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THE FUNCTIONAL IMPORTANCES OF KEY POSITIONS OF THE WT 3K PEPTIDE CORRESPONDING TO THE 506 AND 508 T-CELL RECEPTORS AND ITS IMPLICATIONS FOR NEGATIVE SELECTION

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Abstract

The generation of the T cell receptor (TCR) repertoire is an essential process for the adaptive immune system. The development of a functional TCR involves random genetic recombination in which a diverse group of TCRs must be developed while avoiding combinations that would target self-antigens. By investigating a WT peptide and its variations between two TCRs we are able to perceive the differences between the two, demonstrating key points essential to both TCRs along with key functional locations that are distinct for each TCR. Through this research we begin to understand the structural variation that presents itself during the development of separate TCRs.

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Background

It is essential for all organisms to protect themselves from potentially dangerous and pervasive pathogens. Microbes and viruses pose a constant threat that necessitates mechanisms, such as the innate and adaptive immune systems, which are designed to eliminate these pathogens. During development, it is imperative that the immune system distinguishes between self and non-self antigens. Errors made during development could result in immunodeficiencies and/or autoimmunity. The process of immune development is of particular interest to understand medical conditions that are a result of insufficient immunity or autoimmune responses.

Innate Immunity

When a foreign substance enters the body, the innate immune system is the first and most broad line of defense. The innate immune system is comprised of a variety of leukocytes, white blood cells that have developed from hematopoietic stem cells in bone marrow, such as natural killer (NK) cells, mast cells, macrophages, and neutrophils that all interact to eliminate foreign entities.

Foreign particles and viruses typically enter the cell through pinocytosis, similar to endocytosis in which cells take up solutions from their environment. A similar process used for taking up larger volumes of solutions, particles, or nutrients is called phagocytosis. Macrophages, developed from circulating monocytes, have developed a different use for phagocytosis, however, which use this process to take up potentially dangerous substances for degradation.

All immune responses must take into consideration the difference between self and non-self particles, and the innate immune system does so with pattern-recognition receptors (PRRs), such as Toll-like receptors. The innate immune system identifies non-self pathogens by having developed a large variety of these receptors tuned to recognize patterns and motifs that are typically conserved on microbial organisms, pathogen-associated molecular patterns (PAMPs), which provide an integral function to the microbe (Janeway et al., 1992; Aderem & Underhill, 1999; Akira, 2006). These patterns are not conserved, however, on larger eukaryotic organisms-allowing the innate immune system to ignore self molecules.

Neutrophils, along with eosinophils and basophils, are considered granulocytes. Granulocytes contain granules in the cytoplasm that contain chemicals for the purpose of killing or inhibiting the development of bacteria. These granules include molecules such as defensins, proteolytic enzymes, and lysozyme. When a neutrophil takes up a bacterium it's lysosomes and granules empty out their toxic elements into the ingested microbe. Like macrophages, neutrophils are also phagocytic, but constitute a considerably larger quantity of the leukocyte population, and hence are usually the first cells of the immune system to target infections.

One key attribute of the innate immune system's phagocytosis approach is the use of opsonin, a protein in the plasma that binds to bacteria 'opsonising' them to promote the adhesive properties between the bacterium and the macrophage by binding its complement receptors (Aderem & Underhill, 1999).

Since phagocytes such as macrophages typically reside in the bloodstream, an inflammatory response is necessary to recruit them to the infected area. This inflammatory response is often initiated by mast cells. Unlike other leukocytes, mature mast cells exist only in tissues without infection, and they congregate around nerves and blood vessels. They have

demonstrated adhesive properties to laminin, fibronectin, and other components of the connective tissue basement membrane, an extracellular matrix located under the epithelial cells forming the skin of internal and external surfaces (Metcalfe et al., 1997). Histamine secreted from mast cells can initiate an inflammatory response which will open up areas around the endothelial cells to allow plasma to flow in, along with the phagocytes. Damaged cells can also cause the release of cytokines and leukotrienes which attract other immune cells.

Natural killer (NK) cells are the innate immune system's way of dealing with viral infections before the adaptive immune system is underway, and are known to induce apoptosis in the virally infected cells. NK cells can be initiated by cytokines and chemokines, such as IFN-α, that can be produced by infected cells or other lymphocytes of the innate system like macrophages or dendritic cells. "Activation receptors" on NK cells are specific for viral molecules, and can use the mechanisms that viruses typically use against the adaptive system against them. (French et al., 2003)

Dendritic cells (DCs) are considered mediators of innate and adaptive immune mechanisms (fig. 1). DCs exist throughout the body, but are concentrated most on the skin and mucosal surfaces. When the pattern recognition receptor (PRR) of a DC recognizes a pathogenic threat, the DC processes the antigen to present it as a peptide on its MHC class I and II molecules. The DC's to migrate from the periphery to the lymph nodes with their new found antigens. Upon reaching a lymph node, DC's present antigen to both naïve T cells and B cells, and induce differentiation and begin the adaptive immune response (Banchereau et al., 2000; Steinman, 2006; Kobayashi, 2007).

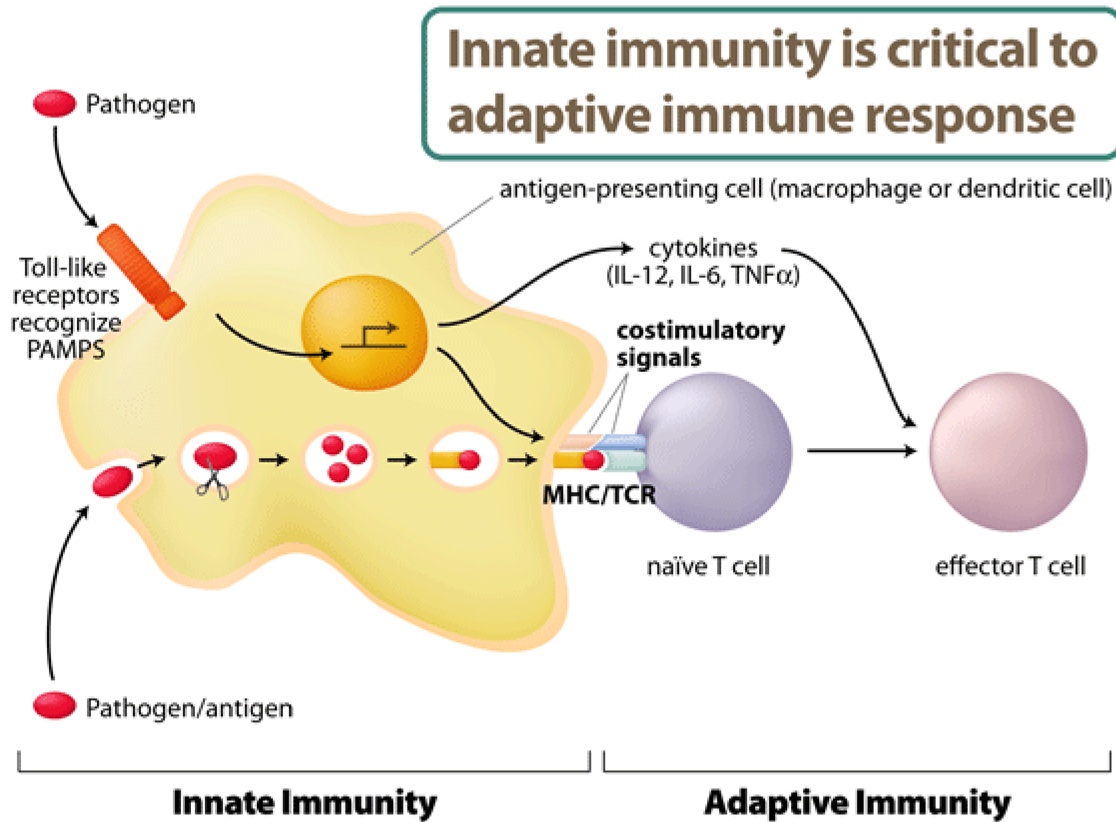

Fig. 1: Factors in innate immunity have a significant impact on the progression of the adaptive immune response and play an active role in its initiation. (Courtesy of Kobayashi, 2007: http://research.dfci.harvard.edu/innate/innate.html)

Adaptive Immunity & T-Cell Development

Unlike in innate immunity, adaptive immunity reacts in a manner much more specific towards foreign substances, and has the capability of providing a more long-term solution to infection by producing 'memory' type effector cells. The adaptive immune system consists of B and T cells that circulate in the blood and lymphatic system. The naïve cells circulate until they are activated by antigen, and then they develop into effector cells that proceed to eliminate the pathogen. Almost all cells are capable of presenting antigen to B and T cells through MHC I,

although DCs and B cells function especially well to activate T cells and are described as antigen presenting cells (APCs).

One of the main purposes of B cells is to create antibodies which are intended to locate and disable pathogenic material. The types of antibodies developed are characterized by a variety of immunoglobulins equipped to deal with specific pathogenic antigens. While T cells require the antigen to be presented in conjunction with self-MHC, the receptors on a B cell can identify the antigen on their own.

While B cells remain in the bone marrow to mature, and the T cells mature in the thymus, they both share some similar attributes in regards to their receptors. The receptors of B and T cells are surface membrane proteins that develop in large quantities on the cell's surface. The genetic coding for receptors on B cells and T cells is not the same per individual cell; only one variation per cell is produced, making each type of receptor very specific against certain pathogens.

B cells can interact with T cells by presenting the antigens they find on a major histocompatibility complex (MHC) class II. The MHC class II receptor presents a fragment of the antigen to T helper cells which are characterized by being able to bind to MHC class II with a coreceptor, CD4. The CD4+ T cells secrete cytokines, such as interleukin 4 (IL4) that promote further B cell development. B cells can develop into plasma cells to secrete antibodies and remain as memory cells in case of a future infection involving the same pathogenic antigen (Cassell and Schwartz, 1994).

Two types of T cells are produced in the thymus, characterized by two types of T cell receptors (TCRs): αβTCR and γδTCR. T cells of the αβTCR are more common and better understood than those of the γδTCR. Unlike T cells with αβTCRs which migrate through the

lymphatic system and lymphoid organs, those with γδTCRs tend to migrate to the epithelial layers of tissues in the body and react somewhat faster to antigen, similar to an innate response (Xiong and Raulet., 2007). The repertoire of γδTCRs is considered smaller than that of αβTCRs, and they do not need MHC class I or class II receptors to recognize antigens and hence do not express the coreceptor CD4 or CD8. Although γδTCRs stem from the precursors normally associated with adaptive immunity their function more closely resembles that of innate immunity.

The two main types of mature αβTCR T cells are cytotoxic killer cells, which express the CD8 surface glycoprotein, and helper cells, which express the CD4 surface glycoprotein. Both types of T cells, CD8 and CD4, utilize specific T-cell receptors to recognize foreign peptides in conjunction with the major histocompatibility complex of one of two types: class I and class II respectively. After contact with the peptide/MHC complex, T cells divide and produce cytokines, such as IFNγ and TNFα, to further stimulate cells or induce apoptosis.

The two types of αβT cells, CD4 and CD8, have separate functions. Aside from assisting the development of B cells, CD4 T cells can also present to CD8 T cells, secrete lymphokines, and stimulate macrophages. CD8 T cells typically initiate apoptosis in the infected cells. To do this they have a transmembrane protein known as Fas ligand which is upregulated upon recognition of the antigen. This Fas ligand binds to a Fas receptor that most eukaryotic cells have that instructs the cell to self-destruct (Tateyama et al., 2000). CD8 T cells can also initiate infected cell destruction by sending granzymes into the cell which initiate a cascade leading to the degradation of the cell's DNA.

In order for T cell development to be successful, the precursor cells must be exposed to separate environments within the thymus. The initial state of the precursor cells is called double

negative because they lack both the CD4 and CD8 coreceptors. There are several stages within the double negative stage in which CD44 can be detected, followed by CD25 and then just CD25 as they move through the thymus. The TCR β chain then begins formation at this point (Germain, 2002).

During T-cell generation and maturation, the variable domains of TCRs are assembled by what is known as V(D)J recombination. The three segments involved, V (the variable gene segment), D (the diversity gene segment), and J (the joining gene segment) are responsible for variation within the immune process, along with providing a checkpoint for the immune system's development (de Villertay et al., 2003; Kedzierska et al., 2008). These V, D, and J segments are the exons coding for the variable regions for the TCR, and immunoglobulins for T cells and B cells, respectively.

The genetic recombination of V(D)J begins with double stranded DNA breaks initiated at the V, D, J segments and at recombination signal sequences, that are detected by a conserved heptamer domain adjacent to the coding segment and separated by a non-conserved twelve or twenty-three base pair gap from the conserved nonomer. Recombination occurs only between two recombination signal sequences separated by different lengths. The V segment is added only after the preceding D and J arrangement (Bassing et al., 2002). This 12/23 rule prevents unproductive joining of J-J or V-V complexes. Even so, about two thirds of the rearrangements produced are not in frame, and hence are unproductive due to the arbitrary nature of the diversification process. Successful arrangements produce the TCRβ chain which then forms a complex with the TCRα, formed from a V and J segment, at the double negative stage (Steen et al., 1997; Bassing et al., 2002). In total, a TCRαβ is combined from five segments: Vα, Vβ, Jα, Jβ, and Dβ.

Rag 1 and Rag 2, the recombinant activating proteins, can distinguish the recombination signal sequences on the V, D, and J DNA segments and act in concert as a heterodimer to initiate rearrangement (Bassing et al., 2002, Kedzierska et al., 2008). This somatic recombination of the V, D and J segments are essential to the development of the appropriate and variable configurations of B and T cell receptors (Fig. 2).

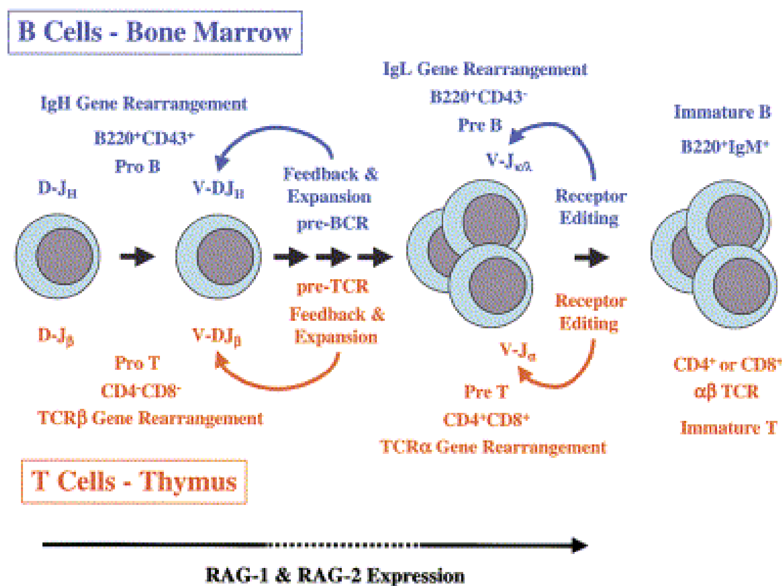

Fig. 2: RAG expression is essential to the development of B cells and T cell repertoire. (Courtesy of Bassing et al., 2002)

Rag-1 recognizes the nonamer of the RSS (recombination signal sequence), and the Rag proteins proceed to align the two regions targeted for binding. The biochemical reactions within the complex lead to the formation of a coding joint and signal joint. The coding end is used to add or subtract nucleotides, while the signal end forms a complex with recombinase and ‘protects’ the development of the coding end (Agrawal and Schatz., 1997).

Rag’s participation in somatic rearrangement of the TCR encoding genes is imperative to the development of a diverse TCR repertoire. Even with a mutation just in Rag’s TCRβ gene prevents T cell development from proceeding to the double positive stage, and leaves only double negative premature thymocytes (Robey and Fowlkes., 1994).

Once the rearrangement of the TCRβ chain is complete, thymocytes continue to the double positive stage in which they have both CD4 and CD8 coreceptors. It is at this stage that TCRα chain undergoes rearrangement and form the TCRαβ with the TCRβ chain.

Upon the construction of the TCRαβ the double positive cells undergo a test for response to self-MHC presented by cortical epithelial cells. Any receptors restricted to self-MHC complexed with self-peptide produce signals that down regulate Rag expression and allow the T cell to proceed into the medulla for negative selection (Starr et al., 2003). The thymocytes must remain engaged to the MHC for several days before proceeding. The other T cells that do not respond to the self-MHC, however, will die due to neglect. Upon migration into the medulla, thymocytes binding strongly to self-MHC complexed with self peptide will be negatively selected.

Both positive and negative selection involves self-MHC in conjunction with self-peptide. While positive selection selects for the T cells that react to this complex, negative selection eliminates the possibility of an autoimmune response being triggered by selecting against response to self-MHC with self-peptide. This counter-intuitive procession has led to the development of a variety of hypotheses and paradigms.

Hypotheses on the Variation of TCR Signals

As previously mentioned, T cells that recognize self peptide are eliminated during the developmental process during the negative selection stage, yet self peptide in conjunction with the MHC is essential to proceed through positive selection. It is clear that separate and distinct

conditions must be required for each instance or else there would be no mature T cell development. There are several different hypotheses that have been proposed to explain this phenomenon.

One of the more general theories suggests that it is the affinity between the TCR and the MHC that ascertains the occurrence of either positive or negative selection. This perception indicates that weak interaction between the two receptors is sufficient for the follow through of positive selection, while interactions above a certain threshold target those cells for apoptosis due to negative selection (Robey and Fowlkes., 1994). Another possibility that has been presented indicates that high affinity reactions can be correlated with both positive and negative selection, and that the variation between the two paths lies in certain qualitative differences of the various self-peptide/MHC complexes (Robey and Fowlkes., 1994).

One more recent hypothesis presents the possibility that thymocytes must receive signals through the TCR from the CD28 co-stimulatory pathway expressing CD80/CD86 for negative selection. This theory further indicates that thymocytes that recognize peptide/MHC complexes presented on the epithelial thymic stromal cells, which are deficient in the co-stimulatory molecules, are permitted through to be selected further on the peripheral co-stimulatory hematopoietic cells (Nakagawa et al., 1998; Lucas et al., 1999).

Another model is founded on the quality of a signal, and takes into consideration the variation of responses influenced by agonists, partial agonist and antagonists. Only small structural variations can determine whether a ligand causes a reaction to be identified as agonist, partial agonist, or antagonist in nature. In this theory variation in the intracellular signals produced by the agonist could encourage proliferation, while apoptosis is the result of a partial

agonist or antagonist signal that may only be slightly different from the structure or signal of the agonist (Combadiere et al., 1998; Lucas et al., 1999).

One particularly interesting hypothesis is based on two quantitative thresholds, as opposed to one in the first hypothesis, in regards to the signals presented from the TCR that exists for the purpose of determining whether a cell will continue on or be eliminated. Anything below the lowest threshold is eliminated due to neglect, and anything above the second highest threshold is eliminated in negative selection. Only those thymocytes that receive a signal within the two thresholds are allowed to survive and move on to positive selection. This model describes the signal to be a combinatory result of TCR affinity for ligand/peptide, ligand density, avidity and co-signaling attributes (Sebzda et al., 1994).

Yet another study demonstrated that different concentrations of a particular peptide could result in either positive or negative selection. They selected a transgenic mouse line (327) that expressed TCR specificity to a viral peptide (LCMV-gp) bred with β_2-microglobulin KOs (β_2M^-) that deterred positive selection. When testing concentrations of the viral peptide it was proposed that the signals for positive selection or negative selection would be reflected by the quantity of transgenic TCRs that engage the peptide where a high concentration of peptide was predicted to produce a higher level of TCR interaction and hence negative selection, while positive selection could be associated with a lower concentration of TCR interaction and a smaller amount of peptide. Vα2 staining was utilized in association with an augmentation of TCR density and positive selection (Sebzda et al., 1994).

Previous & Current Studies of the Huseby Lab

Previously, Eric Huseby and colleagues performed studies on positively selected T cells in mice that had limited functionality of negative selection, and WT mice with fully functioning negative selection. Those T cells having proceeded through a more lenient negative selection demonstrated to be cross-reactive for peptide and MHC, and the following process of negative selection forms the final MHC and peptide specificity of mature peripheral T cells. His studies led to the conclusion that negative selection selects for responses initiated from the foreign peptide in conjunction with the self-MHC allele, as opposed to foreign MHC alleles and the TCR segments are genetically predisposed to respond to conserved attributes of all MHCs (Huseby et al., 2005).

In his studies, T cells originating from the C57BL/6 mouse strain, with two separate transgenic T-cell lines B3K 506 and B3K 508, were compared for cross reactivity with the more lenient negative selectivity of TCRs originating from IA^b-SP lines. The C57BL/6 T cells were not cross reactive while some TCRs from the IA^b-SP proved to be significantly cross reactive. The results of the experimentation was summarized in a set of color coded blocks that diagrams the structure of the outward-facing sequence, subject to TCR recognition, of the IA^b-3K complex (Huseby et al., 2005: fig 3). The blocks are colored coded to indicate the level of activation that is maintained despite amino acid substitutions and recognizes areas of the 506 and 508 lines that are more or less sensitive (Huseby et al., 2005). Although his studies covered a vast spectrum of mutations and responses associated with them for the 3K peptide, it did not delve into the specific individual differences of the peptide variants that are used in this MQP study which will further gauge variations between the two TCRs.

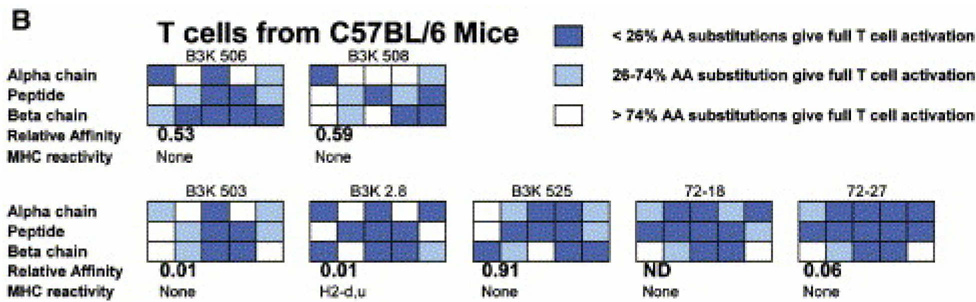

Fig. 3: Locations within the WT 3K peptide in which amino acid substitutions still maintain full T cell activation and have little effect on the peptide's potential. (Courtesy of Huseby et al., 2005)

The current MQP focuses on some of these previously studied mutants of the 3K WT peptide and uses two different transgenic T-cell lines so that comparisons can be made from the different responses to the mutations from the double positive to the single positive T cells, and between the two TCR types. The goal of this MQP is to be able to identify patterns or differences in the way the two different TCRs recognize the WT peptide that is proven to initiate a response in the two lines.

Previously the Huseby lab calculated the association rates (K_a), dissociation rates (K_d), strength of the TCR-peptide-MHC binding (free energy of binding, ΔG), and change in free energy of binding ($\Delta\Delta G$) for P1-A, P2-A, P3-A, P5-A, and P8-A for the 506 and 508 murine line TCRs (Table 1) (Huseby et al., 2006). This previous data, along with the data gathered on these peptides within the latest MQP research was used to determine the implications of the results.

TCR	I-A^b + 3K^a	K_d (μM)	K_a (1/M)	k_a (1/s × M)	k_d (1/s)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
B3K506	WT	7	136,986	102,997	0.8	−7.0	0.0
B3K506	P−1 A	26	38,462	100,288	2.6	−6.3	0.7
B3K506	P−1 L	122	8,200	ND	ND	−5.3	1.7
B3K506	P−1 K	101	9,900	61,182	6.2	−5.4	1.6
B3K506	P2 A	278	3,597	ND	ND	−4.8	2.2
B3K506	P3 A	>550	<1,818	ND	ND	>−4.4	>2.6
B3K506	P5 A	>550	<1,818	ND	ND	>−4.4	>2.6
B3K506	P5 R	11	95,100	79,575	0.8	−6.8	0.2
B3K506	P8 A	92	10,870	39,022	3.6	−5.5	1.5
B3K506	P8 G	69	14,400	67,872	4.7	−5.7	1.3
B3K506	P8 L	256	3,906	ND	ND	−4.9	2.1
B3K506	P8 Q	114	8,772	ND	ND	−5.4	1.6
B3K508	WT	29	34,130	11,766	0.3	−6.2	0.0
B3K508	P−1 A	>550	<1,818	ND	ND	>−4.4	>1.7
B3K508	P−1 L	>550	<1,818	ND	ND	>−4.4	>1.7
B3K508	P−1 K	>550	<1,818	ND	ND	>−4.4	>1.7
B3K508	P2 A	175	5,714	ND	ND	−5.1	1.1
B3K508	P3 A	>550	<1,818	ND	ND	>−4.4	>1.7
B3K508	P5 A	>550	<1,818	ND	ND	>−4.4	>1.7
B3K508	P5 R	93	10,700	11,610	1.1	−5.5	0.7
B3K508	P8 A	>550	<1,818	ND	ND	>−4.4	>1.7

Table 1: Affinity and kinetic calculations of various peptide variants of WT 3K TCRs by method of surface plasmon resonance. (Courtesy of Huseby et al., 2006)

Project Purpose

The purpose of this project is to compare the activation of developing thymocytes and proliferation of mature peripheral T cells for two different transgenic T cell receptors, B3K508 and B3K506. This will be done using a peptide recognized by both TCRs, referred to as the wild type 3K peptide, and six variants of this peptide that range and differ in their affinity for each TCR. This comparison will identify the functional differences and similarities between the two TCRs which will allow for a better understanding of negative selection. The T cells were isolated from TCR-transgenic mice bred onto a RAG-/- background which will prevent the development of any other TCR variation.

Methods

Transgenic Mice

T cells were isolated from Rag knockouts of C57BL/6 mice reactive to I-A^b + 3K peptide. The two strains of mice we used expressed TCR types B3K506 and B3k508. These mice were exposed to 6 variations of the wild type MHC-peptide along with the WT 3K peptide itself.

Thymocyte Preparation

Thymocytes were derived from four thymuses from the B3K508 transgenic Rag -/- mice and three thymuses from the B3K506 transgenic Rag -/- mice. Mice were euthanized by means of CO_2 asphyxiation, and were then laid down on their backs for dissection. Cuts were made up the upper torso to the neck, and the thymuses were carefully removed and placed in separate 15 ml conical tubes containing 5 ml of RPMI medium.

The end of a syringe tube was used to break up the thymus and remove the epithelial layer in a tissue culture plate with RPMI. The thymocytes were spun down and resuspended in a hypotonic solution for the purpose of eliminating erythrocytes, and the remaining cells were then neutralized with the addition of an equal amount of RPMI. 10^6 thymocytes were added per well along with 5×10^4 B7/I + IA^b-No Peptide. This culture was allowed to incubate overnight, and then the cells were washed and stained with antibodies for CD4, CD8, and CD69.

Splenocyte Preparation

Splenocytes were derived from four spleens from the B3K508 transgenic Rag -/- mice and three spleens from the B3K506 transgenic Rag -/-. Mice were euthanized with CO_2 asphyxiation, and laid on their sides for dissection. Cuts were made in the left side, and the spleens were removed and placed in separate 15 ml conical tubes containing 5 ml RPMI.

The end of a syringe tube was used to break up the spleen, and the epithelial layer was removed and placed in a tissue culture plate with RPMI. The splenocytes were spun down and resuspended in Gey's solution, and neutralized. 1×10^5 spleen cells were incubated for 48 hours along with the titration of peptides. The B3K508 cells were given 2×10^5 Mitomycin C treated C57BL/6 splenocytes as feeder cells.

Titering WT Peptide and Variants

WT 3K peptide and variants had been previously constructed from the e_α peptide sequence (Huseby et al., 2006). The seven peptides were titered in duplicate starting with 10 μl/μg and increasing to $1/10^9$ μl/μg. This dilution series was performed per peptide and per transgenic TCR type, and was combined with RPMI for media in four 96 well plates.

Cell Counts

Thymocytes and splenocytes were counted using a hemocytometer.

Flow Cytometry

A Florescence Activated Cell Sorter (FACS) was used to measure and analyze the surface molecules of cells through a beam of light that detects fluorescences of the cells. This

was used to detect the CD4 and CD8 coreceptors, and CD69 upregulation, with the use of PerCP associated with CD4, FITC associated with CD8, and PE associated with CD69. The instrument used was a Becton Dickinson FACSCalibur, and the data was collected on CellQuest Pro software and analyzed on FlowJo. 30,000 events were run for each well.

Several comparisons were made regarding the single positives and double positives. Thymocytes were gated on live verses dead cells for double positive, CD4+CD8+, T cells for one analysis. For another analysis data was gated on live cells with further separate gates for CD4+ and CD4+CD8+ which were analyzed based on CD69 presence.

Proliferation Assay & Radioactive Labeling

After a 48 hour incubation, radioactive thymidine (^{3}H-thymidine) was added, and the thymocytes were allowed to incubate for another 16 hours. The cells incorporated the radioactive thymidine into their DNA and the radioactivity increased with each cell division, thus the counts per minute (CPM) tritium was a measure of cell proliferation.

After the 16 hour incubation the cells were harvested. The cells were washed out of the wells by multiple rinses. The cells were burst in hypotonic buffer and were relieved of their DNA which passed through a filter membrane which collected intact DNA. A higher proliferation was marked by a higher quantity of cells harvested and a higher radioactivity in the DNA collected. The filter membrane was dried and the amount of radioactivity was counted on a scintillation counter as ‘counts per minute’.

Results

The main goal of this research project was to determine crucial portions of the presented peptide to the individual TCR types, and compare the differences of their varying properties to better understand the TCR repertoire and the process of negative T cell selection. Along with this information, the level and type of functionality that is lost or gained can be perceived through the analysis of the qualitative effects.

Six alanine substitutions of the wild type (WT) 3K peptide were developed previously (Huseby et al., 2006). The WT 3K peptide is a good antigen to use for these purposes as both types of transgenic TCRs, 506 and 508, react in response to it with the same degree of effectiveness. The responses to these variants and the WT 3K peptide itself were examined by FACS analysis of thymocytes and by a proliferation assay of exposed splenocytes.

Peptide-MHC Combinations in Thymocytes

The FACS data from the 506 TCR transgenic mice and the 508 TCR transgenic mice were normalized to the maximum and minimum levels of the results from exposure to the WT 3K peptide. The maximum and minimum data points used in these calculations are shown in Table II. The data points were taken from an average of the individual results, with the line of standard deviation graphed to convey the data with as much accuracy as possible.

Table II: The WT 3K maximum and minimum values used to normalize the graphed FACS data for thymocytes with the formula: (X-min)/(max-min)*100 to formulate it into a percentage.

Data Type	Max	Min	Data Type	Max	Min
506 SP CD69 UpReg	74.1	18.2	**508 SP CD69 UpReg**	68.2	12.8
506 DP CD69 UpReg	25.7	1.75	**508 DP CD69 UpReg**	38.9	0.89
506 DP Live	84.1	74.8	**508 DP Live**	87.8	44.4

As seen in figures 4 and 5, the cell death of double positive cells is shown to correlate with the CD69 upregulation in both double positive and single positive cells, and lessens as the peptide concentration increases. These figures also present that, generally, the CD69 expression tends to be increase in single positives over double positives for higher concentrations of that peptide.

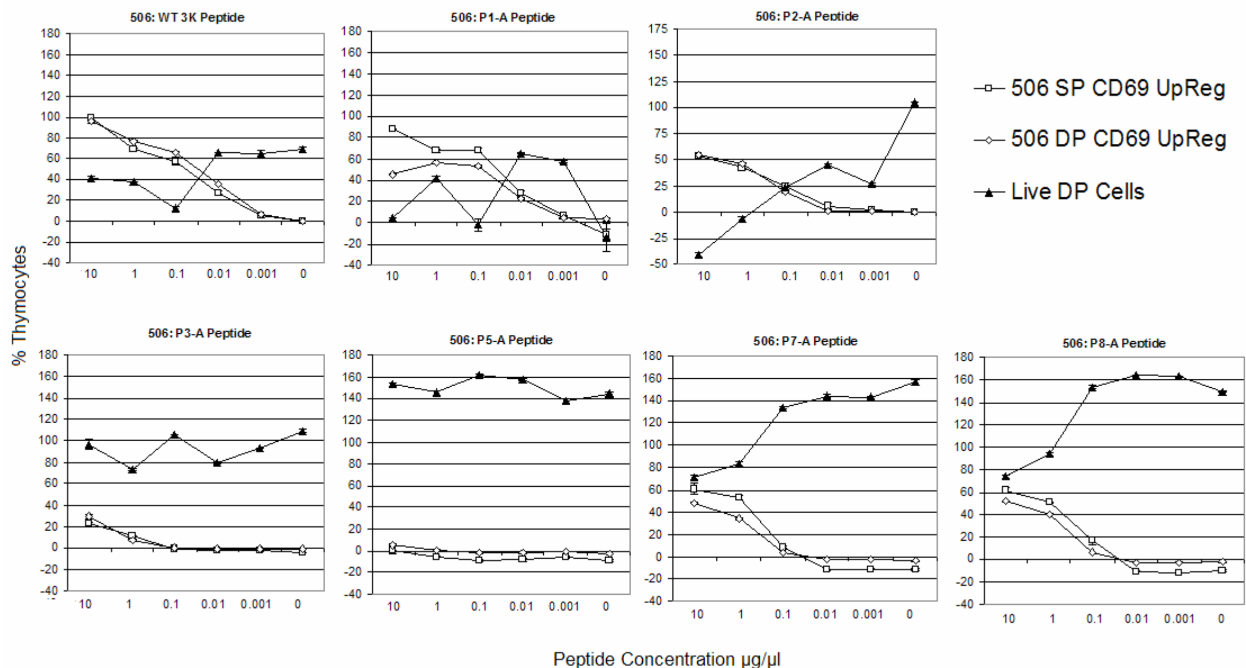

Fig. 4: FACS data from thymocytes stained for CD69, CD8, and CD4 expression when the 506 TCR is presented with the WT 3k peptide and the six variants. The above data was normalized to the 506-WT 3K CD69 expression levels and the WT 3K levels of remaining live cells.

For the 506 TCR (fig. 4) the alanine substitutions at the first and second positions are the least detrimental of the set of variants. The amount of dead cells related to exposure to the alanine substitution in the first position increases by nearly double when compared to the death rate associated with the 3K WT peptide at the highest concentration (10 μg/μl), and the percentage of cells expressing CD69 for double positive and single positive cells follow a similar path as the cells exposed to the 3K WT but with a notable increase in CD69 for single positives relative to that for double positives. The peptide with the alanine substitution in the second position follows the pattern of the 3K WT, but the percent of remaining live cells and CD69 upregulation at the highest concentration begins at a much lower level, while the difference between the single positive and double positive CD69 expression is difficult to discern.

The 3K variant with alanine at position-3 (P3-A) and P5-A were proven to cause the most significant loss in effectiveness for the 506 TCR. The P3-A mutant for the 506 TCR demonstrates higher levels of remaining live cells and much lower levels CD69 upregulation. Although there is some variation on dead cells for this particular mutation even high concentrations at 10 μg/μl maintain a larger amount of remaining live cells than that of the 3K WT. The P5-A mutant demonstrates itself to be a significant hindrance to the normal activity of the 3K WT peptide as not only was there negligible CD69 upregulation, but the live cells remained well above the WT 3K normalization setting at 100%, never dropping below 135%. Although the levels of CD69 expression in single positives versus double positives were very similar, there was a tendency for the single positives to show levels below that of the double positives like the results of other, more agonistic, variant peptides at very low concentrations.

The P7-A mutant, for the 506 TCR, started out causing cell death in about 30% less double positive cells than the 3K WT peptide, but with at least 40% less CD69 presentation on

both SP and DP cells. P7-A does, however, mimic the trademark feature of CD69 expression prevailing in excess in the single positives over the double positives at the higher concentrations. This mutant loses its functionality which is nearly completely dissipated by the 0.01 μg/μl, at which time the CD69 upregulation for single positives dips below the level of that for the double positives where it remains.

The P8-A mutant started out having eliminated very few cells, nearly doubling the amount of cells left alive when compared to the WT 3K. Cell death becomes comparatively negligible at a concentration of 0.1 μg/μl, and the same is the case with the CD69 expression at 0.01 μg/μl which follows the same pattern as the P7-A variant.

Of all these mutants, it is interesting to note that the P2-A variant demonstrated itself to cause the least decrease in functionality of all the mutants for the 506 TCR that follows data describing this peptide as killing almost 40% more double positive cells than the WT 3K going far to suggest that this mutant may actually enhance the WT 3K peptide for negative selection as seen in the increased amount of cell death at the highest concentration relative to the influence of the WT peptide. The CD69 expression still remains sub-optimal, however, at 50% that of the WT 3K even at 10 μg/μl.

The 508 TCR, seen in fig. 5, shows some difference in the effects of its variants. The 3K WT peptide for this TCR model followed the expected presentation with the quantity of living DP T cells dwindling with the increase in peptide concentration. Compared to the 506 TCR however there seemed to be more single positive cells upregulating CD69 at the concentration of 0.1 μg/μl and lower.

Unlike how the P1-A mutant presented its effects for the 506 transgenic TCR, for the 508 transgenic TCR, the results for that variant resembled a response more closely associated with a partial agonist, similar to the results seen in the outcome of 506 transgenic TCR exposed to P7-A

or P8-A. While the P1-A variant showed a WT-like functionality for 506 the same peptide variant for 508 at a peptide concentration of 0.1 μg/μl lost all noticeable functionality.

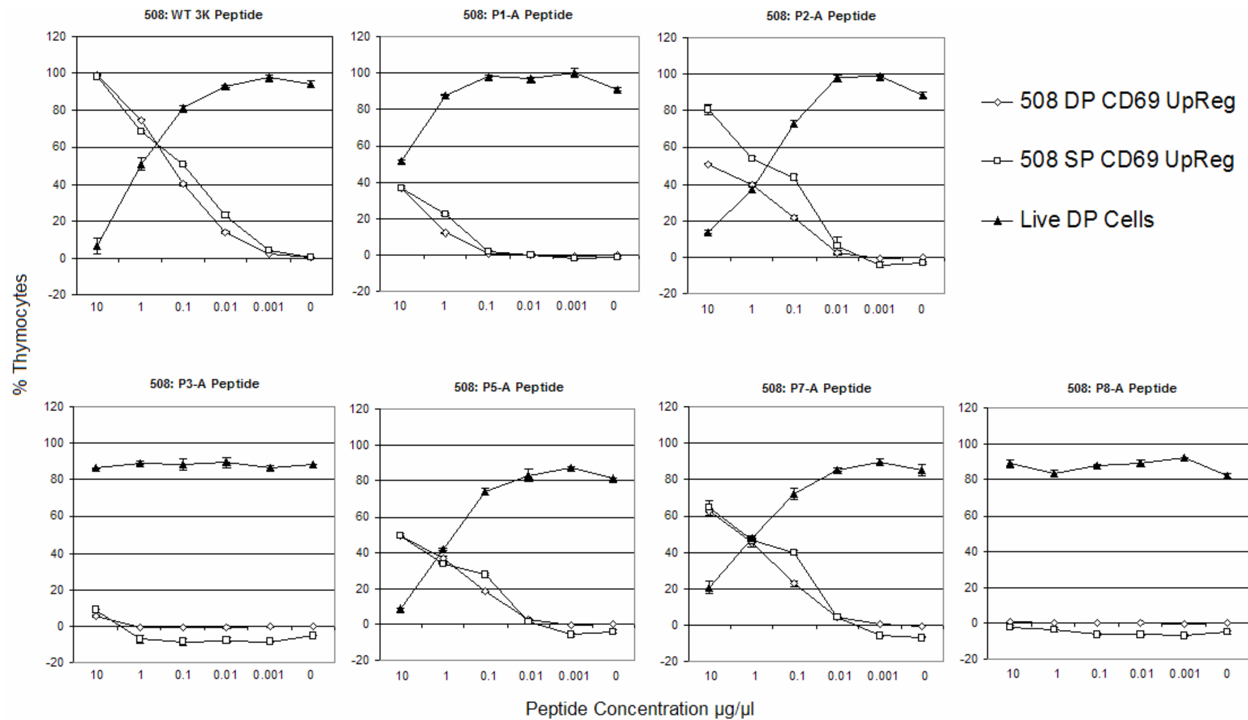

Fig. 5: FACS analysis of 508 TCR transgenic thymocytes when exposed to the WT 3K peptide and its six peptide variants. The above data was normalized to the 508-WT 3K CD69 expression levels and the WT 3K levels of remaining live cells.

Variants P7-A and P2-A had the least degrading effect on the normal functionality of the WT peptide for the 508 TCR while P5-A had a lesser degrading effect when compared to its functionality for the 506 TCR. The P7-A variant is one of the alanine substitutions that seemed to have only a small negative effect on the capability of the WT 3K standards for the 508 TCR. The response instigated by the P2-A mutant for the 508 TCR also followed a very clear path concerning the percent of thymocyte death with an almost full agonistic response although not as much as the results of P7-A in regards to the single positive CD69 upregulation. The responses for both are completely abrogated by 0.01 μg/μl. The P5-A variant shows much more of a response in regards to the 508 TCR than it did in regards to the 506 TCR when it had a nearly

completely nonagonistic response. Like P2-A variant for the 506 TCR, the P5-A variant for 508 maintains some agonistic functionality. The only difference between the P2-A variant from 506 and the P5-A variant from 508 responses is that P2-A from 506 dips below the 0% normalization for the level of remaining live cells while the P5-A variant does not.

Variants P3-A and P8-A were the most nonfunctional variants for the 508 TCR. The remaining live cells linger between 80-100% consistently for all peptide concentrations. The P3-A variant severely damaged the typical functionality of the peptide showing a constant level of live cells, indicating a complete disablement of negative selection along with a CD69 upregulation that did not exceed 15% of the WT 3K's even at the highest concentration in the 508 TCR much like it did in the 506 TCR, indicating that this position is essential to the interaction of the peptide/MHC complex to the TCR. Variant P8-A is, by far, the most debilitating of all the peptide variants for the 508 TCR, and no significant change in CD69 concentration or cell death can be identified even at the highest concentration. Interestingly, this case is not as extreme for the 506 TCR in which the P8-A variant at least invokes a partial agonistic response, as opposed to the complete non agonistic presentation as seen for the 508 TCR.

Peptide-MHC Effect in Splenocyte Proliferation

The results of the cell proliferation assay are presented in fig 6 where the effectiveness of each peptide variant to induce a developmental response is further verified. The proliferation demonstrated by the splenocytes can be seen to correlate with the upregulation of CD69 and cell

death that was indicative of negative selection in the thymocyte data. This demonstrates that positive selection is taking place in these cells as well.

Proliferation, as expected, is most prevalent in response to the WT 3K peptide presentation, and this response peaks around the 0.01 µg/µl to 0.001 µg/µl peptide concentrations before dropping drastically. In regards to the data from the 508 TCR transgenic model, the P2-A and P7-A peptides follow this route most closely in comparison to the other mutants, but drop off in effectiveness at 1 µg/µl and 0.1 µg/µl, respectively. The P1-A peptide for the 506 TCR is most dominant in retaining WT 3K-like agonistic function and only loses its function at 0.001 µg/µl.

The results of variants P1-A and P5A show that their CPM remains at a level less than half that of the WT 3K peptide for the 508 TCR, while the partial agonists for the 506 TCR typically lie at least 50k CPM above that level, allowing its partial agonists to stay above the half-way point of the WT 3K's CPM at the highest concentration of 100 ug/ul.

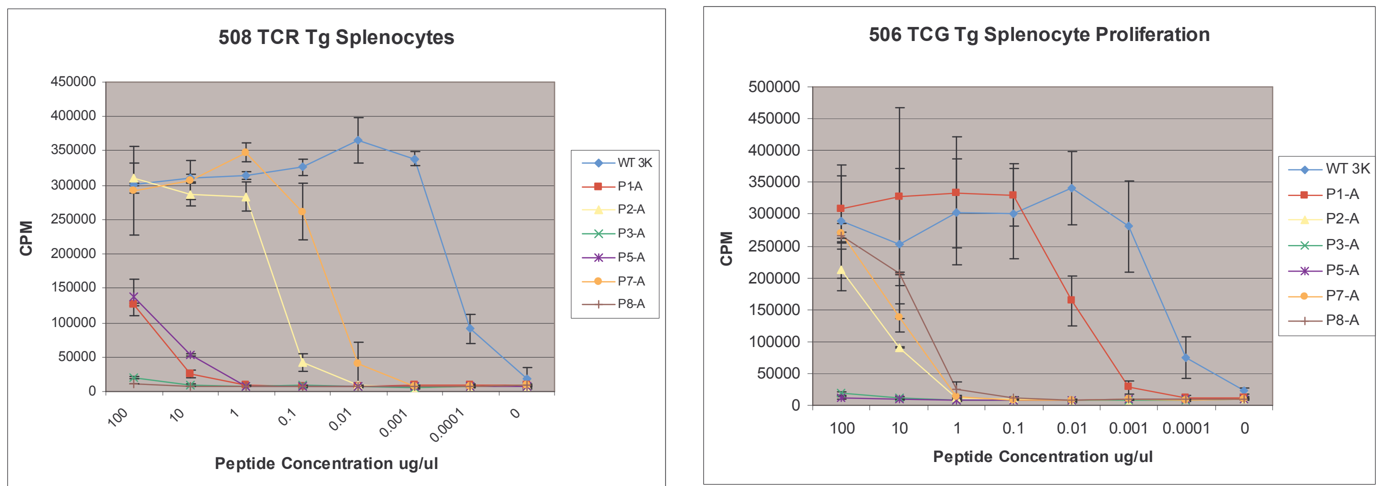

Fig. 6: The proliferation assays, where counts per minute (CPM) gauges the level of cell proliferation in targeted splenocytes for the WT 3K peptide and variants, are show here.

Functional Implications of Peptide Variants and 'Hot Spots'

Although the peptide variants, in conjunction with the 506 and 508 TCRs, follow the same general pattern in both the proliferation assays and the FACS analysis, there are some interesting differences that can be seen for the peptides that result in a more half-agonist response. One such instance of this is the P7-A variant for the 508 TCR. When the FACS data of variants P7-A and P5-A are analyzed, the two variants do not appear as though they instigate a dissimilar response. However, when the proliferation data is presented, the gap between the CPM for P7-A and for P5-A is much more significant.

Both the FACS and proliferation data for the 506 TCR is a bit more turbid, fluctuating somewhat for the WT 3K, P1-A and P3-A data, than that of 508, but it still can be gathered that the results of P2-A, P7-A, and P8-A exposure can be differentiated more significantly and to a different degree in the proliferation data than the FACS data when being viewed by CD69 upregulation. P7-A and P8-A, however, were demonstrated to have nearly identical functional capabilities in both the proliferation assay and the FACS.

As previously mentioned, the most notable attribute of the 506 P2-A peptide is the large amount of dead cells when compared to the WT 3K of the FACS analysis at the highest peptide concentration. Other peptides that display such a significant amount of cell death, such as the P1-A peptide for 506 and P2-A for 508, also show a significant level of proliferation. 506's P2-A, however, shows only mediocre levels of proliferation despite having had such a significant role in promoting cell death. P5-A of the 508 follows this same trend as well.

Seeing that 508's P5-A and 506's P2-A are capable of influencing such as great degree of negative selection as demonstrated by FACS analysis and yet are shown to significantly deter the level of proliferation marks these two locations as points key to the progression through positive

selection. This clearly presents a case in which the increase in functionality regarding negative selection is not congruent with the level of functionality regarding positive selection.

In the FACS analysis of 506's P3-A and P5-A mutants, P3-A demonstrates a very suboptimal level of CD69 upregulation and negligible levels of cell death, while P5-A has no significant indication of CD69 upregulation of cell death whatsoever. These variants severely cripple the normal functionality yet their differences are clear: one is a poor partial agonist while the other is a definite case of nonagonist inability. However, the proliferation data show both as completely nonfunctional in regards to their effect on positive selection. This marks the position of P3 to be an extreme determinant in regards to both positive and negative selection which its close companion, P5, lies closer as the essential point for positive selection as well only for the 506 TCR

There was not much difference in the FACS and proliferation results for 506's P1-A, P7-A, and P8-A. P1-A stayed in the close vicinity of the WT 3K's range, and P7 & P8 appeared to have equally degrading effects for both positive and negative selection. This data marks the P1 position as relatively unimportant to the 506 TCR, and marks P7 and P8 as not absolutely imperative to general function, like that of P3 and P5, but important to maintain full agonistic capabilities.

The peptide position that follows the pattern of 506's P7-A and P8-A for the 508 TCR is the P1-A peptide. For the 508 TCR the substitution at P1 degrades function to that of a partial agonist for both positive and negative selection. Interestingly, the P5-A peptide demonstrates equal effectiveness as P1-A did in regards to positive selection but was significantly more capable, with WT 3K-like levels of cell death, when negative selection was taken into consideration as demonstrated in the FACS analysis. This data taken together marks the position

of P5 to be crucial only for the full function, mostly in regards to positive selection, as the level of negative selection it induces is very similar to WT levels.

The P2-A and P7-A variants for the 508 TCR are both very similar in their functional capabilities for both negative and positive selection, similar to the peptide positions 7 and 8 for the 506 TCR- except with a higher overall functionality. This reveals P7, specifically, to be of greater importance to the 506 TCR than the 508 TCR for both the purpose of negative and positive selection to equal degrees. Although the results for P2-A exposure follow the same general directions as the P7-A for 508 its effects on the 506 TCR model are shifted. While P2-A increases negative selection and partially disables the WT function of positive selection for the 506 TCR its function at the higher concentrations for the 508 TCR mimic that of the WT 3K peptide with only the negative effects being demonstrated around the 0.1 μg/μl concentration. Although some level of enhancement in the negative selection process is identified in the P2-A results from the 506 data, this is not detected in the 508 version. This position appears to mark a relatively unimportant point for the 508 TCR, but a somewhat more crucial location for full positive selection functionality for the 506 TCR.

The P3-A and P8-A variants proved completely debilitating for the function of positive and negative selection for the 508 TCR with P3-A only being slightly less-so in regards to negative selection. The results of P3-A exposure was the same for both 508 and 506, while the P8-A for 508 was most similar to the P5-A for 506. This indicates that the third position is imperative for both types of TCRs and is not open for variation when the repertoire is being constructed. P8-A for 508, like P5-A for 506, is more important for positive selection.

The clear TCR functional ‘hot spots’ are P5 for the 506 TCR, and P8 for the 508 TCR, and P3 for both of them. The importance of position 3 is conserved through the development of

both TCRs while the other ‘hot spot’ is more capable of variation. No locations are conserved between the two variants whose alterations do nothing to damage the typical functionality, although positions 2 and 7 appear to, at most, degrade the functionality to the state of a partial agonist.

The FACS analysis also reveals whether there is an increase or decrease in affinity for the peptide as it proceeds through development from the double positive stage to the single positive stage. The data involving the upregulation of CD69 in double positive and single positive cells sticks close together but some differences can be identified.

The WT 3K peptide appears to instigate a similar response in the double positive and single positive cells with the single positive gaining slightly more affinity for the peptide between the concentrations of 1 and 0.1 μg/μl for the 508 TCR, and only at the highest concentration of 10 μg/μl for the 506 TCR. Even so, the only increase in affinity that can be detected is very slight.

The single positive cells increase their affinity for the 508 TCR for the P2-A variant to the highest degree, but seem to increase only at one particular concentration for the three partial agonists: P1-A, P5-A, and P7-A for the 508 TCR. The point at which P1-A’s response is increased in single positives is at 1 μg/μl while both P5-A and P7-A instigate a relatively higher response from the single positives only at 0.1 μg/μl. Increased affinity for a larger range of concentrations can be identified in the responses by the 506 TCR with the P7-A and P8-A variants in which the single positive cells are more sensitive to these peptides from the highest concentration at 10 μg/μl to 0.1 μg/μl whereupon the sensitivity recedes below that of the double positive cells.

The cases in which the double positives instigate a more significant response are usually located in the variants that have a more nonagonist response, such as P8-A for the 508 TCR and P3-A for both the 508 and 506 TCRs. This is also the case for lower concentrations of those successfully instigating a partial agonistic response.

Through these studies we have identified the P3 as a major functional 'hot spot' that is conserved between the two TCRs marking itself as having very important structural significance. Along with this finding, the locations of partial agonists and the variation between the two TCRs were identified and associated with not only different levels of functionality but different aspects of functionality as well. These mappings of structural significance and the identification of the conserved P3 location may further assist in finding patterns and uncovering the method of development of these TCRs, and how the repertoire designs these successful TCRs.

Implications on Negative Selection and TCR Comparisons

The P1-A variant of the 506 TCR has a K_d and K_a much closer to that of the WT 3K peptide of the 508 TCR with 26 μM for K_a instead of 29 μM for the WT3K and 38,462 K_a instead of 34,130 K_a, making the K_a of P1-A larger than that of the WT 3K, and the K_d a bit smaller. For this variant there is a very notable increase in the affinity of the single positives over that of the double positives, and there appears to be a substantial level of negative selection taking place especially at the highest (10 μl) concentration. The level of proliferation is also quite high, but drops off after 0.1 μg/μl instead of after 0.01 μg/μl like in the 506 WT.

The P1-A variant of the 508 TCR has an extremely large K_d of over 550 μM, and an extremely low K_a of under 1,818, marking it as a variant of poor affinity. At the highest concentration it demonstrates is a level of cell death half that of the WT 3K for this TCR and

CD69 levels under 40%. However, there is still more affinity towards the peptide at the higher concentrations mostly at 1 μg/μl. There is also extremely low but notable proliferation never exceeding 150k CPM.

Other peptide variants that have very low K_d's and K_a's for the 508 TCR are: P3-A, P5-A and P8-A. All of them exemplify a K_d above 550 μM and a K_a below 1,818. The P3-A and P8-A variants both exemplify the behavior of a nonagonist except for a slight increase in CD69 expression at the highest concentration for P3-A. Aside from that, the level of living cells remain relatively constant along with the CD69 levels within which the double positives maintain a low level just above that of the single positives, as negative selection is not taking place. For these two variants there is also no evidence of positive selection, as the proliferation data describes any growth as negligible. Interestingly, P5-A actually demonstrates itself to have a much greater response than the others despite its low affinity. The P5-A follows the same pattern of negative selection as does the WT 3K peptide but with half the level of CD69 upregulation. It also exemplifies proliferation levels like that of the P1-A variant. This implies that, despite the high K_d and low K_a, these variants are capable of significant negative selection, even with suboptimal CD69 expression and limited capability in regards to positive selection.

The two variants of the 506 TCR with K_d's over 550 μM and K_a's under 1,818 are P3-A and P5-A, and behave more similarly to what would be expected of low affinity peptides. Both show decreased cell death and CD69 expression, along with no evidence of proliferation. Only the P3-A peptide shows some evidence of negative selection at the 10 μg/μl concentration, and even then the single positives remain below the double positives in CD69 upregulation.

The P2-A variant for 508 has a K_d of 175 μM and K_a of 5,714, and invokes a predictable partial agonist response. Notably the single positives display significantly more CD69 expression

than the double positives. The level of dead cells resulting from this peptide is similar to that of the WT 3K and 508 P5-A. The major difference that separates P2-A from P5-A is the higher level of single positives CD69 expression ad the much higher, WT 3K-like, level of proliferation.

The 506 P2-A, with a K_d of 278 μM and K_a of 3,597, had an overall lower level of CD69 upregulation but good evidence of negative selection and some proliferation, just over half that of the WT at the highest concentration. The difference between the CD69 expression in double positives and single positives were very similar, not showing a very strong variation between the two as may be expected of a partial agonist displaying the lowest but still notable amount of proliferation.

The P8-A and P7-A data for the 506 TCR appear almost identical. Unfortunately only the kinetic data for the P8-A is available for comparison. The P8-A peptide has a K_d of 92 μM and a K_a of 10,870. It does not invoke much negative selection as the level of live cells remains fairly high even at the 10 μg/μl peptide concentration. The level of CD69 upregulation is at about half that as for the WT 3K peptide and is increased in the single positives over the double positives until 0.1 μg/μl, the same concentration at which all evidence of negative selection is lost. P8-A also demonstrates levels of proliferation starting in close vicinity to that of the WT 3K peptide at the highest concentration but degrading in that function after 10 μg/μl.

P2-A has more evidence of proliferation than 506's P2-A and P-A with the same 300k CPM at 100 μg/μl as the WT 3K despite it dropping off early right after 1 μg/μl. 506's P1-A, however, was adept at positive selection and has the lowest K_d and highest K_a of all the variants of both 508 and 506 TCRs.

Of these four variants the P2-A of the 506 TCR has the greatest K_d and lowest K_a. P2-A of the 506 TCR also has the most negligible difference between the CD69 upregulation of the

single positives and double positives, and lowest level of proliferation. What is the most similar however is the total level of CD69 expression. 506's P2-A also exemplifies a high level of negative selection.

Following P2-A of the 506 TCR is the P2-A of the 508 TCR which has a K_d of 175 and K_a of 5,714, which demonstrates itself to have more single positives sensitized to this peptide as compared to double positives by more than 25% at the highest concentration, a greater difference than even the WT 3K peptide demonstrates.

The two peptide variants with the lowest K_d and highest K_a, 506's P1-A and P8-A, are only similar in that the level of CD69 that is expressed is relatively the same and increased for single positives over double positives. P1-A demonstrates more cell death and proliferation indicating that it is for the P1-A peptide that negative and positive selection remains the most functional in. These two peptide variants are closest in K_d and K_a and are even exposed to the same TCR yet have very different attributes. There is one very significant difference between the two peptide kinetic and affinity calculations, however. That is the $\Delta\Delta G$. The ΔG, free energy of binding, are not much different as P1-A has a ΔG of -6.3, and P8-A has a ΔG of -6.8 but the $\Delta\Delta G$ has more significance in it's difference which is 0.7 for P1-A and 1.5 for P8-A. Unfortunately this is not revealing as the $\Delta\Delta G$ for 506's P2-A is 1.1, higher than P1-A, yet this peptide displays more negative selection function.

The major difference between the two TCRs was that the 508 was more capable of maintaining some function in variants with highly unfavorable binding kinetics. Yet, for the most part the relation of affinities and activities followed the same set of 'rules' that were derived from these similarities.

The clearest conclusion that can be made from this data is that the lower the K_d and higher the K_a the more significantly single positive cells are sensitized to that particular peptide. This is most clearly demonstrated for the 506 TCR but is also evidental when viewing the peptide variants with the 508 TCR.

It is also apparent that the K_d and K_a do not need to be favorable for negative or even positive selection to occur. The P5-A variant of the 508 TCR has an unfavorable K_d, K_a, ΔG, and $\Delta\Delta G$ yet participates in nearly the same amount of negative selection as the WT 3K peptide, and is proven only to be disabled in it's function for positive selection and the sensitization of single positives over double positives. The P1-A variant is also an example of this but displays more limited functionality in negative selection and more limited CD69 expression than P5-A. 506's P3-A and P5-A along with 508's P3-A and P8-A, which all have unfavorable K_d's and K_a's do indicate an increased probability of a compete loss in functionality including both negative selection and positive selection for such a state.

Another determination that can be extracted from this data is the fact that the concentration of peptide determines how well it functions or whether it functions at all. At the highest concentrations there is a lot more evidence of CD69 upregulation and both negative and positive selection, as the peptides and TCRs has a much greater chance of interaction.

This data brings forth a theory regarding the process of negative selection. The peptide interaction induces negative selection when there is a greater amount of signal being produced and is not necessarily completely dependent on high affinity between the TCR and peptide-MHC, although this is proven to be helpful as the TCR-peptide-MHC interaction is required for this process. The cells remaining after negative selection are enhanced in their sensitivity for the peptide when that peptide did have a high affinity, however. Because the low affinity peptides

for the 506 did not undergo negative selection, while some for the 508 did, it is possible that negative selection for some TCRs is more lenient than for others.

Discussion

The data extracted from subjecting the two TCRs to the 3K WT and six variants permits a more accurate gauging of each position's functional importance along with how those functions relate to previously determined kinetic and affinity data, allowing the identification of patterns between the two that works as another step towards understanding the mechanisms of negative T cell selection. In summary, we determined that low affinity peptides always result in less CD69 expression, including relatively less expression where single positive cells are concerned as compared to double negatives, and these low affinity peptides do not always result in less cell death even though many may not undergo negative or positive section. Along with these results, it was also noted that an increase in concentration induces higher activity and CD69 expression in single positives likely due to the greatly increased chance of TCR-peptide-MHC interaction at such concentrations. Some of the recent data collected can be seen in table III where the points at which functionality is significantly compromised are presented. The affinity and kinetics data used is shown in table IV.

Peptide Concentration at which CD69 expression is below 10% (ug/ul)				Peptide Concentration at which the CPM is below 100k (ug/ul)	
	506	**508**		**506**	**508**
WT 3K	0.001	0.001		0.0001	0.0001
P1-A	0.001	0.01		0.001	10
P2-A	0.01	0.01		10	0.01
P3-A	1	10 (max)		100 (max)	100 (max)
P5-A	10 (max)	0.01		100 (max)	10
P7-A	0.1	0.01		1	0.01
P8-A	0.1	10 (max)		1	100 (max)

Table III: A summarization of the functionality displayed for the WT 3K peptide and its six variants for CD69 expression, indicating the peptide's encounter and successful attachment to the TCR, and the CPM of the proliferation assay describing the functional capabilities as directly related to positive selection.

TCR	I-Ab + 3Ka	K_d	K_a	ΔG	ΔΔG
B3K506	**WT 3K**	7	136,986	-7	0
B3K506	**P1-A**	26	38,462	-6.3	0.7
B3K506	**P2-A**	278	3,597	-4.8	2.2
B3K506	**P3-A**	>550	<1,818	>-4.4	>2.6
B3K506	**P5-A**	>550	<1,818	>-4.4	>2.6
B3K506	**P8-A**	92	10,870	-5.5	1.5
B3K508	**WT 3K**	29	34,130	-6.2	0
B3K508	**P1-A**	>550	<1,818	>-4.4	>1.7
B3K508	**P2-A**	175	5,714	-5.1	1.1
B3K508	**P3-A**	>550	<1,818	>-4.4	>1.7
B3K508	**P5-A**	>550	<1,818	>-4.4	>1.7
B3K508	**P8-A**	>550	<1,818	>-4.4	>1.7

Table IV: Affinity and kinetic calculations that were used to further interpret the data derived from the FACS and Proliferation Assay for the two TCRs, 506 and 508, the WT peptide and it's alanine variants.

The data obtained follows the well established fact that maturing T cells, although specific to the MHC-peptide complex, do have some ability to recognize some variants. Notably some variants, as P5-A for the 508 TCR and P2-A for the 506 were found to be, would instigate a much higher amount of negative selection over positive selection which can be identified through the examination of double positive cell death and the level of proliferation. The fact that this was never the other way around to the same extent supports the idea that it is negative selection that determines the final TCR specificity. This likelihood has been previously indicated through the analysis of cross-reactive TCRs from mice with limited negative selection capabilities in the Huseby lab (Huseby et al., 2006).

The conclusions developed through the analyses support one hypothesis presented by Robey and Fowlkes, 1994, that suggests that reactions of a higher affinity can be associated with both positive and negative selection with the real determinants lying in the qualitative aspects of

the peptide-MHC complexes. Since most peptides resulted in both positive and negative selection and it was the qualitative attributes, individual residues, that could determine the function of the entire complex, this hypothesis is upheld. The hypothesis suggested by Lucas et al., 1999 was also partially supported as the data agreed with his presentation that small structural variations determine the functional fate of the ligand.

There were a couple hypotheses however that were not supported by this data. Sebzda et al., 1994 suggests a theory that different concentrations of a peptide can determine whether positive or negative selection take place. This hypothesis suggested that positive selection was associated with a lower concentration of TCR-peptide-MHC interaction or a lower quantity of peptide and vice versa for negative selection. This was not seen in the collected data, as positive selection appeared to require the same high concentrations that negative selection required. Sebzda et al., 1994 also presented the theory of two quantitative thresholds. Although it is still possible for this hypothesis to be true, these thresholds could not be identified in the data that was collected.

Because of the nature of the alanine amino acid, such as it's small size and ambivalent character, it could allow a more flexible or lenient formation of the rest of the peptide. This could affect the binding energy by allowing for another amino acid's positional change. It is because of this that more amino acid substitutions should be explored in future experiments.

It has been established that it is not just the peptide that the TCR responds to, but also portions of the MHC (Robey and Fowlkes, 1994; Starr et al., 2003; Huseby et al., 2006). This opens up the possibility that an investigation regarding variation in MHC alleles along with these peptide changes may further establish which alterations are truly essential to any activity, and

which variants are pushed forward to TCR recognition by the MHC allele that is presenting them.

Data has previously indicated that there are amino acid residues of the MHC-peptide that deter TCR binding (Huseby, 2006) and because negative selection is known to eliminate developing T-cells with higher affinity to this MHC-peptide complex, the MHC itself is a variable that ought to be taken into consideration. Even with this factor weighed in however the peptide is the most variable of the complex and hence should still be considered as containing the most determining of the influential residues.

It is unfortunate that we did not have the K_d, K_a, ΔG, or $\Delta\Delta G$ data for the P7-A variant, it would probably be very revealing as P7-A had very different activities between the two TCRs, but mimicked the P8-A result for the 506 almost perfectly. Obtaining these calculations would be a good next step and could reinforce a lot of the data here or open up new possibilities.

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