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MECHANISMS AND MODELS OF PERIPHERAL CD4 T CELL SELF-TOLERANCE

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1. ABSTRACT

Each response made by our immune system is either to promote or to prevent immune reactivity. That the immune system can successfully achieve both goals under specific biological conditions depends on central and peripheral tolerance mechanisms. Over the years, various experimental systems have been generated to study peripheral CD4 T cell tolerance. The experimental approaches are clearly diverse, but can be broadly categorized into those involving "tolerogenic" antigen injections, the use of transgenic mice (antigen transgenics, TCR transgenics and the combination thereof) and transplantation models. Results of these studies suggested a number of potential mechanisms mediating peripheral CD4 T cell tolerance, including clonal deletion, anergy and immune regulation. While the available systems all presented with their own limitations, they nevertheless provided a foundation from which our understanding of peripheral CD4 T cell tolerance will be built. In addition, previous studies done to examine CD8 T cell tolerance left with us some potentially helpful clues and ideas about how CD4 T cell tolerance should be studied. However, our current understanding on the immunity/tolerance decision made by CD4 T cells in the periphery remains incomplete. Evidence accumulated thus far favors the view that peripheral tolerance, unlike central tolerance, may not primarily determine the selection of specificities within the baseline T cell repertoire but may instead regulate cellular responses within the repertoire, in terms of both expansion/contraction and differentiation. Nevertheless, more robust and refined models need to be developed before we can make definitive conclusions about the potential role of peripheral tolerance in repertoire selection mediated by deletion of self-reactive cells.

2. INTRODUCTION

Our immune system is a biological marvel. Everyday, it interacts with both the external and the internal environment and determines what responses should occur in the presence of specific conditions. To the immune system, a response can be manifested in one of many different ways, though that response is made for one of two purposes: "to promote or to prevent immune reactivity" (1). Each response, whether it is the clearance of an invading pathogen or the removal of autoreactive lymphocytes, comprises a series of biological events that are conceivably executed to maintain the host in homeostasis both internally and in relation to its environment.

The immune system has a selective reactivity, responding strongly to pathogenic agents and generally not to self-tissues. This property has been attributed to a selection of T lymphocytes, resulting in the physical and/or functional elimination of those cells with specificity for self-antigens. Historically, central (thymic) tolerance has been considered to be largely responsible for shaping a T cell repertoire known for its specificity and its

discriminatory nature. Thymocytes undergoing maturation and development in the thymus are subject to positive and negative selection. An overwhelming majority of thymocytes do not survive the selection events. In particular, negative selection is important in regulating immune reactivity, as thymocytes bearing high-affinity T cell receptors (TCRs) against thymus-derived host antigens are eliminated in the thymus to prevent autoreactivity in the periphery. The expectation that there would be self-antigens in the periphery that are not expressed in the thymus, and experiments that appeared to demonstrate tolerizability of mature T cells raised doubts about the thymic capacity to act as the sole regulator of autoreactivity and led to the idea of peripheral mechanisms of tolerance. While studies relating to the autoimmune regulator gene (AIRE) demonstrating the expression of peripheral tissue antigens in the thymus (2-5) provide renewed support for central tolerance as the sole necessary tolerance mechanism, they do not exclude the contribution by potential mechanisms of peripheral tolerance. There is a large amount of data suggesting the existence of peripheral tolerance mechanisms that contribute to the overall state of self-tolerance. Hence, negative selection of self-reactive T lymphocytes ("tolerance") may occur during thymocyte maturation in the central lymphoid organs, as well as later in the periphery with mature T cells. What is not clear is whether these mechanisms primarily deal with low-avidity T cells (6,7) that escape central tolerance (despite the presence of their cognate antigens in the thymus) or with high-avidity T cells specific to peripheral antigens not present in the thymus. For the purposes of the current discussion we will focus on CD4 T cell tolerance to antigens found only in the periphery and not encountered in the thymus. It is not that we consider the low-avidity "escapees" to be insignificant, but rather that these cells are likely to be maintained in an inactive state simply by clonal competitive effects (8) (revealed under lymphopenic conditions; example the regulatory T cell (T_{reg}) phenomenon) rather than by the tolerance mechanisms that evolved to silence high-affinity T cells reactive to tissue specific antigens. Regulatory T cells, their history and supporting experiments, have been reviewed in numerous publications recently and we will therefore not cover them extensively here except in terms of their impact on modeling of the peripheral immunity/tolerance decision. Also, discussions of the intracellular signals and factors involved in "tolerogenic" signaling will not be discussed as they have been detailed recently elsewhere (9).

An understanding of the mechanism(s) of peripheral tolerance could lead to new avenues for therapeutic interventions in conditions where tolerance to specific antigens/tissues is needed. Since CD4 T cells control a considerable portion of both B cell and CD8 T cell reactivity (10-13), elucidation of tolerance mechanisms in the CD4 compartment will be crucial to understanding tolerance in general. Peripheral tolerance in CD4 T cells represents a unique problem since these cells are only capable of seeing antigen on the relatively few cell types that express MHC class II (antigen presenting cells (APCs), B cells and endothelial cells) under normal circumstances and the peptide antigens they functionally see are derived largely from proteins taken up from the endocytic pathway, rather than from proteins made within the cell (14,15).

In this contribution we will discuss various models that propose to explain the general rules determining peripheral CD4 T cell tolerance versus immunity and the specific mechanisms of tolerance involved. We will evaluate the limitations in existing systems that have been used to test CD4 T cell tolerance models/mechanisms and look at future approaches that may help refine our understanding of peripheral tolerance.

3. THE MODELS

In broad terms, ideas about how lymphocyte selection leads to tolerance of self-tissues have moved from a focus on the timing of antigen exposure in the life of the animal, to the timing of antigen exposure in the life of the lymphocyte together with the regulation of selection by antigen nonspecific signals. Tolerance due to antigen exposure early in development (fetal or neonatal period) began with the ideas of Burnet and the neonatal tolerance studies of Billingham, Brent and Medawar (16). Because of the elegant simplicity of the early tolerance window idea, it has been maintained in a small number of current models (17-19) despite accumulating evidence against this view. For example in one study tolerance appears only to be established later in life (20). In this system tolerance to a peripherally expressed beta cell transgenic antigen was apparent in adult but not young animals based on the induction of diabetes upon infection with a virus expressing the antigen in young but not adult mice. It could be argued, however, that the response induced by injecting the same dose of virus into a young versus an old mouse is not a valid comparison (21). In addition, a number of studies have shown that a graft given before the development of the immune system still leads to rejection of that tissue (22-28). This is even true for grafts mismatched for as little as one minor histocompatibility (minor-H) antigen (29), and argues strongly against a tolerance window early in life. Instead the most popular current view is that tolerance of self is primarily established by self-antigen encounter during lymphocyte development (central tolerance, as originally proposed by Lederberg (30)) and secondarily by various mechanisms in the periphery. There are of course proponents of the various extremes, including those viewing self-tolerance as almost completely determined centrally (AIRE etc.) (2-4) and those proposing tolerance to be largely dealt with in the periphery (31). What follows is a brief description of the key aspects of various models, as they apply to CD4 T cell tolerance.

Most of the current models of peripheral T cell tolerance (see Table 1; we would appreciate suggestions for other models that could be included here) can be divided into three categories, those that propose 1. that tolerance is the default pathway in the absence of second signals; 2. that tolerance results from actively induced signals (rather than just antigen in the absence of second signals); and 3. that most peripheral antigens are ignored, only those widely distributed in lymphoid tissue at high enough doses induce

Table 1. Selected models and their characteristics as applied to peripheral CD4 T cell tolerance

Model	Immunity	Peripheral Tolerance	Key Determining Factor(s)
Associative Recognition	Ag with T cell help; linked recognition	Ag without T cell help (default); absence of linked recognition	Timing of Ag exposure
Costimulation: Infectious non-self	Ag with costimulation	Ag without costimulation (default)	[1] PAMPS trigger costimulation
Costimulation: Danger	Ag with costimulation	Ag without costimulation (default)	[2] Endogenous signals trigger costimulation
Costimulation-Coinhibition	Ag without coinhibition; costimulation amplifies	Ag with induced coinhibitory ligands/signals (feedback/quiescence)	Not yet defined
Regulatory T cells	Ag without suppression	Ag recognition in presence of eT_{reg}; linked recognition	[3]Timing of Ag exposure
Tuning	Signals above threshold required	Continuous signals that only change slowly	Rate of change in many undefined stimuli
Ignorance	Ag in localized lymph tissue	Only when Ag in high dose throughout lymphatics	Ag location and dose

[1] PAMPS are pathogen associated molecular patterns that are recognized by recipient Toll-like receptors on APCs. [2] Stressed or damaged cells, but not healthy or apoptotic cells, release molecules that trigger activation of APCs. [3] This determining factor is taken from the model of Coutinho and colleagues, and does not necessarily represent a consensus view in the T_{reg} field (eT_{reg} = effector T_{reg}).

tolerance. These in essence are passively, actively and spatially determined mechanisms of tolerance.

3.1. Antigen encounter without rescue signals (signal 1 without 2)

3.1.1. Associative antigen recognition (T cell help)

The cornerstone of this model is the proposal that all lymphocytes acting in a conventional adaptive immune response (including CD4 and CD8 T cells and B cells) require two antigen specific signals for their induction (17,32,33). The first signal comes from antigen engagement of the lymphocyte antigen receptor (TCR or BCR) and the second signal comes from an effector T helper cell that is also recognizing a linked epitope of the antigen (hence associative antigen recognition) (34). In the absence of a signal from an effector helper, antigen receptor engagement leads to tolerance (via deletion). Thus, in this model the key to self-tolerance is the induction of tolerance in self-specific helper T cells due to the absence of second signals from effector helpers recognizing self-antigen in a linked fashion. The absence of effector helpers when the immune system is first generated early in life allows the establishment of tolerance (an early window of time where antigen encounter can only lead to tolerance and not immunity). The tolerance established early due to deletion of self-specific helpers is maintained throughout life as each new helper that is generated enters a peripheral environment lacking self-specific helpers. The postulate that helpers themselves need help generates a chicken and egg conundrum, namely, where did the first effector helper come from? Cohn and Langman proposed that there is a slow time-dependent spontaneous generation of effector T helpers and that self-reactive helpers would encounter their self-antigen prior to differentiating into an effector and therefore would be killed. This model is supported by the numerous studies showing that B cells and CD8 T cells often require linked T cell help for their differentiation into effectors (35-37) and these cells can be tolerized when encountering antigen in the absence of T cell help (38,39). There are also a few interesting studies suggesting a requirement for T cell help in generating helper T cell responses (40), although these studies and the concepts therein have not impacted on the current design of experiments in immunology. There are downsides to the associative recognition model in terms of providing a general explanation for self-tolerance. These include the requirement for additional assumptions to explain helper independent lymphocyte responses and the seemingly unstoppable autoimmunity that would occur each time a newly generated (not yet tolerized) self-specific helper T cell recognizes its antigen on an APC that is also presenting peptides of a pathogen to an effector anti-pathogen helper T cell. We would suggest that the linked recognition of epitopes intrinsic to T cell help and the associative antigen recognition model are perfectly suited to control the effector class (including memory (41)) and magnitude of immune responses, and probably play little if any role in determining somatic selection of the baseline repertoire. Because effector molecules of one class can inhibit the function of another, it is critical that the class of immune response is coherent. This can only be achieved by having class regulated through a cellular communication, and determined on an antigen-by-antigen basis. In contrast, tolerance can be achieved on an epitope-by-epitope basis. Furthermore, when B cells and CD4 and CD8 T cells encounter antigen in the absence of a collaborating cell type (e.g. in B cell deficient mice, or upon CD4 T cell depletion), the responding cell no longer is capable of receiving or inducing the appropriate signals from the collaborating cell when it is returned to the system (42). In this way the factors controlling the class of the initial response are maintained upon subsequent antigen encounters. In this view the experiments interpreted as demonstrating tolerance due to the absence of T cell help may instead reflect the presence only of a primary response in a class that was not measured, and a lack of memory induction (41). The lack of a cell intrinsic capacity to generate secondary responses probably arose only after this function evolved to be under the control of T helper cells, long after the primary tolerance mechanism(s) had been established.

3.1.2. Costimulation-based models

While associative recognition helped in understanding B cell activation, the difficulties in explaining the generation of the first effector T helper and other considerations led to the current rise of costimulation-based models. However, the idea that antigen nonspecific signals from APCs provided costimulatory signals for T cell activation originated with Lafferty and Cunningham's model that developed from an explanation for the stronger responses to allogeneic compared to xenogeneic cells (43). They hypothesized that stimulator cells (APCs) presented antigens to cognate lymphocytes (signal 1) in the presence of a second (costimulatory) signal supplied by the APCs. Recognition of signal 1 alone by lymphocytes resulted in their inactivation. Because of a lack of experimental support, however, their model was largely ignored and did not gain popularity for many years until a surprising observation was noted by Jenkins and Schwartz (44). Using chemically treated splenocytes as stimulators, they found the responding T cell population to become unresponsive, suggesting that the chemical treatment impaired the ability of APCs to provide additional signals to responding T cells that had recognized cognate antigen/MHC complexes. This finding was crucial to the further experimental development of costimulation-based models.

A key weakness of the early costimulation models was the lack of control over the expression of costimulatory molecules by APCs. Since APCs are equally capable of presenting host-derived as well as foreign antigens, it is unclear how an APC can distinguish between the two types of antigens and express costimulatory molecules appropriately to activate cognate T cells. To solve this problem, Janeway and subsequently Matzinger offered alternative solutions. Janeway proposed that APCs expressed receptors (now known as pattern recognition receptors or PRRs) recognizing evolutionarily conserved molecular motifs found in bacteria, and the recognition of these structures resulted in APC activation and expression of costimulatory molecules (45,46). Hence, self-nonself discrimination was based on self being noninfectious while antigens treated as nonself included only those antigens with an infectious component. Indeed, experimental support of his model arrived with cloning a few years later of the first Toll-like receptor (TLR), a PRR (47), and since then other TLRs specific for various pathogen-associated molecular patterns (PAMPs) have also been identified (48).

While Janeway's model potentially explains a number of important observations, including the need for adjuvants to enhance the immunogenicity of antigens, it failed to account for other findings, such as immunity towards viral infections (where in many cases PRRs may not play a role) and organ transplants. Consequently, Matzinger proposed another costimulation-based model known as the Danger model (49) that now abandons self-nonself discrimination as the key factor in the immunity/tolerance decision (50,51). She hypothesized that cells of tissues subject to injury or stress would release endogenous "danger" signals that could activate APC to become costimulatory, thereby allowing antigens presented on APCs to be recognized by cognate T cells in an immunogenic context. In addition to observations explainable by Janeway's model, her model offered reasons as to why established tumors and organ transplants could be tolerated or rejected by the host immune system. Moreover, experimental data in support of the Danger model have also arisen over the years (29,52-58).

3.2. Antigen encounter with additional signals (signals 1 and 3)

3.2.1. Coinhibition

The above "passive" models suggest that the absence of a signal (costimulation or T helper signals) leads to tolerance; antigen receptor engagement alone is a negative signal. Sinclair developed an alternative model in which the antigen receptor signal is positive. In this model costimulation serves as an amplifier of immune responses rather than a reverser of antigen receptor negative signals (consistent with recent *in vitro* studies (59)), and costimulation is counterbalanced by receptors that mediate negative coinhibitory signals. Coinhibition is negative signals mediated by antigen nonspecific receptors that work in concert with antigen specific signals through the antigen receptor (AgR). Coinhibition is the opposite of costimulation and in some cases has confusingly been called "negative costimulation". Under quiescent conditions, where costimulatory signals are limited, coinhibition may predominate (for example, due to the higher affinity of CTLA-4/B7 interaction compared to CD28/B7). The concept of coinhibitory receptors derived from ideas about the mechanisms of antibody feedback (60) a number of years before costimulation arrived on the scene. It originated with Sinclair's proposal (61) that B cells have receptors for the Fc portion of IgG (FcR) and that coaggregation of the AgR and the FcR shuts off the B cell. The B cell FcR (Fc-gammaRIIb) was thus the first coinhibitory receptor described, and it was found to have a motif in its intracytoplasmic domain common to many subsequently described coinhibitory receptors (62,63). Coinhibition was expanded to be part of a general model of immune regulation in T and B cells (64-66) involving the balance between costimulation and coinhibition by numerous receptors (some examples include CTLA-4, FAS, PD-1, CD5, CD22, CD72, IFNgamma-R). Thus, coinhibitory signals can be delivered by end products such as antibody and cytokines or by receptor-ligand interactions between cells. There are now many studies that seem consistent with a role for coinhibitory receptors in peripheral CD4 T cell tolerance, as demonstrated by blocking the coinhibitor or eliminating it by gene knockout (67-69). Very recently another coinhibitor in CD4 T cells, Tim-3, has been added to the list (70,71). Tim-3 is expressed on the surface of T_H1 cells and its ligand (Tim-3L) is expressed by both T_H1 and T_H2 cells and some dendritic cells and macrophages but not B cells. Blockade of Tim-3 with Tim-3 fusion proteins blocks tolerance in these cells induced by injection of high dose antigen (70) or by costimulatory blockade strategies in transplantation (71). Whether Tim-3 truly plays any role in the establishment of natural peripheral self-tolerance is not yet clear. This will require analysis of tolerance to naturally or transgenically expressed peripheral self-antigens in Tim-3 knockout mice or in transfer models with Tim-3 blockade.

Coinhibition seems to have become the paradigm for CD4 T cell tolerance and tolerance in general (72), and the signal 1 alone models of peripheral tolerance are clearly antiquated. However, there needs to be developed a clear definition of what controls coinhibition if we are to fully understand how it contributes to the immunity/tolerance decision or other potential functions it may have (73). While there are some clear and simple hypotheses for the control of costimulation (control by "danger" signals or PAMPs) there is not yet a corresponding concept for the control of coinhibition. Nevertheless, augmenting coinhibitory signals already shows potential as a strategy for tolerance induction in transplantation (74-76).

3.2.2. Regulatory T cells

Suppression or regulation could be considered a specific subset of coinhibitory signals, as suppression is also the induction of tolerance via antigen recognition in the presence of tolerogenic antigen nonspecific co-signals. Models that attribute peripheral tolerance to regulatory T (T_{reg}) cells simply restrict the job of delivering coinhibitory signals to a specialized T cell subset. However, while the suppressive mechanism may be antigen nonspecific (e.g. cytokines transforming growth factor-beta or interleukin-10), T_{reg} cells add an additional dimension in that the coinhibitory signals are delivered by antigen specific cells. T_{reg} cells, like helper T cells, may act through linked recognition of antigenic determinants. Since triggering of T_{reg} cells is antigen specific, the obvious goal would be to devise a model whereby T_{reg} cells suppress self- but not foreign reactive T cells. However, surprisingly few had even made an attempt, with the only clear model being that proposed by Coutinho and colleagues (77). This model suggests that newly generated T cells from the thymus are only sensitive to suppression by T_{reg} cells for a short period. In this way most foreign reactive T cells would proceed to the stage where they cannot be suppressed, while self-reactive T cells would encounter their cognate self-antigen and T_{reg} cells during the phase of sensitivity to suppression. Unfortunately this model is not supported by the existing data (50). Although it seems possible to generate a T_{reg}-based model for peripheral tolerance versus immunity, there is accumulating evidence that T_{reg} cells suppress anti-microbial responses just as effectively as anti-self responses (78,79). Recently it was shown that TLR ligands (LPS and CpG) triggering dendritic cells could overcome T_{reg}-mediated suppression (80). This effect was in part mediated by the production of interleukin-6. However, again the dramatic effects were in the ability of the T_{reg} cells to suppress a foreign antigen (OVA) triggered response. Thus, T_{reg} cells have little preference for suppressing self-reactive cells as opposed to foreign reactive ones. It therefore appears that T_{reg} cells do not contribute to the decision making process (self versus foreign/pathogen) that is based on somatic selection of lymphocyte clones. The data suggested that even in the presence of T_{reg} cells this decision seems largely to be governed by the activation of APCs. However, other data seemed to indicate an opposing role for TLR ligands when at higher concentration. It was recently shown that T_{reg} cells express a number of TLRs, one of which is the LPS receptor TLR4, and in the presence of LPS the T_{reg} cells could become activated to control inflammatory responses (81). This would suggest that in the course of an immune response against an invading bacterial pathogen, for instance, that T_{reg} cells are important in regulating the magnitude of the anti-pathogen response such that it remains protective in terms of ridding the pathogen but not so strong that there is excessive bystander damage to host tissues (81,82). In this way, T_{reg} cells may be part of a negative feedback process that limits the overall magnitude of any response, whether it is self- or pathogen specific. They may also play a role in the maintenance of memory (83).

While previous failures in identifying genetic loci corresponding to Treg cell function led to the downfall of the Gershon and Kondo initiated concept of Treg (or suppressor T cells as used during that time period) existence (84,85), the recent discovery of Foxp3 as a key transcriptional factor for Treg cell development (86) arguably provides the strongest evidence yet that Treg cells exist as a critical differentiation stage or even as a distinct cell type in the overall immunologic repertoire. This discovery offers strong support to previous studies demonstrating the production of Treg cells via thymic selection (87), as well as sheds light on studies that argue for Treg cell generation by multiple distinct pathways (88). Defects in Foxp3 lead to lymphoproliferation and autoimmunity. However, similar to defects in CTLA-4 and Fas, it is not clear whether these autoimmune diseases truly represent a breakdown in peripheral tolerance to tissues specific antigens or simply a generalized defect in lymphocyte control such that nonspecific tissue damaging inflammatory events ensue.

3.2.3. T cell tuning

While the theory of tuning activation thresholds was developed many years ago (89,90) it has only been experimentally examined recently in terms of its relevance to peripheral CD4 T cell tolerance. The basis of this model is the postulate that T cells are able to constantly "tune" (adjust) their signaling based on the ambient level of signals present in the environment. This tuning sets the threshold level of signals required to activate a T cell upon subsequent encounters with cognate antigen. In the face of changing levels of antigen the T cell retunes and resets the threshold. In this view, the trigger for a response is the rapid change in levels of signals (including antigen levels) such that the T cell does not have sufficient time to readjust the threshold needed for activation. Evidence that CD4 T cells within a monoclonal repertoire can make such adaptations based on the level of persistent antigen presented was recently provided (91,92). That the level of tolerance attained may vary depending on the level of antigen thus appears likely. However, this appears more to be a detail of mechanism rather than the basis of an explanation for the negative selection of self-reactive T cells and the maintenance of pathogen specific cells. Since rapid changes in the level of antigen alone are not sufficient to trigger a response, it seems more likely that it is the change in the level of other signals (e.g. costimulatory signals) that is key.

3.3. Ignorance

In terms of self-tolerance, the ignorance model of Zinkernagel and colleagues suggests that tolerance to self is induced when any antigen is presented for a sufficient time

and dose throughout lymphoid tissue (1). Thus all self-antigens that normally are found within the central and peripheral lymphoid tissues will induce tolerance. The ignorance model provides a novel explanation for the regulation of immunity in the periphery. It suggests that peripheral tissue specific self-antigens are ignored because they reside in parenchymal tissues and are not expressed or presented by migratory cells in lymph nodes at sufficient levels. Induction of a productive T cell response only occurs when antigen reaches a localized lymphoid tissue in sufficient amounts for a sufficient period of time. Encounter with antigen outside lymphoid tissue leads neither to immunity nor tolerance. Although ignorance of peripheral antigens has been suggested experimentally (93-97), it may only exist for some peripheral self-antigens. It remains unclear whether ignorance of peripheral antigens is a generalizable phenomenon or a rare exception. However, the current evidence appears to favor tolerance rather than ignorance of most transgenically or naturally expressed peripheral self-antigens presented in the context of both class I (98) and class II (99). The proposition that CD4 T cells are ignorant of tissue restricted peripheral self-antigens is perhaps the most dubious aspect of Zinkernagel's model. If self-reactive T cells are ignorant rather than tolerant, each time a peripheral tissue is infected the self-antigens of that tissue will be taken up by local APCs and crosspresented in the draining lymph node resulting in immunity. Since CD4 cells, in the absence of a CD8 response, are capable of significant tissue destruction, autoimmune disease would be extremely frequent in an immune system governed by ignorance.

4. GENERAL EXPERIMENTAL APPROACHES TO ANALYZE PERIPHERAL CD4 T CELL TOLERANCE

It has been difficult to study the acquisition of peripheral tolerance in CD4 T cells *in vivo*, therefore much of the early studies were done *in vitro* with T cell clones. As predicted by the two-signal model of Lafferty and Cunningham (43), these data suggested that helper T cell activation requires a signal triggered by specific antigen (peptide/MHC) binding to the TCR and antigen nonspecific signal(s), termed costimulation, delivered by the APC. In the absence of costimulation the T cell clones instead became unresponsive (anergic) (44). However, it is not clear that the *in vitro* models reflect the tolerance mechanisms occurring *in vivo.* While anergic T cell clones do not proliferate or secrete IL-2, their production of chemokines is not reduced (100). Furthermore, it has not been possible to anergize primary naïve T cells *in vitro*; only previously primed cells are susceptible to anergy induction (101). Thus, it will be important to determine what mechanisms of peripheral tolerance act *in vivo.* In contrast to the many elegant studies of T helper cell tolerance to ubiquitous self-antigens (102,103) and breakdowns in tolerance (104-108), there have been few studies of the successful establishment of peripheral tolerance (tolerance to antigens not present in the thymus) in CD4 T cells. The study of *in vivo* CD4 T cell tolerance to peripheral tissue specific antigens has been faced with two major hurdles, the lack of clearly identified tissue specific antigens and the absence of methods to follow the antigen specific T cells in the sea of T cells specific to other antigens. Survival of viral superantigen reactive T cells in bone marrow chimeric mice provided a system to study the tolerant state in peripheral CD4 T cells (109). However, since the antigens were also encountered in the thymus this model did not allow the study of tolerance to uniquely peripheral antigens. By blocking thymocyte encounter with superantigens using antibodies to I-E, and releasing the autoreactive cells into the periphery, Jones and colleagues were able to study the establishment of peripheral tolerance once antibody treatment had been terminated (110). Under these conditions there was a dramatic deletion of the relevant Vbeta expressing CD4 and CD8 T cells. The generalizability of this strong peripheral deletion is however questionable as the widespread distribution of the deleting antigen does not reflect the distribution or dose that is likely for normal tissue specific peripheral antigens. One way to overcome the "absence of antigen" problem has been to simply inject an antigen into an animal and assume that if unresponsiveness is induced, that the mechanisms involved reflect those that would normally occur during tolerance to tissue specific peripheral self-antigens (111-113). However, this is a large assumption as the distribution of an injected antigen, timing of its presence in ontogeny, quantity, and cells presenting the antigen, are all likely to be different from the natural situation. In addition, the apparent tolerance associated with intravenous injection of soluble antigens/peptides may reflect the characteristically short half-life of antigens administered in this way, a characteristic that is certainly unlikely to mimic most true peripheral self-antigens. Furthermore, both *in vivo* and *in vitro* anergic states in terms of proliferation, can be associated with intact helper and killer cell effector functions (114-116). An approach that removed many hidden assumptions was the development of antigen transgenic mice using tissue specific promoters. However, this approach has not always been successful in generating a truly peripheral antigen, as thymic expression often occurs, making it difficult to analyze peripheral tolerance independent from previous antigen encounter in the thymus (117-119).

Another approach to study peripheral tolerance, one that arrived serendipitously rather than through planned tolerance studies, is the use of certain gene knockout and spontaneous mutant mice that appear to develop autoimmune disease. Examples include the spontaneous *lpr* and *gld* mutants (120), and knockouts of CTLA-4 (121). Studies in these systems have provided some experimental support for the view that coinhibitory molecules are critical for peripheral tolerance. However, these knockouts display generalized defects, including in some cases a loss of tolerance to ubiquitous nuclear autoantigens. They have not been shown to have a particular loss of tolerance to natural tissue specific antigens that would lead to tissue specific autoimmune diseases such as Type-1 Diabetes. Thus, it is not fully clear whether these molecules are involved in the establishment of tolerance to tissue specific antigens in the periphery or alternatively play a role in homeostatic mechanisms or regulation of effector class. More recently, knockouts for other molecules, such as PD1

(69), have revealed coinhibitors that may more directly play a role in tolerance to tissue specific peripheral antigens.

4.1. Transgenics

4.1.1. Antigen transgenics

Initially MHC molecules were the antigens of choice for peripheral transgenic expression. This is because there is a high frequency of cells reactive to an allogeneic MHC and T cells reactive with certain MHC molecules can be tracked because they use certain TCR Vbeta families. Lo and colleagues expressed MHC class II molecules on beta cells or acinar cells of the pancreas (122-124) and studied the TCR Vbeta family that reacted with the MHC class II. While they found that the T cells were not deleted but instead became anergic (124,125), others who generated similar models did not find anergy (126-128). While thymus expression of the transgene may have been responsible for the anergic state, the discrepancies between the various studies raises a difficulty in interpreting MHC transgenic mouse models. Unlike normal peripheral self-antigens, transgenic MHC molecules on a peripheral tissue are in effect hundreds of antigens because there is no central tolerance to the transgenic MHC and its multitude of associated peptides. Another major drawback of MHC transgenic studies relates to the MHC restricted nature of antigen recognition by T cells and as a consequence the lack of recognition of the transgenic antigens via crosspresentation (indirect presentation). T cells specific for intact transgenic MHC molecules are not able to recognize the processed peptides of the transgenic MHC that are crosspresented by the recipient's APCs and MHC. While crosspresentation had been seen many years ago (129), evidence has only appeared recently that it may play an important role in peripheral tolerance to tissue restricted antigens (99,130-133). Thus, MHC transgenics miss any tolerogenic effects via crosspresentation, a process that allows tissue APCs to pick up and present tissue specific antigens. However, initial studies using mice with transgenic expression of LCMV glycoprotein in the pancreas, where both pathways of presentation could occur, appeared to show that T cells were ignorant rather than tolerant (93). The reason for ignorance was unclear, but may reflect an important role for the level of antigen expression, as tolerance has been observed in other systems (134).

4.1.2. TCR plus antigen transgenics

Difficulties in visualizing tolerance mechanisms can be overcome by using mice with a monoclonal T cell population specific for a self-antigen. This has been achieved with TCR transgenic (Tg) mice that are crossed to SCID mice or RAG knockout (KO) mice in order to prevent endogenous TCR gene rearrangement. However, few studies have used such mice to analyze peripheral tolerance in CD4 T cells. In some studies TCR Tg mice specific for hemagglutinin (HA) in $I\text{-}E^d$ were combined with viral HA antigen Tg mice, expressing HA on hematopoietic cells or numerous tissues (99,135,136). Antigen expression in the thymus could be detected thus necessitating transfer of TCR Tg cells into antigen expressing hosts. In both studies hyporesponsiveness *in vitro* (anergy) was observed. However, these studies are difficult to interpret for a number of reasons. The TCR Tg mouse employed in one set of studies was not on a SCID or RAG-KO background (99,136), and therefore some of the T cells would have expressed endogenous TCRs as well as the Tg TCR. Expression of a second TCR is likely to alter the outcome of antigen encounter (137), as has already been shown for antigen encounter within the thymus (138). Indeed, using the same TCR Tg along with different HA antigen transgenics (87,139), escape from negative selection in the thymus in T cells expressing endogenous TCRalpha chains was found (140). In these studies it could not be established whether the tolerant state in the periphery was true peripheral tolerance or a state induced during non-deletional encounter with antigen in the thymus. Another difficulty lies in the site of expression of the target antigen. It is becoming clear that the outcome of antigen presentation (tolerance or immunity) is greatly influenced by the type of cell presenting the antigen (21,141-144). In the Lanoue *et al*. study the vast majority of cells presenting the self-antigen were B cells, not the cell type that would be expected to present naturally occurring peripheral self-antigens. The quantity and wide distribution of antigen in all of these studies are likely to have influenced the outcome. Most truly peripheral (not present in the thymus) self-antigens would not have a wide distribution but instead would be found within a particular organ/tissue or the lymph node draining that tissue (130,145). Similar difficulties due to antigen/tissue distribution and/or endogenous TCR gene rearrangements also apply in earlier work with systemically injected peptides (113) and in other recent studies of CD4 T cell tolerance (91,146). However, such studies of tolerance to systemic antigens may have relevance for understanding tolerance to a small subset of systemic self proteins that putatively only arise later in life after the full development of the T cell repertoire (113). A study by Forster *et al.* may provide the best hints about peripheral CD4 T cell tolerance. Using Tg mice expressing SV40 T antigen (Tag) in the pancreas and Tg CD4 T cells specific to Tag (147), they found that the T cells were not deleted in the thymus early in life but were either deleted or partially anergic in the periphery. The tolerance was only partial, as insulitis without diabetes was present and tolerance did not occur when the T cell population was monoclonal (endogenous TCRs excluded by crossing to a RAG-KO background). Thus, Forster's study was the first to eliminate the confounding variable of endogenous TCR expression when analyzing CD4 T cell tolerance using TCR Tg cells. However, since interactions between T cells of different specificities greatly influence the outcome, it is not clear that such studies with a monoclonal repertoire will reflect the outcomes/mechanisms that occur under the physiologic conditions of a polyclonal repertoire. Clarification of these issues will require seeding small numbers of monoclonal (RAG-KO) TCR transgenic T cells into a normal repertoire and tracking their fate.

TCR and antigen transgenics have also been employed to test what signals are involved in self-tolerance. Does tolerance result from antigen receptor engagement alone (signal 1) or are coinhibitory receptors necessary to maintain self-tolerance? While the

lymphoproliferation and systemic autoimmune-like phenotypes in CTLA-4 KO and Fas deficient mice suggest they play a critical role in self-tolerance, there has been little in the way of direct evidence for a preferential loss of peripheral tolerance in these animals (as opposed to a nonspecific heightened responsiveness). Recent work by Abbas and colleagues has begun to address this issue by transferring wild type versus CTLA-4 KO CD4 anti-HEL TCR Tg T cells (not on RAG-KO background) into mice expressing HEL in pancreatic beta cells with subsequent immunization using HEL in various adjuvants (148). They compared T cell activity at the site draining the neo-self-antigen and at the site where HEL was introduced in immunizing form. Deficiency in CTLA-4 led to increased accumulation of transgenic T cells in the lymph node draining the pancreas but not draining the site of immunization, and this accumulation was associated with ensuing autoimmune diabetes. However, diabetes could be induced even with wild type Tg T cells if stronger adjuvants were employed. These data suggest the possibility that the coinhibitor CTLA-4 may function to allow tolerance to self-tissues to be maintained in the face of concomitant immunity by T cells crossreactive to pathogen and self-epitopes. Thus, the balance between costimulation and coinhibition can be tipped towards immunity/autoimmunity with sufficiently strong adjuvants such as CFA. Further experiments are needed to fully determine if coinhibitory signals from CTLA-4 or other coinhibitors act constitutively or are regulated such that they act predominantly to prevent self- but not pathogen specific reactivity. The critical question is whether the immunity/tolerance decision is determined by constitutive coinhibition together with inducible costimulation (i.e. costimulation alone is the determining factor since coinhibition is always active) or by a system where both coinhibition and costimulation are inducible, a situation where both can play a determining role.

One common feature to all *in vivo* tolerance studies using TCR Tg mice thus far is that the T cells that are eventually deemed to be tolerant (whether anergized or deleted) arrive at this state after having made what looks like a conventional immune response, including both proliferation and differentiation (at least in terms of changes in surface markers). This is not what one would predict if peripheral tolerance were simply about deleting self-reactive specificities from the repertoire (why waste energy proliferating?). It instead suggests that the peripheral immunity/tolerance decision is one of determining the magnitude of response, and not a question of whether there will be a response. The response versus no response decision may instead be determined by the presence or absence of a given specificity in the repertoire, a property largely if not completely determined during thymocyte selection. We cannot however rule out the possibility that peripheral deletion does substantially shape the repertoire of T cell specificities. If this is the case, proliferation prior to deletion (tolerance induction) may indicate that the gap that evolved between tolerogenic and immunogenic presentation is quite narrow. In the context of a costimulation-based model this would mean that it is not a black and white situation of self-antigens presented on resting APCs and pathogen antigens on activated (costimulatory) APCs. Instead, naïve T cells may have to "add" up the sum of a number of encounters with antigen/APC before a final decision between tolerance and immunity can be made. In this way, for example, the summation of encounter with 8 resting and 2 activated APCs presenting antigen will lead to some cell division but eventual tolerance, while encounter with 5 resting and 5 activated APC may lead to sustained immunity. There may also be a summation in signals from separate sequential costimulators that prevents premature termination of the response (149). We do not yet have a handle on how wide the divide is between tolerogenic and immunogenic presentation. Elucidation of these quantitative aspects will increase the precision of our description of immunity versus tolerance, but whether they will also force a change in overall conceptual models remains to be seen (150).

4.2. Transplantation models

Expression of antigens under the control of tissue specific promoters has provided a way to study peripheral tolerance to a defined antigen without the knowledge of natural tissue specific antigens. The approach however is not without some drawbacks. It is not always clear that the antigens are expressed at physiologic levels or in appropriate sites and the forced expression of an antigen not normally expressed by a particular cell type may have adverse affects on the function/viability of the cell independent of any immune response (122,127). Another less traveled path to generate a model peripheral antigen that may overcome some of these deficiencies is to graft a tissue naturally expressing its own histocompatibility antigens. The obvious difficulty with using grafts as the source of a model peripheral antigen is that grafts normally induce immunity rather than tolerance. However, they provide an excellent model with which to test our ideas of peripheral tolerance mechanisms, for if we truly understand what it is that makes grafts but not self-tissues trigger an immune response, it should be feasible to block the trigger and generate tolerance. Indeed there have been a number of situations in which acceptance of a foreign (mismatched) transplant has been achieved and apparent tolerance induced without chronic immunosuppression. The most successful method of inducing transplantation tolerance is through the generation of systemic chimerism with donor cells. However, the tolerance in this situation is predominantly central rather than peripheral and thus generally not relevant to the present discussion. Instead we will briefly discuss approaches that have focused on blocking costimulation. They include depletion of donor APCs, antibody/fusion protein mediated blockade of costimulatory receptors/ligands and removing the signals that lead to activation of APCs and upregulation of costimulatory molecules.

Donor leukocytes have long been considered important in stimulating an anti-graft response. One primary difference between a graft and a normal peripheral self tissue that may contribute to the different way these two entities are treated by the immune system is the presence of APCs in the graft (but not the self-tissue) that express antigens to which the immune system of the

recipient is not centrally tolerant. In contrast, the parenchymal tissue of both the graft and a peripheral self-tissue expresses antigens to which recipient T cells are not centrally tolerant. Thus simply eliminating the donor APCs should put the graft and the self-tissue on a more equal footing. Depletion of donor APCs from transplants leaves only parenchymal tissue, and not resident APCs, expressing the graft antigens. Thus, the distribution of cells expressing graft antigens becomes the same as for cells expressing a peripheral self-antigen. However, donor APC depletion is unlikely to fully mimic natural peripheral tolerance. It leads to graft acceptance with many MHC mismatched grafts (151-159) but not reliably with MHC matched minor-H antigen mismatched grafts (160). Similar to the difficulties encountered with MHC transgenic studies (see section 4.1.1), donor APC depletion does not take into account the natural role of crosspresentation (termed indirect presentation in the context of transplantation). When MHC matched, both donor and recipient APCs are able to present donor minor-H antigens in the same haplotype of MHC. Therefore, removing the donor APCs would not fully block presentation of donor antigens to T cells capable of directly recognizing the graft. Crosspresentation would still occur and would be capable of activating T cells that can directly recognize donor cells and their peptide/MHC complexes. In contrast, with MHC mismatched grafts, removal of APCs leads to a loss of function because T cells with direct specificity/restriction for donor MHC cannot be primed by the indirect pathway, and conversely, the T cells capable of recognizing donor antigens in recipient MHC (indirectly primed T cells) are not able to directly recognize the donor tissue and reject it (for further discussion of these issues see ref.(161)). In the long-term, indirect presentation may be tolerogenic, but as before, the T cells that directly recognize the mismatched donor MHC antigens cannot recognize the donor antigens when presented indirectly in recipient MHC molecules. Thus, MHC mismatched donor APC depleted grafts do not induce tolerance in T cells that directly recognize graft antigens (159).

A way to tackle the contribution of both donor and recipient APCs is to block their ability to send costimulatory signals. The view that inducible costimulatory signals provided by APCs are necessary for a functional immune response has become the leading paradigm (45,162-166). Blockade of some of the costimulatory pathways (CD28/B7 and CD40/CD40L) has achieved some success in various transplant models (167-173). The mechanism of graft acceptance/tolerance achieved by this method may involve both deletion of reactive cells and class regulation (immune deviation) of the remaining cells (166,171,174-178). However, this method has not been fully successful in inducing long-term donor specific tolerance (as defined by challenge with second donor grafts) under the most stringent conditions. This may reflect the possibility of other costimulatory pathways not blocked by the treatment taking effect (179,180) or that the mechanisms of peripheral tolerance do not have the capacity to deal with the large repertoire of responding cells in the alloreactive population. The synergistic effects of recipient lymphocyte depletion and costimulation blockade support the latter possibility. One of the most promising recent approaches combines lymphocyte depletion and blockade of T cell growth factor signals generated by interleukin-2 (IL-2) and interleukin-15 (IL-15) using lytic receptor agonist (IL-2) and antagonist (IL-15) fusion proteins in combination with rapamycin to block IL-2 induced proliferative signals while maintaining IL-2's pro-apoptotic activity (181). This method indicates that blockade of signals downstream of costimulation can also provide a means to achieve tolerance.

Another approach to make peripheral graft antigens be treated like self-antigens is to reduce the signals that generate the costimulation that triggers graft rejection. This approach is based on the view that costimulatory signals are stimulated by one of two general sources. As discussed earlier costimulatory signals may be triggered either by recognition of PAMPs by TLRs or by endogenous signals from stressed or damaged cells. In the latter situation graft rejection could be explained by the surgical tissue damage that is likely to trigger costimulation in both donor and recipient APCs. However, since it is known that the repertoire of T cells responding to a fully allogeneic graft includes memory cells it is likely that these cells were triggered by crossreactive antigens that themselves were associated with inducers of costimulation. Thus, in this case it does not seem possible to block all the relevant costimulation inducers since some of the costimulatory activation of T cells had occurred prior to transplantation. To generate a model where all potential inducers of costimulation triggered rejection can be controlled it seems necessary to use a graft that has only a small number of minor-H mismatches. In this way the response of a naïve anti-graft repertoire may be analyzed for conditions that lead to peripheral tolerance. We recently took this approach and asked whether allowing a single minor-H (male H-Y) antigen graft to heal into an immunodeficient recipient, so that the tissue damage associated APC activating signals had time to dissipate, would allow the graft to be treated as self. We found that even long healed grafts were rejected when the recipients immune system was reconstituted by hematopoietic stem cell transfer (28,29). Thus, surprisingly the graft antigens were not treated like self-antigens despite the small mismatch and despite allowing the graft to heal prior to allowing the immune system to encounter it. Rejection may have been due to some long-term abnormalities in the grafted tissue (mRNA analysis of graft tissue provided some support for this possibility (29)) or due to the origin of the grafted tissue. We and others had chosen skin grafts for these types of experiments and it may be difficult to eliminate all inducers of costimulation from an external tissue such as skin, given its continuous exposure to microbes and potential tissue damage (scratching etc). A recent study supports this latter possibility as female recipient mice were unable to reject male skin grafts from donors defective in the TLR signal transduction pathway (182). Thus, a more critical test will require allowing an internally placed graft (e.g. heart, islets) to heal and then see if it is able to induce peripheral tolerance in a newly generated immune system.

Waldmann and colleagues published a series of data demonstrating dominant peripheral CD4 T cell

tolerance to multiple minor-H mismatched skin grafts that were transplanted onto recipients given a tolerance inducing antibody regimen (consisting of varying combinations of depleting and non-depleting anti-CD4, -CD8, and anti-CD40L antibodies). The involvement of a dominant mechanism involving linked suppression was supported, in one study, by long-term acceptance of (donor x 3rd party)F1 skin grafts (183) and by transfer of the tolerant state to naïve T cells in a second study (184). In a third study, they showed that tolerated skin grafts carried passenger T cells capable of preventing rejection (185). While the combination of these studies clearly provided evidence for a robust dominant tolerance to multiple minor-H grafts, it is not yet clear why the antibody treatments induced such a state or whether this state in any way mimics the mechanisms naturally employed in the development of peripheral self-tolerance. Nevertheless, application of infectious tolerance mechanisms would be ideal for clinical tolerance protocols in transplantation and autoimmune disease as these mechanisms alleviate the necessity of knowing all the target antigens involved; tolerance should spread to any unrecognized donor- or auto-antigens.

4.3. Can CD8 T cell tolerance studies teach us anything about CD4 T cell tolerance?

Some of the early studies of CD8 T cell tolerance suggested that the immune system is ignorant of peripheral self-antigens (93,94,186). In another system, the transfer of Tg T cells specific to a widely distributed antigen in the recipient (anti-H-Y CD8 T cells into a male mouse) resulted in expansion and then deletion of the transferred cells (187). However the deletion was not complete and did not result in a reduction of T cell numbers below the number originally injected. The remaining cells appeared anergic. In a different model, TCR transgenic CD8 T cells specific to K^b expressed on liver were not ignorant or anergic but instead induced autoimmune tissue destruction and underwent deletion (188). Evident tissue destruction and deletion was also the result when sufficiently high numbers of TCR transgenic T cells recognizing OVA/MHC class I complexes were transferred into hosts that transgenically expressed OVA in islets (98,130). It is not clear whether all aspects of these studies may be generalized to CD4 T cells tolerance. However, it is likely that increased frequencies of autoreactive cells will lead to tissue destruction when CD4 cells are employed just as in the above CD8 T cell experiments. In other studies CD8 T cell peripheral tolerance has been associated with downregulation of the CD8 coreceptor (189). Such downregulation has not been observed in studies with CD4 T cells.

While we have thus far discussed tolerance in simplified terms as an “all or none” phenomenon, the reality is of course that there will be a threshold of avidity for tolerance and self-specific cells of sufficiently low avidity will escape the tolerance mechanisms. The functional relevance of such cells is highlighted by studies of Sherman and colleagues (190). They described an aspect of CD8 tolerance that is likely to be generalizable to CD4 T cells. Tg mice with HA expressed in the pancreas were functionally tolerant of HA in that they accepted an HA-expressing tumor while non-transgenic mice did not. However, immunization with virus expressing HA led to the ability to reject the tumor even in HA transgenic mice. The rejection was associated with the production of low-avidity CTL specific to HA that presumably eliminated the tumor and yet did not cause destruction of the HA expressing beta cells of the pancreas. This suggests that tolerance versus immunity is not simply a consequence of clonal selection of receptor specificities resulting in the physical or functional elimination of self-reactive cells. Self-reactive cells can be functional at eliminating an invader (in this case a tumor) without significant destruction of self-tissues. Thus, the maintenance of such low-avidity self-reactive T cells if crossreactive to an epitope of a pathogen may provide a survival benefit. Tolerance of self-tissue may be maintained either because of its “healthy” state (no APC activating signals) or because of a lower expression of the relevant epitope.

Existing models of CD8 T cell tolerance not only provide potential clues as to the mechanisms that may be operational in CD4 T cell tolerance but they also suggest new ways to study CD4 T cell tolerance. Pregnancy as a model for examining peripheral tolerance is one such example and it is arguably a very natural (less manipulated) system for studying peripheral tolerance mechanisms. Since the appearance of fetal antigens only occurs long after the generation of the maternal T cell repertoire, central tolerance cannot play a primary role in the acceptance of the fetus. It is therefore possible that mechanisms of tolerance to the fetus may be similar to mechanisms involved in tolerance to certain self-antigens that have been hypothesized to appear only late in ontogeny (191). MHC class I restricted TCR Tg mouse strains have made it possible to monitor anti-paternal CD8 T cells during pregnancy (no published data are yet available for class II-restricted T cells). These studies found that maternal tolerance to fetal alloantigens was transient involving a temporary reduction/deletion of cognate T cells (192) and induction of unresponsiveness in those T cells that did not undergo deletion (193).

5. CONCLUSIONS AND PERSPECTIVES

Given the above discussion of mechanistic studies and models of peripheral CD4 T cell tolerance are we any closer to solving the puzzle of self-nonself discrimination or the danger-nondanger discrimination? In the past 15 years a number of critical tools have been developed that have allowed some light to be shed on the nature of peripheral tolerance. Nevertheless, some of the key issues remain elusive and may only be resolved when further refinements in the model systems are developed. The ideal system for studying mechanisms of peripheral CD4 T cell tolerance would have the following characteristics: 1. expression/presentation of a defined peptide antigen (not allo-MHC) restricted to a single peripheral tissue, and whose timing of expression in the life of the animal can be controlled; 2. methods of studying MHC II-restricted T cell effector responses to the peripheral antigen (not just proliferation); 3. methods of

following the fate of the antigen specific T cells expressing a single TCR (a condition not fulfilled by the TCR transgenics that allow endogenous TCR expression); and 4. defined conditions where the antigen induces tolerance versus immunity. Most of the studies reviewed herein demonstrate some aspects of the ideal model but none fulfills all the criteria. Consequently, there remains some doubt as to the generalizability of the conclusions drawn in each model system. Did the immune system really evolve multiple mechanisms of peripheral tolerance (deletion, anergy, regulation etc.) or is it simply our variable experimental systems that lead to the variable answers while the natural course included only a single chosen mechanism? In terms of the physical or functional selection of the T cell repertoire into the good clones (anti-pathogen) and the bad clones (anti-self), no compelling argument has been put forward to account for the multiple mechanisms observed experimentally. Once the first peripheral selection mechanism to arise was in place there would seem to be little selective pressure for an additional mechanism. From this point of view, the additional mechanisms apparent in various models may have a different function, perhaps in controlling the expansion/contraction or differentiation of clones from the already determined steady state repertoire (a concept put forward by Cohn (33)). This control of the magnitude and class of response could equally well be important in maintaining the integrity of the individual, and defects in these mechanisms could lead to autodestruction. Thus, it may be possible to define the mechanism(s) involved in the selection of the baseline repertoire, and separate mechanisms that control the use of the clones within that repertoire. It is still controversial whether the baseline peripheral repertoire is largely if not completely shaped by the central selection events or if peripheral selection also plays a large role. If the latter is not the case, then control of expansion/contraction and differentiation of the established baseline repertoire is the sole peripheral means of preventing autoimmune disease in the periphery. The conventional view instead would be that both central and peripheral tolerance determine the clonal specificity of the baseline repertoire. Which view is correct can only be answered by determining whether or not the frequency of autoreactive cells is higher in the pool of recent thymic emigrants when compared to long established peripheral T cells. An even more challenging area for the future will be the definition of the function of each costimulator and coinhibitor. It is unlikely that each serves an overlapping (redundant) role, and elucidation of each of their specific functions is likely to give us a much clearer picture of both peripheral tolerance and the regulation of class of immune responses.

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