDR3 loops dominating binding to the pMHC (16). The crystal structure of another autoreactive TCR involved in diabetes complexed with its auto-pMHC showed abnormal, highly peptide-centric interactions, with at least 69% of TCR-pMHC contacts made with the peptide (17). It is speculated that altered binding properties allow the autoreactive TCR to escape thymic negative selection. Likewise, it seems reasonable that dual TCR T cells also exhibit altered binding in alloreactivity since they can contain a TCR that also evades thymic selection pressures, as it does not need to mediate positive selection and can mask another TCR from negative selection. That

altered binding seems to predominantly involve the peptide is not surprising. With germline affinity governing CDR1 and CDR2 interactions with the MHC, the T cell is left with CDR3 loops to exhibit more flexibility in binding the peptide or entire pMHC surface.

It would be interesting to further investigate dual TCR T cells in autoimmunity. Our results showed decreased TCR$\alpha^{+/-}$ T cell binding to the EAE autoantigen, MOG/I-A^b. Though previous work did not see any differences in disease after EAE induction, that study used a proteolipoprotein peptide to immunize the TCR$\alpha^{+/-}$ mice on a different background (SJL) compared to ours (B6). It is possible that TCR$\alpha^{+/-}$ mice on the B6 background immunized with the MOG peptide epitope bound by a decreased frequency of TCR$\alpha^{+/-}$ T cells would elicit differences in EAE disease. Additionally the course of disease in SJL mice versus B6 mice is different, with the former exhibiting relapsing-remitting disease and the latter showing chronic symptoms without remission (18-20). This distinction could lead to different results when using TCR$\alpha^{+/-}$ mice on the SJL and B6 backgrounds.

Expression of an additional class II MHC molecule elicits diverse effects on different immune responses

There is evidence that MHC molecules present during selection can shape the alloreactive T cell repertoire, with positive selection resulting in a highly alloreactive T cell population that becomes restricted by negative selection (21), the selecting MHC affecting the avidity of T cells for alloantigen (22), and homology of the self and allogeneic MHC impacting allopeptide recognition (23). It is unknown what impact on T cell alloreactivity ensues from increasing the

types of MHC expressed. Studies have looked at the effect of MHC number on the T cell response to pathogens and in autoimmunity, using mice expressing an additional MHC class II molecule (ie. comparing mice with both I-E and I-A vs. mice with just I-A) as well as comparing MHC heterozygote mice versus MHC homozygote mice. Results from these studies have shown that a more complex MHC repertoire can be beneficial and detrimental in pathogen resistance and protection from autoimmunity, depending on the pathogen and autoimmune model (24-36).

We were interested in investigating the impact on alloreactivity from increasing the number of MHC molecules expressed. We wondered whether adding more MHC would increase positive selection and alloreactivity, or whether it would enhance negative selection and negate the effects of positive selection. To address this, we examined B6.E$^+$ mice, which have an *E*α^k transgene and thus express an additional class II MHC (I-E^b, I-A^b) compared to B6 mice (I-A^b only). B6.E$^+$ CD4$^+$ T cell response to H-2^d and H-2^s APCs in MLR experiments showed a significantly decreased alloresponse compared to B6. In addition to an impact on alloreactivity, the addition of I-E^b also affected other types of immune responses, altering the cytokine production in LCMV infection and enhancing EAE possibly through decreasing Treg cells. We think our results are consistent with enhanced negative selection from adding I-E^b that dominates any effects from increased positive selection, thus resulting in decreased alloreactivity and Treg cells as well as an altered LCMV-specific T cell repertoire. Our work is novel because previous studies did not examine whether adding a particular MHC elicits distinct effects on different T cell responses, only investigating the impact on one disease model. We now know that diverse effects result from adding I-E^b, including a hitherto unappreciated impact on alloreactivity.

In the future, it would be insightful to understand the extent of decreased alloreactivity of B6.E^+ T cells. Currently, we know that I-E^b expression diminishes the naive alloreactive T cell frequency to H-2^d and H-2^s APCs. Does I-E^b affect the T cell response to individual peptides? pMHC tetramers with I-A^d, I-E^d, or I-A^s MHC could be used to address this. Additionally, whether B6.E^+ T cells elicit diminished alloreactivity *in vivo* would be interesting to determine. GVHD or solid organ transplant models could be performed to assess this, potentially yielding clinically relevant insight into the extent T cells selected in an environment with one additional MHC molecule can mediate GVHD or rejection. Finally, though we believe our results of decreased alloreactivity as well as Tregs in autoimmunity support a negative selection effect from I-E^b, it is possible that positive selection is at play. To investigate this, and rule out positive selection-mediated impacts, bone marrow chimera experiments could be performed in the future. Different combinations of B6 and B6.E^+ bone marrow and irradiated recipient mice would be set up and used for MLR experiments to determine if I-E^b expression on radio-sensitive bone marrow cells (that participate in negative selection) or radio-resistant thymic stromal cells (involved in positive selection) is required to select out T cells with decreased alloreactivity.

To conclude, our work has shown that $CD4^+$ T cells recognize their allostimulatory pMHC ligands with a high degree of allopeptide specificity, weak affinity similar to conventional recognition, increased flexibility of binding upon expression of dual TCRs, and diminished reactivity when T cells come from an environment with additional MHC expressed. Moving forward, the field of T cell alloreactivity would benefit from structural comparisons of $CD4^+$ T cell alloreactivity with conventional recognition as well as more work on allopeptide recognition and *in vivo* alloreactivity of T cells coming from mice expressing increased types of MHC

molecules.

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Investigate how $CD4^+$ T cell alloreactivity compares to conventional recognition. Examine the contribution of dual TCR T cells and MHC repertoire to alloreactivity.

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Studied the effects of the *Listeria monocytogenes* pentose phosphate pathway on the host immune response.

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Publications:

Ni, P. P., Y. Wang, and P. M. Allen. 2014. Both positive and negative effects on immune responses by expression of a second class II MHC molecule. Submitted.

Ni, P. P., B. Solomon, C. S. Hsieh, P. M. Allen, and G. P. Morris. 2014. The ability to rearrange dual TCRs enhances positive selection, leading to increased allo- and autoreactive T cell repertoires. Submitted.

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Conference Abstracts:

Ni, P. P., P. M. Allen, and G. P. Morris. Generation of secondary TCRs is critical for T cell development and the formation of an alloreactive T cell repertoire.
American Association of Immunologists Meeting 2013, Oral presentation and poster

Ni, P. P. and P. M. Allen. A direct comparison of alloreactive and conventional T cell recognition.
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