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The self-obsession of T cells: how TCR signaling thresholds affect fate decisions in the thymus and effector function in the periphery

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Abstract

Self-reactivity was once seen as a potential characteristic of T cells that was eliminated by clonal selection to protect the host from autoimmune pathology. We now appreciate that the T cell repertoire is in fact, broadly self-reactive, one could even say self-centered. The strength with which a T cell reacts to self ligands, and the environmental context that this reaction occurs in, influences almost every aspect of T cell biology: from development to differentiation to effector function. In this review we highlight recent advances and discoveries that relate to T cell self-reactivity, with a particular emphasis on TCR signaling thresholds.

Introduction

The physical elimination of self-reactive lymphocyte clones (clonal deletion) is a central tenet of the clonal selection theory, and has been a major focus of immunologists since introduction of the concept by F. Macfarlane Burnet and Peter Medawar in the 1950s. Indeed, many self-reactive clones are eliminated each day in the thymus, and from numerous studies of TCR transgenic mice crossed to self-antigen expressing mice, we consider clonal deletion to be a particularly efficient process¹. Given this, it would be natural to assume that clonal deletion plays a central role in immunological tolerance, and that the remainder of T cells that exist in healthy animals are not self-reactive. The former assumption is not readily apparent from the experimental literature, however, and the latter is clearly not the case. In the first part of this review, we discuss how pervasive clonal deletion is, how important it is for overall immunological tolerance, and how T cells interpret low and high affinity interactions to result in life or death fates respectively. Next, we discuss how strong TCR signals can also induce the differentiation of unique lineages, including regulatory T cell (T_{reg}) cells, invariant natural killer T cells (iNKT cells), and intraepithelial lymphocytes (IEL), in the thymus. Finally, we discuss how the “weak” interactions of T cells with self, which are selected for by positive selection, yield a repertoire of naïve T cells with substantial heterogeneity in their ability to respond to foreign antigens.

Clonal deletion in the thymus

For T cells, clonal deletion occurs in the thymus, and is most efficient for clones that have high affinity for self-antigens presented by professional APC, such as dendritic cells¹. That deleted clones have a higher affinity for self-p/MHC than positively selected clones has been

extensively validated in both monoclonal and polyclonal experimental models, although it has been unclear precisely how many clones achieve this high signaling threshold and become deleted, relative to the number that are positively selected. It had been widely assumed that the number of clones that interact with any given peptide/MHC (p/MHC) complex with high affinity (and are deleted) would be smaller than the number of clones that could interact with low affinity (and are positively selected), because the CDR3 region of the TCR is produced by random assortment and non-templated nucleotide addition. However, several groups recently addressed this question with new approaches, and their data suggest that many more clones undergo clonal deletion in the thymus than positive selection. Two groups used an approach that focused on *Bim* deficient mice, which have impaired clonal deletion. These groups used novel transgenic (Nur77^{GFP})² or endogenous (Helios)³ markers to enumerate the strongly signaled cells that are generated in mice lacking the pro-apoptotic molecule Bim. They reported that 55%³ – 57%² of all signaled thymocytes at the double positive (DP) stage in the cortex are deleted, and that another roughly 50% of the positively selected single positive (SP) cells were subsequently deleted in the medulla. Thus more than three quarters of the cells that respond to self-p/MHC in the thymus are deleted. These studies were remarkably concordant with those generated by a completely different approach, where a synchronous cohort of thymocytes developing in normal mice was analyzed, and mathematical modeling of death and differentiation was used to explain the numbers of thymocytes at each stage. That data suggested 75% of cells that start selection fail to complete it⁴. All of these data favor the notion that the TCR repertoire has a germline encoded bias toward recognition of MHC molecules⁵, rather than a bias that is strictly rendered by thymic selection processes⁶. Although it is worth emphasizing that while the T cell repertoire is overtly MHC reactive on the whole, thymic selection processes further skew it toward recognition of the specific MHC alleles present in the individual.

Since many self-reactive clones are eliminated each day in the thymus, and we consider clonal deletion to be a particularly efficient process from the study of TCR transgenics, we might assume that clonal deletion plays an essential role in immunological tolerance. However, studies have attempted to evaluate clonal deletion from the perspective of a given self-antigen, and these reports, which used p/MHC tetramers to ask how many self-antigen reactive clones are present in animals that do or do not express the self-antigen, suggested that deletion may not be particularly efficient. Bounead *et al.* found only a 3 fold reduction in the number of male specific cells in male versus female mice using male-antigen/D^b tetramers in TCR β chain transgenic mice⁷, and similar results were observed for a tissue-specific antigen in a different TCR β chain transgenic model⁸. Although it might be argued that TCR β transgenic mice are not a physiologic model because of their elevated frequency of reactive T cells, this type of decrease was also seen in studies that used enrichment techniques to enumerate Class II restricted self-reactive T cells mice with a normal polyclonal T cell repertoire^{9, 10}. One consideration is that all of these studies proposed a lower TCR avidity of the remaining T cells, consistent with a lower tetramer staining intensity. Two of the studies included TCR repertoire analysis and found that certain receptor specificities were indeed eliminated from the tetramer binding pool^{7, 10}. Thus from the perspective of a given high affinity receptor, clonal deletion may be as highly efficient in the polyclonal pool as it is in monoclonal TCR transgenic models. On the other hand, from

the perspective of numbers of T cells that can react to a particular self-p/MHC complex, clonal deletion is incomplete. While tetramers are useful for comparative studies of defined self-reactive T cell populations in mice with specific mutations¹¹, or in autoimmune prone strains¹², and transgenic models are useful for exploring deletion mechanisms in animal models, the true measure of clonal deletion efficiency cannot be addressed using either approach alone. In the future, technology will likely allow a comprehensive evaluation of clonal deletion where self-specific T cells are defined at the level of the sequenced TCR repertoire.

Irrespective of the precise degree of deletion efficiency, it is somewhat surprising that there are no autoimmune diseases where impaired clonal deletion has been shown to be a pathogenic mechanism. It had been assumed that Aire deficiency in APECED patients reflects autoimmunity due to a failure to clonally delete self-reactive T cells¹³. However, new evidence suggests that Aire is also crucial for the development of tissue specific regulatory T cells¹⁴, so this inherited autoimmune syndrome may represent a more complex loss of tolerance. It is remarkable that Bim deficient mice, which have a profound increase in the number of self-reactive clones²³, do not have widespread autoimmunity, even in a sensitized screen using hematopoietic reconstitution of RAG1^{-/-} mice¹⁵. In part this is because clonal deletion is not required to purge the repertoire of clones reactive to ubiquitous antigens¹⁶. The reason for this has to do with the very nature of differential TCR signaling in the thymus: In DP thymocytes, high affinity ligands cause a strong signal of short duration that results in rapid induction of apoptosis^{17, 18}. In contrast, low affinity ligands have a preferential ability to result in sustained signals, which are required for positive selection^{18, 19}. Thus strong stimuli at the DP stage, even when they fail to induce apoptosis, do not support positive selection and inclusion of those clones in the repertoire¹⁶. Once cells undergo positive selection and encounter different self-antigens in the medulla, they are again susceptible to deletion. However, in this case, if apoptosis is prevented, autoreactive clones will accumulate²⁰. Therefore any autoimmune pathology that would result from a failure of clonal deletion, such as in Bim deficient mice, is predicted to be focused on tissue specific antigens. That such mice do not display autoimmunity suggests that high frequencies of self-reactive T cells can be controlled by other tolerance mechanisms. Interestingly, double deficiency in Bim and another pro-apoptotic protein, Puma, was recently shown to result in spontaneous multi-organ autoimmunity¹⁵. Thus, a very extensive (perhaps complete) impairment of clonal deletion is presumably required to breakdown the protective effects of tolerance mechanisms such as clonal anergy and extrinsic regulation by regulatory (T_{reg}) cells.

Specialized mechanisms are at play in the cortex, to select T cells with low self-reactivity

T cell progenitors undergo TCR gene rearrangement in the thymus. As soon as an intact TCR $\alpha\beta$ heterodimer is expressed on the cell surface, the clone is exposed to potential ligands in its environment. The specific environment where this first recognition event occurs in the thymus is the thymic cortex, which is a highly specialized environment established by the activities of the transcription factor Foxn1. This factor is conserved

throughout evolution in species that have a thymus²¹, and speaks to the importance of the “first-exposure” environment in establishing a repertoire of T cells that can function properly in the animal. It has long been known that cortical, or CD4/CD8 double positive (DP) thymocytes, are very sensitive to TCR ligands and can respond to low affinity ligands more readily than single positive (SP) thymocytes²² (Figure 1). DP cells have a dramatically different gene expression profile compared to SP cells, and at least some of these genes are presumed to account for this enhanced sensitivity. Several genes have been identified that are highly expressed in DP but reduced or absent in mature T cells, and that function to support positive selection and/or tuning. These include *Themis*²³, *Tespa1*²⁴, and *Scn4b*²⁵, which encode proteins involved in TCR proximal signaling events. *Scn4b* encodes the regulatory subunit of a voltage gated sodium channel (VGSC). Inhibition of the VGSC inhibited Ca^{++} influx and positive selection in thymocytes²⁵. Furthermore, ectopic VGSC expression in mature T cells allowed them to respond to low affinity ligands, thus VGSCs appear to be crucial for the enhanced sensitivity of thymocytes (Figure 1). Both positive and negative selection invoke Ca^{++} signaling, although the patterns are distinct, which was demonstrated directly in thymic tissue using two-photon microscopy recently²⁶. Interestingly, store-operated Ca^{++} entry, which is crucial for mature T cell responses, is dispensable for positive selection of conventional T cells²⁷. Rather, store-operated Ca^{++} entry seems to function in DP thymocytes to promote “agonist-selection” of T cells (inKT cells, Treg cells, and IEL)²⁷—discussed further below. Thus mechanistically distinct Ca^{++} responses are at play when thymocytes perceive weak versus strong TCR signals. It was recently shown that positive selection causes a gradual increase in the basal level of intracellular Ca^{++} ²⁸. This may also contribute to the tuning that thymocytes experience, as there are data from other biological contexts that elevated basal Ca^{++} correlates with reduced receptor-evoked Ca^{++} responses²⁹.

The precise signaling functions of *Tespa1* and *Themis* remain to be fully elucidated, although *Tespa1* deficiency also influences Ca^{++} signaling²⁴. An important recent study showed that *Themis*-deficiency allows low affinity ligands to elicit negative selection-like signaling characteristics³⁰. Thus *Themis* acts to attenuate or modify weak signals through the TCR to facilitate positive selection. Likewise, *Schnurri-2*, which encodes a zinc finger protein that possibly acts downstream of *Themis*, was shown to inhibit apoptosis in response to positive selection signals in DP thymocytes³¹. These unique signals transduced by the TCR during positive selection must be sustained for a surprisingly long time. Blocking the TCR signal 24 – 48 hours after initiation with anti-MHC antibodies¹⁹, ERK inhibitors¹⁹, Zap-70 inhibitors¹⁸, or by induced loss of Zap-70³², all were shown to impact positive selection. Intriguingly, cortex to medulla migration was recently shown to occur about 12–24 hours after positive selection initiation²⁸, (Figure 1) suggesting that at least some of this sustained signaling may be occurring in the medulla. Nonetheless, there is little evidence that medullary epithelial cells³³ or any MHC ligands in the medulla³⁴ are needed for positive selection, so this issue needs further investigation.

It is important to consider how multiple genes that act at different levels of the signaling pathway are regulated in concert to tune TCR responsiveness. The microRNA miR-181a is highly expressed in DP thymocytes but not SP, and was shown to repress expression of

several phosphatases, including SHP-2, PTPN22, and DUSP5/6, to alter TCR responsiveness^{35, 36}. Its ectopic expression in mature T cells enhanced their sensitivity³⁵, and its loss in DP thymocytes impaired clonal deletion³⁷ and agonist selection of iNKT cells³⁶. In contrast, the expression of a negative regulatory proteins, such as CD45 and CD5, increase during T cell development³⁸ (Figure 1). CD5 is an interesting membrane protein that can negatively regulate signaling, yet its expression level seems to be directly proportional to the signal strength initially perceived³⁹. Although the precise mechanisms of how these changes over time act together to influence the signal network remain to be fully elucidated, it is clear that specific genetic mechanisms exist to allow cortical thymocytes to preferentially respond to low affinity TCR ligands, and to do so in such a way as to not undergo apoptosis.

Not only are cortical thymocytes specialized for selection, the cortical antigen presenting cells (APCs) are as well (Figure 2). Cortical thymic epithelial cells (cTEC) display a distinct repertoire of peptides compared to other APC. The cTEC unique peptides, which have been called “private peptides”, are apparently crucial for selection of the repertoire, since deletion of cTEC unique genes such as *Psmbl1* (encoding the $\beta 5t$ subunit of the proteasome), *CtsL* (encoding cathepsin L), or *Prss16* (encoding thymus-specific serine protease or TSSP) resulted in reduced CD8 or CD4 T cell numbers [reviewed in⁴⁰]. In the case of $\beta 5t$, this unique private peptide diversity was suggested to be important for positive selection, rather than reducing the impact of negative selection (either of which could result in reduced T cell numbers)⁴¹. Interestingly, naïve CD8 T cells that were CD5^{lo} and had lower reactivity for self were selectively affected by $\beta 5t$ deficiency, suggesting that private ligands on cTEC enhance selection of T cells with low affinity for self⁴¹. How having a distinct peptide repertoire expressed by cTEC favors positive selection was discussed recently⁴⁰, and will not be reviewed in detail here. Suffice it to say that the naïve T cell repertoire in normal animals contains clones with a range of reactivity to self (CD5^{lo} to CD5^{hi}). In the steady state, these clones do not respond to their selecting self-ligands, either because the specific peptides are not presented by peripheral APC, and/or because the TCR signaling threshold was adjusted by tuning. It is important to stress that positive selection does not absolutely require expression of private peptides – indeed, cells selected on “public peptides” (those shared between cTEC and other cell types) have the opportunity to reencounter those ligands in the periphery, leading to enhanced basal TCR signaling (Figure 3A). Recent studies showed that a relatively abundant self-pMHC was capable of positively selecting CD4 T cells of defined foreign antigen specificity⁴², in support of this concept. What affect having a range of self-reactivity within the naïve repertoire has on peripheral T cell function is discussed in greater detail in the section on self-reactivity in the periphery, below.

Strong self-reactivity drives multiple T cell fates in the thymus: T_{reg} cells, IEL, and iNKT cells

While it is abundantly evident that strong TCR signals can drive clonal deletion, increasing evidence suggests that strong TCR signals can also drive the survival and differentiation of other T cell populations, including foxP3+ T_{reg} cells, invariant natural killer T cells (iNKT) and intraepithelial lymphocytes (IEL). Regarding T_{reg} cells, support for the concept of

agonist selection has been extensively reviewed elsewhere^{43,44}, but the seminal observations are: 1) co-expression of an agonist ligand in TCR transgenic mice promotes T_{reg} cell development⁴⁵, at least in some models, 2) FoxP3+ T_{reg} cells have an overlapping repertoire with autoreactive T cells derived from FoxP3-deficient mice⁴⁶, and 3) T_{reg} cells show signs of stronger and/or more recent activation in both the thymus and periphery of TCR signal reporter mice⁴⁷. These data have led to the frequent discussion of a model where T_{reg} cell selection is supported optimally by TCR affinities for self-p/MHCII that are intermediate between positive selection and clonal deletion. Recently Hsieh and colleagues examined a panel of six TCRs with varying degrees of reactivity for a model antigen and then studied the thymic development of cells with transgenic and/or retrogenic expression of those TCRs in mice that expressed the model antigen as a self-antigen. They observed a direct correlation between the degree of antigen reactivity and T_{reg} cell development; and negative selection was apparent only with the most self-reactive TCRs. This would support the model stated above. However, the range of reactivities over which they observed T_{reg} cell development was surprisingly broad, providing an explanation for the observed overlap between the TCR repertoires of T_{reg} and non-T_{reg} cells⁴³, and suggesting that either the TCR signaling thresholds between positive selection, clonal deletion, and T_{reg} cell development are not rigid, or that natural variation in signaling capacity between cells with the identical TCR is large. Theoretical modeling posits an integration of different signal thresholds together with loss of TCR sensitivity over time that allows the divergent fate outcomes of positive selection and T_{reg} cell induction, which is an interesting idea that will need further experimental testing⁴⁸. In the future, it will also be important to have a comprehensive understanding of the TCR repertoire overlap between conventional CD4 T cells, T_{reg} cells, and MHCII-restricted deleted TCRs to fully understand the role of the antigen receptor.

The medulla is a specialized environment for T_{reg} cell induction

Transgenic and retrogenic models using TCRs cloned from T_{reg} cells have confirmed the crucial role of antigen receptor signaling⁴⁹, yet also made clear that a particular TCR specificity alone is not sufficient to induce T_{reg} cell differentiation, since T_{reg} cell generation was only detectable at low precursor frequencies, and never observed to be complete (where 100% of the cells were foxP3+). Thus, there is a limiting “niche” for T_{reg} cell development, whether it be at the level of competition for TCR ligands or due to competition for other factors. In addition to TCR signaling, T_{reg} cell differentiation is known to require multiple other factors, including CD28, IL-2, TGFβ, PI3K signaling (reviewed in⁴⁴), and as recently described, TNFR superfamily co-stimulation⁵⁰. The availability of one or more of these factors may account for the recent observation that T_{reg} cell development is dependent on medullary epithelial cells³³ (Figure 2). An intriguing idea recently proposed is that thymocyte apoptosis may enhance the generation of regulatory T cells through production of TGFβ by phagocytic cells, particularly in the medulla⁵¹. Likewise, certain TNFR ligands, like OX40L and GITRL are also expressed on medullary APC⁵⁰. Furthermore, it may be an intrinsic property of medullary thymocytes to respond to these cues, since semi-mature SP thymocytes that reside in the medulla were shown to have enhanced susceptibility to foxP3 induction⁵². This may reflect the requirement for TNFR signaling, as TNFR families member, such as GITR are upregulated only in medullary thymocytes, through a TAK1

dependent process during positive selection⁵⁰. In this context, it is interesting to consider that tumor infiltrating self-antigen specific T_{reg} cells were shown to be dependent on AIRE, expressed in medullary epithelial cells¹⁴. Although AIRE deficient animals are not profoundly lacking T_{reg} cells, the extent to which the T_{reg} cell repertoire is affected by AIRE deficiency, and whether this contributes to the autoimmune pathogenesis in mice and humans, is now of great interest.

New data on iNKT cells is consistent with a threshold model

iNKT cells have a unique semi-invariant V α 14-J α 18 TCR that recognizes lipid molecules presented by CD1d. They develop in the thymus, like other $\alpha\beta$ T cells, but have many distinctions from conventional T cells, including their dependence on SLAM family member signals, and the use of other DP thymocytes as APC which makes the cortex a unique environment for their development (Figure 2). iNKT cells have a previously activated phenotype, like T_{reg} cells, which suggested they may perceive strong TCR signals during development. Indeed, analysis of Egr2⁵³ and Nur77⁴⁷ reporter mice was consistent with this idea. A crucial role for strong signaling comes from observations that iNKT cell development required a full complement and ITAMs⁵⁴, and was abrogated in mice lacking miR-181a/b³⁶. Recently, Bedel and colleagues more fully explored the extent to which TCR affinity for agonist self-ligands controlled iNKT cell differentiation⁵⁵. They created a TCR β chain transgenic animal using a TCR β chain that increased the affinity for self-lipid/CD1d complexes when paired with canonical V α 14-J α 18 rearrangements. iNKT cells that expressed the high affinity canonical V α 14-J α 18 rearrangements did not mature in these TCR β chain transgenic mice, proving that iNKT cells are subject to clonal deletion. A small proportion of cells escaped deletion in these mice, and were shown to utilize a non-canonical V α 14-J α 18 rearrangement with a 40 fold lower affinity for self-ligands. However those TCRs did not lead to the efficient induction of the iNKT-lineage specific transcription factor PLZF, and thus displayed aberrant proliferation, trafficking, and effector function⁵⁵. This study thus suggests that proper iNKT cell differentiation requires a specific window of TCR signal perception, together with other anatomically restricted lineage induction factors, much like T_{reg} cells (Figure 2).

Some highly self-reactive clones divert to IEL

The intestinal epithelium is interspersed with a heterogeneous lymphocyte population. IEL expressing an $\alpha\beta$ TCR and CD8 $\alpha\alpha$ are referred to as natural IELs and are thought to be important for mucosal tolerance and homeostasis⁵⁶. Such natural IELs are generally thought to be derived from thymic precursors and to require TGF β ⁵⁷ and IL-15⁵⁸ for survival. This natural IEL pool has an activated phenotype, is known to contain 'forbidden clones', and TCR transgenics models suggested that IEL were dependent on high affinity TCR interactions in the thymus⁵⁹. More recently, it was shown that rescuing cells from thymic clonal deletion through deficiency in CD28 or transgenic overexpression of bcl-2, led to increased IEL development⁶⁰, suggesting that the precursors of gut IEL are cells destined for clonal deletion in the thymus. It is challenging to understand why cells destined for deletion in the thymus would sometimes traffic to the gut and adopt this fate. Nor do we understand how frequently it occurs and whether it is stochastic or regulated by specific mechanisms.

Nonetheless there are a large number of such cells in the gut, and their potential to play an important role in mucosal tolerance and homeostasis merits further examination.

Self-reactivity in the periphery and naïve T cell homeostasis

The majority of thymocytes that emerge after thymic selection are “conventional” naïve TCR $\alpha\beta$ T cells rather than the agonist selected populations discussed above. Considerable evidence suggests that these naïve T cells maintain a low-level response to self-p/MHC ligands, and that those interactions are important for sustaining a basal TCR signal and naïve T cell homeostasis. At the same time, accumulating evidence indicates that the strength of the T cell interaction with self-p/MHC is not uniform among naïve T cell clones, and that even within this “low affinity” range, the extent of self-recognition has a substantial impact on the T cell’s ability to respond to homeostatic cues and to react against foreign p/MHC ligands in an immune response. Hence, like developing thymocytes, a continuum in intensity of self-awareness has significance for function and maintenance of the peripheral naïve T cell pool. Furthermore, as will be discussed below, accumulating data suggest the impact of self-pMHC interactions in the periphery is not identical for CD4 and CD8 T cells, suggesting an unexpected layer of subset specialization.

It has been known for many years that naïve CD8 and CD4 T cells exhibit a basal TCR signal (manifest as partial phosphorylation of the TCR ζ chain), and that this is lost with deprivation of self-MHCI and MHCII molecules respectively^{61, 62, 63, 64}. Likewise, naïve T cells express a basal level of GFP in Nur77^{GFP} transgenic mice, but this expression is rapidly lost when naïve CD4 T cells are deprived of self-MHCII molecules⁴⁷. However, the significance of these weak TCR signals has been more difficult to discern. Initial studies on T cell homeostasis suggested an essential role for self-p/MHC ligands in naïve T cell survival^{65, 66} but interpreting these findings became more complicated when it was discovered that self-p/MHC recognition is one of the cues that induces naïve T cells to proliferate and acquire memory-phenotype in situations of lymphopenia (which were often employed in studies on T cell homeostasis)^{61, 67}. There is general consensus that self-p/MHC recognition provokes lymphopenia-induced proliferation (LIP) of both CD4 and CD8 T cells, and that similar TCR encounters are needed for naïve CD8 T cell survival in a lymphoreplete environment. Determining whether CD4 T cell survival requires TCR binding to self-pMHC Class II has proven more controversial^{61, 67}, however comprehensive studies by Martin and colleagues suggest a more complex picture in which competition with CD8 T cells (but not other CD4 T cells) enforces dependence on self-pMHC Class II for naïve CD4 T cell survival, although the molecular mechanisms for this regulation are currently unclear⁶⁸. Also, while memory CD8 T cell maintenance appears completely independent of self-p/MHC recognition (or even of TCR expression)^{61, 67}, studies using a TCR loss model show that a subset of memory-phenotype CD4 T cells continue to depend on TCR signals for maintenance⁶⁹.

An uneven playing field: heterogeneity in self-sensitivity leads to heterogeneity in naïve T activation potential

Part of the difficulty in getting a clear impression of how self-p/MHC affects T cell homeostasis is that not all naïve T cells respond to homeostatic cues in the same way: Analysis of TCR transgenic models showed radically divergent capacity of naïve CD8 and CD4 T cells to proliferate and acquire memory-like properties in LIP models⁶⁷. Likewise, there was notable variability in the capacity of different TCR transgenic naïve CD8 T cells to respond to the cytokines IL-7 and IL-2^{70, 71}. Relating this heterogeneity to self-p/MHC recognition is inherently difficult. For conventional T cells, the affinity of the TCR for self-p/MHC ligands is predicted to be very low. The p/MHC complexes that drive thymic positive selection are known to bind the TCR with low affinity (and similar ligands can promote naïve T cell homeostasis), and it is likely that multiple self-peptides can engage a given TCR in such interactions, making it impractical to identify and characterize all relevant self-p/MHC complexes in a physiological setting^{72, 73}.

Fortunately, studies in mice have revealed valuable phenotypic markers that correlate with the intensity of TCR recognition of self. Of these, the molecule CD5, has proven the most reliable (Figure 3A). Analysis of TCR transgenic and polyclonal naïve T cells (of either CD4 or CD8 subsets) revealed that increasing levels of CD5 expression correlate positively with: a) degree of basal TCR ζ phosphorylation^{63, 64, 74}; b) capacity to rapidly produce IL-2 and induce ERK phosphorylation following T cell stimulation⁶³ and c) capacity of T cells to undergo LIP^{71, 75, 76}. We have observed that CD5^{hi} naïve CD4 and CD8 T cells also show increased expression of GFP expression in Nur77^{GFP} mice, and that CD5^{hi} and CD5^{lo} naïve CD8 T cells differ in gene expression profiles⁷⁷, suggesting that the CD5^{hi} pool has improved readiness for activation and functional differentiation (see below). CD5 itself is a cell surface molecule that can, through associated SHP-1, negatively regulate TCR signals, and has been proposed to function as a rheostat in dampening TCR signaling during thymic development³⁹. Indeed, CD5 expression levels are set during thymic development and are typically maintained in the periphery^{63, 78} although deprivation of T cell interaction with self-p/MHC in the periphery can cause a decline in CD5 expression⁷⁹. Since it is T cells with the highest levels of CD5 that appear to show the strongest reactivity to self-p/MHC, however, its proposed rheostat function appears insufficient to normalize TCR reactivity for all clones, making this marker valuable for isolating cells with different levels of active self-sensitivity. Studies using CD5-deficient TCR transgenic T cells support the concept that this molecule restrains TCR signaling, yet CD5 loss did not effectively normalize the differential responses of clones that are normally selected into CD5^{hi} and CD5^{lo} pools^{63, 64}. Hence, though CD5 expression levels have proven to be an effective and useful maker for naïve T cell subsetting, the functional relevance of CD5 *per se* is controversial.

Intriguingly, differences emerge in comparison between naïve CD4 and CD8 T cell populations (Figure 3B). Expression levels of GFP in Nur77^{GFP} reporter mice are greater in naïve CD4 T cells than in naïve CD8 T cells⁴⁷ and there is a notably greater range in resting p-TCR ζ levels when comparing CD5^{lo} and CD5^{hi} populations in the CD4 pool, rather than the CD8 subset⁶⁴. Further, while pharmacologically induced IL-2 production increases with

CD5 expression levels on both populations, the overall expression is notably greater in the CD4 subset⁶³. On the other hand, CD5^{hi} naïve CD8 T cells are provoked into proliferation when exposed to sustained high dose IL-2 stimulation (in the absence of foreign pMHC ligands), while this response was not observed for naïve CD4 T cells (regardless of their CD5 expression levels). That cytokine-induced proliferation was found to correlate with preferential localization of CD122 (the IL-2Rbeta chain) to lipid rafts in CD5^{hi} CD8 T cells⁷¹, and hence the failure of naïve CD4 T cells to engage in this response may reflect the lower expression levels of CD122 on CD4 subsets compared to their CD8 counterparts. We observed that CD5^{hi} naïve CD8 T cells show elevated expression of factors associated with early T cell activation (including T-bet, Eomes, CXCR3, Xcl-1), and patterns of gene expression that indicate improved preparation for responsiveness and cell division⁷⁷, yet such changes have not been reported for naïve CD4 T cell subsets. Thus there may be additional distinct features of the CD5^{hi} and CD5^{lo} populations from naïve CD4 versus CD8 T cell populations.

Although basal TCR signaling, and associated CD5 expression levels, are considered to reflect the intensity of self-recognition by naïve T cells, this does not necessarily mean they reflect the affinity/avidity of the TCR interaction with self-p/MHC. Instead, these signals might reflect the tissue distribution of suitable self-p/MHC ligands (Figure 3A). Whether a given T cell would encounter the relevant self-p/MHC frequently or rarely might dictate its steady state basal TCR signal. A potential reflection of this is the finding that selection of some CD8 TCR transgenic clones strongly depends on $\beta 5t$ expression, while others are independent of $\beta 5t$. Intriguingly, this generally correlates with the CD5 express status of the T cells – with CD5^{hi} cells being less dependent on $\beta 5t$ ⁸⁰. Consistent with this, the CD8+ T cells selected in $\beta 5t^{-/-}$ animals are enriched for the CD5^{hi} population. It is tempting to conclude that cells selected on self-MHC molecules containing $\beta 5t$ -independent “public peptides” may encounter the very same pMHC complex on other cells in the periphery (imparting a basal TCR signal), while this would be less likely to occur for cells selected on ligands containing private $\beta 5t$ -dependent peptides. It is currently unclear whether the same pattern applies to CD4 T cells selected on private versus public peptides presented by Class II MHC ligands. This concept was discussed in a recent review⁴⁰, and hence will not be further explored here.

Self-sensitivity regulates foreign antigen reactivity

Although the degree of self-p/MHC sensitivity impacts TCR basal signaling intensity, gene expression patterns and the proliferative response to lymphopenia, those differences, perhaps surprisingly, do not seem to affect steady state survival of naïve T cells – hence, studies on CD5^{hi} and CD5^{lo} polyclonal CD4 and CD8 T cells reported similar maintenance (and preservation of their CD5^{hi/lo} status) after adoptive transfer into a lymphoreplete environment^{63, 64, 77}. This suggests that a minimum TCR signal suffices for T cell survival, while greater self-awareness provides naïve CD8 T cells with increased sensitivity to homeostatic opportunities.

However, multiple studies suggest the response to foreign antigen is influenced by naïve T cell self-p/MHC sensitivity – though the exact nature and basis of that effect remains

controversial. Studies from Mandl et al. reported that CD5 expression levels on naïve CD4 T cells not only corresponded with the level of basal TCR signaling but also predicted the capacity of these cells to bind specific foreign p/MHC ligands (as revealed by p/MHC tetramer staining)⁶⁴. This led to the intriguing hypothesis that the interaction with self-pMHC during thymic positive selection not only provides a signal for T cell maturation, but also produces T cells better able to bind foreign pMHC. However, other studies reached divergent conclusions. Allen and colleagues generated two TCR transgenic strains, which bound the same foreign pMHC ligand with very similar TCR affinity, and yet were selected into CD5^{hi} or CD5^{lo} populations (with corresponding differences in basal TCR signaling)^{63, 81}. Likewise, studies on TCR transgenic and normal polyclonal CD8 T cells did not indicate a consistent difference in foreign p/MHC tetramer binding to CD5^{hi} versus CD5^{lo} populations⁷⁷.

There is better consensus with the concept that the intensity of the basal encounter with self-pMHC impacts reactivity to foreign antigen. There are two aspects of this that have been explored –the contribution of self-pMHC recognition during foreign antigen encounter itself, and the effects of self-pMHC on intrinsic properties of naïve T cells prior to encounter with foreign antigen. Davis and colleagues first introduced the concept that non-stimulatory self-pMHC complexes may serve as “co-agonists” to assist the T cell response to foreign pMHC ligands^{82, 83}. However, it remains unclear whether co-agonists contribute to all T cell responses, and even in situations where a co-agonist role could be observed there was considerable controversy over whether co-agonist encounters were peptide specific or not, with divergent conclusions being drawn for CD4 and CD8 T cells^{82, 83}. A recent report from Gascoigne’s group nicely resolves this discrepancy by showing that there are two modes of co-agonist contribution, in which the significance of the TCR affinity for the co-agonist pMHC depends critically on the strength of co-receptor binding to the foreign pMHC target⁸⁴ – since the CD8 co-receptor is thought to have a higher affinity for Class I MHC molecules (albeit not all alleles) compared to the CD4-Class II MHC interaction, TCR specificity for co-agonist ligands may be a much more stringent limitation for CD4 compared to CD8 T cells⁸⁴.

Three recent studies investigated the foreign antigen-specific response of CD5^{hi} versus CD5^{lo} naïve CD4 T cells^{63, 64} and naïve CD8 T cells⁷⁷, using a combination of polyclonal and TCR transgenic model systems. These reports included studies on the properties of CD5^{hi} and CD5^{lo} naïve T cells prior to antigen encounter, and in situations where a co-agonist role could be excluded, in order to determine whether and how self-pMHC sensitivity (reflected as CD5 expression levels) alters the *intrinsic* capacity of naïve T cells to respond to foreign ligands. As discussed above, CD5^{hi} populations of both CD4 and CD8 naïve T cell subsets show evidence of the increased basal TCR signaling and improved functional characteristics, and this was found to correlate with greater expansion in response to foreign antigen in two of those studies^{64, 77}. While Mandl *et al.* correlated this response pattern with changes in TCR binding to foreign pMHC by CD5^{hi} naïve CD4 T cells^{64, 77}, Fulton and colleagues found that the CD5^{hi} naïve CD8 T cells exhibited more efficient clonal recruitment into the immune response, and superior augmentation of their response by inflammatory cues⁷⁷. In apparent contrast to both of these reports, studies using two TCR

transgenic strains that recognize the same foreign pMHC ligand but were positively selected into CD5^{hi} and CD5^{lo} populations, exhibited a different pattern, in which the CD5^{lo} clones displayed greater expansion than the CD5^{hi} group during the primary immune response^{63, 81}. A possible resolution of these discrepancies reflects the concept that the CD5^{hi} population in naïve CD4 T cells may maintain a higher basal activation state than CD5^{hi} naïve CD8 T cells (Figure 3B) – and hence may be more susceptible to activation induced cell death pathways after strong stimulation. Indeed, Allen and colleagues suggested that the poor expansion of their CD5^{hi} TCR transgenic clone was not due to ineffective early activation but rather to IL-2 driven apoptosis⁶³. In this context, is interesting to note that, compared to polyclonal cells, the CD5 levels on this “CD5^{hi}” TCR transgenic clone were greater than those on TCR transgenic clones analyzed by Mandl et al.^{63, 64} potentially suggesting an even greater degree of self-pMHC reactivity. Hence, superior intrinsic sensitivity in the CD5^{hi} naïve T cell pool may (at least in the CD4 subset) come at the cost of increased vulnerability to elimination mechanisms. Furthermore, it is worth noting that in secondary immune responses, Persaud *et al.* reported that the CD5^{hi} clones expanded to a greater extent than the CD5^{lo} population⁶³. Whether this arises from avoidance of IL-2 induced cell death pathways during the recall response or some other mechanism is unclear.

While these reports are consistent in that the CD5^{hi} populations of naïve CD4 and CD8 T cells possess enhanced intrinsic reactivity toward foreign pMHC stimulation, the underlying mechanism and immediate consequences for clonal recruitment and expansion are harder to predict and may depend on where cells lie on the scale of self-reactivity.

In conclusion, T cells experience a broad range of self-reactivity continually; from the time they first express a surface receptor in the thymus, and throughout their lifespan and residency in peripheral lymphoid organs or tissues. The developmental stage of the T cell, as well as the affinity and co-stimulatory/cytokine context of these interactions, all impact what kind of response is ultimately made and maintained. Thus self-interactions are crucial to the sophisticated and highly controlled adaptive immune response.

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Figure 1. TCR sensitivity changes as cells mature from cortical (DP) to medullary (SP) thymocytes

DP thymocytes reside in the cortex and respond efficiently to both low and high affinity TCR ligands. High affinity ligands can trigger apoptosis (clonal deletion), whereas low affinity ligands are more likely to induce survival and differentiation (positive selection). 12–24 hours after positive selection, DP thymocytes migrate to the medulla. At the same time they begin differentiating to the SP stage—a process that is not complete for another 1–2 days. In the medulla, SP thymocytes remain responsive to high affinity ligands, but their response to low affinity ligands decreases, a process sometimes referred to as “**tuning**”. DP thymocytes have a unique gene expression profile that serves two purposes. First, it allows them to interpret graded TCR signals in a digital fashion, to induce life or death in the cortex. The protein Themis is hypothesized to be crucial for this feature. Second, it allows them to respond more efficiently than SP thymocytes to low affinity ligands. Evidence suggests that the protein Tespa1, the voltage gated sodium channel (VGSC) and the microRNA mir181a all contribute to this property. Mir181a represses expression of several phosphatases (SHP-2, PTPN22, and DUSP5/6) that are known to negatively regulate TCR signaling. Another phosphatase (CD45) and a cell surface protein known to interact with phosphatases (CD5) are upregulated in SP thymocyte independently of mir181a. These gene

expression changes, together with an increase in the basal level of intracellular Ca^{++} , are all thought to contribute to the developmental tuning of the TCR response.

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Figure 2. The anatomic context of TCR signaling is crucial for thymocyte fate

Cortex: TCR signal strength is central in DP thymocyte fate outcome, but extrinsic factors provided by antigen presenting cells in the cortex are also key. Weak interactions between the nascent TCR and self-pMHC complexes on cortical thymic epithelial cells (cTEC) are crucial for positive selection. The pMHC repertoire expressed by cTEC is unique, due to the function of several genes involved in endogenous or endosomal proteolysis, such as $\beta 5T$, TSSP, and cathepsin L. Although this unique repertoire is essential for efficient positive selection, we do not fully understand why. Strong interactions between the newly expressed TCR and self-pMHC complexes in the cortex classically induces apoptosis (clonal deletion) and co-stimulation by B7 family members enhances this outcome. Strong TCR signals can also induce cells to become IEL precursors (IELp), although it remains unclear how frequently this occurs (relative to deletion), if it requires unique extrinsic factors, and whether IELp express a transcription factor that acts as a “master-regulator” of this fate. In the case of iNKT cells, other DP thymocytes expressing CD1d are a crucial APC. The semi-invariant iNKT TCR must recognize a high affinity CD1d/lipid ligand, but SAP-dependent signals from homotypic interactions between SLAM family members on DP thymocytes is also required. These integrated signals result in the upregulation of PLZF.

Medulla: The cells positively selected by weak TCR signals in the cortex will migrate to the medulla and, by downregulating the inappropriate co-receptor, will form the medullary SP thymocyte pool. SP thymocytes interact with distinct APC, including medullary TEC (mTEC), which express the nuclear factor AIRE. As AIRE promotes the expression of tissue-specific antigens, mTEC also display a unique pMHC repertoire. Strong interactions between the TCR and new pMHC complexes displayed by mTEC or DC classically induce apoptosis (clonal deletion), and both express B7 family members as a source of co-stimulation. Strong TCR signals can also induce CD4⁺ SP cells to become T_{reg} cells, through the induction of the transcription factor FoxP3. T_{reg} cell induction requires signals from TNFR family members, including OX40 and GITR, the ligands of which are expressed on mTEC. T_{reg} cell induction also requires TGFβ, which is abundantly produced by mTEC. Like for IELp cells in the cortex, it remains unclear how frequently T_{reg} cell induction occurs (relative to deletion). SP thymocytes that continue to weakly recognize self-pMHC ligands ultimately emigrate from the thymus and form the naïve T cell pool in peripheral lymphoid organs.

Figure 3. Self-reactivity establishes the activation potential of naïve T cells

A. Naïve T cells, as a population, display a heterogeneous level of CD5, and this heterogeneity reflects the strength with which the TCR of a particular clone recognizes self-pMHC. This was confirmed using Nur77^{GFP} mice, where CD5 expression levels correlate with GFP expression. An additional means by which CD5 heterogeneity may arise is by recognition of public versus private self-pMHC. We refer to public self-pMHC as those that are displayed by both positive-selecting APC in the thymic cortex (cTEC) and dendritic cells in the periphery. Private self-pMHC are those displayed only by cTEC, due to their unique

expression of proteolysis factors. A clone selected on private pMHC will fail to continue to recognize the same pMHC in the periphery, resulting in lower CD5 levels. CD5^{hi} clones have a higher level of basal TCR ζ phosphorylation and altered gene expression patterns compared to CD5^{lo} clones, factors that contribute to their activation potential.

B. CD5 expression levels on naïve T cells correlate with their ability to respond to foreign antigens during an immune response. CD5^{lo} cells are less efficiently recruited into immune responses, produce IL-2 more slowly, and undergo less clonal expansion. Interestingly, naïve CD4 T cells have an overall higher level of CD5 (and GFP in Nur77^{GFP} mice) than naïve CD8 T cells and are more effective at rapidly producing IL-2. Because CD4 T cells can undergo activation induced cell death (AICD) when over-stimulated, the CD4 clones with the very highest CD5 levels will not be as well represented in memory response.