Title: When three is not a crowd: A crossregulation model of the dynamics and repertoire selection of regulatory CD4 T cells

Authors: Jorge Carneiro¹, Kalet Leon¹², Íris Caramalho¹, Carline van den Dool¹, Rui Gardner¹, Vanessa Oliveira¹, Marie-Louise Bergman¹, Nuno Sepúlveda¹, Tiago Paixão¹, Jose Faro¹³, Jocelyne Demengeot¹

Affiliation:
1. Instituto Gulbenkian de Ciência, Oeiras, Portugal
2. Centro de Inmunologia Molecular, Habana, Cuba.
3. Universidade de Vigo, Vigo, Spain

Correspondence to:
Jorge Carneiro
Estudos Avançados
Instituto Gulbenkian de Ciência
Apartado 14
2781-901 Oeiras
Portugal
Phone: (351) 214 407 920
Fax: (351) 214 407 973
Email: jcarneir@igc.gulbenkian.pt
Summary

Regulatory CD4 T (T\textsubscript{R}) cells, enriched in the CD25 pool of healthy individuals, mediate natural tolerance and prevent autoimmune diseases. Despite their fundamental and potential clinical significance, T\textsubscript{R} cells have not yet been incorporated in a coherent theory of the immune system. This article reviews experimental evidence and theoretical arguments supporting a model of T\textsubscript{R} cell dynamics, uncovering some of its most relevant biological implications. According to this model, the persistence and expansion of T\textsubscript{R} cell populations depend strictly on specific interactions they make with APCs and conventional T\textsubscript{E} cells. This three-partner crossregulation imposes that T\textsubscript{R} cells feed on the specific autoimmune activities they suppress, with implications ranging from their interactions with other cells, to their repertoire selection in the periphery and in the thymus, and to the relationship between these cells and the innate immune system. These implications stem from the basic prediction that the peripheral dynamics sorts the CD4 repertoire into two subsets: a less diverse set of small clones of autoreactive T\textsubscript{E} and T\textsubscript{R} cells that regulate each other’s growth, and a more diverse set of barely autoreactive T\textsubscript{E} clones, whose expansion is limited only by APC availability. It is argued that such partitioning of the repertoire sets the ground for self-nonself discrimination.

Keywords: Mathematical model; Regulatory CD4+CD25+Foxp3+ T cells; Crossregulation model; Repertoire selection
Introduction

Regulatory CD4 T cells, that express FoxP3 (1-3) and are enriched in the CD25 pool of healthy individuals (4), have been gaining increasing relevance in immunology (5). Many lines of evidence indicate that these cells play a key role in the development of natural tolerance and in the prevention of autoimmune pathologies, by controlling the activation and proliferation of other autoreactive lymphocytes (4, 6). The functional significance of these cells has broadened as they were shown to modulate the immune response against pathogens preventing the associated immunopathology (7-10), and to prevent rejection of transplants (11-13).

The hypothesis that suppressor or regulatory T cells orchestrate the immune system is not new (see for example the editorial in (14)) and yet a theory incorporating these cells and their function is still lacking. The paucity of theoretically framed hypothesis adds up to other difficulties in dealing with regulatory T cells, namely the inability to isolate regulatory T cells, and the difficulty to design and interpret quantitative experiments to assess their function.

This article reviews a mathematical modeling approach to regulatory T cells, based on the hypothesis, put forward by Leon et al (15), according to which the persistence and expansion of regulatory T cell populations depend on the interactions these cells make both with APCs, and the T cells they suppress. This three-partner crossregulation model, which “forces” regulatory T cells to “feed” on the specific (auto)immune activities they suppress, has far reaching implications for the immunobiology of these cells, ranging from the interactions they make with other cells, to their population dynamics and repertoire selection in the periphery, to the constraints of thymic selection, or the relationship between these cells and the innate immune system.

Natural tolerance, clonal selection, and regulatory T cells
Natural tolerance refers to the status of absence of harmful immune responses against body components observed in healthy individuals. The developmental processes leading to natural tolerance are so robust, so “natural” that for many years they were cast aside from immunology (see Grabar (16)). The biological significance of natural tolerance becomes most patent upon its failure during autoimmune diseases. The risk of autoimmunity cannot be disassociated from the capacity of the immune system to cope with diverse and fast evolving pathogens (17). The latter is achieved by setting up a vast and diverse repertoire of antigen receptors expressed by lymphocytes, which as a whole is capable of recognizing any possible antigen. Indeed, most lymphocytes have a unique antigen receptor generated by VDJ recombination in lymphocyte precursors. The randomness in the generation of antigen receptors makes it inescapable that lymphocytes with receptors recognizing body antigens are also made. These autoreactive lymphocytes can potentially cause autoimmune diseases if their activation and clonal expansion in the periphery is not prevented. The question is how is this avoided in naturally tolerant individuals?

According to Burnet’s original clonal selection theory (18) expansion of autoreactive lymphocyte clones and autoimmunity would be avoided by deleting autoreactive lymphocytes from the repertoire once and for all during embryonic development. This possibility was dismissed by the fact that the generation of lymphocytes is a life long process in mammals. Following an early suggestion by Lederberg (19) deletion of potentially self-destructive lymphocytes was reformulated as an aspect of lymphopoiesis, and accordingly immature lymphocytes expressing an autoreactive receptor are deleted either in the thymus or in the bone marrow (20, 21). Cohn and Langman (17) solved the problem of tolerance to peripheral antigens not expressed in these lymphopoietic organs, through the two-signal model, which predicts efficient deletion of autoreactive lymphocytes during circulation.

Central or peripheral deletion of immature cells play certainly an important role, but alone cannot explain natural tolerance. The major shortcoming of deletion models is the well-documented presence of significant numbers of mature autoreactive lymphocytes in the periphery of normal healthy animals. Many different experiments have demonstrated that these autoreactive cells could undergo clonal expansion and cause disease, if they were not under the control of specific regulatory
T cells. It is worth recalling here some of these experiments as they reveal important properties of the processes leading to the development and maintenance of natural tolerance.

Animals thymectomized during a short time window after birth, but not thereafter, develop in adulthood a pathologic autoimmune syndrome characterized by infiltration of multiple organs (22, 23). Although these observations could suggest a special wave of regulatory T cell production in the thymus during the perinatal period (24) there is now evidence that, in mice, regulatory T cells are produced in the thymus throughout life (25-28). Most likely, neonatal thymectomy produces an imbalance of regulatory T cells in the seed of cells colonizing the periphery (29), and this imbalance is critically amplified by the peripheral cell population dynamics (30). Indeed, reconstituting neonatally thymectomized animals with CD25+CD4+ regulatory T cells from healthy adult animals prevents the autoimmune syndrome triggered by neonatal thymectomy (31).

Adoptive transfers of the total peripheral CD4 T cell pool into syngeneic thymectomized animals reconstitute (partial) immunocompetence and also natural tolerance in the recipients. This indicates that if the thymus is fundamental for the development of natural tolerance it may play a lesser role in its maintenance during adulthood. Natural tolerance will not be reconstituted in the recipients upon transfer of CD4 T cell subsets that do not have a poised proportion of regulatory T cells. Thus, large clonal expansions and autoimmune pathology are observed in empty animals transferred with few numbers of CD4 T cells (our unpublished observations) or with CD25- (4), CD45Rb<sup>high</sup> (32-34), or Foxp3- (35) CD4 T cell subsets that are poor in regulatory T cells. This autoimmune lymphoproliferative pathology is not observed in animals reconstituted with the complementary CD4 subsets, which are enriched in regulatory T cells, or with poised mixtures of regulatory enriched and impoverished subsets. In these recipients, the clonal expansion of T cells is controlled, thus reaching a steady state (36-38). Regulatory T cell enriched pools from donors lacking specific tissues fail to prevent autoimmune responses against those tissues in the recipients (25), which indicates that regulatory T cells are antigen (or at least tissue) specific, and that their persistence as a population requires continuous stimulation by peripheral antigens. Collectively, these observations indicate that the robustness of natural tolerance in the adults is associated to the density-dependent interactions
regulatory T cell populations make with other T cell and APC populations (15). It also shows that very strong perturbations to the T cell equilibrium proportions may be amplified through clonal expansion leading to autoimmunity.

This disequilibrium between regulatory T cells and their targets leading to autoimmunity may be elicited, not only by direct perturbations to the T cell proportions, but also indirectly by perturbing other leukocytes. Autoimmune responses to body antigens can be triggered in adult animals by immunization with adjuvants (reviewed in (39)) that entail massive local inflammation, and therefore strong perturbations to the innate immune system. Intriguingly, reverting a situation of overt autoimmunity in adulthood seems more difficult to achieve than to imbalance a situation of natural tolerance.

The conditions necessary to achieve tolerance to histo-incompatible transplants grafted during embryonic life, further emphasize the role of thymic selection and peripheral lymphocyte dynamics in the establishment of natural tolerance to body tissues. Most histo-incompatible tissues successfully grafted during embryonic life will be rejected once immunocompetence develops, the only exceptions being the hematopoietic tissue (40) and thymic epithelium (41, 42). These two tissues are able to induce tolerance to themselves, and also to other tissues from the same donor (40-42). Both hematopoietic cells and thymic epithelium are involved in positive and negative selection and MHC restriction of CD4 T cells in the thymus, which suggests that thymic selection preconfigures the repertoire to facilitate the development of natural tolerance to the ensemble of peripheral MHC-peptides; while failure in preconfiguring the repertoire in this way may lead to tissue rejection.

Based on the Crossregulation Model described in the next section, we will sketch in the following sections a picture of the immune system, trying to portray and give a coherent tentative explanation to all the above properties of natural tolerance. Before doing so, it must be stressed that it will be assumed throughout this review that to establish natural tolerance it is sufficient to prevent clonal expansion of autoreactive CD4 T lymphocytes. This simple view is appropriate because it turns the regulation of autoimmunity into a problem of lymphocyte population dynamics, which can be clarified by studying the conditions for the equilibrium between potentially pathogenic and regulatory T cells. However, it is also important to acknowledge from the outset that some important aspects of natural tolerance might
be lost by assuming this somewhat traditional “clonal selection” view, since autoimmune pathology can be prevented by regulatory T cells even in the presence of an already large “clone” of autoreactive T cells (43).

**The Crossregulation Model**

Leon et al (15) argued that the large body of data on adoptive transfers of tolerance implies a bistable dynamic system, and that this is a natural expectation if the persistence and expansion of regulatory T cell populations depend on the target T cells they suppress. Further studies (44-48) led to the consolidation of a hypothesis for the dynamics of regulatory T cells and the role of these cells in natural tolerance and in self-nonself discrimination. This hypothesis is embodied in the Crossregulation Model outlined in this section, and succinctly proposes that specific autoreactive regulatory T cells “feed” on the very same autoimmune dynamics that they suppress.

**General Biological Principles**

Every model obeys some general principles that guide its design. The Crossregulation Model is based on two general principles that are essential for the integrity and viability of multicellular organisms. First, the persistence of any cell lineage or tissue requires that its cells make recurrent interactions with other cells within the organism; cells that fail to make intercellular interactions will die by apoptosis. Second, the homeostatic turnover of cells in a lineage or tissue involves density dependent feedback mechanisms controlling cell cycle. These feedback mechanisms are mediated by indirect interactions among cells (such as competition for limited survival and growth factors of molecular or cellular nature) or by direct interactions, such as contact inhibition. In the immune system these general principles of multicellular organization must be reconciled with the capacity of leucocytes to undergo activation, proliferation, and differentiation in response to pathogens (49, 50).

Postulates of the Model
The Crossregulation Model describes the peripheral lymphocyte population dynamics taking into account three mutually interacting cell types: (a) antigen presenting cells (APCs) displaying membrane MHC-peptide complexes; (b) effector T (T_E) cells that can potentially induce autoimmunity or build immune responses to foreign pathogens depending on their specificity; and (c) regulatory T (T_R) cells which suppress proliferation of effector T cells with similar specificities, preventing their clonal expansion.

The model, in its conceptual or mathematical formulations, requires a set of postulates that summarize the life cycle of these cells and interactions they make with each other. These postulates are the following:

1. APCs in the body can be collectively classified as different populations of equivalent APCs. Thus, each APC in a particular population presents the same set of peptides, being regarded as equivalent as far as recognition by and conjugation with T cells is concerned.

2. Each APC population is in a stationary state being continuously renewed from precursors.

3. T_E and T_R cells are also classified as different populations according to their clonal specificity. For the purpose of this paper, it is more relevant to aggregate these cells into populations of equivalent clones with respect to their interactions with the APC populations.

4. T_E and T_R cells are exported as such by the thymus where they differentiate from precursors after productive rearrangement of their TCR genes, at some time during positive and negative selection. The quantitative contribution of peripheral differentiation is neglected here.

5. Quiescent or resting T_E and T_R cells are slowly lost by apoptosis in the periphery.

6. Activation of T_E and T_R cells to perform functions and to progress through the cell cycle requires interactions with APCs presenting cognate antigens (cognate APCs, for short), and depends on interactions these T cells make with each other.

7. T_E and T_R cells interact indirectly by competition for access to cognate APCs and more directly by molecular processes that require the co-localization of both cells in physical domains in the vicinity of these cognate APCs. We will call these domains APC-dependent interaction foci (or foci, for short). The simplest form of these foci is the multicellular conjugates studied in Leon et al. (15). Interactions in
the same foci guarantees some degree of specificity in these interactions: only T_E and T_R cells recognizing peptides on the same APC will partake the same foci and therefore interact with each other.

8. Proliferation of specific T_E cell populations is promoted by productive interactions with cognate APC populations, and may be suppressed by regulatory T cells if the APCs present also their cognate peptides.

9. T_R cell proliferation depends on interactions with both APCs and T_E cells co-localized in the same foci.

Minimal Mathematical Formulation of the Model

In this review we illustrate the insights gained with the Crossregulation Model using one of its simplest mathematical forms, namely the scenario where the APCs can only form multicellular conjugates with a maximum of two T cells, and crossregulatory interactions between T_E and T_R cells are restricted to these multicellular conjugates (fig. 1). Although this is clearly an unrealistic setting, it captures most of the qualitative properties of more realistic scenarios, as discussed in the next section. The reaction diagram in fig. 1 can be translated into the following set of differential equations describing the time evolution of the densities of T_E and T_R cell populations with the same specificity, respectively E and R, that are driven by a single cognate APC population, and that is at a fixed density.

\[
\frac{dE}{dt} = p_E \cdot E_A - d_E \cdot E \tag{1}
\]

\[
\frac{dR}{dt} = p_R \cdot R_A - d_R \cdot R \tag{2}
\]

where E_A and R_A are the densities of activated T_E and T_R cells in multicellular conjugates. The parameters p_E and p_R are the proliferation rates of activated T_E and T_R cells, respectively. The parameters d_E and d_R are the death rates of T_E and T_R cells.

The densities of activated T_E and T_R cells are calculated in a stepwise manner (15, 48). First, the equilibrium density of T cells in conjugates, denoted C, is obtained as a function of the total density of T cells, T=E+R, and the total density of APC conjugation sites, A. This is computed considering that the reactions of conjugate formation and dissociation, which happen at a much faster time scale than the changes in T cell population sizes, are at equilibrium with constant K. The expression for the
conjugates (eqn. 3) implies that they initially increase linearly with both APC and T cell density, and saturate when either of the two populations becomes limited.

\[
C = \frac{1 + K(A + T) - \sqrt{(1 + K(A + T))^2 - 4ATK^2}}{2K}
\]  

(3)

The densities of \(T_E\) and \(T_R\) conjugates, respectively \(E_C\) and \(R_C\), are obtained through the corresponding portions of the conjugate densities.

\[
E_C = \frac{E}{T} C, \quad R_C = \frac{R}{T} C
\]  

(4)

Considering the fractions of conjugation sites per APC that are occupied by \(T_E\) and \(T_R\) cells at equilibrium, respectively \(\varepsilon\) and \(\rho\) (eqn 5):

\[
\varepsilon = \frac{E_C}{A}, \quad \rho = \frac{R_C}{A}
\]  

(5)

the density of activated cells is finally obtained according to the following expressions:

\[
E_A = E_C \left(1 - \frac{2\rho}{2 - \varepsilon}\right)
\]  

(6)

\[
R_A = R_C \left(\frac{2\varepsilon}{2 - \rho}\right)
\]  

(7)

that take into account the stoichiometries of the conjugates indicated in the diagram of fig. 1. The factors in brackets in eqns 6 and 7 are, respectively, the probability that a conjugated \(T_E\) cell has no neighboring \(T_R\) cell (i.e. being alone in the conjugate or with another \(T_E\) cell), and the probability that a conjugated \(T_R\) cell has a neighboring \(T_E\) cell. These expressions are based on a multinomial approximation that is valid given that the total number of sites (summed over the APCs) is much larger than the number of sites per APC. More rigorous and general expressions, based on the hypergeometric distributions, were derived in the original references (15, 46).

Standard Behavior

Leon et al. (15) have shown that the particular dynamical behavior set into play in the Crossregulation Model is determined by two key composite parameters, representing the effective "growth indexes" of \(T_R\) and \(T_E\) cell populations. These two parameters
are directly proportional to the basic parameters controlling population growth, namely conjugation constants, density of APCs, maximum proliferation rate per activated T cell, and inversely proportional to the death rate of the corresponding population. The values of $T_R$ and $T_E$ growth indexes define four parameter regimes according to the resulting dynamic behavior. A systematic analysis of the possible parameter regimes can be found in the original publication (15). For the present purposes, it suffices to focus on the parameter regime where a system composed of two populations of $T_E$ and $T_R$ cells, driven by a sufficiently large population of APCs, shows a bistable behavior. In particular, the system can evolve either into an equilibrium state in which only $T_E$ but not $T_R$ cells are present, or into a state in which both cell types coexist in a stable balance, depending on the composition of the seeding mixture of populations (fig. 2). The system develops into the $T_R$ and $T_E$ coexistence state, provided that the seeding population has sufficient $T_R$ cells (fig. 2a). Otherwise, if $T_R$ cells are initially “outnumbered”, $T_E$ cells will competitively exclude them from the system (fig. 2b). One of the key aspects of the model is that the interactions between $T_E$ and $T_R$ cells depend on the density of the APC population, and thus $T_R$ cells can also be “outnumbered” by the APCs themselves. A sufficiently large number of APCs will dilute away the direct T cell interactions, giving $T_E$ cells a chance to competitively exclude $T_R$ cells (fig. 2c).

In this model, (auto)immunity and tolerance to a given antigen are interpreted, respectively, as the competitive exclusion of $T_R$ cells by the expansion of the $T_E$ cell population, which becomes limited only by APC availability, and the poised coexistence of both $T_E$ and $T_R$ cell populations. As argued before (15, 48), healthy immune systems necessarily operate in the bi-stable regime. This is the only condition compatible with the observations that significant numbers of both $T_R$ and $T_E$ cells can be recovered from healthy animals, and that the incidence of autoimmune pathology or tolerance in adoptively reconstituted recipients can be modulated by changing the proportions and absolute numbers of $T_R$ and $T_E$ cell-enriched populations.

**Cellular and molecular mechanisms of crossregulation between regulatory and effector T cells**
Since the original hint at the crossregulation hypothesis (15), a considerable amount of information has accumulated concerning the life cycle of CD25+ T<sub>R</sub> cells, and the interactions they make with other cells. In this section we will briefly review these findings. The bottom-line is that there is ample evidence for the existence of APC-dependent crossregulation of autoreactive T<sub>R</sub> and T<sub>E</sub> cell populations, as captured in the model, though the molecular and cellular details are hitherto unclear.

Crossregulatory interactions between regulatory and effector T cells <i>in vitro</i>

Irrespective of the underlying mechanisms, APC-dependent crossregulation of T<sub>E</sub> and T<sub>R</sub> cell proliferation can be readily observed <i>in vitro</i>, using an experimental design that allows one to follow the proliferation of both cell types independently (fig.3). T<sub>R</sub>-enriched CD25+CD4+ T cells (for short CD25+ T<sub>R</sub> cells) isolated from healthy animals are unable to proliferate when stimulated <i>in vitro</i> with APCs and anti-CD3 (51, 52). And yet, these regulatory cells do proliferate when T<sub>E</sub>-enriched CD25-CD24+ T cells (for short CD25- T<sub>E</sub> cells) are added to the culture, as can be assessed by CFSE-delabeling (fig. 3b). Moreover, the proliferation of T<sub>R</sub> and T<sub>E</sub> cell populations in these co-cultures is strongly correlated, indicating that the two cell types are using the same growth factors. Since the collective proliferation increases with the T<sub>E</sub>:T<sub>R</sub> cell proportion at which the co-cultures are seeded, it follows that T<sub>E</sub> cells are producing growth factors shared by both cell types, and the production (or availability) of these growth factors is inhibited by T<sub>R</sub> cells in a dose dependent manner.

IL-2 is one of the growth factors mediating the crossregulation between T<sub>E</sub> and T<sub>R</sub> cells observed <i>in vitro</i> (51-53). Most of the proliferation of freshly isolated T cells is dependent on autocrine/paracrine IL-2 produced by the T cells themselves. CD25+ T<sub>R</sub> cells, which do not proliferate when stimulated in similar conditions, do not transcribe the IL-2 gene. Notwithstanding, CD25+ T<sub>R</sub> cells, expressing a high affinity receptor for IL-2, do proliferate when supplied with exogenous IL-2 (51, 52). Anti-IL-2 blocking antibodies inhibit the proliferation in co-cultures in a dose-dependent manner (54). Finally, CD25+ T cells suppress IL-2 transcription by CD25- T cells (55) and/or the availability of this shared cytokine in co-cultures (54), completing the crossregulation interaction scheme.
The molecular and cellular details of the suppression mechanism are controversial. Studies using transwell culture systems and specific blocking antibodies have been used to minimize the role of paracrine suppressive cytokines, concluding in favor of a pathway dependent on direct cell-to-cell interactions between activated CD25+ T cells and target CD25- T cells (52). This conclusion, however, is not fully warranted because suppressive cytokines can act in a juxtracrine manner that may not be effective at the relatively long distances of transwell cultures, or that may not be inhibited with antibody concentrations optimized to block paracrine pathways. Convincing evidence show that a significant part of the suppression observed *in vitro* is due to the consumption of IL-2 by the CD25+ TR cells (54). The fact that expression of the IL-2 gene is “autocatalytic” implies that the overall concentration of IL-2 available in cell cultures might be critically sensitive to IL-2 consumption by CD25+ TR cells, as discussed by (53) and suggested by mathematical models (56, 57). However, suppression of IL-2 messenger RNA is still observed in co-cultures of CD25+ TR cells and CD25- TE cells supplemented with exogenous IL-2, despite the bypass of the proliferation blockade (55). This inhibition of IL-2 transcription implicates mechanisms of suppression other than IL-2 consumption.

In summary, TE and TR cells crossregulate each other’s proliferation *in vitro*, through the crossregulation of IL-2 production and availability via yet unresolved interactions. Other growth factors may play minor roles in crossregulation of proliferation. CD25+ TR cells stimulated with anti-CD3, with or without APCs, proliferate in response to a variety of γc-family cytokines, namely IL-4 (58) and IL-15 (our unpublished observations). Some of these cytokines, namely IL-4, can be directly produced by cells within the CD25- TE subset (59). CD25- TE cells may also induce APCs to upregulate growth promoting factors, as it has been documented for other stimulatory interactions among T cells (60).

Crossregulatory interactions between regulatory and effector T cells *in vivo*

Several lines of evidence indicate that the mutual interactions between TE and TR cell populations, which underlie the Crossregulation Model, are also relevant *in vivo*. The immunopathology observed in immunodeficient animals reconstituted with CD25- TE cells isolated from healthy donors is concomitant with uncontrolled proliferation (4,
38). In animals receiving CD25+ T_R cells alone, or animals receiving both CD25- T_E and CD25+ T_R cells, the T cell proliferation is less pronounced and controlled; consequently, the total T cell population reaches an equilibrium state with lower cell numbers (37, 38). Furthermore, in recipients reconstituted with CD25- T_E and CD25+ T_R cells, the size of the T_R population recovered at steady state increases with that of the activated T_E population (37, 38), indicating that T_R cell proliferation depends on T_E cells. Although the clear interpretation of these experiments is complicated by the fact the cell populations are clonally heterogeneous, the results are non-trivially compatible with the Crossregulation Model (15, 48). Hence, the controlled expansion observed in recipients of CD25+ cells is only compatible with the model, if CD25+ T_E cells are co-purified and carried along with CD25+ T_R cells into the recipients, where they become the source of shared growth factors. In fact, recent observations on the relative frequency CD25+Foxp3- T cells, presumably bona fide activated T_E cells, within the pool of CD4+CD25+ T cells of healthy mice (28) gives support to this additional assumption.

Corroborating the relevance of the observations in vitro, IL-2 has also been implicated in the crossregulation of T_E and T_R cell population dynamics in vivo. Mice genetically deficient in IL-2 signaling, namely IL-2-/- (61), CD25-/- (62), IL-2 receptor β-chain (63), STAT-5-/- (64, 65), and JAK-3-/- (65) mutants, display uncontrolled lymphoproliferation and autoimmune pathology. Regulatory T cell density is significantly decreased in these animals (65, 66). Adoptive transfers of CD25+ T cells isolated from wild type donors restores regulation of T cell dynamics and prevents disease in mutant animals (37, 65, 67). Collectively, these observations indicate that the persistence and expansion of T_R cell populations in vivo are strongly dependent on IL-2 signaling. As non-regulatory T cells are the major source of this cytokine in vivo, IL-2 must be one of the major T_E-dependent growth factors shared by T_E and T_R cells. Although it would be tempting to assume here a simple scenario where IL-2 is THE only growth factor underlying all the postulates in the Crossregulation Model, this hypothesis would fall short. The expansion of non-regulatory T cells observed in animals with compromised IL-2 signaling is necessarily mediated by other, most likely autocrine, growth factors. Likewise, wild type T_E cells resort to other autocrine growth factors whose expression is likely coordinated with that of IL-2. In contrast, IL-2 seems to be the main growth factor that T_E cells provide
to T<sub>R</sub> cells. This was captured in the model by making the proliferation rate of activated T<sub>E</sub> cells greater than that of activated T<sub>R</sub> cells (p<sub>E</sub>＞p<sub>R</sub>). Coherence with the postulates of the model requires the collective expression of all autocrine growth factors to be suppressed; otherwise, if T<sub>R</sub> cells would only suppress the subset of growth factors they depend on, they would always be outcompeted by effector T cells (15).

As to the actual mechanism of suppression in vivo the information is still rather incomplete. It is unlikely that all autocrine growth factors produced by T<sub>E</sub> cells would be suppressed by IL-2 consumption alone, unless their expression is dependent on IL-2. Accordingly, cytokines such as IL-10 (38, 68) and TGF-β (69, 70), which are potent modulators of gene expression in target cells, have been implicated as likely mediators of T<sub>R</sub>-dependent suppression of T<sub>E</sub> cell proliferation and function in vivo. Direct cell-to-cell interactions among T cells, as proposed for in vitro systems, may also play a role as suggested by the implication of GITR (71, 72) and CTLA-4 (73, 74) in suppression. However, direct cell-to-cell interactions among T cells have been recently questioned based on imaging of APC, T<sub>R</sub> and T<sub>E</sub> cell movement and encounters in vivo (75), which indicate that there are less frequent stable conjugates between T cells than between T cells and APCs. This suggests that APCs mediate signals between T cells serving as a temporal bridge (75). Alternatively, T<sub>R</sub> cells may leave a transient trail of suppressive molecules in the foci surrounding the APC that suppress T<sub>E</sub> cells when they later pass by.

APC-dependence of interactions among antigen specific T cells

As mentioned above, the Crossregulation Model postulates that growth, survival, and suppression signals exchanged by T<sub>E</sub> and T<sub>R</sub> cells require the physical co-localization of these cells in interaction foci, whose capacity depends on the APC density. On the one hand, this confers antigen specificity to the interactions, and on the other hand, brings forth the possibility that changes in the number of APCs affect the balance between T<sub>E</sub> and T<sub>R</sub> cells.

Recent advances in two-photon confocal microscopy support this assumption. Both antigen-specific T<sub>E</sub> and T<sub>R</sub> cells swarm around cognate APCs, with which they can form stable conjugates (75). This local increase in the density of antigen specific
T cells is expected to favor and make possible interactions among T cells, which would otherwise not meet frequently enough, due to the low densities in the rest of the body. This need for increase in local density is required for direct cell-to-cell interactions as well as to those interactions that are mediated by soluble factors acting in paracrine or juxtacrine models.

Localization of T cell interactions, whether resulting in proliferation or suppression, around cognate APCs is also imposed by the transient, labile expression of their molecular mediators. Thus, most above-mentioned paracrine regulatory molecules, which are candidates to play the role of T E cell derived growth factors shared with T R cells, or to play the role of a suppressive signal, are transiently expressed by T cells upon productive interactions with cognate APCs. This is well documented for growth promoting cytokines, such as IL-2 and other γc-family members, as well as for suppressive cytokines like IL-10 and TGF-β. The expression of the corresponding receptors by T cells is also TCR-dependent and labile. The best example being the labile expression of CD25 itself (37, 38, 76) which is definitively involved in allowing T R cells to benefit from IL-2 produced by T E cells, and may also be involved in suppressing proliferation via IL-2 consumption, as discussed above. Even when interactions between T cells are mediated through the modification of APCs, irrespective of growth promoting or inhibiting interactions, the effect can be very localized provided that the APCs remain in place, or if their modification is transient.

Further support to the notion that antigen-specific interactions between T R and T E cells operating in vivo (43, 77, 78) are restricted to APC-dependent foci is provided by several reports, showing that two T cell populations will only interfere with each other if they have the same specificity or if their cognate antigens are presented by the same APCs, or co-localized in the same tissue (79-83).

Notwithstanding these facts and considerations, in vitro studies (84) originated a common assertion that regulatory T cells are antigen non-specific. Definitively, regulatory CD4 T cells do not recognize the antigen on their target T cells since the latter lack MHC class II molecules. And yet, suppression will be effectively antigen-specific in vivo if the rate-limiting step is the antigen-dependent co-localization of the suppressor and target cells. Antigen-nonspecific bystander suppression is most likely observed in vitro when the experimentalist forces the massive co-localization of
regulatory and target cells in the bottom of the culture plate wells, reproducing artificially what in vivo could only be achieved by antigen- and TCR-dependent mechanisms.

Several molecular mechanisms may lead to the same cell population dynamics.

In the previous subsections we evoked different hypotheses about the molecular details of the crossregulatory interactions between APCs, regulatory T cells and their targets. The different hypotheses for the mechanism of suppression are illustrated in fig. 4, where the temporal order and stoichiometry of the key intermediate steps are portrayed. Despite their differences, all the reactions have the same input and output. It is therefore expected that these different cell interaction mechanisms may produce the same collective behavior and the same cell population dynamics, provided that the intermediate steps are sufficiently fast.

In order to better understand how each cell interaction mechanism impinges on the collective behavior of the population, we explored individual cell-oriented simulation systems that take into account geometric and spatial constraints, not easily captured in differential equations. In these simulations, individual cells are regarded as hybrid microagents (85, 86), treated as soft spheres with internal state and state transition rules. The spheres undergo random walks in Real 3D-space, and can adhere to each other, building non-stoichiometric aggregates. These aggregates move and rotate randomly, and the magnitude of translation and rotation decreases with the size of the aggregate, as suggested by experimental observations. To make these simulations realistic, the motion and adhesion parameters have been parameterized so that the simulations can reproduce the motion and aggregation patterns observed when APCs, T<sub>e</sub> and T<sub>r</sub> cells are cultured in vitro, and imaged by confocal microscopy (fig. 5a). In these simulations, the interactions each cell makes determine changes in its state transition rules. The internal state, in turn, determines if the cell dies or divides, and how it interacts with other cells. By defining the rules by which the internal state changes, one can simulate the different hypotheses. As it turns out, simulations based on each of the hypotheses listed in fig.4, can qualitatively reproduce the experimental CFSE-profiles depicted in fig. 3b, under plausible parameter regimes. For example, the results illustrated in fig. 5b were obtained using
simulations of the hypothesis portrayed in fig. 4c. According to this hypothesis the APC-dependent activation and production of growth factors by \( T_E \) cells is inhibited by \( T_R \) cells via direct cell-to-cell contact, and this inhibitory capacity is induced transiently in \( T_R \) cells following their contact with the APC.

A systematic analysis of the capacity of the different mechanisms to explain the quantitative details of \textit{in vitro} suppression assays will be reported elsewhere (Gardner, et al. in preparation). For the present purpose it is only necessary to say that the Crossregulation Model describes, without loss of generality, the cell population dynamics that is expected from all these different molecular mechanisms. In other words, at the slow time scale at which the average T cell population densities change by death and proliferation one can bracket all the fast intermediate steps indicated in fig. 4 to obtain the “aggregated” suppression reaction of fig. 1.

Supported by these results, in the next section we use modeling and simulation of the Crossregulation Model, described via eqn.1-7, to understand the selection of the repertoire of \( T_E \) and \( T_R \) cells \textit{in vivo}.

**Peripheral selection of the preimmune repertoire dependent on crossregulation**

What maintains the size and diversity of regulatory T cell populations in a steady state immune system? Are all regulatory T cells specific for self-antigens? Or instead, are there regulatory T cells against any possible antigen, including pathogens? If so, how can protective immune responses be mounted? These are the kind of questions that are being debated in the literature, and for which immunological common sense and current theories provide no clear a priori answer. It turns out that the Crossregulation Model makes some qualitative predictions about the repertoire of regulatory T cells, thus generating testable answers to these questions.

Dependence on APC density

The Crossregulation Model posits that the persistence and expansion of T cell populations require recurrent interactions with APCs that lead to activation and, if growth factors are locally available, to cell proliferation. This proliferation is necessary to compensate the slow loss of quiescent cells. The model assumes that
APCs are limited in number, and therefore T cells will compete for this resource reaching a steady state (15, 87, 88). While both $T_E$ and $T_R$ cells need productive APC conjugations for activation, $T_E$ cells produce autocrine growth factors and $T_R$ cells do not. Therefore, $T_E$ cells once activated by APCs can proliferate driven by their own autocrine growth factors, while $T_R$ cells, in addition to a productive APC conjugation, will need paracrine or juxtacrine growth factors produced by $T_E$ cells in their vicinity. These simple principles imply that there is a critical APC density, say $a_E$, that is necessary and sufficient to sustain a $T_E$ cell population (fig. 6-top). The persistence of a $T_R$ cell population requires a higher density of APCs, say $a_R$; only APC densities above this value can sustain a sufficiently large density of a growth factor producing $T_E$ cell population in the neighborhood of the $T_R$ cells (fig. 6-top). Based on these simple cell population principles it is expected, as shown below, that the peripheral T cell repertoire can be naturally partitioned in fractions with and without regulatory T cells, according to the degree of autoreactivity.

Stationary state in clonally diverse $T_E$ and $T_R$ cell populations

Understanding the T cell population and repertoire dynamics seems a daunting task. $T_E$ and $T_R$ cells belong to many different clones. Each clone of T cells will have its own set of cognate APCs, which most likely will partially overlap with those other clones. Furthermore, APCs are also heterogeneous, consisting of many populations of equivalent cells. APCs in each population will present a particular set of peptides, and each peptide will be at its own specific concentration. This peptide set may overlap partially with the peptide set of other APC classes. How can we grasp the properties of this complicated network of interacting cell populations?

Many of these complications may be fairly reduced if one considers only the cell population dynamics at the steady state in a preimmune individual. At least this is what would be expected according to the principles of population dynamics underlying the Crossregulation Model. Leon et al. (46) demonstrated that each population of equivalent APCs can either sustain a single clone (or set of equivalent clones) of $T_E$ cells, or, alternatively, sustain a single clone (or set of equivalent clones) of $T_E$ cells in equilibrium with a single clone (or set of equivalent clones) of $T_R$ cells. This is so because, according to the model, all the T cells that recognize
some peptides on the same APC will compete for conjugation sites, and the most efficient clones of each T_E or T_R type, will exclude their respective competitors.

Competitive exclusion implies that, to some approximation, one can regard the steady state composition of the peripheral repertoire as a set of “operationally” independent pairs of T_E and T_R clonal populations, in equilibrium with their own population of (equivalent) cognate APCs. How good is this approximation? To address this issue, we performed simulations where many T_E and T_R cells clones and APC populations classes, were initially networked in non-trivial ways, and allowed to reach equilibrium. The results of these simulations will be reported elsewhere (Sepulveda et al. In preparation). It turns out that in these simulations, after competitive exclusion has purged the repertoire from less efficient clones, the final connectivity is effectively one T_E cell clone to its cognate APCs, or one pair of T_E and T_R clones with their cognate APCs. A simplified setting where the whole repertoire is made of many independent pairs of T_E and T_R clones driven by a distinct cognate APC population, recapitulates the basic behavior of the more complex simulations, and is, therefore, used in the following sections to illustrate the implications of the Crossregulation Model.

Partitioning of the repertoire in subsets with and without regulatory T cells

The Crossregulation Model predicts that the peripheral repertoire of CD4 T cells in healthy preimmune individuals can be naturally partitioned in three subsets of lymphocytes according to the density of the corresponding cognate APCs (fig. 7).

The first subset is composed of all the lymphocytes that do not meet their rare cognate APCs frequently enough during circulation through the body. Essentially these would be recent thymic immigrants waiting to die, and their total number should be determined by the ratio between thymic production and peripheral death rates. Within this lymphocyte set, the average clonal size is 1 cell. The proportions of T_R and T_E cells should be the ones produced by the thymus if the two cell types have identical death rates, or otherwise balanced towards the cell type decaying at a slower pace. T_E cells produce autocrine growth factors, and can divide upon sporadic encounters with cognate APCs, and therefore, as a population, they will decay more slowly than T_R cells, even if the “intrinsic” life-span of the two cell types are the
same. This set of peripheral lymphocytes would be lost in thymectomized adult animals.

The second subset of specificities corresponds to T cells that are driven to proliferate by populations of cognate APCs whose density in the body can sustain $T_e$ cells but not $T_r$ cells (i.e. APC density larger then $a_e$ but lower than $a_r$). The clonal sizes within this T cell subset would be proportional to the limiting APC population, being small but larger than a single cell. Because lymphocytes compete for APCs, only one clone (or a class of equivalent clones) would remain in the system; the remaining clones would be competitively excluded or reduced to the baseline level determined by thymic export and death rate. Within this population, there would be mostly $T_e$ cells. $T_r$ cells with the same specificity would not be sustained because they would not encounter APCs and $T_e$ cells, on which they depend, often enough to compensate their death rate. The $T_e$ cells within this subset would persist upon adult thymectomy but the residual $T_r$ cells would be lost.

The third subset of lymphocyte specificities corresponds to T cell clones that recognize a sufficiently dense cognate APC population that can sustain both $T_e$ and $T_r$ cells (i.e. cognate APCs at densities higher then $a_r$). The clonal sizes of either $T_r$ or $T_e$ cells in this subset would be relatively small because $T_r$ cells would prevent clonal expansion. Diversity would also be limited, as in the previous subset, due to competitive exclusion. The proportions of $T_e$ and $T_r$ cells would be biased towards the latter, and this bias would increase with the number of stimulatory APCs that sustain them (fig. 7). Both $T_e$ and $T_r$ cells within this set should persist upon adult thymectomy.

Tolerance to self requires that $T_r$ cells control all autoreactive T cells that would otherwise cause autoimmunity. It is reasonable to assume that $T_e$ cells that are in equilibrium with cognate APCs at low densities (lower than $a_p$) are not sufficiently expanded to cause autoimmunity, and only $T_e$ cell specificities driven by cognate APCs at high density (larger than $a_p$) can trigger autoimmunity. Under these conditions, a globally tolerant steady state can only be reached if all the peripheral T clonal populations are seeded with enough regulatory T cells. In the simulations illustrated in fig. 7b this was guaranteed by seeding each population with 1/3 of $T_r$ cells, irrespective of their specificity.
The Basic Ground for Self-nonself Discrimination

The partitioning of the preimmune repertoire described above is plausible given that it follows naturally from the experimentally supported assumptions on crossregulation, and that it implies that the peripheral repertoire of (Foxp3+) T_{R} cells should only partially overlap with that of (Foxp3-) T_{E} cell, and should be, on average, more biased towards body antigens, as recently demonstrated (reviewed by Kim & Rudensky (89)). But does this have any biologically meaningful consequences?

Consider that the peripheral repertoire of healthy adult animals would in fact be partitioned as portrayed in fig. 7. The frequency distribution of specificities over their cognate APCs produces (by construction) a reasonable output: none of the T cell populations driven by a high density of cognate APCs are locked in the equilibrium containing exclusively effector cells, the total T_{E} cells are a minor fraction of the total T cells (fig. 7c piechart on the left), and the diversity of T_{R} cells is only a fraction of the total diversity of the resident peripheral pool (fig. 7c piechart on the right).

Suppose now that a new antigen would be introduced in the body under conditions that generate a new ensemble of APCs presenting its peptides. Since this new antigen has not participated in the selection of the preimmune repertoire, it is fair to assume that all T cell populations in the repertoire, in the fractions with and without T_{R} cells, are likely candidates to recognize its peptides. Under these conditions, the pool of T cells responding to the new antigen corresponds to a random sample from the set of available clonal specificities. Most likely, T_{R} cells will be a minority in this pool, and therefore responding cells will proliferate and evolve towards the equilibrium where T_{E} cells eventually outcompete T_{R} cells.

This means that the repertoire partitioning, which follows naturally from the Crossregulation Model, gains a whole new meaning in terms of the capacity of the immune system to be tolerant to body antigens, and to respond to the remaining universe of antigens. In other words, the natural partition of the repertoire sets the ground for self-nonself discrimination.

**Constraints on Thymic Selection and Maturation of Regulatory T cells**
The thymus plays a key role in self-tolerance. This section addresses the possibility that thymic selection and differentiation of regulatory T cells from immature thymocytes are controlled in a way that they synergize with peripheral selection to produce an appropriate partitioning of the preimmune repertoire. The upshot is that there is some redundancy in thymic and peripheral selection, and this redundancy increases the robustness of repertoire partitioning and natural tolerance.

How can thymic selection influence the density of APCs that can stimulate a given clone of T cells in the periphery? The density of cognate APCs available to a T cell clone in the periphery increases with the crossreactivity of the TCR and with the ubiquity of the cognate peptides. On the one hand, the more crossreactive, degenerate, or promiscuous a TCR is the larger the set of its cognate peptides, and therefore the larger the set of APCs that can present cognate peptides. On the other hand, if the TCR of a clone recognizes ubiquitous peptides, then this clone can be, at least potentially, stimulated by many APCs. In the thymus, positive selection keeps in the repertoire only those immature thymocytes whose TCRs are sufficiently crossreactive to recognize some peptides expressed locally, and perhaps a minimum density of APCs presenting these peptides (90). In turn, negative selection deletes from the repertoire those immature thymocytes, bearing TCRs that either recognize ubiquitous antigens or that are excessively multireactive. The overall process of thymic selection is expected to produce and export to the periphery an emergent repertoire, in which clonal specificities are distributed over the density of peripheral cognate APCs as a bell-shaped curve (illustrated in fig. 7a and fig. 8). The median and the variance of this distribution must therefore depend on the thresholds and stringency of positive and negative selection processes.

The other relevant question is: how is the frequency of regulatory T cells at which the periphery is seeded controlled in the thymus? This is basically a question about the mechanism of generating T<sub>r</sub> cells. Commitment of immature thymocytes to the regulatory phenotype may happen by many different non-mutually exclusive mechanisms, ranging from stochastic cell-fate decisions followed by selection, to directed differentiation or “instruction”. Both selection and instruction may be mediated by a combination of TCR-dependent (24, 27, 91) and independent mechanisms, including IL-2 receptor dependent signals (67). The fraction of
regulatory T cells that seeds the periphery can be therefore modulated both in TCR-specificity dependent and independent ways.

Constraints on positive and negative selection, and on thymic differentiation of regulatory T cells

Thymic selection can potentially shape the frequency distribution of T cell clonal specificities over cognate APC densities in many ways that are compatible with a globally tolerant steady state (fig. 8). This is possible due to the robustness of the peripheral selection process that sorts the repertoire in portions with and without T<sub>r</sub> cells, and ensures that the highest autoreactivities are restricted to the regulated portion.

In a scenario where thymic selection is less stringent and the avidity thresholds for positive and negative selection of the TCR are further apart, the distribution of the emergent repertoire over the density of peripheral cognate APCs is shifted to the right and broader than in the previous scenario (fig.7). Less stringent selection means that thymopoiesis will have a better yield in terms of the number and diversity of mature cells per precursor. However, a less stringent selection also implies a higher frequency at which the thymus exports clonal specificities driven by more dense cognate APCs in the periphery — i.e. highly autoreactive or crossreactive clonal specificities. This in turn increases the frequency at which autoreactive populations are locked in the regulatory-cell free equilibrium, interpreted as autoimmunity (fig.8a). The risk of autoimmunity might nevertheless be kept under control in conditions that relax selection stringency (fig.8d compared to fig.7) through a compensatory increase in the rate at which the thymus generates regulatory T cells (fig.8c,d and e). Potentially, this compensatory increase in regulatory cell generation rate may be mediated by tuning the efficiency of TCR-specific or nonspecific mechanisms (fig.8b, d, and e).

From the point of view of ensuring tolerance to self, a scenario in which the thymus exports regulatory T cells with a fixed (specificity-independent) probability (fig.8b) might be equivalent to a scenario in which the thymus exports preferentially autoreactive or multireactive regulatory T cells (fig. 8d). And yet, for the same number of precursors, one expects that there will be less clonal specificities of T<sub>r</sub>
cells able to respond to invading pathogens, in the first scenario (fig. 8b) than in the second scenario (fig. 8d). This is so because the crossregulation dynamics in the periphery purges from the repertoire those regulatory T clones that are not sufficiently autoreactive, and therefore do not find dense enough cognate APCs populations. The TCR-dependent maturation of regulatory T cells in the thymus is therefore more flexible, and deals better with a tradeoff between generation of diversity and reliability of natural tolerance. Whether or not there is an advantage in generating regulatory T cells via a TCR-dependent mechanism depends therefore on how much diversity is necessary to protect against pathogens, and also on how many clonal specificities would be lost by committing to regulatory function immature lymphocytes whose progeny will not persist in the periphery.

A mechanism in which thymic selection biases the repertoire of regulatory T cells towards those TCRs that are highly autoreactive or crossreactive, or recognize ubiquitous body antigens (as depicted in fig. 8) was proposed before (24), and more recently shown to operate in mice (27, 91). This indicates that at least in mice there might be a trade-off between diversity and tolerance induction. An intriguing possibility is that animals with larger bodies, which have higher absolute number of precursors and therefore can produce more TCR variants per unit of time, might get away without resorting to a TCR-dependent mechanism of generating regulatory T cells in the thymus. Therefore, it is not unlikely that there might be differences across species in terms of the constraints on thymic selection. For example humans might have lost, or perhaps never have evolved, mechanisms of regulatory T cell maturation in the thymus that depend on TCR signaling, as mice seem to have done.

It is worth stressing that, according to the model, specific $T_R$ cells will only colonize the periphery if $T_E$ cells of the same specificity are also there, which implies that the thymus should produce specificity-matched pairs of $T_E$ and $T_R$ cells, as it was shown in TCR-transgenic animals (27, 92).

Finally, this section would not be properly concluded without emphasizing that the thymus can only produce an appropriate distribution of specificities over the density of cognate APCs in the periphery if there is some correlation between what is presented in the thymus and what is presented in the periphery. This is achieved partially, as mentioned in the introduction and discussed before (46), by biasing the emergent TCR specificities towards the recognition of MHC peptide sets present both
in the thymus and in the periphery. This correlation is further promoted by the AIRE gene product that makes constitutive tissue-specific antigens available in the thymus (93, 94). This promiscuous expression of peripheral antigens mediates deletion of immature thymocytes (95), and may elicit the maturation to the regulatory phenotype (96). AIRE mutant mice (97), which display generalized autoimmune pathology, would correspond in our model to a scenario where the repertoire distribution is broader and shifted to higher APC densities than the wild type (the scenarios illustrated in fig. 8a,c and fig. 7, respectively). The finding that AIRE protein mediates the expression of peripheral antigens in local clusters of epithelial cells implies that deletion or instruction to the regulatory phenotype is inherently stochastic: among all the immature thymocytes bearing a specific TCR only those that occasionally migrate close to these clusters of epithelial cells can be deleted or instructed to become regulatory.

Coordination with the Innate Immune System

Invertebrates have highly evolved mechanisms of defense against pathogens. Some of these mechanisms are still built-in in the innate immune system of vertebrates, most likely readapted to operate in coordination with the adaptive lymphocyte system. As discussed above, the partitioning of the T cell repertoire, as expected from the Crossregulation Model, provides a robust mechanism underlying some kind of self-nonself discrimination, i.e. natural tolerance to self and adaptive immune responses to exogenous antigens unrelated to self. The model suggests also how innate mechanisms can synergize with crossregulation of T lymphocytes to fight infection, to reinforce tolerance to self, and to deal with conflicting situations such as infections with pathogens expressing antigens that mimic self.

The direct recognition of molecular structures of pathogens by innate receptors on APCs and their precursors plays a well-accepted role in promoting immune responses (98-100). From the onset of an infection, these receptors trigger the proliferation and differentiation of APC precursors causing an avalanche of new APCs that present antigens from the pathogen in the draining lymph nodes. These APCs are “new” not only because they contain new antigens from the pathogen, but also because they likely present profiles of self-peptides that are different from self-
peptide profiles displayed by the preimmune APCs. These changes in self-peptide profiles may be instrumental in facilitating immune responses by rendering pre-existing dominant T_R clones “blind” to these new APCs (46).

The local upsurge of these new APCs drives the expansion of a pool of T cell clones sampled from the equilibrium preimmune repertoire. In most of the cases, regulatory T cells will be outnumbered in this pool, and although they might interfere with the rate of clonal expansion of the responding T_E cells, they will not prevent the response. However, in those rare situations in which the microorganism mimics self, T_R cells may predominate in the pool of antigen-specific T cells. In these situations of mimicry, as discussed before (47), the outcome will depend on speed and magnitude of the rise in APC density, triggered by the innate mechanisms, relative to the proportion of T_R cells preset in the preimmune repertoire. Whatever the proportion of protective T_R cells is, they may be diluted in APC-dependent interaction foci, if the influx of APCs is sufficiently fast and large. In contrast, if the influx is slow and gradual enough, a fraction of T_R cells in the responding pool may slowly adapt its size and control the expansion of T_E cells. The innate APC responses and T cell crossregulation may be co-adapted in vertebrates, so that among the microorganisms mimicking the host, those that make fast and overwhelming infections will override self-tolerance and eventually trigger autoimmunity, while those that grow slowly within the body might be assimilated.

The causal relationship between infections and autoimmunity is a long-standing puzzle. A direct correlation between the incidence of autoimmunity and infections is to be expected from antigen mimicry, according to the classical models and also, as just seen, in the present models of regulatory T cells, in the case of acute overwhelming infections. A few examples of autoimmune disorders, which are documented as being caused by infection with particular pathogens, support this view (7, 8, 39, 101). And yet, there are several experimental animal models where infection appears to prevent the onset of autoimmunity (7, 8, 39, 101). Moreover, some epidemiological studies suggest an inverse correlation between the incidence of autoimmunity and the general prevalence of infections in human populations (102). The Crossregulation Model provides a simple rational for these puzzling observations as discussed in Leon et al. (47). The continuous, gradually increasing exposure to diverse subclinic infections, rapidly controlled by innate and adaptive immune
responses, is expected to lead to a concomitant slow gradual increase in the density of APC presenting self-peptides to autoreactive T cells. This slow increase in APC density renders tolerance more robust, since it implies a gradual increase in the density and in the fraction of T<sub>R</sub> cells (see for example fig. 6 and fig. 7).

The discovery that CD25+CD4+ T<sub>R</sub> cells express some innate receptors for molecular structures of pathogens, such as toll-like receptors (TLR), and proliferate in response to the corresponding ligands (103-105) might have an important biological significance in this context of the coadaptation of innate and adaptive immune systems. This direct effect of microorganisms on regulatory T cells can synergize with the indirect effect that subclinic infections have on APC densities to render tolerance more robust. In particular it might have a fundamental role in keeping a proper balance of T<sub>E</sub> and T<sub>R</sub> within the relative minority of autoreactive T cells that happen to be driven by the growing population of APCs elicited during an acute infection by a pathogen poorly related to self. In the absence of such potential direct signals to T<sub>R</sub> cells by the pathogen, as we have discussed above, the tendency within the pool of responding T cells would be towards a predominance of T<sub>E</sub> cells, and eventually to the competitive exclusion of T<sub>R</sub> cells. This predominance of T<sub>E</sub> within the subset of autoreactive T cells swiping with the response, could switch the equilibrium associated with APCs presenting purely endogenous peptides towards autoimmunity (46). This sort of “epitope spreading” of immunity from pathogens to self-antigens, can be controlled through the direct stimulatory effect that pathogen structures like LPS have on T<sub>R</sub> cells (103), via the maintenance of regulatory cells within the autoreactive cells swiping on the immune response. A similar protective effect could also be mediated by the upregulation of endogenous TLR-ligands locally in the infection foci.

A Critique of the Crossregulation Model and Other Scenarios

The Crossregulation Model describes regulatory T cells, from the cell interactions that control their life cycle to the selection of their repertoire in the thymus and in the periphery, and their contribution to the definition of self. The model submits specific answers to some of the currently open questions about regulatory T cell immunobiology. Thus, under the light of the model, one expects that the most
important source of regulatory T cells should be the thymus, and not peripheral differentiation, given the functional implications of this assumption. The regulatory T cell repertoire emerging from the thymus should be complete and overlapping with that of conventional T cells, though the model is undecided on the fraction of regulatory T cells per specificity. In contrast with the emergent repertoire, the actual repertoire of regulatory T cells residing in the periphery should not cover all the specificities available within the pool of conventional T cells. Instead, it should be restricted to those specificities that see high densities of cognate APCs, and therefore could be associated to autoimmunity. Regulatory cells should therefore be all autoreactive, and only occasionally will some of these cells also recognize antigens from microorganisms. This means that adaptive immune responses to microorganisms are facilitated by the predominance of non-regulatory T cells within the responding pool. The model also indicates that protective immune responses can be mounted to microorganisms that mimic host antigens provided that infection induces locally a massive upsurge in APCs.

Some of these expectations derived from the Crossregulation Model contrast with some of the hypotheses available in the literature and deserve a critical discussion here.

Does size matter? Control of Lymphocyte Activation and Effector Function versus the Control of Clonal Expansion

As mentioned before, we assumed that $T_R$ cells prevent autoimmunity by controlling the clonal expansion of autoreactive CD4 T lymphocytes. In a transgenic animal model for regulatory T cell prevention of spontaneous autoimmune encephalomyelitis (43) the peripheral repertoire mimics a situation where a single clone of autoreactive anti-myelin basic protein T cells represents more than 90% of all the peripheral CD4 pool, and still, a minor population of specific regulatory T cells is able to prevent autoimmune disease. This indicates that controlling clonal expansion of autoreactive T cells may be sufficient, though not necessary, to control autoimmunity. In these animals, other mechanisms must operate to ensure tolerance despite the high frequency of responding cells. These additional mechanisms are not captured in the
Crossregulation Model. The crossregulation mechanism and the implied partitioning of the repertoire, must therefore be understood as a first filter before other mechanisms of peripheral tolerance induction, or class regulation, are allowed to operate.

Central versus peripheral Generation of Regulatory T cells

We neglected the contribution of peripheral differentiation of regulatory T cells, despite some indications that regulatory T cell differentiation may take place in the periphery (106-109). Often, one cannot rule out the possibility that thymic-derived T<sub>r</sub> cells assist the <i>de novo</i> generation of regulatory T cells from naïve cells in the periphery. With this in mind, we have modeled this scenario by adding T<sub>r</sub>-dependent peripheral differentiation of effector T cells to the regulatory phenotype, and the results are equivalent to the ones described here ((15, 46); unpublished results). However, in some reports <i>de novo</i> generation of T<sub>r</sub> cells cannot be explained this way unless one assumes that TCR transgenics in a Rag deficient background may have residual amounts of regulatory T cells. Although this matter deserves further definitive clarification, the fact that thymic differentiation of T<sub>r</sub> cells is so well-established, and together with our theoretical results, lead us to the conviction that the mechanisms of peripheral differentiation play a secondary role.

Specificity of the growth factors regulatory T cell populations

Burroughs et al (57) proposed a model of regulatory T cell dynamics that has some of the features of the Crossregulation Model presented here, but differs significantly in that they propose that regulatory T cells would be sustained by a, hitherto unidentified, regulatory T cell specific growth factor. The availability of this growth factor in different tissues would allow each tissue to sustain a population of regulatory T cells at a particular density. This tissue specific density of regulatory T cells would set a threshold on the density of IL-2 producing cells that would have to be reached before an immune response would kick in. In our model, these effects might also exist though the regulatory T cell population is controlled indirectly via tissue specific effector T cells.
Regulatory T cells express specific transcription factors, such as Foxp3, which could lead to the upregulation of unique cytokine receptors, not expressed by naïve or effector T cells. However, this scenario has not been detected by microarray analysis of CD4 subsets (72, 110-112). In addition to this empirical argument, one can raise an argument of principle against regulatory T cell specific growth factors. Pathogens are notorious for exploring and utilizing any “fault line” in the immune system of the host. A pathogen that would incorporate in its genome a regulatory-specific growth factor of the host, or would somehow promote its hyperexpression by the infected tissues, could compromise the immune response. This fault-line is not available to pathogens in the model we proposed here, because $T_R$ cell growth depends on the same autocrine/paracrine factors (IL-2 or others) that drive the immune responses by $T_E$ cells. A pathogen that would mess with these putative growth factors could find itself in trouble. On the one hand, decreasing the availability of growth factor might enhance the immune response on the long run following a transient decrease of regulatory T cells. On the other hand, increasing the availability of the growth factor might, at least transiently, enhance directly the expansion of effector T cells. Therefore, the Crossregulation Model, as formulated here, might embody a host protection against this kind of microorganism manipulation of the immune response.

Concluding Remarks

Despite the growing relevance of regulatory T cells and the hope that they might be applied in clinical immunology to improve the management of autoimmune diseases, the truth is that hitherto these cells have not been incorporated into a coherent view of the immune system. Many fundamental questions about regulatory T cells find no tentative answers within the frame of traditional immunological thinking. And yet, submitting tentative answers to open problems, which can be turned into precise testable hypotheses, is what makes theories useful. The absence of theoretically framed hypotheses turns regulatory T cell immunobiology into a cutting-edge, but particularly difficult field.

In this review we explored the hypothesis that specific autoreactive regulatory T cell populations “feed” on the very same autoimmune responses that they suppress, and show that this mechanism provides an integrated explanation for several features
of the immune system. The most important consequence of this Crossregulation Model is that the repertoire of the resident peripheral CD4 population is expected to partition naturally into two pools: a less diverse pool containing small clones of autoreactive $T_E$ and $T_R$ cells that regulate each other’s growth, and a more diverse pool, containing many clones of barely autoreactive $T_E$ cells, whose expansion is limited only by APC availability. The clones in the latter pool are therefore free to mount immune responses to microorganisms antigenically unrelated to the host.

This review was driven by the conviction that modeling is only productive when it manages to bridge theory and experimentation. We hope that the enunciating of several specific hypotheses during the present exploration of the crossregulation model, will serve as a basis for further modeling and experiments.

Why is it then, that three is not a crowd? It should be clear by now that all the described immune behaviors predicted by the model require the engagement of all three cell types – APC, regulatory T cells, and effectors T cells – and depend critically on their relative densities. Therefore, none of these cell types can be factored out without loosing the capacity to explain some relevant aspects of immunobiology. Indeed, many controversies in the literature, ranging from the disparity between in vitro and in vivo observations, to the role of TLR-ligands in promoting or inhibiting regulatory function, may derive from the oversimplification of trying to eliminate any of these cell types, and their respective proportions, from the scene.
References


Figure Legends

Figure 1- The Crossregulation Model. The reactional diagram indicates the events and interactions underlying the dynamics of APCs, T_E cells and T_R cells as assumed in the model. In this simple scenario the APC can only form conjugates with a maximum of two T cells.

Figure 2- Population dynamics according to the Crossregulation Model. a, b, and c- Numerical solutions of the model for different seeding values of variables E and R, and parameter A, respectively 2:2/3:1/3 (a), 2:8/9:1/9 (b) and 16:2/3:1/3 (c). The pies represent the proportions of the indicated cell types in the initial seeding and at the final steady states (the area of the pie is a linear function of the log(A+E+R). Reference parameter values: K=1, dE=dR=0.01, pE=1.1, and pR=1.0. d- Phase space of the model under the same parameters with A=2 (the same as in a and b). The lines are the nullclines uniting points where the individual variables do not change (dR/dt=0 and dE/dt=0 are plain and dashed). The black dots indicate the two stable steady states, and the white dot indicates the unstable saddle point.

Figure 3- Crossregulation among regulatory T cells and effector T cells in the presence of APCs in vitro. a- Experimental design. b- Proportions and CFSE-profiles of T_E and T_R cells. CD4+CD25+ T cells and CD4+CD25- T cells, purified by high-speed cell sorter respectively from Thy1.2 and Thy1.1 C57Bl/6 congenic mice, were used as T_R and T_E cell enriched populations. Both cell populations were labeled with CFSE and set into cultures at the indicated proportions fixing the total input of 10^5 T cells with 2x10^5 T-cell depleted splenocytes as APCs, and anti-CD3 (5 microgram/ml). Proportions and CFSE-fluorescence profiles of the Thy1.1 and Thy1.2 populations were measured after 3 days of culture by flow cytometry. Data from (45).

Figure 4- Candidate molecular mechanisms for APC-dependent suppression of T_E cells by T_R cells. The reactional diagrams indicate the key intermediate steps
involved in different hypotheses for suppression: a- the T\textsubscript{R} cell delivers an inhibitory signal to T\textsubscript{E} cell simultaneously conjugated with the APC; b- the T\textsubscript{R} cell uses the APC as a temporal bridge to deliver a signal to the T\textsubscript{R} cell; c- the T\textsubscript{R} cell, upon activation by the APC, delivers a contact dependent signal to the T\textsubscript{E} cell; d- the activated T\textsubscript{R} cell delivers paracrine signal to the T\textsubscript{E} cell; e- the activated T\textsubscript{R} cell consumes autocrine/paracrine factors required by the activated T\textsubscript{E} cell.

**Figure 5- Individual cell-oriented simulations of in vitro cultures of regulatory T cells, effector T cells, and APCs.** a- Simulations in silico can reproduce patterns of aggregation of the three cell types as observed in real in vitro cultures under different media. Purified CD25+CD4+ T\textsubscript{R}-enriched cells (labeled with CFSE-green), purified CD25-CD4+ T\textsubscript{E}-enriched cells (labeled with CMTMR-red), and irradiated erythrocyte and T-cell depleted APC-enriched leucocytes from peritoneal cavity (labelled with DDAO-blue) were set into cultures in glass-bottom petri-dishes with RPMI-media, with or without methylcellulose, and with anti-CD3. Aggregates were imaged with a two-photon laser-scanning microscopy setup. b- Individual cell-oriented simulations of CFSE-delabeling using aggregation parameters corresponding to RPMI media and state transition rules for the cell life cycle corresponding to hypothesis of fig.4c. Results adapted from (Gardner et al. in preparation).

**Figure 6- Equilibrium densities of specific T\textsubscript{R} and T\textsubscript{E} cell populations as a function of the density of their cognate APCs.** a- Bifurcation diagram of the model representing all possible equilibria. The lines indicate the total equilibrium density of T cells (sum of the variables E+R) as a function of the cognate APC density (parameter A). Solid lines indicate stable equilibria and the dashed line indicates unstable equilibria. The pies indicate the relative proportions of T\textsubscript{E} and T\textsubscript{R} cells at equilibrium (respectively E/(E+R) and R/(E+R)) for the indicated values of A. b- The lines indicate the equilibria that are actually reached by solving the system with fixed initial conditions (solid line: E=2/3 and R=1/3; dashed line: E=1/3 and R=2/3), as a function of the density of cognate APCs. Remaining parameter values as in fig. 2.

**Figure 7– Partition of the repertoire in specificity subsets with and without T\textsubscript{R} cells as a function of the density of cognate APCs.** a- Frequency distribution of specificities over the cognate APC density. Log(A) is a Gaussian with mean -1.1 and
standard deviation 0.6. The lighter and darker regions correspond respectively to $T_E$ and $T_R$ cells. b- Equilibrium composition of $T_E$ and $T_R$ populations in the periphery. White and black dots represent the equilibrium values of $T_E$ and $T_R$ cell density (variables $E$ and $R$) in 100 independent pairs of populations driven by specific cognate APCs whose densities $A$ were randomly sampled from the distribution depicted in (a). c- Equilibrium proportions of $T_E$ and $T_R$ cells in terms of total number of cells (sums of $E$ and $R$ over all populations) and diversity (counting only populations where $E$ or $R$ are greater than 0). Remaining parameter values as in fig. 2.

**Figure 8**– Dependence of the incidence of autoimmunity on the shape of the distribution of clonal specificities over the density of cognate APCs and frequency of regulatory T cells. a- Illustration of “autoimmunity”: at least one $T_E$ clonal specificity, that could be controlled by regulatory $T_R$ cells, reaches the APC-limited state (indicated by the arrows). b,c,d,e- Shape of the emergent repertoire seeding the periphery in terms of the frequency of specificities distributed over the cognate APC density (Log($A$) is a Gaussian with mean -0.9 and standard deviation 0.8). The lighter and darker regions correspond respectively to $T_E$ and $T_R$ cells. Proportions were defined as fixed specificity-independent fraction of $T_R$ cells set at 0.3(3) (c) or 0.6(6) (b), and variable specificity-dependent fraction of $T_R$ defined as the following saturating function of $A$: $m \cdot 10^4 /[0.1 + 10^4]$ where $m$ is 0.6(6) (d) or 0.95 (e). The percentages indicated the incidence of autoimmunity, when the repertoire is seeded with 100 clones drawn randomly from these distributions. Remaining parameter values as in fig. 2.
Crossregulation Model

Figure 1
Figure 2

(a) Seed
(b) Steady State
(c) Time Course
(d) APC
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8