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Relationship between CD4 Tregs and anergy in vivo

Lokesh A. Kalekar[1] and **Daniel L. Mueller**[2]

[1]Department of Medicine and Center for Immunology, University of Minnesota Medical School, Minneapolis, Minnesota, USA

Abstract

Selective suppression of effector $CD4^+$ T cell functions is necessary to prevent immune cell-mediated damage to healthy tissues. This appears especially true during pregnancy or in individuals predisposed to autoimmunity. $Foxp3^+$ regulatory T (T_{reg}) cells and induction of anergy, an acquired state of T cell functional unresponsiveness in $Foxp3^-$ cells, have both been implicated as mechanisms to suppress dangerous immune responses to tissue-restricted self antigens. Anergic $CD4^+$ T cells and T_{reg} cells share a number of phenotypic and mechanistic traits—including the expression of CD73 and folate receptor 4 (FR4), and the epigenetic modification of T_{reg} cell signature genes—and an interesting relationship between these two subsets has recently emerged. In this review, we will compare and contrast these two subsets as well as explore the role of anergy in the generation of peripheral T_{reg} cells.

Introduction

T cell self-tolerance mechanisms can be broadly characterized as central or peripheral (Fig. 1). Central tolerance mechanisms destroy high affinity self-reactive T cells during thymic development, or else induce their differentiation into a regulatory T (T_{reg}) cell lineage (1). Nonetheless, central tolerance appears insufficient to clear all self-reactive T cells and other peripheral tolerance mechanisms are necessary (2, 3). Peripheral tolerance may rely on 1) ignorance, wherein autoreactive T cells never encounter their cognate antigen, 2) deletion, whereby self-specific peripheral T cells are destroyed after TCR engagement, 3) anergy, which is a state of functional unresponsiveness induced upon self antigen recognition, and/or 4) $Foxp3^+$ T_{reg} cell-mediated suppression of dangerous T cell responses against self antigen. Each of these potential tolerance mechanisms has been clearly defined in numerous *in vivo* experimental systems using TCR-transgenic responder T cells—typically at abnormally high cell frequencies. However, much less is known about self-tolerance in the natural polyclonal $CD4^+$ T cell repertoire.

T_{reg} cells control tolerance to some tissue-restricted self antigens

Two recent studies took advantage of experimental antigens (peptides derived from either the P1 bacteriophage Cre recombinase (Cre) or the *Aequorea victoria* enhanced green fluorescent protein (eGFP)) whose expression in mice was directed by transgenic tissue-

[2]Corresponding Author: Daniel L. Mueller (muell002@umn.edu).

[1]Present Address, Department of Dermatology, University of California, San Francisco CA, USA

specific promoters, and additionally made use of pMHCII tetramers to detect polyclonal $CD4^+$ T cells that respond to these model self antigens (4, 5). Both experiments demonstrated that ubiquitous (both thymic and peripheral) expression of self antigen leads to deletion of 60 to 95% of the highest affinity self-specific $CD4^+$ T cells, with the remaining low affinity cells left functionally unresponsive to antigen (Fig. 1). In contrast, restriction of self antigen expression exclusively to non-thymic tissues leads to a tolerance that appears to rely solely on ignorance by naive autoreactive T cells. The strongest evidence for an active peripheral self-tolerance mechanism in these studies came in the form of self antigens that were only weakly expressed in the thymus and, in particular, were otherwise restricted to mucosal tissues (e.g., Cre protein expression in the intestinal brush border driven by a *Vil1-Cre* transgene, or expression in airway Clara cells driven by a *Scgb1a1-Cre* transgene). These antigens induce little central deletion, and instead self-tolerance relies on the generation of self pMHCII-binding $Foxp3^+$ T_{reg} cells (5). Importantly, these T_{reg} cells appear to suppress the conventional $CD4^+$ T cell responses to self antigen, as systemic ablation of $Foxp3^+$ T_{reg} cells at the time of a self antigen immunization led to a restoration of the conventional T cell clonal expansion and cytokine production response to near control levels (5).

In addition to T_{reg} cells, several other suppressor cells have been described such as the Tr1 cells, myeloid-derived suppressor cells (MDSCs), as well as the regulatory B cells. However, it is well established that $Foxp3^+$ T_{reg} cells maintain immune homeostasis and prevent adverse immune responses throughout the lifespan of an individual. Foxp3 is an essential lineage-defining transcription factor of T_{reg} cells (6, 7), as mutations or deletion of *Foxp3* gene lead to impaired generation of T_{reg} cells and cause severe autoimmunity in humans and mice (6, 8). Furthermore, instability of *Foxp3* expression allows for the trans-differentiation of T_{reg} cells to T effector cell lineages capable of causing autoimmunity (9, 10). Although Foxp3 is considered to be the master regulator of T_{reg} cell suppressive function, the expression of *Foxp3* is not sufficient to maintain a stable T_{reg} cell lineage (11, 12). Numerous T_{reg} cell-specific genes are in fact expressed independently of Foxp3 protein, including *Il2ra* (the gene for CD25), *Ctla4, Ikzf4* (Eos), and *Nrp1* (neuropilin 1) (11, 13, 14). These data suggest that additional lineage-defining factors act with Foxp3 to ensure the generation of a stable, functional T_{reg} cell compartment.

Most $Foxp3^+$ T_{reg} cells undergo their terminal differentiation in the thymus and are referred to as thymic T_{reg} (tT_{reg}) cells, whereas others originate in the periphery (particularly at mucosal barrier surfaces exposed to food antigens and commensal organisms) from conventional $Foxp3^-$ $CD4^+$ T cells, and are consequently called peripheral T_{reg} (pT_{reg}) cells (15, 16). It is believed that the self pMHCII-specificity and suppressive functions of these two T_{reg} subsets complement one another in preventing immunopathology (17). tT_{reg} cells are primarily responsible for maintaining general T cell immune homeostasis, while pT_{regs} control immunopathology that is directed against tissue-restricted antigens in mucosal tissues such as the lung and gut (17).

Natural T_{reg} (nT_{reg}) cells are defined as all T_{reg} cells generated *in vivo* and thus include tT_{reg} and some pT_{reg} cells (16). For the case of most nT_{reg} cells, epigenetic changes associated with the development of a unique T_{reg} cell methylome (T_{reg}-me) are thought to be necessary

for stable Foxp3 expression. The T_{reg}-me consists of de-methylated DNA CpG motifs at four T_{reg} cell-related gene loci, including *Tnfrsf18* (the gene for GITR), *Ctla4*, *Ikzf4*, and the *Foxp3* conserved non-coding DNA sequence 2 (CNS2) (18). Therefore, the expression of Foxp3 and the development of the T_{reg}-me are independent and complementary events. TCR signaling is required for T_{reg}-me induction, which in turn helps maintain the function and stability of T_{reg} cells (12, 18). It has been proposed that within the thymus, the strength of TCR signaling controls Foxp3 expression, whereas the duration of signaling establishes the de-methylations that are associated with the T_{reg}-me (12, 18). In contrast, quantitative de-methylation of the T_{reg}-me loci (including *Foxp3* CNS2) appears unnecessary for some pT_{reg} cell differentiation. Rather, *Foxp3* transcription in mucosal tissue T_{reg} cells can be stabilized by *Tgfb1*- and *Smad3*-dependent activation of the conserved non-coding DNA sequence 1 (CNS1) enhancer element (19, 20).

Even though pT_{reg} cells have been found to play an essential role in the suppression of tissue-specific immunopathology in adoptive transfer experiments (17), the experimental data of Malhotra *et al.* (4) and Legoux *et al.* (5) have seemed to point toward a thymic origin for the increased self antigen-specific T_{reg} cells observed when tissue-restricted antigen expression in the thymus is only modest. *Vil1* promoter-driven Cre-specific T_{reg} cells express high levels of Helios and Nrp1, both markers compatible with a thymic T_{reg} cell origin (5). Additionally, genetic disruption of the CNS1 sequence from the *Foxp3* locus fails to inhibit the generation of Cre-specific $Foxp3^+$ T_{reg} cells or restore the functional responsiveness of the conventional Cre-reactive $CD4^+$ T cell population in *Vil1*-Cre transgenic mice (5). Interestingly, $Foxp3^+$ T_{reg} cells specific for eGFP are more frequently detected in the thymus of *Ins2* promoter-driven *eGFP* transgenic mice (4), whereas the number of Cre-specific T_{reg} cells in *Vil1-Cre* and *Scgb1a1-Cre* transgenic mice is similar to the wild type (5). Taken together, the experiments suggest that weak expression of self antigen in the thymus (presumably as a consequence of *Aire*-mediated transcription in medullary thymic epithelial cells) is insufficient to elicit central deletion of all high affinity autoreactive $CD4^+$ T cells, yet facilitates the maintenance of self-tolerance by inducing the differentiation of highly functional self pMHCII-specific T_{reg} cells that suppress peripheral responses. In contrast, CNS1-dependent pT_{reg} cells may be more important to suppress mucosal T cell reactivity to food and commensal antigens introduced only after thymic development has completed.

Anergy induction versus pT_{reg} cell generation – differences and similarities

Notably, neither of these elegant studies suggested a role for anergy in the development of tolerance to peripherally expressed self antigens. Should one conclude, therefore, that the induction and maintenance of anergy are unimportant to natural peripheral self-tolerance? Or is it possible that anergy plays some role in the establishment of self-tolerance in concert with antigen-specific $Foxp3^+$ T_{reg} cells? Historically, anergy has been defined as state of functional inactivation wherein $CD4^+$ T cells lose the capacity to produce growth factors and proliferate in response to pMHCII recognition (21–23). But it has been unclear how long this state lasts and/or whether it is an intermediate state *in vivo*. Anergy can be induced in $CD4^+$ T cells when TCR signaling is unaccompanied by strong CD28 co-stimulatory receptor ligation (24–26). The binding of co-inhibitory receptors such as CTLA4 and PD-1

reinforces the induction and/or maintenance of anergy (27, 28). *In vivo*, anergy is observed in $CD4^+$ T cells following systemic, repeated exposure to a soluble antigen or superantigen in the absence of infection or adjuvant (29). Likewise, the adoptive transfer of naive self-antigen specific $CD4^+$ T cells to normal mice bearing relevant self pMHCII complexes leads to the development of functional unresponsiveness (30, 31). This is in contrast to the adoptive transfer of self-reactive $CD4^+$ T cells into lymphopenic hosts lacking a population of $Foxp3^+$ T_{reg} cells, whereby a failure of anergy induction typically leads to dangerous T effector cell clonal expansion and differentiation, with consequent severe immunopathology (31, 32). It is important to note that there are many independent paths to anergy, which result in different types “functional unresponsiveness” or anergic states. The different ways that assays are performed lead to varied readouts because the measurement of anergy has not been standardized. We define anergy here as defective proliferation in response to pMHCII recognition by CD4 T cells.

Multiple biochemical signaling defects have been ascribed to the $CD4^+$ T cell anergic state, including blocked signaling to Ras and mitogen-activated protein kinases 1 and 8, and impaired expression of functional activator protein 1 (AP-1) transcription factor complexes (21, 33, 34). Up-regulation of counter-regulatory gene products such as *Cblb*, *Dgka*, *Rap1*, *Rnf128*, *Itch*, *Dtx1*, and *Ndrg1* (which in most cases lie downstream of NFAT- and Egr2/Egr3-dependent transactivation) are understood to underlie this development of functional unresponsiveness (35–39). Despite the presence of multiple counter-regulatory molecules and signal transduction defects, anergic $CD4^+$ T cells retain their capacity to recognize pMHCII and undergo anergy reversal in its absence (29).

Anergy is also reversed in the setting of T cell lymphopenia, regardless of the continued presence of antigen (40). This may relate to T_{reg} cell deficiency as well as to increased availability of homeostatic cytokines in lymphopenic hosts. Consistent with this, anergy induction and maintenance are strongly antagonized by IL-2R signaling that leads to downstream PI-3K, Akt, and mTORC1 activation, and that promotes a shift in metabolism away from oxidative phosphorylation and toward aerobic glycolysis (41, 42). The activation, clonal expansion, and differentiation of aggressive $CD4^+$ T effector cells is associated with unique metabolic demands that require mTORC1 activity to integrate environmental, nutrient, and intracellular signaling cues (43). If activated $CD4^+$ T cells fail to increase their glycolytic metabolism due to inefficient mTORC1 activation, they can become anergic (44).

It is worth noting that inhibition of mTORC1 signaling not only induces anergy, but also promotes the differentiation of $Foxp3^+$ T_{reg} cells from conventional precursors and stabilizes the expression of *Foxp3* (45–47). Not surprisingly, T_{reg} cells themselves appear to be anergic, as they do not produce IL-2 or proliferate when stimulated unless exogenesis IL-2 is provided. Nonetheless, mTORC1 activity is also necessary for proliferation by T_{reg} cells. In fact, it has been suggested that alternate activation and subsequent inhibition of mTORC1 facilitate optimal T_{reg} cell function and lineage stabilization (48).

Our own investigations have led to the discovery of two additional anergy factors: ecto 5’-nucleotidase (*Nt5e*; hereafter referred to as CD73) and folate receptor 4 (*Izumo1r*, FR4). Both molecules are found expressed at moderate levels on $Foxp3^+$ T_{reg} cells (as well as at

lower levels on conventional $CD4^+$ naive T cells and T follicular helper cells), but CD73 and FR4 are most highly expressed on conventional antigen-experienced $CD44^{hi}$ $CD4^+$ T cells following the induction of anergy (31, 49, 50). Little is understood about the function of FR4 on T cells; however, the gene encoding FR4 (*Izumo1r*) was recently shown to encode Juno, the receptor for Izumo1 (51). In reproductive biology, Izumo1 expression on sperm and Juno on eggs guide normal sperm-egg fusion during fertilization (51). It remains unclear how the expression of FR4 relates to anergy.

CD73 is an ecto-enzyme that normally acts in tandem with CD39 to convert extracellular ATP to adenosine (52). Extracellular ATP is secreted by activated T cells and also accumulates at sites of tissue ischemia and necrosis (53–56). Although the exact mechanisms remain unclear, it is thought that the CD39/CD73-mediated depletion of extracellular ATP limits the triggering of purinogenic receptors such as P2X7. Activation of P2X7 in some systems stabilizes the expression of the glycolytic gene positive regulator Hif1α (57). CD73-dependent production of extracellular adenosine may also serve to resist glycolytic reprogramming through the suppressive effects of the adenosine A2a receptor and its intracellular second messenger cAMP on mTORC1 activity (54). Note that CD39 is not expressed on anergic conventional $CD4^+$ T cells, and the role that CD73 plays in the induction or maintenance of anergy remains uncertain. Nevertheless, these findings may indicate one mechanism by which $CD39^+$ T_{reg} cells facilitate the induction of anergy through their conversion of extracellular ATP to the more tolerogenic nucleotide adenosine (58, 59).

An anergic polyclonal $CD4^+$ T cell compartment in healthy mice

Tissue-restricted expression of *Aire*-regulated transgenic model antigens has clearly defined self pMHCII-specific $Foxp3^+$ T_{reg} cells as an important barrier to peripheral self-reactivity by $CD4^+$ T cells, whereas no evidence for peripheral anergy induction was obtained. Nonetheless, extrathymic *Aire*-regulated expression of self antigens within secondary lymphoid organs has also been shown to functionally inactivate naïve conventional autoreactive $CD4^+$ TCR-transgenic T cells, as well as promote the accumulation of self antigen-specific T_{reg} cells (60). We have questioned whether the detection of naturally occurring anergic $CD4^+$ T cells has been hindered by the lack of sensitive and specific markers for detection of anergic T cells in the polyclonal repertoire.

Our previous discovery of CD73 and FR4 as reliable surface markers of anergic conventional T cells, together with the development of antigen tetramer technologies by our collaborators, offered us the opportunity to thoroughly investigate tolerant polyclonal $CD4^+$ T cells (31, 49, 50, 61). In healthy B6.g7 mice, insulin B chain (InsB) pMHCII tetramer-binding conventional polyclonal $CD4^+$ T cells were found to be naive in phenotype and generally ignorant of self antigen (49, 50), consistent with the results of Legoux *et al.* (5), and Malhotra *et al.* (4). On the other hand, conventional InsB/I-A^{g7} tetramer-binding polyclonal T cells isolated from the pancreas-draining lymph node of non-diabetic NOD mice demonstrated 1) evidence of previous antigen-recognition (increased CD44 expression), 2) up-regulation of FR4 and CD73, and 3) defective IFNγ production, all consistent with the induction of anergy (49, 50). Notably, InsB/I-A^{g7}–specific $Foxp3^+$ T_{reg}

cells were also identified and found to be similar in number in both syngeneic strains. These data, therefore, generally supported the model that normal naive $CD4^+$ T cells often ignore tissue-restricted self antigens in the presence of a stable self antigen-specific T_{reg} cell compartment. Nonetheless, NOD mice predisposed to autoimmune disease development apparently reveal InsB/I-A^{g7} complexes to the naive peripheral T cell repertoire, and then the anergy mechanism becomes available to maintain self-tolerance.

Of course, these observations in disease-prone NOD mice begged the question of whether anergy develops only when other immune tolerance mechanisms fail. This point was addressed in a series of experiments that examined fetal tolerance in healthy B6 pregnant mice, following mating to syngeneic B6 males made transgenic for a ubiquitously expressed 2W self antigen (62, 63). Clonal expansion of 2W pMHCII-specific $Foxp3^+$ T_{reg} cells is known to be necessary for fetal success in this system (64), with Foxp3 expression apparently stabilized by the CNS1 enhancer element (65). Given that the niche for T_{reg} cells having any particular antigen specificity appears limited (66, 67), and the observations of similar metabolic programming for both the anergic and T_{reg} fates (48), we hypothesized that during pregnancy both anergic T cells and $Foxp3^+$ T_{reg} cells will be present among the 2W-specific T cells. 2W/I-A^b tetramer-binding $CD4^+$ T cells were observed to undergo a 5–fold clonal expansion during pregnancy that resulted in approximately equal numbers of unresponsive $CD44^{hi}$ $FR4^{hi}$ $CD73^{hi}$ anergic T cells and $Foxp3^+$ T_{reg} cells (49). Interestingly, most of the anergic compartment disappeared during the postpartum period, perhaps reflecting a requirement for continuous TCR recognition of fetus-derived 2W/I-A^b complexes to maintain anergy or cell survival. Therefore, normal pregnancy is associated with $CD4^+$ T cell anergy to fetal antigens.

The discovery of functionally unresponsive $CD44^{hi}$ $FR4^{hi}$ $CD73^{hi}$ $CD4^+$ T cells specific for self InsB/I-A^{g7} or fetal 2W/I-A^b complexes lends support to the notion that peripheral self-tolerance can rely on $CD4^+$ T cell anergy. To investigate the generality of these observations, we characterized the natural repertoire of polyclonal conventional $CD4^+$ T cells that express these anergy markers in combination. A subpopulation of $CD44^{hi}$ $FR4^{hi}$ $CD73^{hi}$ $Foxp3^-$ cells was found to make up 2–5% of the polyclonal $CD4^+$ T cell repertoire in the secondary lymphoid organs (but not thymus) of multiple normal mouse strains, and this subpopulation increased with age (49). Furthermore, expression of these markers was found to strongly correlate with proliferative arrest and defective cytokine production, the two hallmarks of anergy. Finally, loss of *Aire*-dependent gene expression and central deletion in the thymus of mutant $Aire^{-/-}$ mice led to an increase in the proportion of peripheral $CD4^+$ T cells that have this anergic phenotype (49).

Evidence of anergy in this polyclonal $CD44^{hi}$ $FR4^{hi}$ $CD73^{hi}$ $CD4^+$ T cell compartment cannot be taken as proof of self antigen-reactivity. However, loss of the anergic phenotype in fetal antigen-specific T cells postpartum following the expulsion of fetal tissues did suggest a requirement for continuous TCR engagement to maintain the anergic state (49). Consistent with this, steady state polyclonal anergic T cells were shown to express high levels of PD-1, CTLA4, CD69, and Nrp1—all molecules whose expression can be induced and/or maintained by persistent TCR engagement. Uniformly increased levels of CD5 and a Nur77 reporter gene in anergic T cells also suggested that these cells have high affinity TCRs

specific for available self pMHCII complexes, similar to *bona fide* self antigen-specific T_{reg} cells (68–70). Additionally, the frequency and number of anergic cells was not different between germ-free mice and specific pathogen free mice from our colony, suggesting that the functional inactivation observed here was not solely in response to commensal antigens (unpublished data). Therefore, we now hypothesize that many or all anergic phenotype $CD4^+$ T cells in secondary lymphoid organs have recently recognized self pMHCII.

Anergy reversal can result in immunopathology or alternatively lead to protective T_{reg} cell differentiation

Additional experiments were designed to formally test the self-reactivity of anergic phenotype $CD4^+$ T cells by transferring them into autoimmune disease-prone *Tcra*$^{-/-}$ mice and observing for the development of immunopathology. Initial experiments failed to demonstrate reliable autoimmune disease development, but instead led to the discovery that polyclonal anergic T cells can trans-differentiate into $Foxp3^+$ T_{reg} cells (49). Following the adoptive transfer of highly purified $Foxp3^-$ $CD44^{hi}$ $FR4^{hi}$ $CD73^{hi}$ $CD4^+$ T cells into lymphopenic *Tcra*$^{-/-}$ mice, as many as 25% of the resulting peripheral $CD4^+$ T cells expressed Foxp3. Analogous to tT_{reg} cells that experience persistent high affinity TCR engagements during their differentiation in the thymus, most of these anergy-derived T_{reg} cells also expressed Nrp1 and demonstrated a fully de-methylated T_{reg}-me (including the *Foxp3* CNS2).

Nrp1 was originally thought to distinguish tT_{reg} cells from peripherally differentiated pT_{reg} cells (71, 72). However, this notion has since been challenged because activated T_{reg} cells (which include both tT_{reg} cells and pT_{reg} cells) as well as inducible T_{reg} cells generated *in vitro* from naive $CD4^+$ T cells can express Nrp1 (73, 74). Similar to natural polyclonal T_{reg} cells, anergy-derived polyclonal T_{reg} cells demonstrated an ability to protect lymphopenic *Tcra*$^{-/-}$ mice from inflammatory bowel disease (49). Anergy-derived T_{reg} cells also suppressed the development of autoimmune arthritis, and demonstrated a capacity to induce anergy in other self antigen-specific $CD4^+$ T cells (49).

Interestingly, the treatment of *Tcra*$^{-/-}$ recipients of *Foxp3*^{DTR} anergic T cells with diphtheria toxin to destroy any developing *Foxp3*-expressing cells not only prevented the accumulation of anergy-derived T_{reg} cells, but also led to the development of severe wasting disease and the generation of tissue-specific autoantibodies, further demonstrating the self-reactivity of naturally anergic polyclonal $CD4^+$ T cells (49). Taken together, the data suggest that anergic phenotype polyclonal $CD4^+$ T cells have potentially dangerous TCRs that are specific for peripheral self pMHCII complexes, but these TCRs also make them ideal progenitor cells for the peripheral differentiation of $Foxp3^+$ T_{reg} cells. Furthermore, these experiments indicate that anergy-derived T_{reg} cells cannot be readily distinguished from tT_{reg} cells based on phenotype or T_{reg}-me. Therefore, it is conceivable that some of the $Nrp1^+$ $Foxp3^+$ T_{reg} cells that preferentially expanded during the course of self antigen immunization by Malhotra *et al.* (4) and Legoux *et al.* (5) were originally conventional $CD4^+$ T cells that had undergone anergy induction following peripheral recognition of the same tissue-restricted self antigen.

Role of Neuropilin-1 in the generation of anergy-derived T_{reg} cell progenitors

Nrp1 is a transmembrane glycoprotein on the surface of many cell types, including T_{reg} cells, dendritic cells (DCs), NKT cells, neurons, and endothelial cells (71, 72, 77) and its function is important for axonal guidance in the developing nervous system (78). Nrp1 is a co-receptor for the soluble class 3 semaphorins (79), but can also promote angiogenesis by binding to vascular endothelial growth factor (VEGF) (77). More recently, $Nrp1^+$ T_{reg} cells have been shown to bind to semaphorin-4a on plasmacytoid DC, with subsequent recruitment of PTEN and inhibition of downstream Akt and mTORC1 signaling pathways (75, 80). The stability of the T_{reg} cell lineage is maintained by a Nrp1:semaphorin-4a axis, and Nrp1 can induce the expression of T_{reg} cell lineage-related genes independently of Foxp3 (75, 81).

As described above, a majority of polyclonal anergic $CD4^+$ T cells express high levels of Nrp1 and demonstrate a unique pattern of partial T_{reg}-me DNA de-methylation. Consistent with this coordinate expression of Nrp1 and de-methylation of the T_{reg}-me, $FR4^+$ $CD73^+$ anergic $CD4^+$ T cells sorted for high Nrp1 expression and transferred to lymphopenic *Tcra*$^{-/-}$ hosts were found to be most efficient for the generation of $Foxp3^+$ T_{reg} cells (49). Conversely, the $Nrp1^-$ fraction of anergic T cells was found to be a poor source of T_{reg} cell progenitors, and preferentially differentiated toward a T_H17 lineage that caused wasting disease following adoptive transfer to *Tcra*$^{-/-}$ mice ((49) and unpublished data). A previous study similarly showed that autoreactive $CD4^+$ T cells lacking Nrp1 would induce a more severe form of experimental autoimmune encephalitis (EAE), with their $Nrp1^-$ $CD4^+$ T cells also appearing biased towards the T_H17 lineage (80). Loss of Nrp1 expression on T_{reg} cells limits the nuclear localization of Foxo1/3a, leads to a failure of Foxp3 expression, and allows for the up-regulation of transcription factors such as RORγt that facilitate T_H17 differentiation (75). Thus, the skewing of $Nrp1^-$ polyclonal anergic $CD4^+$ T cells towards a T_H17 fate suggests that a similar $Nrp1^-$ directed genetic program may govern lineage selection in both anergic T cells and T_{reg} cells.

It remains unclear whether Nrp1 expression is essential for the induction and/or the maintenance of the anergic phenotype. Nrp1 was previously implicated in immunological synapse formation, and on T_{reg} cells Nrp1 enhances the duration of the T_{reg} interactions with dendritic cells (DCs) resulting in higher sensitivity to small amounts of antigen (82). How the TCR repertoire and self pMHCII-specificity of anergic T cells relates to this expression of Nrp1 remains uncertain. Nrp1 has also been shown to directly induce the expression of CD73 in T_{reg} cells (75). Nevertheless, our analysis of fetal antigen-specific anergic T cells suggested that Nrp1 is expressed only after the anergic phenotype ($CD73^{hi}$ $FR4^{hi}$) is established. Only half of fetal antigen-specific $FR4^{hi}$ $CD73^{hi}$ $CD4^+$ T cells expressed Nrp1 at day 10 of gestation (unpublished data), whereas Nrp1 expression on anergic phenotype T cells increased to 80–90% by day 18 ((49) and unpublished data). Thus, the level of Nrp1 on anergic T cells may simply indicate the degree of unresponsiveness. Alternatively, Nrp1 expression on anergic T cells may reinforce longer interactions with self pMHCII on DCs to promote quantitative de-methylation of T_{reg}-me genes and induce the expression of Foxp3.

Conclusions

Despite recent advances in biological therapy, the treatment for autoimmune diseases remains problematic. For instance, the treatment of rheumatoid arthritis (RA) with drugs that suppress aberrant immune responses to self antigens still poses risk for infection, as these drugs can have off-target effects leading to the suppression of T cells that recognize and destroy pathogens (2). A better approach for the treatment of autoimmune disorders would be to reinforce a stable T cell tolerance to self-antigens, while maintaining full responsiveness to non self-antigens.

Anergy, a state of long-term functional unresponsiveness, is one such peripheral tolerance mechanism that has been studied extensively. Nevertheless, until recently it has never been shown that anergy can be induced in self antigen-reactive $CD4^+$ T cells that escape negative selection in the thymus, mainly because of a lack of identifying markers specific for anergy development. It has also remained uncertain as to why the immune system would maintain viable anergic T cells long-term. Our experiments have made use of a panel of predictive markers for anergy development in the natural polyclonal $CD4^+$ T cell repertoire—CD44 expression to identify antigen-experienced T cells, the absence of Foxp3 expression to exclude T_{reg} cells, and a combination of elevated CD73 and FR4. The results now suggest that $CD4^+$ T cells that have persistently recognized peripheral self pMHCII enter a $CD44^{hi}$ $CD73^{hi}$ $FR4^{hi}$ unresponsive state. Moreover, anergy cannot be sustained in the absence of self antigen recognition or in the setting of T_{reg} deficiency. Anergy reversal can lead to the differentiation of functional T_{reg} cells that suppress autoimmune disease or, alternatively, potentially pathogenic $T_{eff\text{-}mem}$ cells. Finally, the up-regulation of Nrp1 expression on anergic T cells is predictive of a partially de-methylated T_{reg}-me and serves as a marker for T_{reg} cell progenitors (Fig. 1).

Self antigen-specific T_{reg} cell generation from anergic T cells is now reminiscent of the *in vivo* "infectious tolerance" model previously proposed by Kendal and Waldmann (75). Infectious tolerance is described as a process during which a "tolerant" state is passed on from one group of lymphocytes to another. Newly tolerant T cells would then reprogram, survey the immune system, and pass on their tolerant state to other T cell populations to continuously maintain self-tolerance. Since T_{reg} cells are important for inducing and/or maintaining anergy (31) and anergic T cells in turn can alter their epigenetic and transcriptional programs to become T_{reg} cells (49), anergic T cells may represent the intermediate reprogramming stage before themselves becoming surveying T_{reg} cells that maintain self-tolerance.

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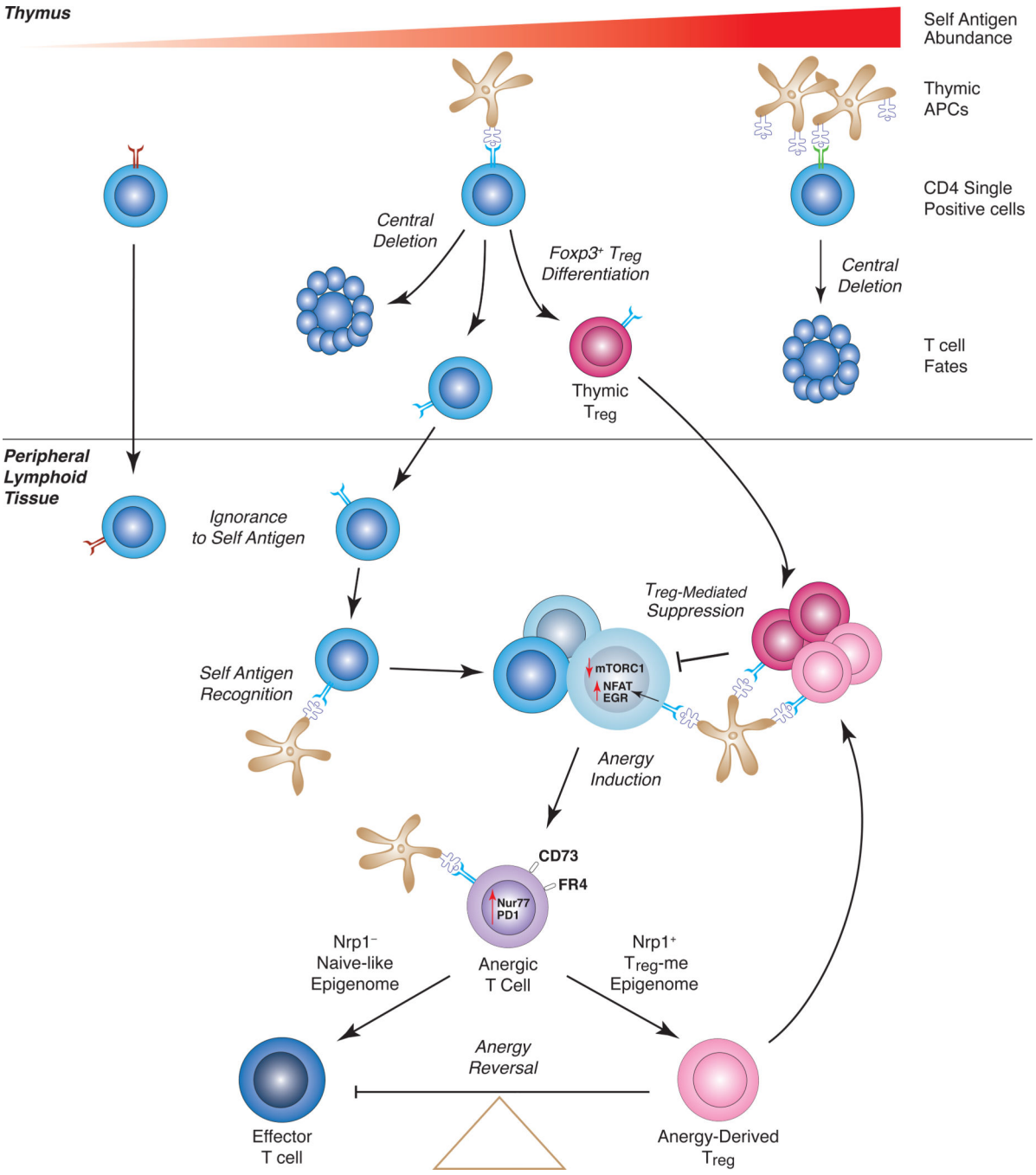

Figure 1. The Role of Anergy in pTreg Generation – A Model

CD4 single-positive thymocytes specific for tissue-restricted self antigens can experience one of several fates. Abundant thymic self pMHCII presentation leads to central deletion. If self pMHCII abundance is very low, thymocytes escape to the periphery and often ignore the antigen. For the case of intermediate self pMHCII complex abundance, some thymocytes will die, some will differentiate to a T_{reg} cell fate, and some will escape the thymus. Self antigen-specific $CD4^+$ T cells that escape into the periphery can recognize peripheral self pMHCII, and through the suppressive actions of T_{reg} cells become anergic cells that express

CD73 and FR4. Persistent antigen encounter induces Nrp1 expression and partial de-methylation of the T_{reg}-me in some of the anergic T cells. $Nrp1^+$ Anergic T cells then become precursors for T_{reg} cell differentiation. Upon conversion to a stable pT_{reg} cell lineage, anergy-derived T_{reg} cells join the tT_{reg} cell pool to suppress immunopathology and reinforce anergy induction.