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Selection of self-reactive T cells in the thymus

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Abstract

On the whole, the healthy adaptive immune system is responsive to foreign antigens, and tolerant to self. However many individual lymphocytes have, and even require, substantial self-reactivity for their particular functions in immunity. In this review, we discuss several populations of lymphocytes that are thought to experience agonist stimulation through the TCR during selection: nTreg cells, iNKT cells, nIELs, and nTh17s. We discuss the nature of this self-reactivity, how it compares with conventional T cells, and why it is important for overall immune health. We also outline molecular pathways unique to each lineage, and consider possible commonalities to their development and survival.

Keywords

Tolerance; Thymic selection; invariant natural killer T cell; intraepithelial lymphocytes; regulatory T cell; natural Th17

The central paradigm of low self-reactivity shaping and maintaining the naïve CD4 and CD8 T cell repertoire

The development of a functional repertoire of T cells requires TCR specificity based selection events that initiate in the thymus. The most abundant products of this process are conventional naïve CD4 and CD8 αβ T cells, produced at a rate of approximately 1–2x10^6/day in a young mouse. The combined actions of positive (1) and negative (2) selection produce a T cell repertoire that is both MHC restricted and non-self reactive. Selection initiates at the double positive (DP) stage in the specialized microenvironment of the thymic cortex. DP thymocytes are acutely sensitive to TCR stimulation (3, 4), and the bulk of evidence suggests that this sensitivity facilitates the perception of low affinity self-peptide/MHC ligands presented by cortical epithelial cells. Although the precise affinity range (5), abundance (1), and uniqueness of the low affinity positive selection ligands (6) continues to be debated, most agree that perception of high affinity ligands at this stage will trigger clonal deletion by direct induction of apoptosis. Failure to receive the appropriate TCR signal required for positive selection results in a default "death by neglect" program (see Figure 1).

The most widely used models for studying selection have employed TCR transgenic mice that also express the corresponding cognate antigen (7). These models clearly show the deletion of DP thymocytes following interactions with high affinity self-antigens. Although

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these models have suggested that clonal deletion is massively efficient (8), approaches that measure the polyclonal repertoire specific to a given self-antigen suggest more modest reductions (9, 10). Indeed no one has accurately estimated the fraction of the positively selected repertoire that is clonally deleted. Although estimates of 50–65% have been made, they are limited by their underlying assumptions, e.g. that only bone marrow derived cells mediate clonal deletion (11) or that the repertoire selected on a single pMHC is representative of the whole repertoire (12, 13).

T cells that have undergone both positive and negative selection then leave the thymus and enter the periphery where they persist by receiving homeostatic signals. A diverse naïve T cell repertoire is maintained by interactions with low affinity self-peptide-MHC molecules and limited IL-7 in the periphery (14, 15). When animals are lymphopenic, these same interactions facilitate cellular proliferation—so called lymphopenia-induced proliferation or LIP — likely due to an increase in the amount of available IL-7. It is thought that the TCR affinity of the naïve T cell for self-peptide-MHC molecules dictates the rate of LIP, with the most highly self-reactive T cells undergoing the greatest expansion. However, the role of TCR affinity for self in LIP mainly derives from the study of TCR transgenics, where the "affinity for self" is inferred based on a combination of the rate of proliferation, selection efficiency in the thymus, and the level of CD5 expression (16, 17). As apparent from the somewhat circular logic applied here, the field greatly needs tools to more directly gauge the TCR affinity for self, particularly amongst polyclonal populations.

Evidence for high TCR self-reactivity in certain T cell populations: agonist selection

T cell precursors that interact with high affinity for self-peptides are generally deleted. However, not only do some cells seem to escape this fate, their specific function in the immune system depends on these so-called "agonist" interactions. These include natural T regulatory (nTreg) cells, invariant natural killer T (iNKT) cells, natural CD8αα+ intraepithelial T cells that reside in the gut (nIEL), and natural T helper 17 (nTh17) cells. What is the evidence that these subsets have higher self-reactivity?

nTreg cell self reactivity

nTreg cells play an important role in suppressing autoreactivity and are hypothesized to have self-reactive TCRs although to date, no one has identified a specific self-peptide ligand(s) of a naturally occurring nTreg cell (18, 19). Nonetheless, the higher self-reactivity of nTreg cells has been inferred from a number of different lines of evidence. First, nTreg cells in the thymus have an activated phenotype highlighted by elevated expression of the activation marker CD25, the α chain of the IL-2 receptor. They also have intermediate levels of CD44, a surface protein associated with antigenic experience in C57BL/6 mice, and express GITR. This activated phenotype indirectly suggests that nTreg cells have encountered their cognate antigen during development (Table I). Further evidence supporting agonist selection came from experiments using TCR transgenic models. In a landmark study, Jordan and colleagues showed that nTreg cell development was enhanced in TS1 TCR transgenic mice (specific for influenza hemagglutinin (HA)) when they were crossed to HA transgenic mice (20). Furthermore, when the TS1 TCR was altered to have low affinity for HA, thymocytes no longer developed into nTreg cells. However, HA specific T cells (TS1 TCR) were deleted when they were crossed to mice that expressed HA encoded by a different transgene, suggesting that where and when the self-peptide is expressed impacts the fate of the T cell (21). This general observation was subsequently made in several TCR transgenic strain combinations (reviewed in (22)), but not all. Additionally, TCR transgenic mice with TCRs

specific for a foreign antigen crossed to a RAG deficient background generally lack nTreg cells unless they are crossed to mice that express the cognate antigen.

The idea that nTreg TCRs have higher reactivity for self would imply that the nTreg TCR repertoire would be distinct from conventional T cells. Indeed, several studies showed that the TCR repertoire of nTreg cells was diverse and predominantly distinct from that of non-nTreg cells (23–25). However, there is some overlap, from which it has been argued that self-reactivity can play only a limited role in nTreg cell development (26), and generally highlights the difficulty of making precise inferences about development based solely on repertoire studies.

It has been difficult to directly demonstrate that the T cell receptors expressed by nTreg cells are self-reactive. nTreg cells do not proliferate in a standard mixed lymphocyte reaction as they are inherently non-proliferative, and overt self-reactivity was not detected in T cell hybridomas expressing nTreg TCR (26). However, T cells transduced with nTreg TCR were shown to undergo enhanced homeostatic expansion in normal or lymphopenic recipients (23, 27). These observations suggest that nTreg cell self-reactivity is either limited to rare tissue specific antigens or is of an intermediate, weaker level, compared to TCR reactivity to foreign antigens. As might be expected, the creation of nTreg TCR transgenic mice provided interesting insight into nTreg cell development. Two groups independently created transgenic mice with TCR genes cloned from naturally occurring nTreg cells (28, 29). Their analysis showed that precursor frequency dramatically affected the likelihood that a given progenitor would develop into a Foxp3+ nTreg cell. Efficient nTreg cell development only occurred at very low precursor frequencies, suggesting that nTreg cells compete for crucial developmental factor(s). The limiting factor could be a rare high affinity self-antigen, or it could be a TCR extrinsic factor (such as IL-2 or co-stimulation, discussed below).

Recently, a novel fluorescent reporter mouse was created where GFP was inserted into the start site of a TCR immediate early gene (*Nr4a1* or Nur77) (30). Analysis of this mouse suggested that GFP levels specifically reflect TCR signal strength in T cells. In such $Nur77^{GFP}$ mice, polyclonal nTreg cells showed a higher level of GFP than non-nTreg cells, consistent with an overall higher self-reactivity of the nTreg cell repertoire. Interestingly, when crossed to nTreg TCR transgenic mice, GFP levels were only higher when the precursor frequency was very low, suggesting that nTreg clones compete for rare self-ligands during development.

Overall, the picture emerging is that at the population level, nTreg cells do have an overall enhanced self-reactivity compared to conventional T cells. However, each TCR does not have a digital propensity to become a nTreg or conventional T cell. Rather, their development is probabilistic, with the likelihood that a given TCR will become a nTreg cell being dependent on cellular competition and possibly other environmental factors (discussed below) (31). This contrasts with clonal deletion, which is thought to have a sharp and well-defined affinity threshold (5), and is thought not to be greatly influenced by competition or environmental factors. However, it should be noted that much of the work on clonal deletion thresholds has been done with Class I restricted CD8 T cell progenitors, whereas nTreg cell development is a Class II restricted event. Indeed, a critical unanswered issue is whether nTreg cell development and clonal deletion occur at similar or distinct thresholds. Was a nTreg cell progenitor “rescued” from clonal deletion? Or is the self-reactivity of nTreg cells, while high compared to non-nTreg cells, still beneath the threshold for clonal elimination? Further work examining clonal deletion and nTreg cell selection in the same experimental systems will be needed in the future to address this question.

iNKT cell self-reactivity

iNKT cells are a minor lineage of T cells that express a semi-invariant TCR and recognize lipid antigens in the context of the MHC class I –like molecule, CD1d (32). They are important regulators of immunity, but unlike nTreg cells, they can either suppress or enhance immune responses, depending on what cytokines they produce, and where (33). iNKT cells develop from DP progenitors and are thought to undergo positive and negative selection similar to conventional T cell progenitors, except in response to self-lipid antigens, and not self-peptide antigens (34). Like nTreg cells, iNKT cells are thought to experience agonist selection in the thymus. They have an antigen-experienced phenotype, being CD44 positive, but are not CD25 positive. They express intermediate levels of the activation marker CD69, and diverse expression of NK cell markers, including NK1.1, DX5, and NKG2D (Table I) (35). iNKT TCR transgenic mice have a markedly smaller thymus in the presence of CD1d than in the absence (36) suggesting that some clonal deletion occurs in addition to iNKT cell maturation during development, consistent with the notion of agonist selection. Nonetheless, iNKT cells can be further deleted by exposure to high affinity exogenous lipids like αGalCer or DC overexpressing CD1d (37). Thus it is possible that iNKT cell selection is similar to that proposed for nTreg cell selection, where agonist lipids select iNKT cells but very strong agonists can lead to deletion.

Like nTreg cells, the precise self-ligands involved in iNKT cell selection are not completely defined, although there is more knowledge about this for iNKT cells than for nTreg cells. The expression of CD1d specifically on DP thymocytes is required for iNKT cell selection and development (38). Bendelac and colleagues identified one potential iNKT cell selecting ligand expressed in the thymus as isoglobotrihexosylceramide (iGb3) (39). Mice deficient in β-hexosaminidase B, which is important for the production of iGb3 in lysosomes, showed a major defect in iNKT cell development and thus argued the importance of iGb3 in iNKT cell development. However, other work suggested that iGb3 is not the only glycosphingolipid deficient in mice lacking β-hexosaminidase B, which have a broad defect in glycosphingolipid processing (40). Mice deficient in iGb3 synthase, which is crucial for iGb3 biosynthesis, have normal iNKT cell development (41). Yet iGb3 shapes the repertoire in a manner similar to that which occurs naturally (42). Thus iGb3 may not be the sole selecting ligand, but is likely to be a relevant self-ligand *in vivo*. iGb3 pulsed DCs can stimulate CD69 expression and proliferation in Vα14 Tg mice (42), IL-2 production in iNKT hybridomas (or human iNKT cells) (39, 43), and activation of iNKT cells *in vivo* (44), thus it may be considered an agonist ligand, yet iGb3/CD1d tetramers do not stain iNKT cells (39). Furthermore, data suggest that APCs do not consistently display the same set of lipid ligands, and that activation associated changes in cellular lipid metabolism can alter the display (44). Further work is needed to understand the nature and timing of self-lipid recognition in iNKT cell selection and homeostasis.

In support of an agonist selection mechanism, iNKT cells developing in the thymus expressed a high level of GFP in Nur77^{GFP} mice. Interestingly, the level of GFP in peripheral iNKT cells was very low (30). It is possible that iNKT cells see an agonist self-ligand displayed by DP in the thymus, but that same ligand may not be constitutively displayed in the periphery. Alternatively, iNKT cells in the periphery may have become desensitized to their selecting ligand(s). Interestingly, while the development of iNKT cells requires CD1d, their survival and function does not (45).

CD8αα+ T cell self-reactivity

Intraepithelial lymphocytes (IELs) of the gut are enriched in T cells that express CD8αα homodimers (CD8αα+ T cells), recently referred to as natural IEL or nIEL (46). They are

thought to play an important role in immunity and tolerance at the mucosal surfaces of the body. CD8αα+ T cells can express either γδ or αβ TCRs. We will focus here on TCRαβ + CD8αα+ nIEL cells, since their specificity and selection has been more widely studied.

The site(s) of nIEL T cell development is still a matter of debate (47–49). Several studies suggested that the development of nIEL can occur through an extrathymic pathway (50–52). However, compelling evidence suggests that nIEL can and typically do arise from thymic precursors (53–56). It should also be noted that nIEL have remarkable homeostatic proliferation capacity, a feature that can complicate interpretation of various studies (57). Overall, it seems likely that multiple pathways exist for nIEL development, which are more or less prominent under certain conditions or at different periods in development.

nIEL reside between the epithelial cells, most prominently in the small intestine. Like nTreg and iNKT cells, nIEL have an activated phenotype (58) (Table I). Studies using TCR transgenic models suggested that the selection of TCRαβ + nIEL is dependent on agonist interactions in the thymus (54, 59–63). Furthermore, DP thymocytes from TCR transgenic mice that are exposed to high doses of their cognate antigen can differentiate into cells that appear similar to nIEL in thymic organ cultures (64–66, 67, Hogquist, 1998 #176). However, it is unclear if the normal thymic precursors for polyclonal nIEL encounter agonist ligands in the thymus. This is difficult to study because there are no definitive means to identify nIEL precursors in the thymus. One study showed that TL tetramers identified a subset of DP thymocytes that was enriched in progenitors (54). However, other studies contest the notion that nIEL develop via a thymic DP progenitor, instead arguing that CD44+ TCR- DN cells give rise to nIEL (68, 69). Furthermore, if the precursors to nIEL do encounter agonist ligands in the thymus, do they continue this recognition in the tissue? nIEL were recently shown to display high basal calcium levels and be refractory to TCR-dependent calcium-flux induction. Blocking the TCR on γδ+ nIEL *in vivo* led to a decrease of basal calcium suggesting that γδ+ nIEL, at least, are constantly being triggered through the TCR (70).

nTh17 T cell self-reactivity

Mature naïve CD4 T cells can differentiate into several different subsets depending on the microenvironment during stimulation. In the presence of TGF-β +IL-6 they differentiate into Th17 cells (71). Th17 cells secrete a variety of cytokines including IL-17A/F and express the transcription factors RORγt and RORα. Th17 cells are important in protection against extracellular bacteria however aberrant responses to self-antigen can lead to a variety of autoimmune diseases (72). Recently Craft and colleagues have shown evidence of IL-17 producing cells that arise developmentally, in the thymus. These naturally occurring Th17 cells (nTh17) are enriched in the presence of high affinity ligands, illustrated by the use of double transgenic mice (Table I) (73). Similar cells in the thymus and periphery of normal unimmunized mice express an activated phenotype, suggesting that nTh17 cells are selected by agonist peptides.

Finally, it is worth noting that the γδ T cell repertoire is also thought to embody substantial self-reactivity (74). Indeed some γδ T cell subpopulations bear remarkable similarity to αβ T cell subsets, e.g. γδ NKT cells and αβ NKT cells (75). However, less is understood about the specificity of γδ T cells in general, and we refer the reader to other sources for further information (76).

Measuring self-reactivity

Accurately measuring the self-reactivity of polyclonal cell populations has presented a major challenge to understanding T cell homeostasis and regulation. Cell surface markers of T cell

activation have been used in this regard. The transmembrane C-type lectin CD69 is arguably the most widely accepted marker of TCR activation, as expression is low in naïve T cells and rapidly induced in an ERK dependent fashion after TCR activation. Consistent with its potential utility as a marker of self-reactivity, some agonist selected T cell populations do constitutively express CD69 (see Table I). However, CD69 up-regulation is not specific for TCR signaling. Inflammatory stimuli, like those that induce type I interferon and toll-like receptor signaling, result in widespread up-regulation of CD69 on lymphocytes (88). Thus it is unclear if the expression of CD69 on iNKT cells and IELs reflects their constitutive perception of self-stimuli, or of other micro environmental stimuli, e.g. through NK-receptors, TLR or other pattern recognition receptors. For example, CpG induced CD69, but not GFP expression, in Nur77GFP mice (30), and other evidence suggested that antigen specific nIEL express CD69 even in the absence of antigen (89). Thus CD69 has utility as a marker of acute TCR activation in some contexts, but is of limited utility for analysis of polyclonal populations, particularly during infection or in specific tissue environments.

The cell surface glycoprotein CD5 is expressed on thymocytes and mature T cells. Its expression is low on DN thymocytes whereas DP and single positive (SP) thymocytes have intermediate to high levels of CD5 (90). High CD5 expression requires peptide-MHC contact and it has been suggested that the expression of CD5 correlates with the avidity of TCR-MHC-ligand interactions based on the observation that CD5 expression parallels TCR signal strength in thymocytes (90) and was reduced on CD4 T cells when they were deprived of Class II (91) or CD8 T cells deprived of Class I (92). Interestingly, a recent study showed that naïve T cells expressing the highest level of CD5 (and presumably the most self-reactive) were hypersensitive to cytokines (93). Thus CD5 may prove to be a valid experimental means to distinguish self-reactivity in the polyclonal repertoire. Consistent with this, CD5 levels tend to correlate with GFP levels in naïve T cells of Nur77^{GFP} mice (GLS and KAH unpublished data), and nTreg cells express a higher level of CD5 than conventional T cells (94). However, it is not yet clear if CD5 is regulated exclusively by the TCR, or in all T cell subsets, such as iNKT cells (95) or nIELs (96). Furthermore, there is evidence that CD5 plays a role in negatively regulating TCR signaling (97, 98), thus CD5 levels presumably both reflect and alter TCR self-reactivity.

The recent development of a Nur77^{GFP} mouse strain suggests its potential to be a useful tool in studying self-reactivity. GFP expression in the Nur77^{GFP} mice specifically reflected the signal strength of TCR-MHC interactions without being influenced by inflammatory stimuli (30). As discussed above, both nTreg and iNKT cells showed increased GFP levels during selection in the thymus (30). Thus further investigation of other agonist selected cell types including CD8αα+ nIEL and nTh17 cells, and of autoreactive and anergic T cells in various models, using the Nur77^{GFP} mice may be interesting in the future. In summary, there is likely no single perfect means to measure self-reactivity in lymphocyte populations, although combinations of the markers discussed above may be useful.

Molecular mechanisms of agonist selection

Although nTreg cells, iNKT cells, and CD8αα nIELs are selected by agonist interactions, each cell type requires unique cellular and molecular factors to develop. Thus it is not merely the TCR interaction that specifies fate, but the context it is encountered in. Key cytokines and co-stimulatory factors are provided in distinct thymic microenvironments, both to favor survival in the face of strong TCR stimuli, and to specify a functionally unique differentiation program. These unique combinations lead to the expression of distinct transcription factors, which play a dominant role in programming each T cell lineage as outlined in the following sections.

Unique factors in nTreg cell development

One key requirement for nTreg cell development is the interaction of CD28 with its B7 family member ligands (Figure 3). Mice deficient in the co-stimulatory molecule CD28 or CD28 ligands B7-1/B7-2 have decreased thymic nTreg cell numbers and percentages (99–101). The role that co-stimulation plays in nTreg cell development is not completely clear. Some reports suggest that co-stimulation provides a quantitative signal (along with TCR stimulation) that drives a T cell to develop into a nTreg cell. Other models suggest that co-stimulation prevents negative selection and supports nTreg cell development (reviewed in (22)). Regardless, it is clear that CD28/B7 interactions are required for nTreg cell development. B7.1 and B7.2 are primarily expressed on thymic APC, including DC and epithelial cells, and it is interesting to consider the possibility that different thymic APC are involved in selecting nTreg cells versus clonal deletion. A variety of experimental data exist on this topic, also reviewed recently in (22). To date, the collective data do not support a simple model whereby one APC is specialized for nTreg cell induction and another for deletion. However future experiments testing this in more physiologic contexts may change the picture.

NFκB is a transcription factor downstream of several pathways, but most notably in the case of nTreg cells, the CD28/B7 pathway. Therefore, it was not surprising that the development of nTreg cells also required NFκB activation. Specifically the NFκB family member c-Rel, but not NFκB1, is critical for nTreg cell development in the thymus (reviewed in (102)).

Another requirement for nTreg cell development is cytokine signaling, most prominently IL-2. Mice deficient in IL-2Rβ have a significant decrease in nTreg cells (103). The phenotype of mice deficient in IL-2 is less dramatic however, with only a 50 percent decrease of nTreg cells in the thymus (104, 105), but this is due to redundancy with other common γ chain cytokines like IL-15 (106). Thus, IL-2 plays a critical role in nTreg cell development and function (107). A two-step model has been proposed where TCR engagement leads to the expression of the high affinity IL-2 receptor, which ultimately leads to IL-2 induced Foxp3 expression and nTreg cell commitment (Figure 3, steps B & C) (108). Mice that express constitutively active STAT5, a transcription factor downstream of IL-2 signaling, further support the importance of IL-2 in nTreg cell development (109). These mice have increased nTreg cell numbers and a more diverse nTreg cell TCR pool that confirms that IL-2 acts primarily on pre-instructed nTreg precursors.

The master transcription factor for nTreg cells is forkhead box P3 (Foxp3). Foxp3 is not only required for their suppressive capabilities, but also for their development. Mice deficient in *Foxp3* or scurfy mice, which have mutated *Foxp3* gene, developed lethal multi-organ inflammation. The development of multi-organ inflammation was attributed to a defect in thymic nTreg cell development, demonstrated by a specific deletion of *Foxp3* in T cells. Furthermore, adoptive transfer of $Foxp3^+$ T cells into neonates protected *Foxp3* deficient mice from their autoimmune pathology (110, 111). Altogether, it is clear that nTreg cells require Foxp3 for development and it is thought to seal their functional fate.

Unique factors in iNKT cell development

The development of conventional T cells requires antigen presentation by thymic epithelial cells, whereas iNKT cells require lipid presentation by CD1d positive cortical thymocytes (32). Cortical thymocytes not only present lipids to iNKT cells, but also provide many co-stimulatory signals necessary for commitment and development.

The SLAM signaling pathway plays a major role in iNKT cell development (Figure 4). The SLAM family surface receptors SLAM1 and SLAM6 are expressed on cortical thymocytes

and mediate iNKT cell positive selection. Therefore, mice deficient in both SLAM1 and SLAM6 have a defect in iNKT cells (112). Furthermore, mice that lack the SLAM adaptor protein (SAP), which is required for SLAM signaling, are deficient in iNKT cells (113–115). The importance of the SLAM/SAP pathway was also highlighted in a study where MHC class II expression was restricted to cortical thymocytes (116). Conventional MHC class II restricted T cells positively selected by the thymocytes had a phenotype very similar to iNKT cells, and was SAP dependent (117) further suggesting that the SLAM/SAP signaling provided by the cortical thymocytes drives development of this unique lineage (Figure 4).

Downstream of TCR/SLAM interactions are several signaling molecules including the Src tyrosine kinase Fyn. Fyn is required for iNKT cell development, therefore mice deficient in Fyn lack iNKT cells (118, 119). In addition to the Fyn signaling pathway NFκB is critical for iNKT cell development. NFκB is required for downstream signaling of the TCR as well as inhibiting apoptosis. Inhibition of several different NFκB family members (by conditional knockout mice or inhibitors) has significant influence on iNKT cell development (120–123). Early growth response 2 (Egr2) is a transcription factor downstream of the calcineurin-NFAT pathway that is activated upon TCR stimulation. It is required for efficient positive selection, maturation and survival of iNKT cells (124).

Finally, the transcription factor PLZF is expressed in iNKT cells directly after positive selection. It is now clear that PLZF is a master transcription factor for iNKT cells and is required for both their maturation and function (125, 126). Furthermore, iNKT cells from mice deficient in PLZF lose their activated phenotype, have a defect in trafficking, and have impaired effector functions (127). More studies will be required to establish precisely how PLZF is induced in iNKT cells and what are its direct gene targets.

Unique factors in nTh17 cell and CD8αα T cell development

The unique factors required for the development and selection of nTh17 cells and CD8αα+ T cells have yet to be uncovered. It would be tempting to hypothesize that the critical factor required for nTh17 cell development/selection is RORγt, the master regulator of differentiated Th17 cells (128). It has been published that nTh17 cells express RORγt; however, the requirement for RORγt in nTh17 cell development has yet to be established (73). It will be important to dissect RORγt targets in both Th17 cells and nTh17 cells to determine if they are similar or distinct. The unique factors involved in CD8αα+ T cell development are even less clear. Additional studies will need to be done in order to understand the signaling and interactions involved for development and maturation of these cells.

TGF-β common survival/development mechanism in thymus?

The development of nTreg cells, iNKT cells, nTh17 cells, and nIEL occurs in the thymus under conditions that normally drive clonal deletion. It is possible that factors specific for the thymic environment might promote an increased survival threshold in these cell types. One potential common mechanism for survival in the thymus is the cytokine TGF-β (Figure 1). TGF-β is a cytokine that has pleiotropic regulatory effects on many different cell types. Ouyang et al. showed that blocking TGF-β signaling in the thymus led to increased deletion of nTreg cells (129). This sensitivity to apoptosis was also observed when thymocytes from TGF-β RII –deficient mice were stimulated in vitro with anti-CD3. These results as well as the significant reduction of developing nTreg cells in the TGF-β RII-deficient mice suggest that TGF-β plays an important role in nTreg cell development and survival in the thymus (129).

iNKT cell development also requires TGF-β signaling in the thymus. iNKT cells from the thymus express increased levels of both TGF-βRI and TGF-βRII. Furthermore, it was shown, using mice deficient in TGF-β signaling components, that TGF-β is required for protection against apoptosis, lineage expansion, and maturation of iNKT cells (130).

Recent work by Chen and colleagues provided evidence that the TGF-β plays an important role in nIEL development (131). Mice deficient in TGF-β, TGF-β RI or the downstream factor Smad3 have significantly reduced numbers of CD8αα+ nIEL. Furthermore, mice deficient in TGF-β have reduced frequency of a putative nIEL precursor in the thymus, showing that TGF-β signaling plays a critical role in nIEL development.

The recently characterization of nTh17 cells also highlighted the importance of TGF-β in development (73). Using TGF-β R DN mice, Marks et al. showed the development of thymic Th17 cells requires TGF-β signaling.

The requirement for TGF-β in developing nTreg cells, iNKT cells, nTh17 cells, and nIEL provides a potential common mechanism of survival of agonist-selected cells (Figure 1). It is not clear if TGF-β plays a role in differentiation or of cells selected by agonist ligands, or merely a survival role. Further studies will be needed to address this and to elucidate the targets of TGF-β signaling. It is possible that common factors other than TGF-β, not yet elucidated, are also important in survival of cells following agonist interactions.

Functional significance of self-reactivity in peripheral T cell homeostasis and immunity

The usefulness of lymphocytes with reactivity to self-antigens is two-fold: they can both promote immunological tolerance and enhance immunity to foreign antigens. nTreg cells provide a classic example of the ability of self-reactive cells to promote immunological tolerance. Ablation of nTreg cells results in massive lympho- and myelo-proliferation. Mature nTreg cells require stimulation via the TCR to exert their suppressive effects in the periphery, although once activated, can exhibit non-specific suppression (132). Indeed, the high GFP levels in peripheral nTreg cells from Nur77^{GFP} mice suggested that nTreg cells continually encounter stimulatory self-peptides (30)(Figure 3). That certain nTreg cell TCR specificities are more common in one anatomic site than another is consistent with continued TCR ligand recognition driving tissue localization (27). Several effector mechanisms have been elaborated for nTreg cells, including production of suppressive cytokines, reducing stimulatory properties of DC, and conversion of inflammatory ATP to adenosine. It is thought that because of their activated phenotype, nTreg cells are more responsive to self-antigens than naïve T cells and could compete for interactions with APC. Interestingly, there is evidence that nTreg cells integrate environmental cues with self-reactivity to tailor the regulatory response to the type of tissue or to the type of inflammatory response going on (133).

iNKT cells can alter immune responses in multiple ways depending on the type of cytokine response they produce (33), and are often considered “innate immune” cells, as their rapid production of cytokines early during infection can alter immunity. iNKT cells offer protection from certain infections (134), but also may contribute to autoimmune disease (135) and allergic hypersensitivity (136). Some species of pathogens produce lipids that can directly activate iNKT cells through the antigen receptor, including *Sphingomonas* and *Borrelia burgdorferi*. However, iNKT cells become activated during many other bacterial, viral, and fungal infections that are not thought to involve a foreign lipid that stimulates through the iNKT T cell receptor (134). Data are emerging to support the idea that during infection, APC display stimulatory self-lipids to activate iNKT cells, particularly in

combination with inflammatory cytokines (137–140). For example, iNKT cell activation during *Salmonella* infection could be blocked by antibodies to CD1d, despite the fact that the bacteria themselves do not produce stimulatory lipids (137). Recent evidence suggests that this may be accomplished through TLR induced alterations in endogenous lipid catabolism (44). Thus, iNKT cells may represent a scenario where strong TCR stimulation is involved in selection in the thymus, but it does not continue in the periphery (Figure 4). During infection or inflammation, APC can be triggered to display the same or similar stimulatory lipids and thereby activate iNKT cells.

The specific functions of CD8αα$^+$ nIEL are rather poorly defined to date. It is presumed that they survey their environment for danger signals and respond via the production of inflammatory cytokines, cytolysis, or tissue repair. Mice deficient in TCRγδ cells are more susceptible to colitis suggesting that TCRγδ nIEL have an important role in the homeostasis of the epithelium (141, 142). Also, TCRαβ nIEL were capable of preventing development of inflammatory bowel disease (IBD) (143). Is TCR recognition important for this role? nIEL in the intestine clearly exist in a partially activated state (144, 145). They express CD69, various cytolytic molecules, and rapidly produce IFNγ (146). However, despite their partially activated state, they proliferate poorly in response to mitogenic signals. Furthermore, it is not known if the activated state is a consequence of antigen receptor stimulation or other environmental stimuli. Further work including the development of IEL TCR transgenic models may be required to fully understand the role of TCR specificity in IEL function.

Since they were only described recently, little is known about the function of nTh17 cells. However, they were shown to be able to mediate host protection in the liver following toxin-induced hepatitis (73). Protection was mediated through secretion of the cytokine IL-22, previously shown to be protective in the liver (147). Future experiments will be also be needed to understand if TCR recognition of self-peptides is involved in nTh17 function.

In summary, the thymus produces several minor T cell lineages that nonetheless play crucial roles in immunity. The requirement for perception of strong TCR signals during development in the thymus unifies these lineages. The cytokine TGFβ may play a key role in the ability of progenitors to survive such strong signals. However, beyond that, there are very few commonalities in the factors involved in differentiation. Each lineage expresses distinct key transcription factors, which dictate unique tissue localization, effector functions, and roles in immunity.

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Glossary

Agonist selection	positive selection of T cells by agonist ligand, in which cells with overtly self-reactive TCRs are directed into a mature T cell lineage
Nur77 (*Nr4a1*)	immediate early gene up-regulated by TCR stimulation
LIP	lymphopenia-induced proliferation
Clonal deletion	process where self-reactive specific T and B cells are eliminated from the repertoire

iGb3	isoglobotrihexosylceramide
nTh17 cell	natural IL-17 producing CD4 T helper cell that arises in the thymus via agonist selection
nIEL	natural intraepithelial lymphocytes that expresses CD8αα homodimers and resides in mucosal surfaces of the body
iNKT	invariant natural killer T cell that expresses semi-invariant TCRs and recognizes lipid antigens in the context of CD1d

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Side bar

The special case of "innate CD8" T cells

Another unusual population of T cells has sometimes been considered under the umbrella of agonist selection are innate CD8 T cells (77, 78). These cells were first studied in ITK deficient mice, where CD8 SP thymocytes accumulate with a memory phenotype and ability to rapidly produce cytokines (79, 80). Like iNKT cells, the development of this population required selection on hematopoietic cells (80), CD28, and SAP (81). One model was that ITK deficiency increased TCR signaling thresholds such that only those cells with agonist interactions with self would survive (82). Interestingly, subsequent studies identified an expanded population of innate CD8 T cells in multiple other gene deficient or mutant mice, including KLF2, CBP, Id3, and SLP76Y145F (83), several of which would not be predicted to alter TCR signaling thresholds. Key insight came from analysis of innate CD8 T cells in KLF2 deficient mice, where wild-type cells in a KLF2 deficient environment also acquired an innate CD8 T cell phenotype, suggesting an indirect or bystander effect of soluble factors, and not a cell-intrinsic effect (84). Further studies confirmed a similar indirect mechanism operates in ITK, CBP (85), Id3 (86), and SLP76Y145F mice (87). Interestingly, the innate CD8 phenotype results from IL-4 over-produced by αβ or γδ iNKT cells, which expand in all of these strains of mice (83) (Figure 2). In fact, the requirement for iNKT cell produced IL-4 explains why the effect is dependent on hematopoietic cell selection, CD28, and SAP, and not because CD8 T cells themselves have an altered recognition of MHC. In fact, the level of Nur77GFP in CD8 T cells is not increased in KLF2 KO chimeras, where they acquire the innate CD8 phenotype (Y.J. Lee and K.A. Hogquist, unpublished data). Altogether these data show that innate CD8 T cells, although they bear phenotypic and functional resemblance to iNKT cells, and to CD8α+ T cells (64), are not a product of agonist selection in the thymus.

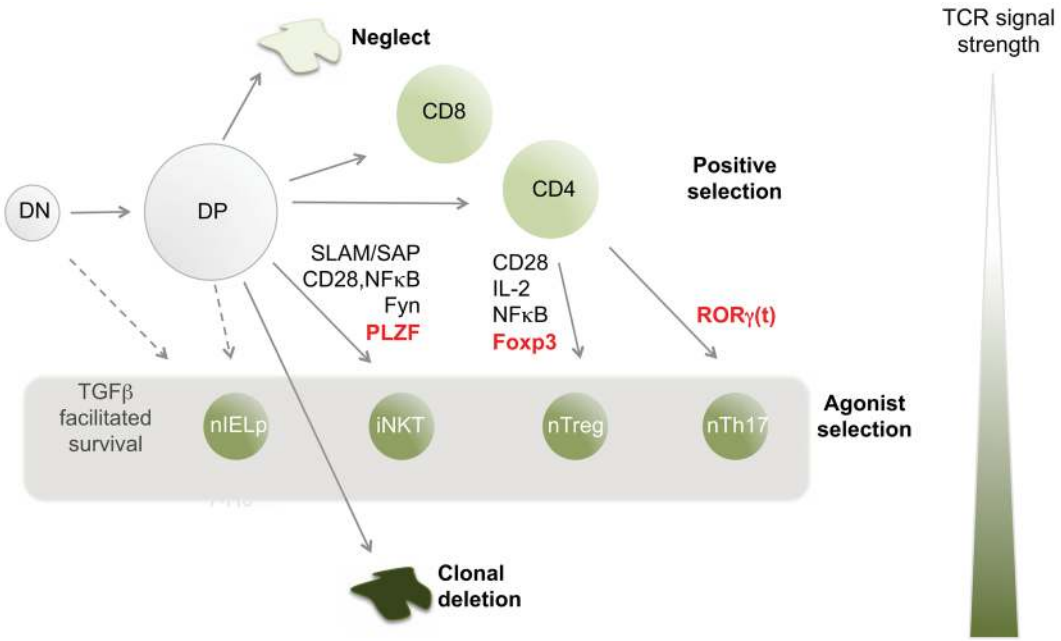

Figure 1. Proposed agonist selection of unique subsets of TCRαβ + T cells in the thymus

The thymus generates a large pool of immature thymocyte progenitors that express clonally distinct αβ TCRs (DP). The reactivity of these TCRs for self-MHC ligands (shown by color scale, darker green being more reactive) plays a defining role in fate. Cells that do not express a TCR, or express a TCR with no ability to react with thymic ligands will die by **neglect**. Those with low affinity undergo **positive selection** to become CD4+ helper or CD8+ killer cells and the major cellular products of the thymus. Those with high affinity undergo **clonal deletion** to preserve self-tolerance. However, several smaller sub-populations of lymphocytes also develop in the thymus: the precursors to CD8αα+ intraepithelial lymphocytes (nIELp), invariant NK T cells (iNKT), natural FoxP3+ regulatory T cells (nTreg) and natural T helper cells that can produce IL-17 (nTh17). Evidence summarized in this review suggests that these four subsets experience stronger interactions with self-ligands during development (**agonist selection**). Each of these subsets requires distinct molecular factors (some of which are listed). They arise at distinct stages; can be Class I, Class II, or CD1d restricted; and can express either or no co-receptor. However, their commonalities include: 1) an activated/memory phenotype, 2) evidence for stimulation by high affinity (agonist) self-ligands during development, 3) a requirement for TGFβ, and 4) regulatory function in immunity.

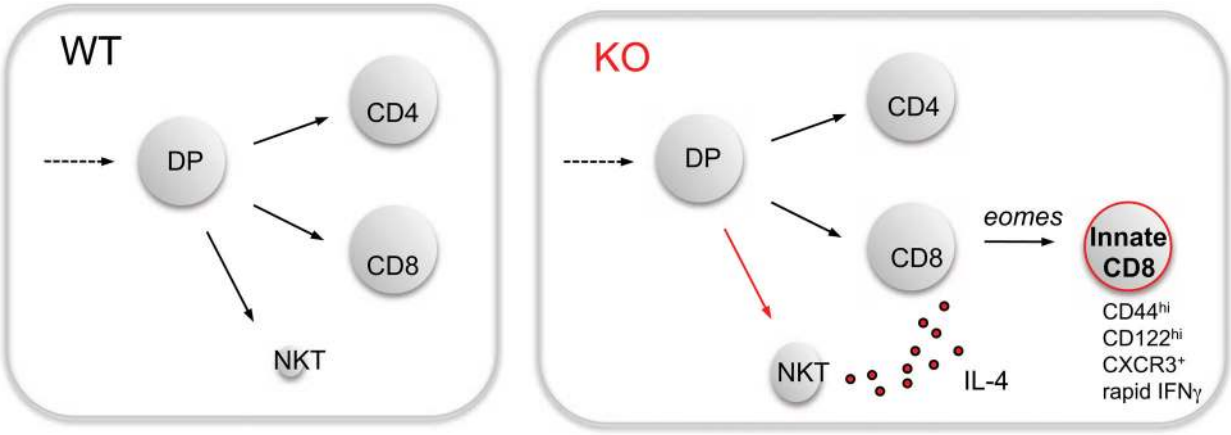

Figure 2. Cross talk between developing iNKT and CD8SP progenitors in the thymus generates innate CD8 T cells

The iNKT, CD4 helper and CD8 killer lineages normally develop largely independently (WT, left panel). However, in some gene deficient or mutant mice (*ITK, KLF2, CDP, Id3*, and *SLP76Y145F*) (KO, right panel) and in some inbred strains (like BALB/c), iNKT cells that constitutively produce IL-4 are expanded. IL-4 acts on developing CD8 lineage cells to upregulate the transcription factor *eomes* and a number of downstream target genes. This leads to the generation of CD8 T cells with a memory phenotype and capacity to rapidly produce cytokines.

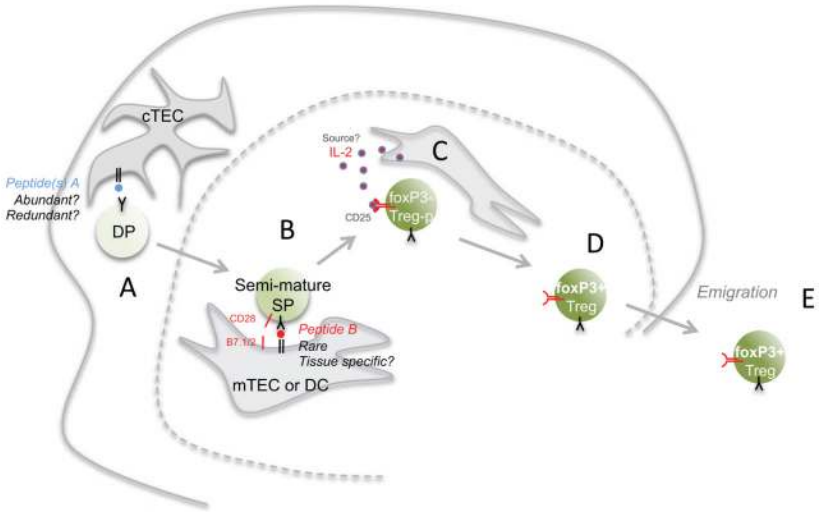

Figure 3. Unique aspects of nTreg cell development in the thymus

A) A double positive (DP) progenitor is positively selected upon interacting with peptides presented by cortical epithelial cells. In this model, we propose that nTreg and conventional T cells are selected in a similar manner in the cortex, *i.e.* via low affinity interactions with abundant self-peptides. A given clone might recognize multiple distinct self-peptides in this affinity range. B) After positive selection, progenitors migrate from the cortex to medulla and differentiate to the semi-mature (HSA^{hi}, $CD62L^{lo}$) stage. If a progenitor encounters self-peptides displayed by B7.1/2+ medullary APC (mTEC or DC) with high affinity, this triggers the upregulation of CD25. C) A CD25+ Foxp3− nTreg cell precursors requires IL-2 for survival and differentiation to the mature Foxp3+ nTreg cell stage. D) Mature, self-reactive nTreg cells emigrate from the thymus to populate the peripheral lymphoid organs. E) In the periphery, nTreg cells continue to perceive higher affinity self-ligands (presumably same or similar to peptide B) and regulate self-tolerance in the steady state.

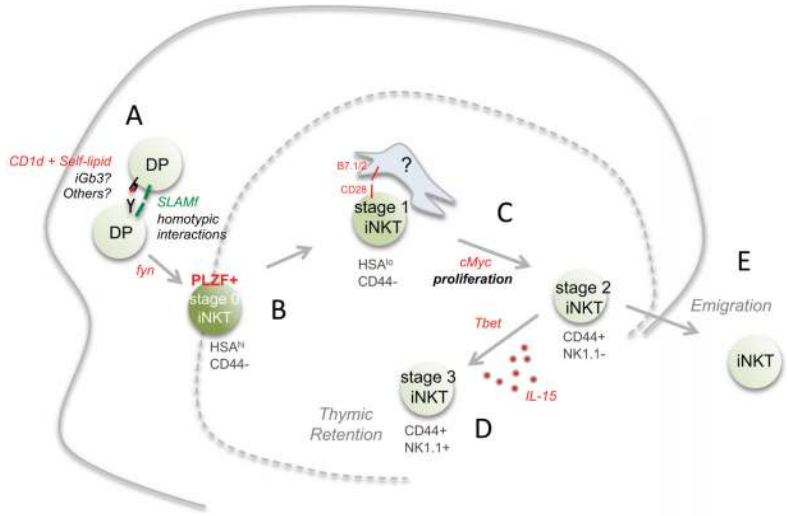

Figure 4. Unique aspects of iNKT cell development in the thymus

A) iNKT cells arise from rare DP progenitors that express a Vα14-Jα18 TCR. Interaction with hematopoetic APC (DP cells) that express CD1d molecules and stimulatory self-lipids, facilitated by SLAM family member interactions, initiate positive selection through the src family kinase fyn. B) The earliest post-selection iNKT cell is an HSA^{hi} "stage 0" cell, which already express the iNKT lineage transcription factor PLZF. As the progenitor develops, it downregulates HSA (stage 1), and upregulates CD44 (stage 2). C) During this time it undergoes cellular proliferation requiring cMyc. The cellular interactions that iNKT cells make in the medulla are not well characterized, but CD28 interaction with B7.1/2+ APC is required for iNKT cell expansion. D) Stage 2 iNKT cells upregulate Tbet and respond to IL-15. The most mature (stage 3) iNKT cell expresses NK1.1 and is retained in the thymus for long periods. E) Alternatively, stage 2 iNKT cells can emigrate from the thymus and populate the spleen and liver. Some evidence suggests that peripheral iNKT cells do not continuously perceive stimulatory self-lipid. Rather, APC can be activated during infection to display stimulatory self-lipids and thus activate iNKT cells.

Table I

Evidence for high self-reactivity in T lymphocyte subpopulations

Subset	Phenotype	Specificity	Repertoire	TCR transgenic systems	Other	GFP level Nur77^{GFP} mice
nTreg	CD44hi CD25+ GITR+	MHCII/self-peptides (generally rare?, tissue-specific?)	Diverse	MCHII restricted TCR Tgs with antigen increase Treg cells	Rapid expansion in lymphopenic recipients	High in progenitors High in mature cells
iNKT	CD44hi CD69+	CD1d-self lipids (iGb3 and others?)	Oligoclonal	Vα14-Jα18 TCR transgenics have reduced thymic cellularity	Peripheral cells can produce cytokines in response to self lipid/ CD1d	High in progenitors Low in mature cells
CD8αα + nIEL	CD44int CD69+	Unknown	Oligoclonal	Increased in MHCI TCR Tgs with antigen	Gut iEL exhibit high basal calcium flux	Low in mature cells
nTh17	CD44hi α4β1+ CCR6+	MHCII/self peptides uncharacterized	?	Increased in MHCII TCR Tgs with antigen		Not determined