Published in final edited form as:
Immunol Res. 2008 ; 41(2): 137–153. doi:10.1007/s12026-008-8017-1.

Negative Regulators in Homeostasis of Naïve Peripheral T Cells

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Abstract

It is now apparent that naïve peripheral T cells are a dynamic population where active processes prevent inappropriate activation while supporting survival. The process of thymic education makes naïve peripheral T cells dependent on interactions with self-MHC for survival. However, as these signals can potentially result in inappropriate activation, various non-redundant, intrinsic negative regulatory molecules including Tob, Nfatc2, and Smad3 actively enforce T cell quiescence. Interactions among these pathways are only now coming to light and may include positive or negative crosstalk. In the case of positive crosstalk, self-MHC initiated signals and intrinsic negative regulatory factors may cooperate to dampen T cell activation and sustain peripheral tolerance in a binary fashion (on-off). In the case of negative crosstalk, self-MHC signals may promote survival through partial activation while intrinsic negative regulatory factors act as rheostats to restrain cell cycle entry and prevent T cells from crossing a threshold that would break tolerance.

Keywords

T cells; MHC; sensitization; desensitization; cell cycle; negative regulation; tolerance

The Influence of Self-MHC in T-Cell Quiescence and Survival

Self-MHC and naïve T cell survival

The role of self-peptides complexed to major histocompatibility complex proteins (self-MHC) to maintain homeostasis of naïve T cells has been the subject of extensive investigation over the past decade. This work has revealed apparent differences in the sensitivity of different T cell subsets to the absence of self-MHC. Perhaps the most informative experiments have attempted to characterize the expansion of peripheral lymphocytes in MHC-replete or MHC-deficient lymphopenic environments. The idea that “space” in the peripheral lymphoid organs drove lymphocyte proliferation arose from the observation that under conditions of lymphopenia such as those produced by non-myeloablative, lymphodepleting regimens in humans or mice, or under conditions encountered by adoptively transferred lymphocytes or bone marrow cells into lymphopenic hosts, T cells would expand without exogenous antigens. It is now apparent that this “lymphopenia-induced proliferation” or “homeostatic proliferation” (HP) results not only from “space”, but also from interactions of T cell receptors (TCR) with self-MHC, and from lessened competition for cytokines including interleukin-7 (IL-7), or in some cases, IL-2 and IL-15 (1–4).

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Lessons from HP

The initial characterization of HP suggested that this process led T cells to acquire memory-like phenotypes (5). T cells generated by HP are not necessarily equivalent to memory T cells generated via responses to foreign antigen, as the frequency of effector cells appears to be lower with less robust molecular signaling profiles in T cells after HP than after response to foreign antigen (6–8). Nevertheless, HP and exposure to foreign antigen can generate functionally equivalent, protective CD8 T cell responses in the presence of CD4 T cell help (9), and many experiments evaluating the functional outcome of HP used unseparated T cell populations, so interpretations could be confounded by the fact that HP favors survival of memory T cells. Specifically, clonal competition promotes expansion of memory T cells at the expense of naïve T cells (10,11); cytokine responsiveness similarly tilts the composition of the reconstituted population towards cells with memory phenotypes (12); and regulatory (CD4/CD25/FoxP3+) T cells (Treg) also can contribute to the balance of naïve and memory cells that repopulate a lymphopenic environment (13). More recent experiments with purified naïve T cells have allowed for the emergence of a model that defines the significance of self-MHC in naïve T cell homeostasis.

The environment that gives rise to HP is an essential determinant of the ultimate phenotype of surviving T cells. Specifically, the tempo of IL-7 mediated HP is slow (14), HP driven by constitutive levels of IL-15 is more rapid, generating memory phenotype cells (4), and HP driven by elevated levels of IL-2/IL-15 is rapid with differentiation into both effector and memory phenotype cells (2). The slow paced (IL-7-dependent) HP seems to be restrained by signals delivered through CD24 expressed in bone marrow derived dendritic cells (DC) (15), but differentiation from naïve T cells to memory phenotype T cells following rapid IL-2 and IL-15-dependent HP is more comparable to events driven by encounter with foreign antigen. In both cases, HP likely induces attenuation of intrinsic negative regulatory pathways that maintain T cells in a non-proliferative state.

Unlike HP in MHC-replete hosts, a majority of naïve T cells die or make at most a few divisions if the lymphopenic periphery is devoid of MHC (16–21), a result consistent with the premise that HP is itself partly driven by recognition of self-MHC complexes. Both CD4 and CD8 T cells undergo HP, although CD4 T cells expand less in response to MHC and CD8 T cells are more sensitive to its absence. In fact, while the requirement for self-MHC in naïve CD8 T cell survival is generally accepted (although it may not apply to all CD8 T cell subsets) (22), the delayed erosion of naïve CD4 T cells in lymphopenic hosts has led to some controversy regarding the significance of self-MHC signals in the survival of these cells.

Self-MHC and CD8 T cell homeostasis

The requirement for Class I MHC in CD8 T cell survival was first inferred by the inability of CD8 thymic emigrants to seed MHC-deficient peripheral organs (16), with the differential capacity of naïve and memory CD8 T cells to survive in an environment devoid of Class I MHC subsequently reported in a system using LCMV as a source of antigen to generate immunological memory (17). The requirement for MHC in survival of naïve CD8 T cells has since been confirmed in both transgenic and polyclonal T cells (see for example (23,24)). An intriguing observation is that CD8 T cells do not die immediately after transfer to MHC-deficient environments. Rather, they survive and can respond to accessory cell signals for at least one week (23), and their eventual death is dependent on expression of the death factor protein Fas and its interaction with Fas ligand (FasL) (24). Rocha et al showed that survival of naïve CD8 cells was not only dependent on MHC expression, but also required the right MHC-restricting element (25). Moreover, while the concept that MHC expression is dispensable for survival of memory CD8 T cells is generally accepted, their experiments also showed that memory cells can only expand when antigen is presented in the context of the right

MHC-restricting element, and at least HY-transgenic memory T cells underwent rapid erosion in an MHC-deficient environment (25). This suggests interactions with self-MHC by different memory T cell clones could result in different outcomes regarding HP and survival.

Self-MHC and CD4 T cell homeostasis

Although inevitably most CD4 T cells will die in an MHC-deficient environment, their half-life is estimated at three to four weeks (20,26). One explanation for this invokes the possibility that the survival requirement for self-MHC is an acquired trait in naïve peripheral CD4 T cells. Specifically, Lantz and colleagues (26) proposed a model that separates naïve peripheral CD4 T cells into 3 stages based specifically on their requirement for self-MHC. Figure 1 illustrates this model, where we have added hypothetical fates for each stage of CD4 T cells after transfer to an MHC-deficient environment. Recent thymic emigrants represent a population of "*Stage 1 CD4 T cells*" that do not divide and have a half-life of about one week, regardless of the presence or absence of MHC. These recent thymic emigrants eventually mature into "*stage 2 CD4 T cells*" that can divide a few times by HP, suggesting maturation from stage 1 to stage 2 is preprogrammed and not driven by recognition of self antigen. "*Stage 3 CD4 T cells*" with a $CD44^{hi}$ phenotype appear about 2 weeks after HP in lymphopenic hosts and require MHC for extensive proliferation (50-fold more in MHC-positive than in MHC-deficient hosts). As a consequence of their proliferative capacity, stage 3, naïve CD4 T cells constitute the majority population (>80%) found after HP in lymphopenic, MHC-replete mice (26).

Self-MHC in desensitization vs. sensitization of naïve T cells

There is an intrinsic paradox to the model described above, since activation of potentially autoreactive T cells with sufficient affinity for self must be restrained while at the same time survival of T cells with lower affinity for self must be supported. Two explanations for the role of self-MHC in naïve peripheral T cell homeostasis have thus been advanced. Grossman and Paul (27) initially proposed that T cells with measurable reactivity for self-antigens were desensitized or 'tuned' by continued interactions in the periphery with MHC and self-peptides against which they were selected in the thymus. This may in part reflect a higher level of the self antigen in the periphery than in the thymus. Several reports indicate that there is a sufficiently high enough threshold for self-antigens in the periphery that autoreactivity can result. MHC dependent peripheral tuning of these T cells prevents this (7,28,29). In an alternative explanation proposed by Stefanova and Germain (30) self-MHC interactions in the periphery would keep T cells in a state of partial activation or 'sensitization' with survival as the outcome, allowing T cells to respond to foreign antigen. Stefanova and Germain base their hypothesis on data showing that interactions between TCR and self-MHC promote modest receptor clustering and CD3ζ chain phosphorylation (20,30), and in the case of CD4 T cells, activation of Rap1 and Rac1 enhancing motility (31).

The observation that T cells undergoing HP in MHC-replete hosts can overcome tolerance could be used to argue in favor of sensitization as the dominant model. Specifically, lymphoid reconstitution by HP can lead to autoimmune phenotypes or T-cell mediated tumor rejection in several experimental models (32–36). However, release of autoreactive cells by HP may be the exception rather than the rule as autoimmunity after HP is generally restricted to strains with a propensity for immune mediated diseases such as the NOD mouse. In the case of tumor rejection, Bracci et al (36) showed that adoptive transfer of tumor-immune spleen cells to mice that were lymphodepleted by cyclophosphamide conditioning led to permanent tumor regression in 100% of mice, but naïve spleen cells were ineffective, suggesting the effect is dependent on T cells with a memory phenotype. We obtained comparable data in two independent models. First, we showed that adoptive transfer of potentially autoreactive T cells from pre-diabetic NOD donors to lymphopenic MHC-replete hosts (NOD-SCID) led to diabetes in 45 days, but adoptive transfer of wild type T cells from C57Bl/6 mice to

lymphopenic MHC-replete hosts (B6-SCID) did not create a diabetic phenotype (Jubala, unpublished). Similarly, adoptive transfer of wild type T cells from C57Bl/6 mice to lymphopenic MHC-replete hosts (B6-SCID) delayed, but did not prevent growth of heterotopic syngeneic Lewis Lung carcinoma[1].

Recent experiments from Reddy's group used a conventional model of graft vs. host disease (GVHD) to test the hypothesis that HP released cells from tolerance. In their system, T cells were allowed to undergo HP for 2 weeks in lethally irradiated syngeneic hosts (B6) or in MHC-replete hosts (B6-SCID) prior to adoptive transfer into allogeneic Balb/c recipients. Intriguingly, allogeneic recipients that received HP T cells showed significantly better survival and less severe clinical GVHD than those that received allogeneic naïve T cells (37). The dampening of GVHD was due to the expanded HP cells and not to residual or newly generated Treg cells, arguing in favor of the desensitization or tuning model, although it is possible that this suppressive effect might be due to unique properties of alloreactivity. The conclusion that naïve, but not memory T cells were responsible for GVHD preceded these experiments (38–40).

The tuning and sensitization models are not mutually exclusive in MHC-replete hosts. Quantum signaling in response to varying avidity of TCR for self-peptides in polyclonal populations might direct tuning of naïve CD4 T cells under high affinity conditions and sensitization under low affinity conditions, respectively, restraining incipient, self-reactive cells and priming naïve cells to react against non-self antigens. Still, tuning and sensitization could be incompatible in the absence of MHC. If the dominant role of MHC is to tune naïve peripheral T cells, the predicted result of adoptive transfer experiments into MHC-deficient hosts would be self-reactivity (autoimmunity), while if the dominant role of MHC is to sensitize low affinity T cells, the predicted result of adoptive transfer experiments into MHC-deficient hosts would be T cell anergy or T cell death. Unfortunately, as mentioned above, both autoreactivity and T cell death have been reported in MHC depleted hosts such that distinguishing between these models may not be possible using this approach.

However, data from such MHC-deficient models provide more support for the tuning hypothesis. Specifically, Bhandoola and colleagues (7) reported rejection of MHC-positive skin grafts in approximately one third of MHC Class II-deficient animals that received naïve CD4 T cells with lymphocytic infiltrates detectable in the skin of all the animals, suggesting both that autoreactive cells could persist in the MHC-deficient environment and that they could then be activated upon encounter with self-MHC. We reached similar conclusions using mice that were doubly deficient for MHC Class I and MHC Class II. Our initial experiments were designed to exploit the predilection for survival of memory cells in the absence of self-MHC. As this model allows enrichment of antigen experienced memory T cells *in vivo* (17), we reasoned that 'parking' T cells from diabetic NOD mice in an MHC-deficient environment would render naïve (and presumably non-autoreactive) T cells unable to survive, leaving behind only the less frequent, antigen-experienced (and presumably autoreactive) T cells. This presented an unparalleled opportunity to study unique properties of diabetogenic T cells. While the experiments in diabetes-prone NOD mice bore out this prediction, we obtained similar results using diabetes-resistant B6 mice. This was somewhat unexpected, and it suggested that the absence of self-MHC led to the generation and/or survival of autoreactive T cells independent from the genetic background or the donor's propensity for autoimmunity.

We expanded this observation to a tumor rejection model that allowed us to test survival and autoreactivity of T cells undergoing HP in an MHC-deficient environment. First, we verified that neither naïve T cells nor antigen experienced T cells (from mice challenged with Lewis

[1]CM Jubala et al, MHC-dependent desensitization of intrinsic anti-self reactivity. Submitted

Lung carcinoma) could mount effective anti-tumor responses after 2 weeks of HP in lymphopenic, MHC-replete hosts. In contrast, our experiments and those of Bracci (36) showed that adoptive transfer of tumor-immune T cells under these conditions led to tumor rejection. Next, we examined the behavior of these cells in MHC-deficient mice. Both naïve T cells and antigen experienced T cells showed delayed tumor growth or outright rejection of tumor challenge in MHC-deficient mice. When present, tumors were infiltrated by CD4 T cells, but persistence of both CD4 and CD8 T cell subsets in this environment required exposure to tumor in the donor. These observations could be explained by release of CD4 T cells from intrinsic negative regulation in the absence of MHC. In other words, the same mechanisms that restrain T cell proliferation might control "tuning". In fact, Liu et al showed CD24 expressed in DCs controls proliferation and survival of CD4 and CD8 T cells transferred to lymphopenic hosts; in the absence of CD24, adoptively transferred T cells undergo unregulated and destructive HP leading to fulminant death (15).

Intrinsic Negative Regulators of T Cell Activation

Tuning might engage pathways that control the transition from G0 to G1 and G1 progression in T cells. It is now apparent that survival and quiescence are actively enforced in T cells (41–47). This paradigm has long been operative in other systems where survival requires signals from a substrate or matrix (avoidance of anoikis) (48,49) and where quiescence is enforced by signals delivered when cells contact each other (50). Although the microenvironment that supports peripheral lymphocytes does not include a fixed matrix, these cells share many other characteristics with cells derived from the embryonic mesoderm (*e.g.*, fibroblasts); hence, survival and quiescence must be enforced by alternative mechanisms.

The process of thymic education dictates that naïve peripheral T cells must interact with self-peptides presented by MHC (*i.e.*, potential autoantigens) in order to survive, but the transition of positively selected (and thus potential autoreactive) thymocytes to the periphery presents an intriguing problem. Naïve, recent thymic emigrants express high levels of T cell receptors, so the avidity for self-MHC might trigger their activation and result in autoimmunity (Figure 1). To prevent such activation, quiescence is enforced by several interrelated negative regulatory pathways of lymphocyte, including molecules that dampen signaling such as Ian5 (51) and by negative transcriptional regulators such as the transducer of ErbB2-1 (Tob1) (52), nuclear factor of activated T cells-c2 (Nfatc2) (43), and Smad3, which is primarily responsible for integrating anti-proliferative and pro-survival signals delivered through the transforming growth factor-β (TGF-β) receptor (53–57).

Ian5 in negative regulation and survival

Ian5 (also known as GTPase of immunity-associated nucleotide binding protein 5 or Gimap5) is a member of a protein family consisting of GTP-binding proteins characterized by a common GTP-binding motif and coiled-coil motifs (58). One of the first lines of evidence that Ian5 was involved in supporting quiescence and survival came from the realization that the lyp mutation responsible for lymphopenia observed in the Biobreeding (BB) diabetes-prone (DP) rat resides in Ian5 (59). Several studies linked members of the IAN gene family with regulation of apoptosis; specifically, mouse Ian5 associates with anti-apoptotic proteins Bcl-2 and Bcl-xL (60) and T cells from Ian5-deficient rats show reduced mitochondrial integrity (61). Thymic development in the BB-DP rat is largely normal, but peripheral CD4+ T cells are reduced in number and peripheral CD8+ T cells are virtually absent. Moreover, the phenotype of surviving peripheral T cells indicates they are recent thymic emigrants (RTE), so death must occur rapidly after thymocytes enter the periphery (51,62,63). On the other hand, the Ian5 knockout mouse shows compromised survival of both peripheral T cells and double positive thymocytes (60).

An important observation linking negative regulation to survival of peripheral T cells was that T cells in the BB-DP rat showed upregulation of the activation proteins CD25 and OX40, increased spontaneous incorporation of BrdU, and reduced expression of the cell cycle inhibitor p27/Kip-1 (63,64). T cells from Ian5-deficient rats spontaneously enter this state of partial or incomplete activation, and this is not simply a response to the lymphopenic environment, but rather it is due to elevated activity of MEK that in turn leads to constitutive activation of NFκB. Moreover, the phenotype is intrinsic to the T cells, as it occurs in bone marrow chimeras where Ian5 mutant T cells develop in a wild type environment (51). Thus, partial T cell activation in Ian5-deficient animals is not a consequence of lymphopenia but is rather part of the stimulus leading to cell death, providing one biochemical link between survival and quiescence pathways (42,51).

Tob1 in negative regulation and survival

Tob1 is a member of the Btg/Tob family of transcriptional repressors (65). Regulation of Tob1 is multifaceted and includes shuttling between the nuclear and cytoplasmic compartments, phosphorylation, and ubiquitin-dependent degradation. These biochemical events are regulated respectively by constitutive nuclear import (mediated by a nuclear localization signal) and nuclear export in response to stimulatory signals (66), phosphorylation by Erk and Jnk mitogen activated protein kinases (67,68), and ubiquitination by SCFSkp2 ubiquitin ligase (69).

In peripheral T cells, Tob1 overexpression enforces quiescence directly by supporting expression of the CDK inhibitor p27/Kip-1 and by silencing the IL-2 promoter, and indirectly by associating with and modulating the activity of SMAD transcription factors (52). Endogenous Tob1 is stabilized by partial antigen stimulation with altered peptide ligands, resulting in anergy (70). Although Tob1 deficiency does not produce gross immune defects, our analyses of T cell receptor signaling in T cells from Tob1-deficient mice show there is a demonstrably more facile progression into activation in Tob1-deficient T cells (Figure 2). Tob1-deficiency also lowers the thresholds for inflammation and tumor recognition *in vivo*. Specifically, when we examined the capacity of Tob1-deficient mice to reject or delay transplantable tumors as a measure of latent autoreactivity, these mice showed increased inflammatory tumor infiltrates (with consequent necrosis), reduced capillary density, and delayed growth of B16 melanoma as compared to wild type C57Bl/6 littermates (Figure 3).

Nfatc2 in negative regulation and survival

Nfatc2 is one of a family of proteins whose transcriptional activity is regulated by a cycle of dephosphorylation (by the calcium-dependent phosphatase calcineurin, or CN) and rephosphorylation (possibly by glycogen synthase kinase-3β, or GSK-3) (71). NFAT dephosphorylation leads to nuclear translocation and DNA binding, and rephosphorylation leads to rapid nuclear export. The role of Nfatc2 as a *bona fide* negative regulator was initially inferred by the observation that Nfatc2-deficient mice had marked splenomegaly and T cell hyper-responsiveness (72).

We identified a possible mechanism to explain the negative regulatory function of Nfatc2 (Figure 2), which functions as a strong repressor of CDK4 expression in isolated peripheral blood T cells and lymphocyte cell lines (43). The functional significance of this finding was confirmed in the Nfatc2-deficient mice that had enhanced CDK4 expression and activity restricted to the peripheral T cell pool, which shows dysregulated expansion in these animals (43,44). An important finding from this work was that the association of Nfatc2 with AP-1 transcription factors was a critical determinant of its activity as a transcriptional activator or a transcriptional repressor.

This concept was subsequently confirmed in a series of elegant experiments by Rao's group showing that low affinity TCR signals (perhaps akin to those seen by autoreactive cells) induce NFATc2 but fail to activate AP-1 or NFkB. This NFAT activation absent AP-1 is tolerogenic, and tolerance is mediated at least in part through enhanced expression of the Itch and Nedd components of the ubiquitination pathway (45,46). Upregulation of these proteins leads to selective degradation of components that are essential for TCR signaling, such as phospholipase C-gamma (PLCγ) and PKCθ with concomitant induction of factors that suppress activation such as Cbl-b. While splenomegaly in the Nfatc2 knockout may result predominantly from failure to pull cells out of cell cycle during the transition from naïve to memory (43), Nfatc2 also might contribute to quiescence of naïve peripheral T cells and may skew their differentiation towards an inducible regulatory phenotype by supporting expression of Foxp3 (reviewed in (73)).

Smad3 in negative regulation and survival

SMAD proteins integrate signals from TGF-β into the system of intrinsic negative regulation (74). CDK inhibitors including Ink4 proteins and p27 are upregulated by TGF-β (75–77), and at least p27 also is strongly induced by Tob1 in lymphocytes (52). TGF-β signaling on naïve T cells leads to cell cycle arrest, apoptosis or prevents development of effector function in a context dependent manner (78–80). Recent findings have shown that it also is essential for the development of the T-helper-17 lineage (81). TGF-β has also been shown to be required for maintaining peripheral CD4/Foxp3 Treg cells, key mediators of peripheral tolerance (82).

The importance of Smad3 as a mediator of TGF-β-dependent T cell growth arrest cannot be overstated. T cells from Smad3-deficient mice have constitutively active phenotypes with consequent immune dysfunction (57) and they are resistant to downregulation of CDK4 upon exposure to TGF-β (83). CDK4 inhibition has been linked to the inability of T cells to respond to cytokine stimulus and enter cell cycle (84) and both Smad3 and retinoblastoma family proteins are directly inhibited by CDK4 phosphorylation (85). Thus, Smad3 inactivation might facilitate transition from the G0 to the G1 phase of the cell cycle, as evidenced by the observation that a mutant Smad3 that was resistant to CDK-phosphorylation rendered T cells resistant to antigenic priming and attenuation of Smad3 by RNA inhibition prevented tolerance induction (54).

TGF-β itself has a profound effect on naïve T cell homeostasis. Inhibition is mediated both upstream and downstream from CDK inhibitors, and while it modulates the activation and survival of both CD4 and CD8 T cell subsets, there are subtle to readily apparent differences in how it affects each of these populations (54,79,83). TGF-β1 deficient mice develop lethal autoimmunity early in life (86,87). Breeding the TGF-β-deficiency into lymphocyte deficient backgrounds showed T cells mediate the lethality phenotype, as the lymphopenic mice did not develop autoimmune disease or inflammation (88,89). Efforts to study the effects of TGF-β signaling on T cells have hence generated mouse models with a spectrum of phenotypes. Generally, TGF-β-mediated signals are dispensable for thymic development (90–93), whereas conditional deletion of TGF-β RII on T cells results in wasting disease and death by 3–5 weeks of age (91,93).

Two recently developed models have made it possible to carefully study the effects of TGF-β signaling on T cells over a longer time span (92,94). These models use a dominant negative form of the TGF-β RII (RII DN) under the control of CD2 or CD4 promoters. CD4 RII DN mice are healthy until 3–4 months of age, when they develop symptoms of autoimmune dysfunction including inflammatory bowel disease. Conversely, CD2 RII DN mice develop polyclonal CD8 T cell hyperproliferation between 2–9 months of age, but do not manifest inflammatory disease. CD8 T cells in the periphery are primarily $CD44^{hi}$ and have a greater percentage of cells in cycle as compared to wild type littermate controls (92). It should be

noted, however, that TGF-β signaling via a TGF-β RII dominant negative transgene does not completely abrogate TGF-β signaling, which may explain why these animals do not have identical phenotypes to those seen in the TGF-β1 knockouts or in the conditional TGF-β deletion mutants.

TGF-β signals may act as counterbalance to IL-15 signals in the development of CD8 T cell memory (95). Specifically, CD8 T cells that do not receive TGF-β signals proliferate and develop a memory phenotype as measured by CD44 expression (92); TGF-β attenuates expression of the IL-2/IL15Rβ receptor (95); and it reduces IL-15 dependent expression of c-myc (96), which is necessary to support homeostasis of CD8 memory phenotype cells (97). Conversely, IL-15 can overcome the anti-proliferative effects of TGF-β on human peripheral blood mononuclear cells, even though these effects are resistant to IL-2 (98). Together, these results illustrate the existence of complex and finely orchestrated interactions between TGF-β-and IL-15-regulated pathways that control T cell survival, proliferation, and progression from naïve to memory phenotypes.

Despite its important cell-intrinsic effects, TGF-β also is important in extrinsic control of autoimmunity as a secreted cytokine and as a membrane-bound form on Treg cells. The extrinsic effects of TGF-β are diminished in the presence of CD28 co-stimulation (99,100), perhaps allowing pathogen specific T cells to respond to contextually presented antigens, while preventing autoreactive cells from becoming activated.

This has presented a heretofore-insurmountable challenge in the development of strategies that employ cancer immunotherapy, where effector T cells are generally self-reactive and co-stimulation may be in short supply (101–103). Creative approaches to ablate these effects such as those used by Koehler et al. to show that CD28 co-stimulation can overcome TGF-β induced suppression of engineered, tumor specific CD8 and CD4 T cells (99) may be necessary to develop successful immunologic approaches to treat cancer as monotherapy, but these also will require strategies to ameliorate adverse autoimmunity that can be exacerbated in the absence of TGF-β signals (104).

Do Common Pathways Control Tuning by Self-MHC and Intrinsic Negative Regulation?

A connection between intrinsic negative regulation and survival is evident from the results described above regarding how Ian5-deficient T cells undergo apoptosis after they enter the peripheral circulation due to their progression to an incomplete state of activation. However, this phenotype is unique to the BB-DP rat, as deficiency in other intrinsic negative regulatory molecules alternatively leads to fulminant autoimmunity (TGF-β RII DN), expansion of the T cell compartment (Nfatc2 knockout) or no appreciable immune phenotype (Tob1 knockout). Tuning might engage molecules that control the transition from G0 to G1 and G1 progression, but there is a paucity of experiments to address the possible connection between these processes. We put this hypothesis to test using adoptive transfer of naive Tob1-knockout cells to MHC-deficient mice that were challenged 2 weeks later with Lewis Lung carcinoma cells. Unlike the results obtained using wild type naïve T cells, Tob1-knockout T cells did not prevent or delay tumor growth in the recipients, and the periphery showed a reverse phenotype with rapid erosion of CD4 T cells and protracted survival of CD8 T cells in peripheral lymphoid organs[1]. This suggests that, much like absence of CD24 in DCs, the loss of intrinsic negative regulation created by knocking out the Tob1 gene shifted the balance of proliferation and death, allowing CD8 T cells to persist but leading to rapid erosion of CD4 T cells.

The dramatically different phenotypes observed upon adoptive transfer of wild type T cells and Tob1-knockout T cells into MHC-deficient mice indicates that MHC-dependent tuning

and intrinsic negative regulation of T cell proliferation use non-redundant and cooperative signaling pathways, and that the consequences of losing one or both pathways are handled differently by naïve peripheral CD4 and CD8 T cells. A mechanistic explanation for these distinct phenotypes has not been elucidated, but co-stimulation is one attractive possibility (73). In this scenario (Figure 4), the survival of CD4 or CD8 T cells in an MHC-barren environment would be consistent with different thresholds for co-stimulation by CD28. Specifically, CD28 signaling is more robust in CD4 cells than in CD8 cells (105–107) and can still be engaged by MHC-deficient dendritic cells (DC) that retain expression of CD80 and/or CD86 (108).

The importance of CD28 for optimal signal transduction has been recognized for some time (109,110). CD28 is not necessary for T cell development in the thymus, and although CD28-deficient mice have reduced T cell help, they still mount effective cytolytic responses (*e.g.*, against virus) (111). During productive interactions between T cells and APCs, the binding of CD28 to CD80 (B7) or CD86 (B7.1) has various signaling consequences. First, this interaction (and those of other adhesion molecules) may stabilize the immunological synapse. Second, CD28 appears to link TCR signaling with the phosphoinositol-3 kinase (PI3K) pathways, either independently or cooperatively (Figure 4) (112). Numerous roles have been characterized for PI3K signaling in T cells; phospholipid-inositol trisphosphate (PIP_3) with phosphorylated inositol at position-3 promotes activation of phospholipid-dependent kinase 1 (PDK1) and protein kinase-B/Akt, which in turn activate mitogen activated protein kinase (MAPK) pathways, protein kinase C-theta (PCKθ), cyclic AMP response element binding protein (CREB), and mammalian target of rapamycin (mTOR), while inhibiting Forkhead Box protein O (FoxO) and glycogen synthase kinase-3 (GSK-3) (112–115). In addition to these signaling consequences, PI3K activation also promotes cell survival by modulating various Bcl-2 family members such as Bad (phosphorylation), Bcl-X_L (upregulation), and Bim (inhibition).

PI3K signaling also seems to impair Fas-dependent death signals. Specifically, T cells that express activated Akt fail to assemble caspase-8 to the death-inducing signaling complex (DISC) (116). The lipid phosphorylation by PI3K is antagonized by the dual-specificity phosphatase PTEN (117), a potent prototypical tumor suppressor protein. Among other defects, developmental PTEN-deficiency in mice results in lymphoma, and haploinsufficiency (loss of a single allele) leads to death by fulminant autoimmunity (118). These PTEN-haploinsufficient mice show defective Fas-mediated apoptosis, and the phenotype of this defect resembles that of mice that carry an activated Akt allele (defective recruitment of caspase-8 to the DISC) (116), establishing a link between PI3K activation and repression of both intrinsic (mitochondrial-dependent) and extrinsic (death factor-dependent) apoptosis. Nevertheless, various alternative mechanisms might explain the differential sensitivity of T cell subsets to self-MHC and the importance of intrinsic negative regulatory factors in sensitization and tuning. We predict that this will be a fertile area of investigation for some time to come.

Acknowledgements

The authors thank Dr. Stephen Jameson for his careful review of the manuscript and helpful suggestions. We regret if meritorious references may have been omitted in the interest of space or brevity. The work was supported by grants R21DK63410, P30CA46934, and R01DK58722 from the National Institutes of Health.

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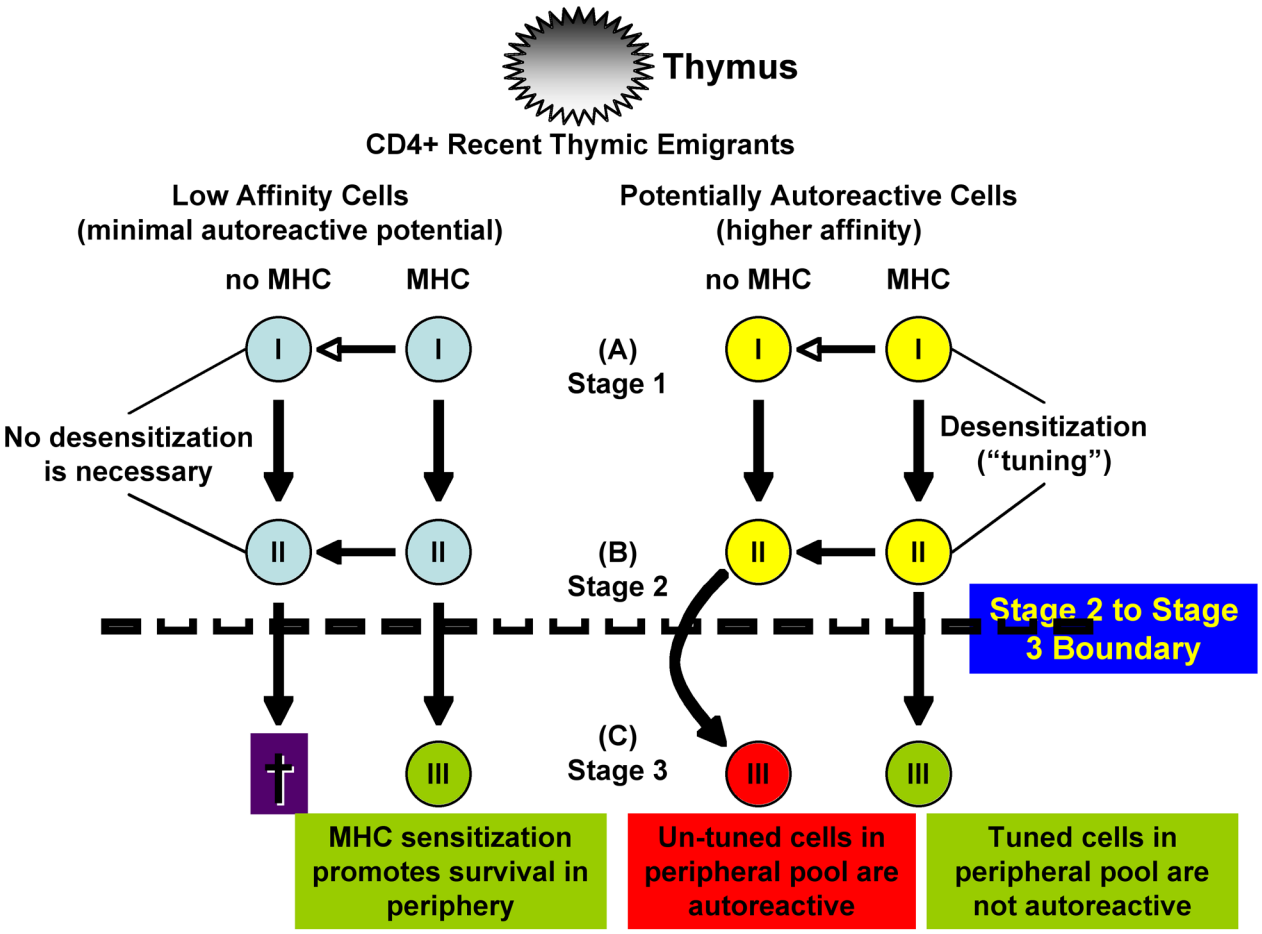

Figure 1. Maturation of CD4 thymic emigrants in the periphery

CD4+ recent thymic emigrants can be subdivided into three stages of post-thymic maturation: Stage-1 cells have a half-life of about one week and do not divide. Maturation from stage-1 to stage-2 appears to be preprogrammed and independent from self MHC, leading stage-2 cells to undergo a few divisions by HP. Conversely, stage-3 cells require MHC for extensive proliferation. Under conditions of HP, $CD44^{hi}$ stage-3 cells appear within ~2 weeks and account for most of the cells seen in MHC-replete mice, but cells that can evade tuning or negative regulation survive in MHC-depleted hosts.

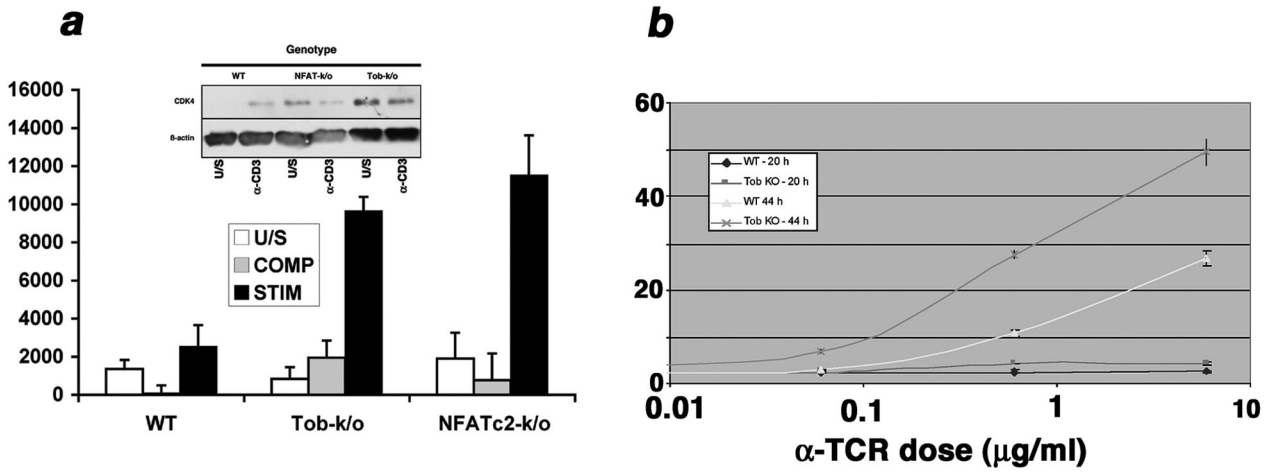

Figure 2. T cells from Tob1-deficient mice are hyper-responsive

(*a*) Competence induction in cells from WT, Tob1-deficient, or NFATc2-deficient mice was established by measuring the ability of cells to respond to limiting quantities of IL-2. Cells were treated without stimulation (U/S), with a limiting dose of anti-CD3 antibody (10 ng/ml, COMP), or with the same dose of antibody in the presence of a limiting dose of IL-2 (0.5 U/ml, STIM). Proliferation was measured using the CyQuant Cell proliferation assay kit. The Y-axis reflects fluorescence absorbance at 485 nm. Inset: steady state CDK4 levels were assessed by immunoblotting in unstimulated cells or in cells treated with anti-CD3 for 6 hr. β-actin was used as a loading control. (*b*) T cells from B6 (WT) or B6 Tob1 knockout mice (KO) were stimulated with increasing doses of anti-CD3. Cells were labeled at induction with CFSE and evaluated after 20 or 44 hours to track cell division. The Y-axis reflects the percent of cells with CFSE dilution measured using flow cytometry.

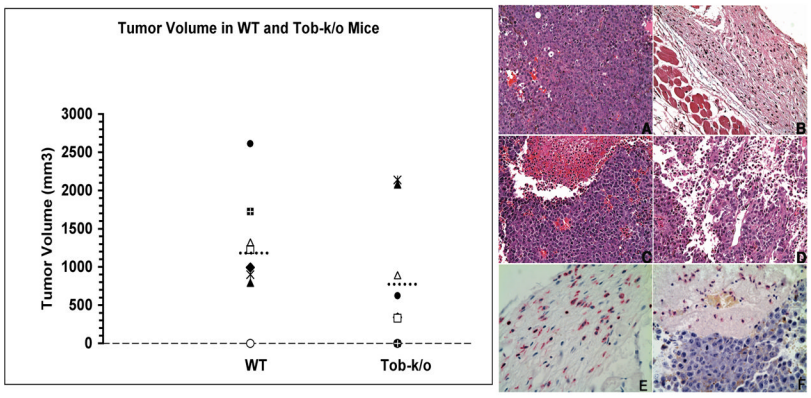

Figure 3. Tumor growth is delayed in Tob1$^{-/-}$ mice

Left: Littermates (8 WT and 8 Tob1-k/o) from Tob1+/− heterozygous breedings were challenged sq with 2.5 × 10^4 B16 melanoma cells. Graph shows 3-D tumor volume for each pair 17 days after challenge. Dashed lines represent mean tumor volume. A trend was present for smaller tumors in Tob1-k/o. *Right*: Representative sq sections from wild type (A) or Tob1-deficient mice (B–F) 21 days after injection with B16 melanoma cells. There is a high mitotic rate, no inflammation, and visible vascularization of tumors in WT mice (A), in contrast to dermal and subcutaneous inflammatory infiltrates (B, C, D), hemorrhagic necrosis (C, D), severe edema and reduced vascularization (D) in Tob$^{-/-}$ mice. (H&E, 200X magnification). (E) and (F) show immunohistochemical staining for leukocytes using CD18 (red-staining cells). (E) is the same tumor as (B). (F) is the same tumor as (C). Note abundant leukocytes in the subcutis, extravasating neutrophils and inflammatory cells infiltrating the tumors (400X magnification). Gross measurable tumor volumes in the mice shown were (A) 1,320 mm^3, (B) 0 mm^3, (C) 891 mm^3, and (D) 340 mm^3.

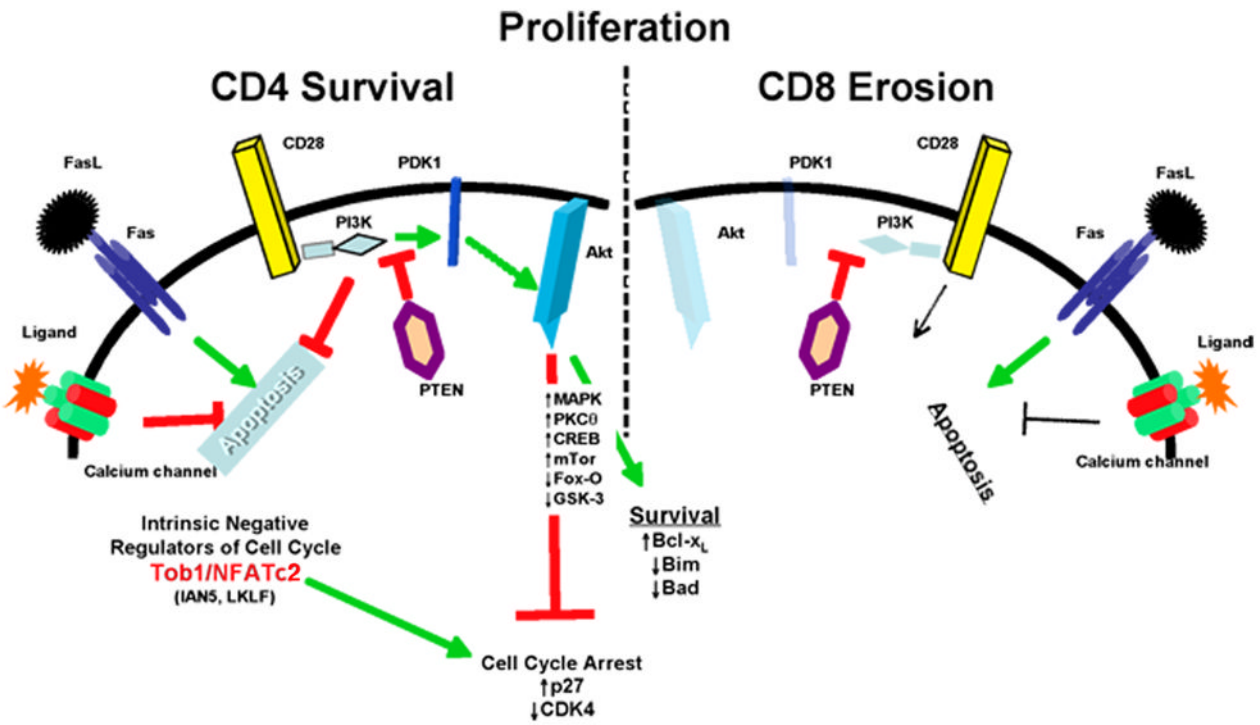

Figure 4. An integrated model of T cell fates in an MHC-less environment

Lymphopenia in MHC-less mice induces proliferation of naïve CD4 and CD8 T cells, leading to upregulation of Fas receptors. We hypothesize that naïve CD4 cells survive because signals initiated by binding CD28, and transmitted mostly through the PI3K-dependent activation of Akt, antagonize the apoptotic impetus from Fas as well as the intrinsic signals from Tob1 and NFAtc2 that enforce cell cycle arrest. At the same time, CD28/PI3K signals tilt the balance of mitochondrial homeostasis towards survival by upregulating Bcl-xL and by downregulating Bim and Bad. PI3K also activates or inhibits additional pathways that similarly favor proliferation and survival. Each of these PI3K-mdeiated events is antagonized by PTEN through dephosphorylation of inositol phospholipids and by dephosphorylation of tyrosine residues in activating kinases. Finally, signals transmitted through calcium channels also increase resistance to Fas-mediated cell death. Naïve CD8 cells, in contrast, do not signal effectively through CD28, and thus show impaired survival when FasL engages Fas signaling. In this context, calcium signals offer only partial or no protection from cell death.