CTMI (2005) 293:43–71

Thymic Commitment of Regulatory T Cells Is a Pathway of TCR-Dependent Selection That Isolates Repertoires Undergoing Positive or Negative Selection

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Abstract The seminal work of Le Douarin and colleagues (Ohki et al. 1987; Ohki et al. 1988; Salaun et al. 1990; Coutinho et al. 1993) first demonstrated that peripheral tissue-specific tolerance is centrally established in the thymus, by epithelial stromal cells (TEC). Subsequent experiments have shown that TEC-tolerance is dominant and mediated by CD4 regulatory T cells (Treg) that are generated intrathymically by recognition of antigens expressed on TECs (Modigliani et al. 1995; Modigliani et al. 1996a). From these and other observations, in 1996 Modigliani and colleagues derived a general model for the establishment and maintenance of

natural tolerance (MM96) (Modigliani et al. 1996b), with two central propositions: (1) T cell receptor (TCR)-dependent sorting of emergent repertoires generates TEC-specific Treg displaying the highest TCR self-affinities below deletion thresholds, thus isolating repertoires undergoing positive and negative selection; (2) Treg are intrathymically committed (and activated) for a unique differentiative pathway with regulatory effector functions. The model explained the embryonic/perinatal time window of natural tolerance acquisition, by developmental programs determining (1) TCR multireactivity, (2) the cellular composition in the thymic stroma (relative abundance of epithelial vs hemopoietic cells), and (3) the dynamics of peripheral lymphocyte pools, built by accumulation of recent thymic emigrants (RTE) that remain recruitable to regulatory functions. We discuss here the MM96 in the light of recent results demonstrating the promiscuous expression of tissue-specific antigens by medullary TECs (Derbinski et al. 2001; Anderson et al. 2002; Gotter et al. 2004) and indicating that Treg represent a unique differentiative pathway (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003), which is adopted by CD4 T cells with high avidity for TEC-antigens (Bensinger et al. 2001; Jordan et al. 2001; Apostolou et al. 2002). In the likelihood that autoimmune diseases (AID) result from Treg deficits, some of which might have a thymic origin, we also speculate on therapeutic strategies aiming at selectively stimulating their de novo production or peripheral function, within recent findings on Treg responses to inflammation (Caramalho et al. 2003; Lopes-Carvalho et al., submitted, Caramalho et al., submitted).

In short, the MM96 argued that natural tolerance is dominant, established and maintained by the activity of Treg, which are selected upon high-affinity recognition of self-ligands on TECs, and committed intrathymically to a unique differentiative pathway geared to anti-inflammatory and antiproliferative effector functions. By postulating the intrathymic deletion of self-reactivities on hemopoietic stromal cells (THC), together with the inability of peripheral resident lymphocytes to engage in the regulatory pathway, the MM96 simultaneously explained the maintenance of responsiveness to non-self in a context of suppression mediating dominant self-tolerance. The major difficulty of the MM96 is related to the apparent tissue specificity of Treg repertoires generated intrathymically. This difficulty has now been principally solved by the work of Hanahan, Kyewski and others (Jolicoeur et al. 1994; Derbinski et al. 2001; Anderson et al. 2002; Gotter et al. 2004), demonstrating the selective expression of a variety of tissue-specific antigens by TECs, in topological patterns that are compatible with the MM96, but difficult to conciliate with recessive tolerance models (Kappler et al. 1987; Kisielow et al. 1988). While the developmentally regulated multireactivity of TCR repertoires (Gavin and Bevan 1995), as well as the peripheral recruitment of Treg among RTE (Modigliani et al. 1996a) might add to this process, it would seem that the establishment of tissue-specific tolerance essentially stems from the "promiscuous expression of tissue antigens" by TEC. The findings of AID resulting from natural mutations (reviewed in Pitkanen and Peterson 2003) or the targeted inactivation (Anderson et al. 2002; Ramsey et al. 2002) of the AIRE transcription factor that regulates promiscuous gene expression on TECs support this conclusion.

The observations on the correlation of natural or forced expression of the Foxp3 transcription factor in CD4 T cells with Treg phenotype and function (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003) provided support for the MM96 contention that Treg represent a unique differentiative pathway that is naturally established inside

the thymus. Furthermore, Caton and colleagues (Jordan et al. 2001), as well as several other groups (Bensinger et al. 2001; Apostolou et al. 2002), have provided direct evidence for our postulate that Treg are selected among differentiating CD4 T cells with high affinity for ligands expressed on TECs (Modigliani et al. 1996b).

Finally, the demonstration by Caramalho et al. that Treg express innate immunity receptors (Caramalho et al. 2003) and respond to pro-inflammatory signals and products of inflammation (Caramalho et al., submitted) brought about a new understanding on the peripheral regulation of Treg function. Together with the observation that Treg also respond to ongoing activities of "naïve/effector" T cells—possibly through the IL-2 produced in these conditions—these findings explain the participation of Treg in all immune responses (Onizuka et al. 1999; Shimizu et al. 1999; Annacker et al. 2001; Curotto de Lafaille et al. 2001; Almeida et al. 2002; Shevach 2002; Bach and Francois Bach 2003; Wood and Sakaguchi 2003; Mittrucker and Kaufmann 2004; Sakaguchi 2004), beyond their fundamental role in ensuring self-tolerance (e.g., Modigliani et al. 1996a; Shevach 2000; Hori et al. 2003; Sakaguchi 2004; Thompson and Powrie 2004). Thus, anti-inflammatory and anti-proliferative Treg are amplified by signals that promote or mediate inflammation and proliferation, accounting for the quality control of responses (Coutinho et al. 2001). In turn, such natural regulation of Treg by immune responses to non-self may well explain the alarming epidemiology of allergic and AID in wealthy societies (Wills-Karp et al. 2001; Bach 2002; Yazdanbakhsh et al. 2002), where a variety of childhood infections have become rare or absent. Thus, it is plausible that Treg were evolutionarily set by a given density of infectious agents in the environment. With hindsight, it is not too surprising that natural Treg performance falls once hygiene, vaccination, and antibiotics suddenly (i.e., 100 years) plunged infectious density to below some critical physiological threshold. As the immune system is not adapted to modern clean conditions of postnatal development, clinical immunologists must now deal with frequent Treg deficiencies (allergies and AID) for which they have no curative or rational treatments. It is essential, therefore, that basic immunologists concentrate on strategies to selectively stimulate the production, survival, and activity of this set of lymphocytes that is instrumental in preventing immune pathology. We have argued that the culprit of this inability of basic research to solve major clinical problems has been the self-righteousness of recessive tolerance champions, from Ehrlich to some of our contemporaries. It is ironical, however, that none of us—including the heretic opponents of horror autotoxicus—had understood that self-tolerance, or its robustness at least, is in part determined by the frequency and intensity of the responses to non-self.

In the evolution of ideas on immunological tolerance, the time might be ripe for some kinds of synthesis. First, conventional theory reduced self-tolerance to negative selection and microbial defense to positive selection, while the MM96 solution was the precise opposite: positive selection of autoreactivities for self-tolerance (Treg) and negative selection (of Treg) for ridding responses. In contrast, it would now appear that positive and negative selection of autoreactive T cells are both necessary to establish either self-tolerance or competence to eliminate microbes, two processes that actually reinforce each other in the maintenance of self-integrity. Second, V-region recognition has generally been held responsible for specific discrimination between what should be either tolerated or eliminated from the organism. In contrast again, it would now seem that both processes of self-tolerance and microbial defense (self/non-self discrimination) also operate on the basis of evolutionarily ancient,

germ-line-encoded innate, nonspecific receptors (Medzhitov and Janeway 2000) capable of a coarse level of self/non-self discrimination (Coutinho 1975). It could thus be interesting to revisit notions of cooperativity between V-regions and such mitogen receptors, both in single cell functions (Coutinho et al. 1974) and in the system's evolution (Coutinho 1975, 1980) as well. After all, major transitions in evolution were cooperative (Maynard-Smith and Szathmary 1995).

1 Introduction

The last few years have witnessed a radical shift in current notions of self-tolerance and autoimmunity. Recessive tolerance, established by negative selection of self-reactive cells, has had the upper hand ever since Ehrlich declared autoantibodies to be dysteleologic. In the 1980s, the discovery of thymic deletion by antibodies to TCR V-betas (Kappler et al. 1987) launched a large volume of work leading to the conclusion that the establishment of self-tolerance and thymic deletion were one and the same process. This was epitomized by von Boehmer's "the thymus selects the useful, neglects the useless and destroys the harmful," the latter being all autoreactive T cells with productive affinities to self-peptide:MHC complexes (von Boehmer et al. 1989). While this Darwinian tautology could not possibly be wrong, it resulted in little or no progress, and tolerance remained the central question in modern immunology.

For a decade or two, a few groups in the world, working at the margin of prevalent concepts, kept producing evidence and arguments for the alternative notion that tolerance is dominant. For these, the putative solution to natural tolerance was turned up-side down: rather than stemming from the elimination of autoreactive T cells, it would require their positive selection and activation. In a turnaround that seemed sudden to many, dating from the International Congress of Immunology of 2001 in Stockholm, Treg and immune regulation have come forth to the limelight, occupying an increasing place in the literature over the last few years. This was received in widely divergent manners. For some, the topic sounds as if suppressor T cells are back, and this is bad news: after a dozen years of abundant phenomenology, suppressor T cells had been driven out of sight by progress in the molecular biology of lymphocytes, and by the efforts of a few who had never been convinced by often irreproducible, nonquantitative *in vitro* assays. Others were interested in dominant tolerance, for they saw "some reasons why that deletion and anergy cannot satisfactorily explain natural tolerance" (Coutinho et al. 1992), underlining the differences between the old suppressor T cell phenomenol-

ogy in response to conventional antigens, and the new *in vivo* evidence for Treg operating in self-tolerance. Yet others, who were part of the previous suppressor T cell journey, gladly joined the new trend, again taking up their *in vitro* suppression assays to describe new markers and mechanisms. A large group adapted to fashion by forgetting experiments and models on recessive tolerance to proclaim their novel conviction of dominant regulation. Few, however, gave enough consideration to the fact that models of dominant tolerance must also explain the time window for tolerance acquisition, how Treg develop and have their repertoires selected throughout life, how autoimmune diseases appear and display such a characteristic range of targets, and how conventional immune responses are produced in a context of suppression. In short, if the approaches to natural tolerance seem to be on the right track, fundamental aspects of the organization of the immune system, including the selection of *available* and *actual* T and B cell repertoires in accordance with self-nonself discrimination, remain unsolved. Most importantly, to the dismay of clinicians, the therapeutic approaches to autoimmune patients are today debatable: immunosuppression, as commanded by the classical views, or immunostimulation, as is now suggested by the novel theories.

As stated earlier (Modigliani et al. 1996b), the original observations of Le Douarin on TEC tolerance to peripheral tissues (Ohki et al. 1987, 1988; Salaun et al. 1990; Coutinho et al. 1993), given the postulate for differential roles of TECs and THCs in the presentation of antigens to developing T lymphocytes, had principally solved the core problems of natural tolerance in the framework of dominant mechanisms mediated by thymically committed Treg (Modigliani et al. 1996a). Four types of recent findings strengthen this conviction:

1. Treg represent a unique differentiative lineage of lymphocytes with intrathymic commitment (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003), as proposed.
2. Treg are selected upon high-avidity TCR recognition of antigens expressed on TECs (Bensinger et al. 2001; Jordan et al. 2001; Apostolou et al. 2002), just as postulated.
3. Tissue-specific antigens are selectively expressed by TECs in a promiscuous manner (Derbinski et al. 2001; Anderson et al. 2002; Gotter et al. 2004), providing a simple explanation for the thymic acquisition of tissue-specific tolerance, and supporting our postulate on the differential roles of TECs and THCs in the generation of Treg repertoires; in addition, the respective genetic and cellular mechanisms remind us, as if it were necessary, of the evolutionary relevance of Treg generation, and may explain the acquisition of natural tolerance to self-antigens that are expressed only after the tolerogenic time window.

4. Treg express innate receptors for inflammation-related ligands (Caramalho et al. 2003) and are amplified by ongoing conventional T cell activity (Almeida et al. 2002), opening new leads to their physiology and putative manipulation.

In this conceptual framework, research must now move on. For the benefit of patients, the immunopharmacology of Treg must be explored, while the time may be ripe to address systemic questions of regulation that remain largely unattended: the developmental co-selection of V-region repertoires and functional classes among lymphocyte subsets, the selection, specificity, and population dynamics of natural antibodies and naturally activated T and B cells, including Treg (Pereira et al. 1985), the basis for the life-long memory of the developmental antigenic self.

2 Somatically Generated T Cell Receptor Repertoires Are Distributed and Continuous

The fate of differentiating T cells is ultimately determined by TCR affinity for intrathymic ligands. Conventional selection models (von Boehmer et al. 1989) assume that increasing self-affinities result in neglect, positive and negative selection, in this order. Randomly generated Variable-regions of T cell receptors (TCR) and antibodies, however, are expected to have an interesting property that is necessary in the evolutionary strategy bringing them forth. Populations of V-regions, provided they are sufficiently large, will have a continuous distribution over whatever quantitative parameter we can consider in their interaction with a given antigenic ligand. Immunologists know this well, and have paid a great deal of attention to affinity distributions and degeneracy when considering thresholds for cellular induction or inactivation. It follows that, for a given fixed set of antigenic ligands (such as the thymic environment of an individual[1]), the affinity distribution of an emerging and sufficiently large V-region population (such as the randomly generated TCR diversity of developing T cells) will be continuous.[2] This means that TCRs will distribute continuously below and above a given threshold of cellular

[1] For the process of establishing self-tolerance by selecting TCR repertoires, it is essential that the set of selective ligands be fixed, and protected from the ever-changing external antigens that may eventually be brought into the thymus. In this context, it would seem critical that TECs do not present external antigens.

[2] It could be argued that developmentally regulated TCR repertoires, which are essentially germ-line, have been evolutionarily selected for pre-set affinities to a given ensemble of ligands, such that they do not conform to a continuous distribution to

selection and, therefore, that TCRs with very similar affinity to the antigenic environment will fall on either side of that threshold. In the case of thymic negative selection, which aims at purging TCRs that might be activated in the periphery, this property of TCR repertoires poses a central problem. Thus, the antigenic environment in the periphery, where the selected cell will perform, is certainly different from that in the thymus, where cells are selected. First of all, the levels of expression of antigenic ligands and their diversity in peripheral tissues, together with the characteristic degeneracy of TCR-ligand interactions (Mason 1998; Wilson et al. 2004), will bring about critical differences. Moreover, these differences will be functionally amplified by the distinct antigenic contexts in the thymus vs peripheral tissues: the architecture of peripheral lymphoid organs, the expression levels of co-stimulatory molecules, the available cytokine and chemokine milieu, all make it inescapable that TCRs, which were just below the threshold for negative selection in the thymus, will be activated in the periphery. This applies for any postulated level of this affinity threshold. The continuous distribution of TCR affinities would thus allow for the positive selection of naïve T cells with anti-self affinities that are too low to be eliminated in the thymic environment, but high enough to be activated in peripheral contexts of higher antigenic and co-stimulation levels (e.g., inflammation). As these models also assume that positively selected T cells leave the thymus uncommitted as to the class of responses they will produce (e.g., helper vs inflammatory, for CD4 cells), this being determined by activation contexts in the periphery, frequent and indiscriminate development of pathogenic autoreactive responses in the periphery seems unavoidable.

There is, thus, a two-sided difficulty with this particular model of thymic selection that remains widely accepted today: unavoidable wobbling in an all-or-none process (cellular selection) that is controlled by a continuous variable (TCR affinity); no coupling of wobbling TCRs to a particular (nonpathogenic) effector function in the gray range of affinities.

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such ligands (Cohn and Langman 1990; Langman and Cohn 1992). On the other hand, the major characteristic of these repertoires is their multireactivity (Coutinho et al. 1995) or promiscuity (Gavin and Bevan 1995), indicating an evolutionary strategy that covers all possibilities and would thus conform to continuous distributions, if with high degeneracy. As argued before (Coutinho 2000), this might be particularly relevant in Treg selection at critical developmental times when TCR repertoires are extremely limited in numbers. Hence, the conventional proposal that embryonic/perinatal multireactivity is favored for reasons of anti-infectious defense makes little sense, since this is ensured by mother-derived passive protection that is paramount at precisely these developmental times.

3
The MM96 Solution to the Thymic Sorting of Emergent TCR Repertoires: Differential Roles of TECs and THCs on Treg Selection

The MM96 has solved these two problems, as it proposes that the highest subdeletional TCR self-affinities necessarily result in (activation and) commitment of selected cells to the regulatory pathway. This postulate provides for a double fail-safe mechanism to avoid pathogenic autoreactivity. On the one hand, the process of sorting the emergent TCR diversity includes the affinity-dependent selection of autoreactive Tregs, which conveniently isolates naïve T cell repertoires to well below autoreactivity thresholds, and provides for protecting cells with self-affinities that necessarily supersede those of aggressive cells. In short, a default pathway selects wobbling TCRs to Tregs. On the other hand, the intrathymic commitment of Treg imposes self-protective functions to the most autoreactive TCR repertoires reaching the periphery, thus excluding their association with other effector functions in the Russian roulette of activation contexts.

The MM96, on the other hand, also explains the apparently contradictory findings that high self-affinity may lead to either deletion or Treg generation, by postulating distinct T cell fates following antigen recognition on either epithelial (TECs) or hemopoietic (THCs) stromal cells. For the same TCR, differentiating T cell fate is determined by the type of presenting cell (APCs) on which it recognizes antigen, a postulate that allows for (developmental) regulation of the generation or relative abundance of Treg by varying the differential composition of the thymic stroma. The suggestion that repertoires of high-affinity autoreactive Treg are predominantly directed at self-antigens expressed by deletion-incompetent TECs, but not by THCs, was derived from Le Douarin's experiments (Ohki et al. 1987, 1988; Salaun et al. 1990; Coutinho et al. 1993). Yet, it has now gained new relevance given the observations that a large set of tissue-specific autoantigens is *selectively* expressed by TECs (Derbinski et al. 2001; Anderson et al. 2002; Gotter et al. 2004). Thus, the MM96 predicts that *thymic selection necessarily produces high-affinity, tissue-specific Treg, which cannot be eliminated by deletion-competent THCs that fail to express that set of autoantigens*. Selective expression of tissue-specific antigens by TECs would thus be the key strategy in the construction of autoreactive Treg repertoires. In contrast, *autoreactive TCRs within the same range of affinities but directed to antigens (also) expressed on THCs are deleted, thus purging Treg functions from the peripheral repertoires of positively selected antigen-reactive T cells that respond to foreign peptides presented by professional APCs. Hence, conventional immune responses would be little, if at all, limited by Treg*. Such a division of labor in TCR repertoire selection, together with the genetic

mechanisms that allow for promiscuous gene expression (Klein and Kyewski 2000; Derbinski et al. 2001; Anderson et al. 2002; Gotter et al. 2004) and T cell-fate decisions (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003) result in a clear predominance of self-tissue-specific Treg and in their under-representation among repertoires directed at non-self antigens and the APCs that handle them. As seen above, this same process contributes to generating the fail-safe mechanism that ensures the absence of potential tissue-specific immune pathology. Thus, productive TCR autoreactivities that are not deleted will necessarily be turned into Treg effector functions, providing for the isolation of autoreactive T cell affinities from those of positively selected T cells.

Within this framework, tolerance to all antigens expressed by THCs has a deletional basis, notwithstanding borderline TCR affinity ranges or cell frequencies, which might always be demonstrated in extreme conditions. This seems to be the case in the experimental system introduced by Medawar and colleagues (Billingham et al. 1953), where tolerance is induced if hemopoietic cells (but not those of other peripheral tissues!) are injected at birth (but not later!) into semi-allogeneic hosts. Thus, if some evidence for dominant, CD4 T cell-dependent mechanisms has been produced (Roser 1989), it seems that Medawar's tolerance essentially results from deletion (Gruchalla and Streilein 1982). Likewise, it would be expected that tolerance to all proteins that are present in circulation at high concentrations, and may be presented by THCs, is recessive as well. This is suggested in classical experiments on physiological tolerance to C5 (Harris et al. 1982; Zal et al. 1994), although Treg may also play a significant role in self-tolerance to this set of antigens (Cairns et al. 1986; Boguniewicz et al. 1989; van den Berg et al. 1991). In contrast, antigens (artificially) introduced in the thymus on cells other than THCs, might be expected to generate Treg and induce dominant tolerance. This has been described for intrathymic grafts of peripheral tissues (Posselt et al. 1990; Gerling et al. 1992; Charlton et al. 1994; Turvey et al. 1999; Salaun et al. 2002), which were shown to overcome and control pathogenic autoimmunity toward the specific tissue.

The model would predict that major deviations from physiology are brought about by alterations in the correct presentation of antigens by TECs or THCs. Treg defects are expected to arise from either deficient promiscuous expression or presentation of tissue antigens by TECs, or else, by their ectopic presentation on THCs (Shih et al. 2004). Likewise, presentation of extrathymic antigens by either cell type may result in pathology, particularly in cases where peripheral self is abnormally expressed by THCs or non-self becomes available on TECs. For example, if peripheral tissue damage releases into circulation tissue-specific proteins that reach the thymus in

concentrations that are high enough to be presented by THCs, Treg deletion ensues and tissue-specific autoimmunity may arise. Conversely, non-self (e.g., viral) antigen presentation by TECs, if selective, would be expected to result in specific Treg generation, and in the inability to eliminate the virus. This is probably a very unusual condition, as it can be expected that the same antigens are also presented by THCs and delete virus-specific Treg. On the other hand, in conditions where extensive depletion of THCs takes place, as could be the case in HIV infection, this hypothesis, however strange, should perhaps not be excluded.

4
Promiscuous Tissue-Antigen Expression in TECs Selects Treg Repertoires: The Origin and Range of Autoimmune Diseases

In autoimmune repertoires, a most interesting question relates to the limited number of clinically identified AID. Thus, recessive tolerance models would predict as many distinct diseases as the number of autoreactive clones, or at least, as the number of autoantigens. The limitation in the range of clinical AID led Cohen to suggest that autoreactive repertoires would be focused onto a subset of autoantigens that he designated as "immunological homunculus" (Cohen and Young 1991). Evidence for these notions has been obtained in the analysis of physiological autoreactivities among natural antibodies in healthy individuals, which are restricted to a subset of autoantigens (Nobrega et al. 1993; Mouthon et al. 1995). As Cohen argues for a kind of dominant tolerance, which does not involve sorting of TCR repertoires between Treg and naïve T cells, this bias in both physiological and pathological autoreactivity had to be explained by global properties of T and B cell repertoires, which remain poorly analyzed. In the context of dominant tolerance mediated by a distinct lymphocyte lineage, however, AID can be attributed to failures in the repertoires or functional competence of Treg with unique TCR repertoires, which are selected by the promiscuous subset of thymic autoantigens. It follows that the range of AID would be determined by the original biases in Treg repertoires—ultimately delineated by the antigenic composition of TECs—irrespective of the peripheral availability of potentially pathogenic effector T and B cells to all sorts of autoantigens. This hypothesis is robust. Because it is based on the dominance of a particular repertoire (the Treg repertoire), it accounts for autoreactive repertoire biases in both physiology and disease, and in both T and B cells. Interestingly, recent observations may be used in support of these notions, as they demonstrate that a given genetic defect (e.g., TGFβ, PD-1 KO), possibly associated with Treg function, will vary in its disease man-

ifestations according to the genetic background (Shull et al. 1992; Dang et al. 1995; Yaswen et al. 1996; Nishimura et al. 1998, 1999, 2001). This had already been described in the seminal observations of Kojima who first analyzed the strain dependence of AID manifestations following newborn thymectomy (Kojima and Prehn 1981). Also here, in conditions where disease does result from limitations in Treg (Sakaguchi et al. 1995), AID manifestations vary with the genetic background, while remaining limited in scope and directed to a few typical targets (e.g., thyroid, stomach, ovary, testicles). In other words, *while Treg generation determines health or disease and sets a specific range of tissue targets (following Treg selection on promiscuous TECs), other genes, possibly truly tissue-specific, will determine the precise disease-associated clonal specificities*. In the frame of the MM96, tissue-antigen expression by TECs (Derbinski et al. 2001; Anderson et al. 2002; Gotter et al. 2004) provides a satisfactory explanation for the limitation in AID syndromes, by describing the set of potentially homuncular self-antigens (Cohen and Young 1991), which represent the targets of Treg repertoires. The nature of Treg deficits would determine the range of disease-associated specificities: for localized failures, pathogenic specificities are those found in the physiological autoreactivity of healthy individuals, modified by helper cell-dependent processes of class-switch and affinity maturation. This seems to be the case for autoantibodies in a variety of AID (Shlomchik et al. 1987). In contrast, it could be expected that generalized defects of Treg result in the indiscriminate production of autoantibodies and pathogenic T cells (e.g., TGFβ KO mice) (Shull et al. 1992; Dang et al. 1995; Yaswen et al. 1996).

Finally, this discussion brings forth the notion that AID pathogeny may well begin with deficits in Treg selection on TECs, as it is highly unlikely that Treg defects will ever result from limitations in somatically and randomly generated TCR repertoires. In short, AID may well be TEC diseases, as direct evidence actually indicates (Forsgren et al. 1991; Thomas-Vaslin et al. 1997; Salaun et al. 2002). If some truth exists in these hypotheses, this would suggest a major shift in current research focus, by seeking AID origin elsewhere than in the peripheral antigenic targets. Evidence for the innocent nature of tissue antigen expression in organ-specific AID has been produced by Holmberg et al. in tetraparental mice (Forsgren et al. 1991). Thus, AID development in embryo-fusion chimeras between an autoimmune (NOD) and a normal strain, in which all tissues were variable mosaics of cells from both origins, correlated with the thymic composition, rather than with that of the peripheral target tissues: if most of the thymus (perhaps TECs) happened to be from the NOD donor, AID developed even when pancreatic islands were normal; in contrast, if the thymus was mostly normal, there was no pathogenic autoimmunity toward NOD islands (Forsgren et al. 1991).

5
Beyond TCR Repertoires: A Unique Differentiative Lineage, That May Represent Class Regulation Operating Intrathymically

Results from Hori et al. and others (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003) brought direct support to the MM96 claim that Tregs represent a unique differentiative lineage, committed upon TCR affinity-dependent activation on TECs. This relationship of Treg commitment (expression of FOXP3) with TEC- and TCR-dependent activation, however, has yet to be ascertained, and it may result from the differential expression of Notch-pathway receptors and/or ligands by TECs (Anderson et al. 2001). For the MM96, the key feature is that Treg cell fate is TCR-dependent and established intrathymically, such that a unique self-reactive repertoire is irreversibly associated with a unique effector function (Zelenay et al. 2005). Accordingly, while the results of specific Foxp3 expression in Treg leave room for extrathymic education of naïve T cells into the Treg pathway (Chen et al. 2003; Cobbold et al. 2004; Fantini et al. 2004; Park et al. 2004; Zheng et al. 2004), they also clearly demonstrate commitment of differentiating CD4 T cells inside the thymus, as predicted (Modigliani et al. 1996b). The MM96 also postulated that Treg "positive selection and functional commitment" inside the thymus was accompanied by cellular activation[3], such that the process would be equivalent to the "class regulation" of naïve CD4 T cells in the periphery (Mosmann et al. 1986). Since the fail-safe mechanism to ensure is the sorting of TCRs to a particular functional class before thymic export, however, the postulate of intrathymic activation of Tregs is dispensable if Tregs are committed to a unique functional lineage upon TCR-dependent selection. It should be noted that the MM96 is entirely based on TCR-affinity selection, and it does not require ad hoc postulates on properties of Treg, such as resistance to negative selection. This alternative would require that an independent differentiative lineage of CD4 T cells is committed prior to selection, and it would thus be incompatible with an appropriate sorting of emergent TCR repertoires into naïve vs Treg classes. For the MM96, cell fate determination results from TCR-dependent selection. If Treg lineage determination is equivalent to the process of Th1/Th2 commitment in the periphery, it could be expected that TCR-dependent selection on TECs, within a given affinity range, activates a genetic cascade, which

[3] Both the MM96 and its present application to recent findings do not depend on a precise definition of positive selection. It is irrelevant whether Tregs are expanded with or after commitment, or whether such TCRs are simply preserved from deletion at a one-to-one ratio between precursor and mature cells. Thus, either alternative finds abundant room within the wide range of emerging TCR affinities to self-antigens expressed by TECs.

is likely mediated by FOXP3 and involves downstream activation of a set of genes associated with Treg development (CD25, CTLA-4, TLR-4, etc.), as well as inactivation of others (e.g., IL-2). In turn, the suspected involvement of the Notch pathway in the generation of Treg (Anastasi et al. 2003; Vigouroux et al. 2003) possibly owing to differential expression of Notch-ligands by TECs and THCs in thymic stroma, could explain the alternative cell fates (Treg commitment vs cell death), upon productive antigen recognition by developing CD4 T cells on either type of presenting cells.

As discussed in the MM96, other mechanisms must account for the putative generation of Treg with specificities for self-antigens outside the thymus. Based on the observations that recent thymic emigrants (RTE) could be recruited in the periphery for entering the regulatory pathway (Modigliani et al. 1996a), a process that Waldmann and colleagues called "infectious tolerance" (Qin et al. 1993), the peripheral activation-dependent commitment of RTE to regulatory functions was proposed (Modigliani et al. 1996b). This would occur when RTE recognize tissue-antigens in the presence of thymically committed Treg with specificities for other antigens expressed on the same cells, providing for some sort of antigen spreading in self-tolerance. Accordingly, linked suppression mediated by Treg has now been demonstrated in a variety of models (Wise et al. 1998; Honey et al. 2000; Thornton and Shevach 2000; Weiner 2001; Jiang et al. 2003; Graca et al. 2004), and the data of Hori and others (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003) leave room for peripheral commitment of naïve T cells to regulatory functions. At first analysis, promiscuous expression of tissue-specific antigens by TECs would seem to solve the problem of functionally uncommitted (and, thus, potentially pathogenic) naïve tissue-specific cells exiting the thymus. Clearly, however, any model of thymic selection must deal with an emergent TCR diversity toward truly tissue-specific antigens that are not included in the promiscuous subset, at least at high levels of expression. Some of these T cells will necessarily be positively selected and seed the periphery, providing for a range of specific pathogenic potential. The physiological existence of such T cells is readily demonstrated by the experimental induction of AID in healthy individuals (Weigle 1980; Wekerle 1992, 1996; Boon et al. 1994) and by direct determinations on their frequencies (Lohse et al. 1996). Hence, natural tolerance requires either the continuous suppression of naïve autoreactive cells by thymic-derived Treg, or else, the more robust mechanism of their peripheral, antigen-dependent recruitment to Treg functions, as suggested in the MM96. Yet several types of experiments have failed to demonstrate peripheral recruitment of tissue-specific Treg (Hori et al. 2002b) in the absence of manipulations interfering with antigen-recognition by CD4 T cells (Graca et al. 2003; Waldmann 2003). While there is solid evidence for antigen-dependent extrathymic

education of naïve T cells to Treg in the latter conditions (Qin et al. 1993; Wise et al. 1998; Honey et al. 2000; Graca et al. 2004), opening great promise in transplantation tolerance, the relevance of infectious dominance for natural tolerance remains unclear.

Interestingly, RTEs, which apparently maintain cell-fate decisions open for some time and are recruitable to the Treg pathway (Modigliani et al. 1996a), may first recognize peripheral antigens on epithelial cells (e.g., at mucosal surfaces). If differentiative rules that apply here are similar to those inside the thymus, this could explain the ease in inducing mucosal tolerance (Wu and Weiner 2003), as well as the findings of dominance in this phenomenon (Weiner 2001; Unger et al. 2003) and of abundant T cells producing TGF-β (a proposed mediator of Treg activity [Fukaura et al. 1996; Weiner 2001]) in the mucosa. Again, particulars of Notch-ligand expression on epithelial cells (Anderson et al. 2001) could apply here as well.

Finally, as discussed below, it is hypothetically plausible that thymically committed Treg are not (all) Class II MHC-restricted. If this were true, and if peripheral recruitment of RTE to the regulatory pathway is a physiologically relevant process, then the Treg population in normal individuals is heterogeneous, containing both thymically committed Treg, as well as cells that have exited the thymus as MHC-restricted, resting naïve T cells. It is perhaps likely that these putative developmental classes of Treg, distinguished by their MHC restriction and, thus, specificity, would also differ in functional competence, patterns of gene expression, markers, population dynamics, and physiological roles. To be confirmed, a period of confusing descriptions and controversies would inevitably occur, which could explain arising disagreements.

6 The Question on the Putative MHC Restriction of Treg: Yet Another Difference Between the Thymic Selection of Treg and Naïve T Cells?

Treg development is far from solved or even principally understood. For example, there is little or no information on the MHC restriction of Treg, if actually these cells are at all MHC-restricted. As MHC restriction results from thymic selection (Bevan 1977; Bevan and Fink 1978; Zinkernagel et al. 1978), there is no a priori reason to exclude that Treg would view antigens as whole proteins, using TCR for an antibody-like recognition of protein surfaces, and remain available for selection in the emergent repertoires, just as occurs for other primitive T cells types (e.g., NK T cells [Bendelac et al. 1997; Taniguchi et al. 2003]) and for conventional T cells exposed to superantigens

(Marrack et al. 1993). Alternatively, Treg may be selected to recognize peptides presented by invariant chaperons, such as HSPs (Gullo and Teoh 2004), in which case their repertoires, while restricted and peptide-specific, would not show MHC-dependent variation. Finally, Treg could be thought to recognize antigenic self-peptides on Class I MHC, as could be indicated by the fact that the promiscuous antigens expressed by TECs are endogenous proteins to the presenting cell and, thus, more likely to engage in this pathway.

On the other hand, if Treg are Class II MHC-restricted, they can only scan tissues under conditions that promote Class II expression (e.g., inflammation), or else, via physiological tissue-antigen transfer to professional APCs, possibly in draining lymph nodes. Either alternative is incompatible with MM96 postulates on thymic selection. Furthermore, the first possibility is also incompatible with the established requirement for peripheral antigen in the physiological survival of Treg (Seddon and Mason 1999; Cozzo et al. 2003; Lerman et al. 2004), while the second poses the central question on how to induce immune responses in a context of dominant suppression, if non-self antigens are presented by the same professional APCs that simultaneously present self-tissue antigens to Treg. In addition, physiological processing and MHC-(cross)presentation of tissue-specific antigens by draining APCs seem to provide conditions that would favor activation of the entire set of tissue peptide-specific autoreactive T cells, which exit the thymus as naïve lymphocytes precisely after selection for MHC-restriction. In short, either Treg and naïve tissue-specific T cells interact on professional APC clusters, in which case responses to self- and non-self-antigens would be equally linked suppressed and self/non-self discrimination jeopardized, or else Treg-dependent suppression relies on other cellular sites or mechanisms. These may be quite diverse, such as Treg-dependent control of tissue immunogenicity or of the expression of tissue-protective genes (Pae et al. 2003). As another nonexclusive scenario, direct interactions of Treg with MHC-restricted naïve T cells may always be possible in species where (activated) CD4 T cells express Class II, and even in mouse if Treg pick up Class II molecules along the course of their thymic development or upon arrival in the periphery. Class II acquisition by activated T cells has been demonstrated (Elliott et al. 1980; Patel et al. 1999; Walker and Mannie 2002; Tsang et al. 2003), and it is expected to preferentially concern Treg as they engage in tissue-specific complexes. These are obviously too many speculations for too few data, but the finding of (some) CD4 T cells in Class II-negative animals, some of which bear Treg markers (Bensinger et al. 2001), could be interpreted by the notion that Treg are also not conventional in regards MHC restriction.

Likewise, little information is available on the age-dependent production of Treg, and on their population dynamics throughout life. These are criti-

cal parameters for understanding the time window in natural tolerance acquisition and the physiopathology of Treg, notably that many AID are first manifested around or soon after puberty. The MM96 suggested that Treg are predominantly produced during embryonic and perinatal life, during the time window of natural tolerance acquisition, precisely when the thymus contains self-antigens exclusively and is secluded with certainty from microbial exposure. As seen above, this was explained by the relative predominance of TEC and THC in the composition of the thymic stroma, the former generating (self-specific) Treg, whereas the latter delete them. In contrast, the findings of autoimmune pathology in animals that are thymectomized 3 days after birth has been interpreted to indicate that Treg production and/or export is antedated by the export of tissue-specific naïve, MHC-restricted T cells (Asano et al. 1996). As argued by others, however, alternative interpretations are possible, as the experiments only show that, under those conditions, the physiological balance between Treg and naïve T cells is biased toward the latter in quantitative terms (Suri-Payer et al. 1999; Dujardin et al. 2004). As peripheral T cell pools after thymectomy are built by proliferation of pre-existing T cells rather than by accumulation of newly-formed T cells exiting the thymus, as in normal conditions (Modigliani et al. 1994), and given the limitations of Treg to expand (Annacker et al. 2001; Almeida et al. 2002; Gavin et al. 2002), it is expected that such bias will always ensue irrespective of a putative Treg excess at the start. Further arguments in this direction can be invoked from the rather precise time requirements for thymectomy (perhaps representing a unique initial ratio of Treg/Tnaïve that, after expansion, would result in pathogenic imbalance), and from the frequencies and limited range of target-organ specificities of autoimmune manifestations. These are individually variable, and often limited to a particular tissue, indicating that enough Treg toward most self-tissues had been produced at the time of thymectomy. In other words, considering all tissues in all individuals thymectomized, autoimmunity is the exception rather than the rule, the strain specificity of the most frequent manifestations perhaps indicating strain-specific lower rates of Treg production for those particular antigens (possibly due to insufficient TEC expression). The medical relevance of this question is obvious, as AID are typically diseases of young adults, often first manifested at puberty, precisely when thymic production declines. Most unfortunately, other than the fact that thymic involution is autonomously controlled by TEC (Ohki et al. 1988), the molecular and cellular bases of this process are not clear, and we are currently unable to regulate (e.g., stimulate) de novo T cell production by either biological or pharmacological means.

7
Selective TLR Expresssion by Treg: Evolutionary Significance and a Possible Handle on Treg Regulation

Caramalho and colleagues have reported the surprising finding that murine Treg express transcripts for seven of nine Toll-like receptors they have studied, and that four of these are not expressed by conventional CD4 T cells, either before or after activation (Caramalho et al. 2003). Furthermore, they have shown that Treg actually respond to pro-inflammatory agents and inflammatory conditions that are known to involve this set of innate receptors (Caramalho et al., submitted). The expression of TLRs on T cells has been extended to humans (Komai-Koma et al. 2004) and, together with the findings of Treg amplification by conventional T cell responses (Almeida et al. 2002; Caramalho et al., submitted), shed new light on the operation of Treg and the general physiological regulation of this cell subset. In addition, these findings could contain the solution for current controversies on Treg markers, on distinct cellular and molecular mechanisms of regulation, eventually, on the range of Treg specificities. Most importantly, they may provide the explanation for the intimate and twofold relationship of infections with autoimmunity: on the one hand, the surprisingly low frequency of autoimmune manifestations accompanying infections, given the wide range of molecular mimicries (Albert and Inman 1999; Rose and Mackay 2000; Benoist and Mathis 2001), on the other hand, the inverse correlation between certain infections and autoimmune diseases (Oldstone and Dixon 1972; Oldstone et al. 1990; Bras and Aguas 1996; Das et al. 1996; Cooke et al. 1999) or atopy (Matricardi et al. 1997, 2000; Bjorksten et al. 1999; Kalliomaki et al. 2001; Zuany-Amorim et al. 2002; Rodriguez et al. 2003), which has been established epidemiologically (Leibowitz et al. 1966; Greenwood 1968; Kurtzke 1995; Matricardi et al. 1997, 2000; Bjorksten et al. 1999; Group 2000; Kalliomaki et al. 2001) and experimentally demonstrated (Oldstone and Dixon 1972; Oldstone et al. 1990; Bras and Aguas 1996; Das et al. 1996; Cooke et al. 1999; Rodriguez et al. 2003). Thus, in acute infections, stimulation of Treg activity by the infectious inflammatory process itself may explain the natural limitation of the pathological process. Accordingly, absence or deficits of Treg number or function invariably result in marked exacerbation of infection-associated immunopathologies. Thus, depending upon the sites colonized by often opportunistic pathogens, Treg deficiency results in either local inflammatory diseases (e.g., bowel, lung, or skin, [Read et al. 2000; Belkaid et al. 2002; Hori et al. 2002a), or in increased severity of systemic symptoms (E. Seixas, unpublished observations). Conversely, a number of spontaneous autoimmune and allergic manifestations are prevented or ameliorated by infection with a wide variety of pathogens (Bach

2001, 2002; Wills-Karp et al. 2001; Yazdanbakhsh et al. 2002). In all observations that are now available, no specificity of Treg to microbial antigens has been described, suggesting that self-specific Tregs are actually stimulated via TLR recognition of microbial mitogens, as well as by the antigen-dependent activation of microbe-specific naïve T cells.

These considerations are obviously related to the epidemiological evidence for the alarming increases in the frequency of allergic and AID in the Western world, and its explanation by the hygiene hypothesis (Strachan 1989; Wills-Karp et al. 2001; Bach 2002; Yazdanbakhsh et al. 2002). The role of Treg and their physiological stimulation by infectious agents indicate a major evolutionary significance of this cell subset and of their responsiveness to innate signals. In turn, this would suggest that modern medicine, which has eradicated—through hygiene, vaccination, and antibiotics—most common childhood infections, now has to face the clinical consequences of a defective natural stimulation of Treg. The obvious response to the present situation is to discover alternative manners to maintain overall Treg levels above disease thresholds. Given the loss of sustained microbial stimulation of Treg in our societies, increased susceptibility to allergies and AID may reveal partial dysfunctions in any of the developmental processes discussed here, which would otherwise pass unnoticed. Thus, promiscuous autoantigen expression by TECs, repertoire selection/cell-fate decisions in the thymus, maturation of effector functions, and Treg population dynamics are all under genetic controls that are likely to show variability in human populations, and may well be read out as autoimmune susceptibility loci. Likewise, the well-established fact that such complex diseases require environmental interactions with a genetic constitution of variable susceptibility may reflect the frequency of subclinical infections, as well as external influences on Treg generation and performance. Many of these processes relate to thymic function and may represent suitable targets for future therapeutic interventions. Finally, all the genetically controlled physiological mechanisms discussed here must follow quantitative rules that are not even considered in the present discussion. Hence, it is also likely that environmental conditions exist that exceed the physiological levels of Treg operation and will, therefore, result in and/or amplify pathogenicity.

In summary, these recent findings on Treg regulation may offer novel targets for therapeutic intervention and a new understanding of the evolution of mechanisms involved in the establishment of natural tolerance.

8
Regulatory T Cells Versus Phenomenology on Regulation

A final note to clarify a very large set of phenomena pertaining to regulation, which are currently attributed to diverse cellular compartments from the regulatory T cells, warrants discussion here. Thus, with the gain in popularity of notions such as physiological autoreactivity (Coutinho 2000; Coutinho et al. 2001) and dominant tolerance (Shevach 2000; Graca et al. 2003; Sakaguchi 2004; Thompson and Powrie 2004), and with the widespread acceptance of Treg, many types of findings are currently attributed to regulation, and the designation of "regulatory" is given to many a cell or molecule! This is certainly unwarranted and confusing. For example, 7S suppression was the very first phenomenon of regulation ever described (Henry and Jerne 1968), but we do not refer to IgG-secreting plasma cells as regulatory. Thus, plasma cell function is antibody secretion; if the antibodies suppress other antibody responses—or enhance them, as is the case for the IgM class—we do not classify the secreting cells as suppressor and helper plasmacytes, respectively. Seemingly, Th1 cells suppress the generation/activity of Th2 cells and vice-versa. Yet, we do not refer to these differentiated stages of helper T cells as regulatory. It would seem appropriate to reserve the designation of "regulatory T cells" to those lymphocytes that specifically differentiate to the particular function of regulating other cells' activities. Conversely, it may well be that regulatory T cells will end up promoting one or another class of immune responses as a consequence of their regulatory activity. Yet, we will continue to refer to them as regulatory T cells, rather than Th2, Th3, or anything else. This is one of the reasons why we prefer the present designation, as opposed to "suppressor T cells". This argument is strengthened by the notion that Treg represent an independent differentiative lineage of T cell, displaying a specific pattern of gene expression, and following specific rules for selection, population dynamics, and operation. In other words, the criterion for demarcation might be the fundamental difference between Treg and all other varieties of CD4 T cell classes or effector types: Treg are committed intrathymically, while all other T cells exit the thymic womb as naïve, functionally uncommitted cells. Moreover, Treg seem to be selected on nominal antigen (if promiscuously expressed) inside the thymus, while other T cells seem to be merely restricted for the recognition of antigens yet to be encountered in the periphery. Having said this, it would be foolish to ignore many of those regulation processes, which are mediated by lymphocytes or other cells that are not born to regulate. These may well contribute to the overall physiological processes of tolerance and regulation of immune responses.

Acknowledgements This work was supported in part by a project grant on malaria from the Fundação Calouste Gulbenkian, by the Fundação para a Ciência e a Tecnologia (Portugal), and by the Centre National de la Recherche Scientifique (France). The Instituto Gulbenkian de Ciência was founded and is supported by the Fundação Calouste Gulbenkian. We thank our colleagues at the Institutes in Oeiras, Nogent, and Paris for many productive discussions.

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