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Mechanisms of self–nonself discrimination and possible clinical relevance

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Abstract

This review discusses different mechanisms that result in immunological tolerance, such as intrathymic deletion of immature T cells, intrathymic and extrathymic generation of regulatory T cells, effector mechanisms of regulatory T cells as well as molecular pathways involved in extrathymic generation of regulatory T cells *in vivo* and *in vitro*. These molecular mechanisms should enable investigators to develop clinical protocols aiming at the specific prevention of unwanted immune responses, thereby replacing indiscriminate immunosuppression that often has fatal consequences.

Keywords

conversion; Foxp3; recessive and dominant tolerance; regulatory T cell; self–nonself discrimination; suppression; TGF-β

Recessive mechanisms of tolerance

The idea that tolerance to self could be due to deletion of immature lymphocytes by self-antigens was first put forward by Burnet [1] and Lederberg [2] and received some support when Medawars group [3] could induce transplantation tolerance in neonatal mice by creating hemopoietic chimerism through injection of allogeneic cells. At that time, however, it was not yet known that the immune system relies on continuous generation of lymphocytes in primary lymphoid organs and, hence, tolerance induction to self must be a continuous process throughout life. Since it was difficult to visualize the deletion of a few cells with antigen receptors for self, new experimental systems were created to address mechanisms of tolerization to self. One approach consisted of the analysis of developing T cells with specificity for so-called superantigens where the specificity rests exclusively with the T-cell receptor (TCR)-β-chain that binds to superantigens in an unconventional way, that is to say, differently from the binding of the αβ-TCR to conventional peptide–MHC complexes. The results from such studies [4] led to the conclusion that superantigens can induce deletion of immature $CD4^+CD8^+$ thymocytes that becomes mostly manifest by the absence of superantigen-specific T cells in the medulla of the thymus [5]. Another approach utilized the injection or supply of anti-CD3 antibodies either *in vivo* or in thymic organ cultures *in vitro*, which resulted in profound depletion of $CD4^+CD8^+$ thymocytes in the thymic cortex. While the *in vivo* results could be criticized because such a maneuver could result in glucocorticoid production that leads to cell death of glucocorticoid-sensitive $CD4^+CD8^+$ cortical thymocytes [6], the *in*

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vitro results in organ culture are not subject to the same criticism and indicate that TCR ligation in the thymic cortex can result in cell death of cortical thymocytes [7].

It is clear, however, that these two approaches employed rather unusual TCR ligands and it remained an open question whether such results could be extrapolated to conventional peptide–MHC complexes. This issue was then addressed in TCR-transgenic mice in which the α- and β-TCR transgenes encoded a receptor recognizing a peptide from a protein encoded by a gene on the male chromosome presented by class I MHC molecules (HY-transgenic mice) [8]. A first comparison of female and male HY-transgenic mice yielded results consistent with deletion of immature $CD4^+CD8^+$ cortical thymocytes in the thymus of male but not female mice. It was, however, realized quite early that the αβ-TCR in these mice was expressed prematurely resulting in lineage diversion of some double-negative $CD4^-CD8^-$ cells into the γδ-lineage prior to reaching the $CD4^+CD8^+$ stage of development [9–11]. Thus, it was argued that the reduction of $CD4^+CD8^+$ thymocytes in male mice could be due to lineage diversion rather than deletion [12]. This argument, however, was anticipated by Swat *et al.*, who isolated $CD4^+CD8^+$ thymocytes from female mice and exposed them to male antigen, which resulted in rapid apoptosis [13]. Similar results were subsequently reported in thymic reaggregation cultures from female HY-transgenic mice supplied with HY peptide [14], confirming that $CD4^+CD8^+$ cortical thymocytes were subject to deletion by conventional peptide–MHC complexes. The 'lineage diversion argument' was further rendered untenable by the observation that male HY mice defective in proapoptotic BCL-2-interacting mediator of cell death (BIM) accumulated $CD4^+CD8^+$ thymocytes in the cortex [15], and that HY mice with mutant MHC molecules that cannot bind CD8 coreceptors likewise accumulated cortical thymocytes, indicating that the elimination of $CD4^+CD8^+$ thymocytes in HY mice was, at least in part, dependent on CD8 coreceptor expression and did not exclusively occur at the $CD4^-CD8^-$ stage of development [16]. Together, these results led to the firm conclusion that tolerance to self-peptide/MHC complexes can involve deletion of $CD4^+CD8^+$ cortical thymocytes [17]. These conclusions were recently confirmed and extended in HY mice with 'on-time' expression of the transgenic TCR in $CD4^+CD8^+$ thymocytes: while initial results in this system were, for unknown reasons, interpreted to indicate that negative selection occurred in the cortico–medullary junction [18], subsequent studies confirmed that deletion could occur in the outer cortex. Interestingly, deletion was largely, but not exclusively, dependent on antigen presentation by dendritic cells (DCs) in the cortex [19]. Thus, at present, there is good agreement that negative selection can affect immature cortical thymocytes as well as immature thymocytes in the medulla [20] and hence the notion of exclusive deletion in the medulla as proposed by Sprent and Kishimoto [21] as well as Nemazee [22] has no experimental basis. At present, there are still lingering arguments that tolerance to self could involve deletion as well as receptor-editing in the thymus [22,23]. To further address the relevance of the receptor-editing argument, studies by Buch *et al.* made use of the HY insertion model, allowing for self-reactive thymocytes to edit their TCR by secondary recombination at the TCRα locus [14]. To this end, the VαJα exon of a male-specific TCR was inserted into the *TCR*α locus, which had undergone Cre-loxP-mediated deletion of the *TCR*δ locus. The results making use of the HY insertion model point to negative selection of autoreactive thymocytes rather than receptor editing, since in female and male mice similar numbers of thymocytes changed their *TCR*α chain by rearrangement, thus arguing that the HY agonist ligand did not specifically induce or prolong rearrangement of endogenous *TCR*α genes.

Therefore, regarding so-called 'recessive' mechanisms of self-tolerance, we presently have firm evidence that it can occur in the form of deletion of very immature thymocytes in the thymic cortex and still-immature thymocytes in the medulla, and deletion as well as anergy of immature and mature T cells whereby in mature T cells anergy and deletion can be preceded by a brief period of proliferation [24].

Ramsdell *et al.*, focusing on tolerance induced by antigens expressed on radioresistant thymic stromal elements, have provided initial evidence that clonal anergy can also be induced intrathymically [25]. In other early studies, we were able to demonstrate that mature T cells from female HY TCR-transgenic mice could be rendered tolerant *in vivo* following transfer into male nude mice [24]. On these cells, a downregulation of TCR and CD8 expression could be observed. Moreover, such cells were shown to be anergic in response to antigenic stimulation and challenged previous data suggesting that clonal anergy could not be reversed following withdrawal of antigen [26,27]. Using the above-outlined HY-transgenic system, we could show that *in vivo* upon removal of antigen, anergic T cells were able to persist for several months in recipient mice and could become fully functional T cells [28].

Mechanisms of Fas–FasL ligand interactions to maintain immunological tolerance to self-antigens have been described to rely on the fact that, following repeated stimulation by their cognate antigen, $CD4^+$ T cells express high levels of Fas and FasL, leading either to killing of themselves or another T cell [29,30]. Studies by Bellgrau *et al.* appeared to support the hypothesis that FasL expression in the testis could act by inducing apoptotic cell death of Fas-expressing recipient T cells following activation by graft antigens [29]. This would be compatible with the notion that FasL might be involved in creating immune privilege in certain tissues.

Furthermore, the authors claimed that such a mechanism may help to limit the number of lymphocytes responding to foreign antigens and in fact may be triggered by abundant self-antigens. However, other available data indicate that Fas is not relevant in negative selection of developing T cells in the thymus, while Fas has been implied to be involved in antigen-dependent deletion of mature T cells [31]. Contrasting work by Zimmermann *et al.*, focusing on the role of Fas and $CD8^+$ T cells after antigen challenge, implicates that tolerance induction as well as peripheral deletion of $CD8^+$ T cells occur in a Fas-independent manner [32]. Thus, the role of the Fas–FasL system in T-cell tolerance is questionable.

In 1989, it was already suggested that the thymus might represent a patchwork quilt of diverse tissues at the level of RNA [33]. In fact, mice transgenic for neo-self-antigens revealed a role of ectopic expression of the respective antigen in the thymus [34,35]. Hanahan described promiscuous expression of antigen in the thymus [35]. In his seminal work focusing on tolerance toward antigens with implication to diabetes, it became obvious that the intrathymic expression of the transgenic β-cell antigen under the control of the insulin promoter can be interpreted as a physiological property of the endogenous insulin gene locus since insulin RNA was also expressed in the thymus of nontransgenic mice. These results raised important questions on the impact of the thymus in the role of tolerance induction toward tissue-specific antigens. Subsequent studies by Klein were supportive of the concept that medullary thymic epithelial cells (mTECs) express a wide range of tissue-specific antigens [36]. This promiscuous gene expression is maintained during the entire period of thymic T-cell output and, thus, suggests a role in tolerance induction to self-antigens. Until now, one molecular regulator of promiscuous gene expression has been identified: the autoimmune regulator (AIRE). In humans, mutation of the gene results in the rare autoimmune polyglandular syndrome type-1 leading to hyperparathyroidism and adrenocortical failure [37]. The use of AIRE-deficient mice revealed that AIRE indeed controls the ectopic expression of a large portion of tissue-specific antigens in mTECs of the thymus [38]. Unexpectedly, in the RIP-Ova/OTII system, expression of ovalbumin under the control of the insulin promoter in mTECs was not influenced by the absence of AIRE, pointing to functions of AIRE beyond promiscuous gene expression and regulation of negative selection [39]. Recent work by Gray *et al.* shows that the $AIRE^+$ mTECs show a high turnover with loss of cells most likely through apoptosis [40]. Thus, AIRE might induce terminal differentiation of mTECs. Cross-presentation of mTEC-derived antigens appears to involve thymic DCs presenting fragments of apoptotic

mTECs [40–42]; however, thus far it is not clear whether, as a rule, these antigens need to be cross-presented for induction of negative selection.

Liston *et al.* argue that AIRE does not operate primarily through an influence on the positive selection of Treg cells [43]. Numbers of $CD4^+CD25^+CD69^-$ cells are apparently normal in AIRE-deficient mice [39]. Furthermore, Tregs exhibited usual Foxp3 transcript and protein levels, as well as suppressive functions *in vitro* and *in vivo* [39]. More importantly, cotransfer experiments with AIRE-negative stroma into thymus-less recipients did not prevent autoimmunity. On the other hand, at a ratio of 4:1, cells from thymus or spleen of wild-type mice could reduce the autoimmunity caused by transfer of AIRE-negative stroma into a thymusless recipient [39]. These results are difficult to interpret and do not necessarily rule out that AIRE has a function in induction of regulatory T cells. In different studies, Derbinski found that promiscuously expressed genes tend to colocalize in clusters of up to 16 genes, encompassing both AIRE-dependent as well as -independent genes [44]. They point to the view that suppression of genes in mTECs is due to accessibility of chromosomal regions to transcription irrespective of tissue-specific differentiation patterns. Until now, however, the molecular mechanisms underlying the role of AIRE in transcriptional regulation are far from clear. Recent data from Koh *et al.* focus on the concept that AIRE may have a role in transcriptional elongation and histone binding with recognition of the amino-terminal tail of histone H3, one of its plant homeodomain fingers being essentially important for mediating its transcriptional control [45]. The AIRE-mediated recruitment of elongation and cotranscriptional mRNA-processing factors to tissue-specific gene promoters may activate a wide range of genes encoding tissue-specific antigens in mTECs.

Apparently, recessive mechanisms of tolerance eliminate mostly lymphocytes with high-affinity TCRs for their ligands, while cells with low-affinity TCRs appear to escape as self-reactive T cells that must be kept in check by so-called ‘dominant’ tolerance mechanisms that involve T regulatory cells.

Intrathymic Treg generation

Following the initial description of the $CD4^+CD25^+$ suppressor or regulatory T-cell subset (Treg), a distinct role of the thymus for their generation has been proposed. Work by Jordan in x-irradiation chimeras using influenza hemagglutinin (HA)-specific CD4 T cells directly showed that expression of the agonist ligand can lead to the commitment of thymocytes to the $CD25^+$ lineage [46]. Similar results were obtained by Apostolou *et al.* in thymus transplantation experiments using the same transgenic system [47] as well as by Walker *et al.* in ovalbumin-specific DO11.10 transgenic mice [48], thus arguing that intrathymic expressions of agonist ligands represents a powerful means for Treg generation [47,49] and suggesting that natural Tregs may express TCRs with high affinity for thymic MHC/self-peptide ligands [50,51].

van Santen *et al.* challenged this conclusion [52]. By the use of a transgenic mouse line allowing for the controlled expression of a T-cell epitope, they hypothesized that diversion of differentiating thymocytes into the Treg cell subset by agonist ligands was nonexistent and concluded that the illusion of induced differentiation was in fact due to the reduced sensitivity of the $CD4^+CD25^+$ subset to agonist-induced clonal deletion [52].

By contrast, Tai and colleagues again underlined the requirement of agonist ligands as well as of CD28 costimulation of developing thymocytes for intrathymic Treg generation [53], in the same transgenic system used by van Santen *et al.* The requirement for costimulation of intrathymically induced Treg is a phenomenon some-what in contrast to peripheral Treg generation, which is most effective under conditions avoiding costimulation (see below). Tai *et al.* referred to the possibility that CD28 stimulation leads to an augmentation of the signaling intensity of low-affinity intrathymic TCR engagement, thereby conferring sufficient signal

strength to allow for Treg generation. Moreover, they showed a pivotal role of the Lck-binding site in the CD28 cytosolic tail for initiating Treg cell differentiation, supporting the hypothesis that binding of Lck to the CD28 cytosolic tail results in an enhancement of the intensity and duration of the antigen-specific TCR signal [54,55]. Their results from mixed bone marrow chimeras led to the conclusion that *in vivo* production of IL-2 did not alter the requirement for intrathymic CD28 costimulation, thus pointing to an IL-2-independent role of CD28 for intrathymic Treg generation.

The possible requirement of IL-2 for thymic Treg cell development has been a controversial issue. Two recent reports demonstrated the presence of polyclonal or monospecific Foxp3-expressing thymic $CD4^+$ T cells in the context of IL-2 or IL-2Rα deficiency, thus pointing to the conclusion that IL-2 is not absolutely required for intrathymic Treg generation [56,57]. However, IL-2 may possess a role in regulating survival and proliferation of $Foxp3^+$ thymocytes, as their numbers are distinctly reduced in *IL-2*$^{-/-}$, *IL-2α*$^{-/-}$ or *IL-2Rβ*$^{-/-}$ mice compared with Tregs from control animals [56,57] and IL-2 plays an essential role in generation of Treg in mice deficient in TGF-β signaling [58].

Data by Aschenbrenner, using reaggregate thymic organ cultures supported the view that thymic epithelial cells (TECs) are sufficient to induce polyclonal Treg cell development and that, in one particular model of Treg induction, antigen does not need to be cross-presented by hematopoietic cells [59]. Nevertheless, mTECs could also be involved in Treg generation [60]. In the experiments by Aschenbrenner *et al.*, one can observe a distinct reduction in the proportion of polyclonal Tregs in the absence of MHC class II molecules on mTECs. Furthermore, the targeting of a model antigen (AIRE–HA) to $AIRE^+$ mTECs resulted in the generation of specific Tregs. These data could support the hypothesis that a reduction of Tregs specific for tissue antigens might contribute to autoimmunity seen in AIRE deficiency. However, as discussed previously, there is currently no direct evidence supporting the involvement of AIRE in the generation of Tregs.

Recent studies by Gavin and Lin point to the hypothesis that there is no requirement of Foxp3 for the initial development of thymocytes into the Treg lineage. Using mice in which the Foxp3-encoding region is replaced by a green fluorescent protein (GFP) reporter, they demonstrate that GFP-positive thymocytes, which do not express the Foxp3 protein, share some characteristics with the Treg phenotype, including anergy. However, these cells do not suppress *in vitro* [61,62]. Thus, Foxp3 seems to be important in stabilizing and sustaining the Treg phenotype as well as to confer Treg suppressor function. Presently, it is not clear how the Treg differentiation program is initiated in the thymus, except that TCR signaling and costimulation are required.

Peripheral induction of Tregs: role of antigen specificity

As indicated above, many self-reactive T cells are deleted in the thymus, pointing to an important role of central tolerance in self–nonself discrimination. Initially, the view that not all antigens are present in the thymus underlined the necessity of peripheral tolerance mechanisms as well. Now that we are aware of ectopic antigen expression in the thymus as well as immigration of antigen-presenting cells [63], one could argue that there is no need for peripheral tolerance mechanisms. However, it has been argued that negative selection affects predominantly cells with high-affinity TCRs [64,65] and, thus, we need peripheral tolerance mechanisms to deal with low-affinity cells.

Furthermore, to maintain local homeostasis, the organism requires the peripheral generation of Tregs in a distinct microenvironment, such as the gut or at those sites chronically exposed to microbes or tumors. Thus, peripherally induced Tregs may have the function to deal with low-affinity autoreactive cells and to limit immune responses to foreign antigens.

Thymic versus peripherally induced Tregs display a close similarity, with regard to their regulatory function, surface phenotype as well as their global gene-expression pattern. Transcriptional profiling of Foxp3-expressing Tregs in comparison with naive or activated T cells demonstrated a distinct number of differentially expressed genes comprising some genes normally upregulated in activated T cells, such as *IL2ra* (CD25), *Ctla4* (CTLA4) and *Tnfrsf18* (glucocorticoid-induced TNF receptor; GITR), representing signature target genes for Foxp3 [66,67].

Recent evidence supports a pivotal role of CTLA4 in $Foxp3^+$ Tregs for their suppressive activity *in vivo* and *in vitro*: the suppression appears to be mediated, at least in part, by CTLA4-dependent downregulation of CD80 and CD86 costimulatory molecules on antigen-presenting cells. CTLA4-defective Tregs are unable to sustain self-tolerance and immune homeostasis, thus pointing to a major role of CTLA4 in controlling the suppressive functions of Tregs [68]. Nevertheless, the signal transduced to DCs as a result of the interaction of CTLA4 with CD80/86 needs to be further examined. It was also reported that CTLA4 could directly control other T cells by binding to their CD80/86 molecules [69]. Perhaps these two mechanisms are not mutually exclusive.

Peripherally generated Tregs can exhibit a long lifespan as resting cells in an intermitotic stage, but independent of further supply of their agonist ligand that induced their formation. This is an important feature because it permits the induction of such cells prospectively to suppress anticipated immune responses. When induced, Tregs encounter their agonist TCR ligand, they rapidly express activation markers and home to antigen-draining lymph nodes where they can undergo considerable expansion [70–72]. Chronic stimulation results in a loss of CD62L, acquisition of CD44 and α_E integrin (CD103) expression. The specific expression profile of adhesion molecules and homing receptors then allows for extravasation and accumulation together with T-effector cells at places of inflammation. Thus, the specificity of suppression is brought about by the specific corecruitment of activated regulatory and regulated CD4 and/or CD8 effector cells in a local milieu. It has been reported that the antigen-specificity of Tregs and T effector cells does not need to match in order to allow for effective immunosuppression as long as the two cell types are co-recruited to the same tissue. Thus, Tregs with one particular antigen specificity are able to suppress a variety of effector cells with different specificity when being colocalized at the same antigen-presenting cell.

The *de novo* generation of Tregs *in vivo* was achieved by following ancient and anecdotal protocols putatively resulting in suppression of specific immune responses. Constant supply of a TCR agonist ligand in the form of a peptide over a 2-week period via implantation of mini-osmotic pumps or by targeting it to steady state DCs using peptide containing fusion antibodies directed against the DEC205 endocytotic receptor were both suitable methods to induce Tregs providing the delivery process did not result in DC activation. The establishment of these protocols added a new dimension to our understanding of antigen-specific tolerance mechanisms [70,72–74]. While the peptide dose was not critical when the peptide $HA_{111–119}$ of influenza A was infused, the amount of the same antigen was crucial with the DEC205 delivery method. This is understandable considering that the peptide will be quickly eliminated in the first case while with DEC205 delivery one sets an antigen deposit that critically influences T-cell activation. It was found that in secondary lymphoid organs, antigen-specific Tregs could be generated from naive T cells, especially under conditions of subimmunogenic antigen presentation (i.e., presented at low amounts by steady-state DCs). The conversion into Tregs was shown to require an intact TGF-βRII on naive T cells and depended on conditions that avoided activation of DCs (costimulation) as well as IL-2 production by the converting cells themselves.

The absence of Treg in TCR-transgenic mice expressing only one particular TCR without coexpression of a TCR agonist ligand supported the concept that Tregs were in fact due to *de novo* generation rather than expansion of already committed Tregs. Once converted, Foxp3-expressing Tregs can be expanded under conditions of immunogenic antigen presentation that induce strong proliferative responses in both Treg and naive T cells [49,72,73].

The hitherto existing difficulties in tracing antigen-specific Tregs in wild-type mice have profoundly limited the understanding of their role in regulating immunity. Antigen-specific T cells are present in minute numbers in wild-type mice; hence until recently, most studies used TCR-transgenic systems to visualize antigen-specific Tregs. We recently attempted to generate HY-specific Tregs with the goal to interfere with HY antigen-specific graft rejection, representing a serious complication in human transplantation of bone marrow from male donors in female recipients. To this end, C57BL/6 female mice were infused with a class II MHC-presented HY peptide. Advantage was taken of the production of MHC class I- and II-specific HY peptide tetramers to visualize male-specific CD8 and CD4 cells. Initially, the data showed that peptide-infused female mice tolerated male skin, spleen cells and bone marrow transplants. Moreover, examination of HY-specific Tregs after male-antigen induced expansion in spleen and draining lymph nodes of male skin-grafted control or peptide-infused mice showed preferential accumulation of HY-specific Tregs in antigen-draining lymph nodes either by local expansion or by specific homing [75]. One needs to consider that this approach has only been proven successful with attempts to convert naive T cells and not preactivated T cells, indicating that it can presumably not be utilized to suppress already established autoimmunity. The HY experiments clearly showed that it is possible to induce prospective tolerance by generating antigen- specific Tregs [75].

In order to maintain functional integrity of Tregs, IL-2 was found to be of crucial importance, since IL-2- or IL-2R-deficient mice lack Tregs and suffer from autoimmune diseases. Adoptive transfer of peripheral Tregs into IL-2-deficient hosts or IL-2-depletion experiments established a nonredundant role of IL-2 signaling for Treg homeostasis in the periphery and lymphoid tissue [56,76]. Moreover, IL-2 has been clearly shown to have an essential role in prolonged peripheral survival of $Foxp3^+$ Tregs [56,57,72,77]. It still remains controversial whether IL-2 signaling plays an essential role in Treg generation or only in survival and expansion of Tregs.

TGF-β-related Treg conversion & suppression

In vivo and especially *in vitro* data point to the essential role of TGF-βRII signaling in generating Tregs. *In vitro* studies have demonstrated that conversion of naive peripheral CD4 T cells into Tregs could be achieved through ligation of the TCR in the presence of TGF-β, suggesting that TGF-β might also play an essential function in Treg development *in vivo* [78, 79].

Besides the role in generation of Tregs and Th17-producing cells, TGF-β also controls CD8 T-cell proliferation. TGF-β plays a role in the Treg-induced inhibition of exocytosis of granules from cytotoxic T lymphocytes (CTLs) [80,81]. These data show that Tregs can suppress already established effector cells late during an immune response. Furthermore, the data demonstrated that such suppression is reversible. *In vitro* experiments using fully differentiated CTLs revealed that TGF-β does not have any effect on cytolysis when added during the effector phase, further supporting the concept that signaling via TGF-β 'sets' conditions for suppression of CD8 T cells rather than representing the main suppressor mechanism by itself. Comparisons of gene-expression profiles of suppressed versus nonsuppressed CD8 T cells in this particular model revealed that the most prominently regulated gene was the 5′-nucleotidase converting 5′-AMP into adenosine suggesting that Tregs can instruct CTLs to inhibit themselves. Compelling data point to an immunosuppressive function of the latter [82]. Within this regard,

recent data imply that adenosine generated from the hydrolysis of nucleotides can exert prominent inhibitory effects primarily mediated through the A2A receptors *in vitro*, which can be additive to cell-contact-dependent mechanisms dictating Treg functions. Thus, further investigations on the coordinated coexpression of CD39/ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) and CD73/ecto-5′-nucleotidase catalyzing the perinuclear generation of adenosine on Tregs and the A2A receptor on activated effector T cells are needed to address the impact of this biochemical signature on Treg function and regulation [82].

Other studies revealed that mice with a T-cell-specific TGF-βRII deletion develop a progressive autoimmune disease with animals finally succumbing to death [83]. Such mice show a significantly increased number of peripherally activated T cells, while in the periphery, Tregs are clearly reduced. Thus, there is evidence that the size and suppressive activity of the peripheral Treg compartment is dependent on TGF-β perhaps also because of promoting Treg conversion under subimmunogenic conditions in addition to maintaining survival and expansion of Foxp3-expressing cells. Despite the observed reduction of the peripheral Treg pool in TGF-βRII-conditional knockout animals, a significant enhancement of the number of cycling Treg cells could be seen in the thymus compared with control mice [83]. It is presently not clear whether the proliferation of TGF-βRII-deficient Tregs is caused by cell-intrinsic or cell-extrinsic mechanims [83,84], but it may depend on exogenous cytokines.

Transient expression of TGF-β using a transgene specifically expressed in pancreatic islets promotes the proliferation and/or generation of $CD4^+CD25^+$ Foxp3-expressing Tregs *in situ* in diabetes-predisposed NOD mice [85]. These data are consistent with the view that TGF-β could play an important role in antigen-driven conversion of Tregs *in vivo*. In fact, it has been shown that pretreatment of T cells *in vitro* with TGF-β reduced the subsequent antigen-specific *in vivo* proliferation to a minimum, thus increasing the conversion rate [86].

Using transfer models, it has been demonstrated that TGF-βRII-deficient T cells are refractory to Treg suppression, which could suggest that TGF-βR signaling is involved in suppression [84,87,88]. However, it remains to be seen whether the TGF-β induced signaling only conditions T cells for other suppressive mechanisms or represents the suppressive mechanism itself.

Role of retinoic acid in enhancing Treg conversion

As described before, mature $CD4^+$ T cells from peripherial lymphoid organs can be converted to $Foxp3^+$ cells under a variety of conditions: chronic suboptimal stimulation by agonist peptide [72,73], after exposure to orally administered agonist ligands [89,90] or under lymphopenic conditions [91]. Recent data support the concept that retinoic acid (RA) has an important role in oral tolerance [89–92]. It is well established that the induction of oral tolerance is associated with the *in situ* production of induced $Foxp3^+$ Tregs. Most interesting in this context was the observation that the conversion of naive T cells to Treg cells took place upon antigen presentation by $CD103^+$ DCs in gut-associated lymphoid tissue that is TGF-β-dependent, and was shown to be augmented by RA and inhibited by inhibitors of retinal dehydrogenase [89, 92]. RA has been characterized as a key regulator of TGF-β-dependent immune responses, helping the conversion of naive $CD4^+$ T cells into Treg cells and inhibiting their development into Th17 cells. Work by Mucida *et al.* has shown that IL-6 inhibits TGF-β-induced Foxp3 induction resulting in Th17 cells, while RA can counteract this inhibition. In *in vitro* studies with naive T cells from *IL-6*$^{-/-}$ and *IL-6Rα*$^{-/-}$ mice, it was noted, however, that RA must have effects in addition to inhibiting IL-6 [93,94]. The *in vitro* experiments aiming at the elucidation of the RA effect have been performed under a variety of conditions with regard to kinetics and concentration of TGF-β and RA, which makes firm conclusions difficult. Intriguingly, Hill *et al.* provided evidence that RA can enhance Foxp3 induction indirectly by inhibiting the release

of factors from $CD4^+CD44^+$ cells arguing that initial studies claiming a direct effect of RA on naive T cells did not purify naive T cells sufficiently [93].

However, our unpublished data show that RA can enhance TGF-β-dependent conversion of naive T cells into Treg by directly counteracting the negative impact on conversion of costimulation and exogenously added cytokines in a retinoic acid receptor α (RAR α)-dependent manner.

The transfer of antigen-specific $CD4^+$ $Foxp3^-$ T cells from OT-II *Rag1*$^{-/-}$ mice into a RARα knockout mouse suggested the existence of an indirect effect of RA *in vivo* by showing suboptimal conversion of these cells into Treg [93]. It still remains to be determined, however, whether under such conditions RA also has a direct effect on highly purified naive T cells as the above-described *in vitro* data would suggest. Further work is required to determine the various molecular pathways by which RA enhances Foxp3 expression.

Impact of aryl hydrocarbon receptor ligands on Treg conversion

Recently, it has also been reported that the aryl hydrocarbon receptor (AhR) can, in a ligand-dependent manner, regulate Treg versus Th17 commitment. AhR is one of several chemical/ligand-dependent intracellular receptors that can stimulate gene transcription in response to xenobiotics, thereby mediating the toxicity of environmental pollutants [95]. AhR ligands include the environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and endogenous molecules such as FICZ (6-formylindolo[3,2-b]carbazole), a tryptophan-derived product thought to be generated in the skin following exposure to ultraviolet light. Furthermore, numerous naturally occurring dietary and endogenous AhR ligands have been identified [95]. TCDD has been reported to promote the differentiation of new antigen-specific Tregs *in vitro* and perhaps also *in vivo*, while on the other hand reducing the number and function of Th17 cells, thus leading to an amelioration of disease symptoms in the experimental autoimmune encephalitis model of multiple sclerosis. On the other hand, FICZ has the opposite effect resulting in a prominent increase of Th17 activity while not influencing Treg functions [96,97]. Until now, however, the pharmacology and pharmacokinetics of AhR ligands such as TCDD or FICZ is far from clear, making the understanding of a physiological role for the AhR in the regulation of Treg/Th17 cells difficult.

Epigenetic regulation of Treg conversion: role of methyltransferases

Epigenetic mechanisms, such as chromatin remodeling, regulate expression by modifying regions of the genome, thus maintaining either gene silencing or activation. Molecular phenomena of epigenetic imprinting include selective demethylation of CpG motifs and histone modifications as shown for cytokine genes [98]. As early as in 1975, Riggs [99] and Holliday [100] proposed that DNA methylation might be responsible for the stable maintenance of a particular gene expression pattern through mitotic cell division. This initial hypothesis has been widely supported and now DNA methylation is accepted as the main contributor to the stability of gene-expression states [98]. Phenotypic analyses of mice with mutations in the various DNA methyltransferase (DNMT) genes have provided mechanistic insight into the role of DNA methylation, while DNMT1 has emerged as the major DNA methyltransferase responsible for the maintenance of DNA methylation in dividing cells [101].

Recent studies provide evidence for a critical role of epigenetic modifications in the *Foxp3* locus. Thus far, the mechanisms responsible for the development of stable Treg lineages were poorly understood. The Treg-specific demethylated region (TSDR) in the *Foxp3* gene, which displays demethylated CpG motifs both in thymic as well as in peripherally induced murine Tregs, correlates with long-term commitment to the Treg lineage [102–104]. Current data support the view that this evolutionarily conserved region is confined to natural thymus-derived

as well as *in vivo*-induced antigen-specific Tregs and might function as a prerequisite for the terminal commitment to the suppressor cell lineage. By contrast, *in vitro*-generated TGF-β-induced Tregs, which despite Foxp3 expression and suppressive properties have not acquired a terminally differentiated phenotype, can loose Foxp3 expression and suppressive activity following restimulation without TGF-β. As a correlation, *in vitro* TGF-β-induced Tregs display only weakly demethylated CpG motifs within the TSDR region of the *Foxp3* locus, supporting an important role of complete demethylation within the conserved region for stable Foxp3 expression. In fact, demethylation of the *Foxp3* locus in the absence of TGF-β can lead to the generation of Foxp3-expressing cells *in vitro* [103].

These data are in agreement with recent work by Kim and Leonard [105], who identified a TCR-responsive enhancer in the *Foxp3* first intron, dependent on a CREB/ATF site overlapping with a CpG island. This CpG-motif containing element is located within the TSDR region described by Floess *et al.* [104], and methylation of this island inversely correlates with CREB binding and expression of Foxp3. Furthermore, the inhibition of methylation by 5-azacytidine or by silencing DNMT1 expression resulted in the induction of Foxp3 expression [90,103,105]. Chromatin immunoprecipitation assay (ChIP) analysis showed that the addition of TGF-β to mouse $CD4^+$ T-cell cultures in the presence of anti-CD3/CD28 and IL-2 results in reduced DNMT1 binding to the *Foxp3* locus [105]. Thus far, it is not clear whether TGF-β-mediated induction of Foxp3 expression solely relies on its impact on inhibiting methyltransferases as DNMT1. In addition, RA has been indicated to induce histone acetylation at the *Foxp3* promoter [92], thereby increasing the stability of Foxp3 expression [106].

Further studies on the molecular pathways that regulate Foxp3 expression are therefore needed to understand induction of Tregs on the molecular level and to design drugs that either permit enhancement of or suppress Treg generation.

Regulatory role of NKT & $CD8^+$ T cells within regulation of self-recognition

Not all regulatory T cells express Foxp3. Recognized more than a decade ago, natural killer (NK)T cells have been indicated to differentiate from mainstream thymic precursors through instructive signals emanating during TCR engagement by CD1d-expressing cortical thymocytes. Thus far, NKT cells are still narrowly defined as a T-cell lineage expressing NK lineage receptors, including NK1.1 in the C57BL/6 background as well as semi-invariant CD1d-restricted α/β-TCRs (type I invariant NKT cells) [107].

Their semi-invariant α/β-TCRs recognize isoglobotrihexosylceramide, a mammalian glycol-sphingolipid, as well as microbial α-glycuronylceramides found in the cell wall of Gram-negative, lipopolysaccharide-negative bacteria. This dual recognition of self and microbial ligands underlies innate-like antimicrobial functions mediated by CD40L induction and massive Th1 and Th2 cytokine and chemokine release [108]. CD1d-restricted NKT cells consist broadly of two groups, in mice invariant NKT cells express a conserved invariant α/β-TCR encoded by the *Vα14* and *Jα18* gene segments paired with a set of Vβ chains [108, 109]. Type II NKT cells (i.e., non invariant NKT cells) have been demonstrated to use variable TCRs and are distinct from the type I $V\alpha14^+$ invariant NKT cells [108]. Studies by Wilson *et al.* follow the hypothesis that dysfunction and/or changes in the frequency of invariant NKT cells are correlated with the development of autoimmunity, in particular autoimmune diabetes, in mice and humans [110]. The molecular basis of their functions are still largely unknown, however, recent findings underscore a potential role of interactions between invariant NKT cells and DCs indicating that invariant NKT cells regulate DC activity to address both proinflammatory as well as tolerogenic immune responses [111]. Furthermore, recent data by Halder *et al.* point to an important role for type II NKT cell-mediated anergy induction in type I NKT cells allowing for suppression of inflammatory liver disease [112]. In these experiments,

activation of type II NKT cells resulted in IL-12- and MIP-2-dependent recruitment of type I invariant NKT cells into mouse livers. These invariant NKT cells were anergic and able to prevent concavalin A-induced hepatitis. In recent years, NKT cell biology has emerged as a new field at the barrier between innate and adaptive immunity. Thus, the reciprocal activation of NKT cell subtypes and DCs as well as the mechanisms and relevant ligands involved to mediate their regulatory capacities in a wide range of immunopathological conditions need further investigations [108].

It is now realized that active regulation in order to maintain self-tolerance is not only a property of $CD4^+$ T cells but also involves $CD8^+$ T cells. Following their initial description almost 40 years ago by Gershon *et al*. [113], $CD8^+$ suppressor cells still remain less well characterized than Foxp3 Tregs, because detailed studies have been hindered by the lack of reliable markers allowing for their identification. Unlike conventional MHC molecules, Qa-1 is expressed preferentially and transiently on activated CD4 T cells. This specific expression pattern assures that only activated CD4 cells expressing Qa-1–peptide complexes induce Qa-1-restricted CD8 regulatory cells [114]. Furthermore, the complex of Qa-1 with Qdm allows for engagement of the CD94/NKG2 receptor expressed on CD8 as well as NK cells, thus leading to an attenuated activity status of these cells associated with reduced $CD8^+$ suppressive activity [115–117].

The mechanisms by which $CD8^+$ Tregs are induced are far from being understood in detail: it has been postulated that mechanisms of costimulation might be involved. It was shown that immunization in combination with anti-CD137 administration results in generation of $CD8^+$ Tregs capable of suppressing induced or spontaneous autoimmune diseases [118,119]. Several mechanisms have been discussed to be involved in the suppressive capacities of $CD8^+$ Tregs, including secretion of cytokines as well as direct lysis. Further experiments pointed to the view that the perforin-mediated cytolytic activity is necessary for suppression by $CD8^+$ Tregs. However, it is not yet clear whether different subpopulations of $CD8^+$ Tregs rely on distinct suppressive mechanisms, depending on the context of the immune reaction [120,121]. A relatively well-established characteristic of CD8 Tregs seems to be, however, their appearance after the peak of the immune response, indicating a distinct role in the immune regulation compared with $Foxp3^+$ Tregs.

Conclusions & future perspective

Mammalian organisms have developed a variety of mechanisms by which the immune system acquires tolerance to self. Elucidation of these mechanisms is of great importance since they may eventually enable the clinician not only to interfere with autoimmunity but also to regulate immune responses such that transplant rejection and allergic responses can be prevented. Over the last two decades, it has become clear that the deletion of immature thymocytes before acquiring functional maturity in the thymic cortex and medulla is one important mechanism that is aided by other mechanisms that permit ectopic expression of tissue antigens in thymic epithelium as well as immigration of antigen-presenting cells from peripheral tissue. It appears that the deletional mechanisms are imperfect and permit the escape of some self-reactive cells that probably have TCRs of relatively low affinity for self. Such escapes can be dealt with by peripheral mechanisms that include deletion as well as reversible anergy, but also so-called ‘dominant’ mechanisms that are executed by regulatory T cells. Tregs are generated *de novo* when confronted with intrathymic agonist ligands and, at least in some cases, the same TCR–ligand can induce deletion as well as generation of Tregs, a decision that is probably regulated by specific microenvironments (niches) in the thymus. However, *de novo* Treg generation is not restricted to the thymus since it can occur in peripheral tissue, for instance under conditions of subimmunogenic antigen presentation. In contrast to *in vitro* generated Tregs, *in vivo*-converted Tregs have a stable phenotype as Foxp3-expressing cells with a characteristic phenotype of surface antigens. The prospective induction of such peripheral Tregs by foreign

antigens permits the establishment of transplantation tolerance and may become clinically relevant to specifically avoid unwanted immune reactions. In order to make this goal more feasible we need to learn more of the molecular pathways of *in vivo* Treg generation to be able to regulate it by suitable drugs. The recent literature has provided some initial insights into *Foxp3* gene regulation and how it can be modulated by drugs raising the hope that one day we may be able to abandon general immune suppression and replace it by schemes that specifically suppress unwanted immune responses without compromising the entire immune system.

Executive summary

Recessive mechanisms of tolerance

- Recessive mechanisms of self-tolerance can occur in the form of deletion of very immature thymocytes in the thymic cortex and still immature thymocytes in the medulla, and deletion as well as anergy of immature and mature T cells.
- In mature T cells, anergy and deletion are preceded by a proliferation and require antigenic stimulation in the absence of costimulation.
- Recessive mechanisms of tolerance appear to mostly eliminate lymphocytes with high-affinity T-cell receptors (TCRs) for their ligands.
- Cells with low-affinity TCRs appear to escape as self-reactive T cells that must be kept in check by dominant tolerance mechanisms involving regulatory T cells.

Intrathymic Treg generation

- The thymus has a crucial role for the generation of the $CD4^+CD25^+$ suppressor T cells or regulatory T cell (Treg) lineage.
- Foxp3 is required to stabilize and sustain the Treg phenotype.
- Molecular pathways of intrathymic Treg differentiation need further clarification, but do depend on TCR signaling and costimulation.

Peripheral induction of Tregs: role of antigen specificity

- Peripheral tolerance mechanisms are needed to deal with low-affinity, self-reactive cells.
- Peripheral Treg induction can be achieved by presenting antigen under subimmunogenic conditions (i.e., in the absence of T-cell activation).
- Thymic versus peripherally induced Tregs are similar with regard to function, surface phenotype and expression of signature genes.
- In our view, IL-2 signaling plays no essential role in Treg generation, but only contributes to their survival.

TGF-β-related Treg conversion & suppression

- *In vivo* and *in vitro* data point to the essential role of TGF-βRII signaling in generating Tregs.
- Conversion of naive peripheral $CD4^+$ T cells into Tregs could be achieved through ligation of the T-cell receptor (TCR) in the presence of TGF-β.
- It is not clear yet whether TGF-β-induced signaling conditions T cells for other suppressive mechanisms or is a suppressive mechanism itself.

Role of retinoic acid in enhancing Treg conversion

- Recent data support an important role for retinoic acid (RA) in oral tolerance.
- RA functions as a key regulator of TGF-β-dependent conversion of naive T cells into Tregs.
- Our data show that RA can work directly on naive T cells by counteracting the inhibitory effect of costimulation and exogenously added cytokines in a RARα-dependent manner.

Impact of aryl hydrocarbon receptor ligands on Treg conversion

- The aryl hydrocarbon receptor (AhR) can, in a ligand-dependent manner, regulate Treg versus Th17 commitment.
- The pharmacology and pharmacokinetics of AhR ligands are not understood.

Epigenetic regulation of Treg conversion: role of methyltransferases

- Epigenetic mechanisms, including selective demethylation of CpG motifs and histone modifications, regulate expression by modifying regions of the genome leading either to gene silencing or activation.
- The Treg-specific demethylated region in the *Foxp3* gene that displays demethylated CpG motifs both in thymic as well as in peripherally induced but not in *in vitro* induced Tregs, correlates with long-term commitment to the Treg lineage.

Regulatory role of natural killer T cells & $CD8^+$ T cells within regulation of self-recognition

- Not all regulatory T cells express Foxp3.
- Natural killer T (NKT) cells are still narrowly defined as a T-cell lineage expressing NK lineage receptors, including NK1.1 as well as semi-invariant CD1d-restricted αβ-TCRs.
- It is controversial whether dysfunction and/or changes in frequency of invariant NKT cells are causally related to development of autoimmunity.
- Mechanism of suppression of $CD8^+$ Tregs might include secretion of cytokines as well as direct lysis.

Conclusions & future perspective

- The prospective induction of peripheral Treg by foreign antigen permits the establishment of transplantation tolerance and may become clinically relevant to avoid unwanted immune responses.
- We need to improve our understanding of the molecular pathways of *in vivo* Treg generation to be able to regulate it by suitable drugs with the future aim to specifically address unwanted immunity without the need to compromise the entire immune system.

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