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## Preferential Recognition of Self Antigens Despite Normal Thymic Deletion of CD4<sup>+</sup> CD25<sup>+</sup> Regulatory T Cells

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Paola Romagnoli, Denis Hudrisier and Joost P. M. van  
Meerwijk

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# Preferential Recognition of Self Antigens Despite Normal Thymic Deletion of CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells<sup>1</sup>

Paola Romagnoli,<sup>2\*</sup> Denis Hudrisier,<sup>\*†</sup> and Joost P. M. van Meerwijk<sup>\*‡‡</sup>

T cell tolerance to self Ags is in part established in the thymus by induction of apoptosis or anergy of potentially autoreactive thymocytes. Some autospecific T cells nevertheless migrate to peripheral lymphoid organs but are kept under control by the recently identified CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell subset. Because these cells inhibit autoimmunity more efficiently than useful non-self Ag-specific immune responses, they are probably autospecific, posing important questions as to how they develop in the thymus. In this study we show that significantly more peripheral CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells recognize self than non-self Ags. However, we also show for a large panel of endogenous superantigens as well as for self peptide/MHC complexes that autospecific CD4<sup>+</sup>CD25<sup>+</sup> thymocyte precursors are normally deleted during ontogeny. Combined, our data firmly establish that the repertoire of regulatory T cells is specifically enriched in autospecific cells despite the fact that their precursors are normally susceptible to thymic deletion. *The Journal of Immunology*, 2002, 168: 1644–1648.

Tolerance of T lymphocytes to self MHC-peptide ligands was thought to be mainly established by thymic deletion of developing autospecific T cells (1). Indeed, in absence of thymic deletion by APCs in bone marrow chimeras, 2- to 3-fold more mature T cells develop (2). Part of these autospecific cells are rendered anergic in the thymus upon recognition of self MHC/peptide ligands expressed by thymic epithelial cells (3). In transgenic mice expressing MHC/peptide complexes in the thymus exclusively on thymic cortical epithelial cells, a T cell repertoire develops that is strongly autoreactive in vitro and in vivo (4–6). However, total elimination of induction of T cell tolerance to self Ags still may not have been achieved in these transgenic mice because lethal graft-vs-host disease in syngeneic animals has not been observed and/or reported (our unpublished results). A third tolerance mechanism originating in the thymus is probably involved in this phenomenon: active (or “dominant”) tolerance assured by regulatory T cells (7–9).

The critical role of active T cell tolerance is known from a variety of experimental systems (reviewed in Refs. 7–11). Rats rendered lymphopenic by thymectomy and split dose irradiation develop autoimmune thyroiditis and diabetes because of absence of regulatory T cells (12, 13). When reconstituted with CD4<sup>+</sup>CD45RB<sup>high</sup> (but not CD4<sup>+</sup> or CD4<sup>+</sup>CD45RB<sup>low</sup>) T cells, athymic rats develop dispersed pathologic lesions (14). SCID mice that had received CD4<sup>+</sup>CD45RB<sup>high</sup> cells developed colitis, whereas coadministration of CD4<sup>+</sup>CD45RB<sup>low</sup> cells inhibited intestinal immunopathology (15, 16). Neonatal thymectomy causes severe autoimmunity, the precise target organ depending on the

mouse strain used. The absence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in thymectomized mice appears to be responsible for the autoimmune manifestations (17). It has recently become clear that the T cells regulating intestinal inflammation have the same CD25<sup>+</sup> phenotype as those inhibiting autoimmunity (18). CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells are involved not only in the inhibition of autoimmunity and intestinal immunopathology but also in the occasional incapacity of T cells to eliminate tumors (19) as well as in tolerance to allogeneic grafts induced under cover of anti-CD4, -CD11a, -CD40 ligand, -CD25, or -CD3 Ab or CTLA4-Ig treatment (11, 20). Combined with reports on deletional and nondeletional peripheral tolerance (1), these data emphasize the redundancy in intra- and extrathymic T cell tolerance mechanisms as well as the major role of regulatory T cells in the control of pathological and physiological immunity.

In vivo data suggest that regulatory T cells are specific for Ags expressed on the target organ. Regulatory T cells can, upon transfer into lymphopenic or neonatally thymectomized animals, inhibit disease. However, when regulatory cells were derived from animals lacking ovaries, testes, prostate, or thyroid gland (male/female, surgical removal, radioactive iodine), tissue-specific autoimmunity could not be inhibited (or was inhibited less efficiently) (reviewed in Ref. 9). Interestingly, thyroid-specific regulatory T cells were found in the thymus but not among peripheral T cells in athymic rats (21), indicating that regulatory T cells require interaction with Ag for their extrathymic survival.

Only sparse data are available concerning thymic selection of regulatory T cells. Their probable exclusively intrathymic origin has been suggested in the neonatal thymectomy-induced autoimmunity model (17). Moreover, thymocytes are known to be more efficient autoimmunity inhibitors than peripheral T cells (reviewed in Ref. 22). In neonatally mouse mammary tumor virus SW-infected BALB/c mice, the number of superantigen-specific Vβ6<sup>+</sup>CD25<sup>+</sup> thymocytes was increased, suggesting that interaction of thymocytes with agonist ligands favors differentiation of regulatory T cells (23). In mice doubly transgenic for influenza hemagglutinin (HA)<sup>3</sup> and an I-E<sup>d</sup>-restricted HA-specific TCR, large numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells developed (24), indicating

\*Institut National de la Santé et de la Recherche Médicale, Claude de Preval Institute, Purpan Hospital, Toulouse, France; †Faculty of Life Sciences, University Toulouse III, Toulouse, France; and ‡Institut Universitaire de France, Paris, France

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<sup>2</sup> Address correspondence and reprint requests to Dr. Paola Romagnoli, Institut National de la Santé et de la Recherche Médicale, Claude de Preval Institute, Purpan Hospital, U395, Institut Fédératif de Recherche 30, BP 3028, 31024 Toulouse Cedex 03, France. E-mail address: Paola.Romagnoli@toulouse.inserm.fr

<sup>3</sup> Abbreviations used in this paper: HA, hemagglutinin; MHC<sup>o</sup>, MHC class I and II deficient; wt, wild type.

a role for agonist ligands in thymic development of regulatory T cells. Because normal developing thymocytes are deleted or rendered anergic upon recognition of (self) Ag, these two reports raise important questions as to how regulatory T cells develop in the thymus.

We have analyzed the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells recognizing self and non-self (allogeneic) MHC/peptide complexes expressed by professional APCs. Our data establish that, although among normal CD4<sup>+</sup> T lymphocytes many more cells are specific for allogeneic than for syngeneic MHC/peptide ligands, the specificity of regulatory CD4<sup>+</sup>CD25<sup>+</sup> cells is strongly biased toward self Ags. To analyze the responsible mechanism(s), we have assessed thymic deletion of CD4<sup>+</sup>CD25<sup>+</sup> thymocyte precursors specific for a large panel of endogenous superantigens. Moreover, using bone marrow chimeras lacking MHC/peptide ligand expression on professional APCs (and therefore deficient in thymic deletion (2)), we have analyzed deletion of MHC/peptide complex-specific CD4<sup>+</sup>CD25<sup>+</sup> thymocyte precursors. In both systems, perfectly normal thymic deletion of regulatory T cell precursors was observed. Therefore, the high frequency of mature auto-specific regulatory T cells is, paradoxically, not caused by resistance to thymic deletion.

## Materials and Methods

### Mice

C57BL/6 and DBA/2 mice were purchased from Janvier (Le Genest St. Isle, France). C57BL/6 mice deficient in MHC expression (MHC<sup>o</sup>) (2) because of targeted deletions in the  $\beta_2$ -microglobulin and IA $\beta^b$  genes (25) were from Centre de Développement des Techniques Avancées-Centre National de la Recherche Scientifique (Orléans, France). All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines (IFR30 approval no. 31-13).

### Abs

The following Abs and secondary reagents were used for phenotypic analysis: FITC- and biotin-labeled anti-TCR $\beta$ ; FITC-labeled anti-TCR V $\beta$ 2, -3, -4, -5.1/5.2, -6, -7, -8.1/8.2, -8.3, -9, -10<sup>b</sup>, -11, -12, -13, -14, and -17<sup>b</sup>; anti-CD25 PE (BD Pharmingen, Heidelberg, Germany); FITC-labeled anti-CD4 (GK1.5); Red 613-labeled anti-CD4 and anti-CD8 (Life Technologies, Cergy Pontoise, France); and streptavidin tricolor (Caltag Laboratories, Burlingame, CA).

### Flow cytometry

Thymi were homogenized, washed in PBS, 2.5% FCS, and 0.02% Na<sub>3</sub>N, incubated with saturating concentrations of Ab for 20 min on ice, and washed before analysis using an Epics XL (Coulter, Marseille, France) or a FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed using WinMDI 2.8 (<http://facs.scripps.edu/software.html>) or CellQuest (BD Biosciences) software. For TCR V $\beta$  analysis, pooled thymocytes of three mice were depleted of CD8<sup>+</sup> cells by treatment with anti-CD8 mAb 31 M (26) and complement (Saxon Europe, Suffolk, U.K.), followed by Lympholyte-M gradient (Cedarlane Laboratories, Hornby, Canada).

### Bone marrow chimeras

Irradiation bone marrow chimeras were generated as previously described (2). In brief, anti-NK1.1 Ab-treated hosts (100  $\mu$ g of PK136 (27) i.p. at days -1 and 0 of reconstitution) were lethally irradiated (850 rad gamma) using a <sup>137</sup>Cs source (700 rad/min) and the next day were reconstituted by retro-orbital i.v. injection of 8–15  $\times$  10<sup>6</sup> bone marrow cells that had been depleted of T cells and NK1.1<sup>+</sup> cells using anti-Thy1 Ab AT83 (28) and PK136 plus C (Saxon Europe). Chimeras were kept on antibiotic-containing drinking water (0.2% bactrim; Roche, Basel, Switzerland) for the complete duration of the experiment (6 wk). Bone marrow chimeras were sublethally irradiated (600 rad gamma, <sup>137</sup>Cs, 700 rad/min) 4 wk after reconstitution. Thymi were analyzed by flow cytometry at day 15 postsublethal irradiation.

### Limiting dilution analysis

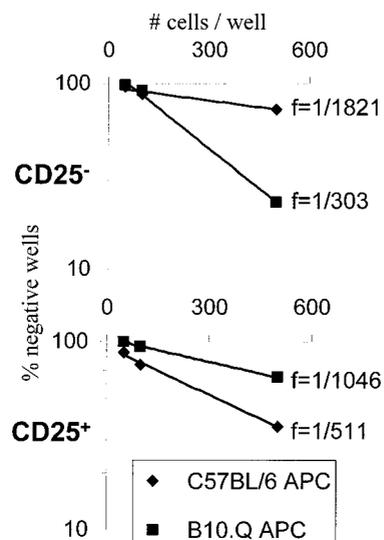
C57BL/6 splenocytes were purified on Lympholyte M gradient (Cedarlane Laboratories). CD8<sup>+</sup> and MHC class II<sup>+</sup> cells were depleted using anti-

CD8 Ab 53.6.7 (29), anti-MHC class II Ab M5/114 (30), and anti-rat IgG-coated magnetic beads (DynaL Biotech, Oslo, Norway). Remaining cells were stained with PE-labeled anti-CD25 mAb PC61 (BD Pharmingen) and FITC-conjugated anti-CD4 mAb GK1.5 (31). CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells were electronically sorted (Coulter Epics Altra) into round-bottom 96-well plates (48 wells/condition) containing 5  $\times$  10<sup>5</sup> T cell-depleted (anti-Thy1 Ab AT83 (28) plus C) irradiated (3000 rad gamma) C57BL/6, B10.Q, or MHC<sup>o</sup> splenocytes in supplemented DMEM glutamax (Life Technologies) containing 100 U/ml IL-2 (supernatant of PMA-stimulated EL4.IL-2 cells (32); American Type Culture Collection, Manassas, VA) and 10% complement-free FCS. Cultures were assessed for proliferation 11 days later by flow cytometry in which a fixed volume of each culture was analyzed for the number of live CD4<sup>+</sup> cells. Cultures were scored as positive if the number of CD4<sup>+</sup> cells was superior to the mean  $\pm$  SD of 24 control cultures containing the same number of T cells and MHC<sup>o</sup> APCs. Precursor frequencies were calculated as previously described (33).

## Results

### The peripheral regulatory T cell population contains a higher frequency of autospecific than alloreactive cells

We analyzed the frequency of regulatory T cells specific for autoantigens and alloantigens presented by APCs. Although regulatory T cells do not proliferate upon in vitro stimulation in absence of cytokines, in presence of high concentrations of IL-2 their “anergic” state can be transiently reversed (23) without loss of suppressive activity (34). Therefore, we performed limiting dilution analysis of normal and regulatory peripheral T cells stimulated with syngeneic, allogeneic, or MHC-deficient APCs in presence of 100 U/ml IL-2 (Fig. 1). The wells in which T cells were stimulated with MHC<sup>o</sup> APCs were used to establish the baseline above which proliferation (as assessed by FACS analysis, as previously described (35)) was considered to be positive (see *Materials and Methods*). Although, as expected among CD4<sup>+</sup>CD25<sup>-</sup> T cells, a significantly higher frequency of alloreactive than autoreactive cells was consistently observed, among CD4<sup>+</sup>CD25<sup>+</sup> cells the frequency of autoreactive cells was reproducibly and significantly higher than that of alloreactive cells (Fig. 1 and Table I). Similar results were obtained for mature CD4<sup>+</sup>CD25<sup>+</sup> vs CD4<sup>+</sup>CD25<sup>-</sup>



**FIGURE 1.** Higher frequency of regulatory T cells specific for self than for non-self MHC/peptide ligands. CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> or CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> splenocytes were electronically sorted into 96-well plates containing C57BL/6 or B10.Q APCs in an IL-2-rich culture medium. Eleven days later, FITC-labeled anti-CD4 mAb was added to each well and accumulation of CD4<sup>+</sup> cells was analyzed by flow cytometry. A typical experiment is shown. f, Calculated precursor frequency.

Table I. Higher frequency of regulatory T cells specific for self-Ags than for non-self MHC/peptide ligands<sup>a</sup>

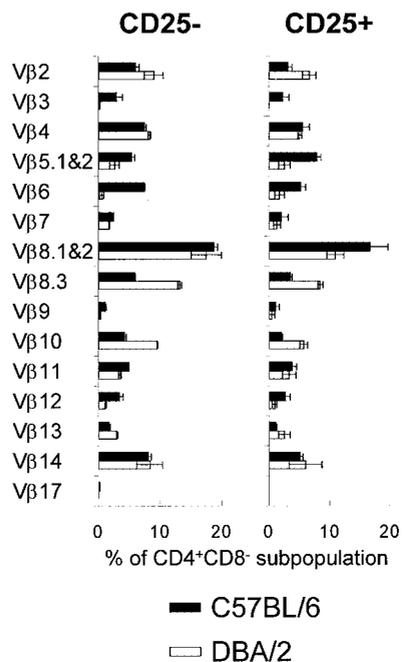
Expt.	APC	CD25 <sup>-</sup>	CD25 <sup>+</sup>
I	B6	1/1821	1/511
	B10.Q	1/303	1/1046
II	B6	ND	1/793
	B10.Q	ND	1/2538
III	B6	1/2857	1/608
	B10.D2	1/319	1/2143

<sup>a</sup> Summary of limiting dilution analysis performed as in Fig. 1.

thymocytes (data not shown). Because the plating efficiencies of CD25<sup>-</sup> and CD25<sup>+</sup> cells are probably not identical (despite the addition of high concentrations of IL-2), no direct comparison between the frequencies of B6-reactive CD25<sup>-</sup> and CD25<sup>+</sup> cells should be made. These data indicate that the repertoire of regulatory T lymphocytes is strongly enriched in autospecific cells, which appears consistent with their critical role in the inhibition of autoimmunity.

#### Superantigens induce deletion of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell precursors

To assess whether the high frequency of autospecific regulatory T cells results from a deficiency of thymic deletion of their precursors, we analyzed susceptibility of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell precursors to endogenous superantigen-mediated deletion. DBA/2 mice express Mtv 1, 6, 7, 8, 11, 13, 14, and 17 and therefore delete Vβ3-, -5-, -6-, -7-, -8.1-, -9-, -11-, and -12-expressing thymocytes (36). Control animals were C57BL/6 mice that do not delete superantigen-specific thymocytes. Pooled thymocytes from three mice were depleted of CD8<sup>+</sup> cells by complement-mediated lysis,

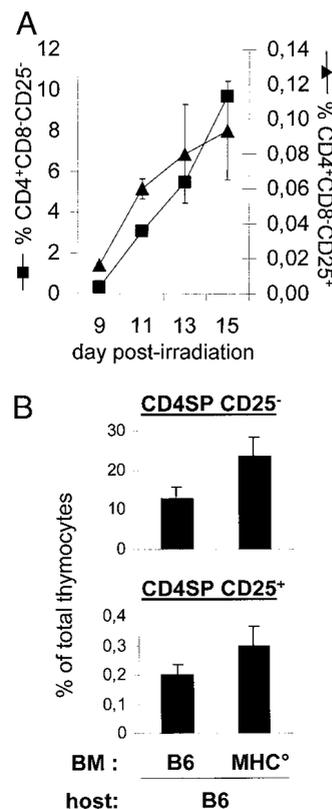


**FIGURE 2.** Normal thymic deletion of endogenous superantigen-specific regulatory T cell precursors. The TCR-Vβ repertoire of CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> C57BL/6 (no superantigen-dependent deletion) and DBA/2 thymocytes was analyzed by flow cytometry using a panel of 15 Vβ-specific Abs. Error bars indicate SD ( $n = 3$ ). The anti-Vβ17 Ab was used as negative control because C57BL/6 and DBA/2 mice do not contain a functional gene for this particular Vβ region.

and the remaining CD8<sup>-</sup> cells were analyzed by flow cytometry using a panel of 15 Abs directed to distinct TCR-Vβ regions. Significantly reduced percentages of Vβ3-, -5-, -6-, -9-, and -12-expressing CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> thymocytes were observed in DBA/2 mice compared with C57BL/6 controls (Fig. 2), whereas deletion of Vβ7 and Vβ11 was much less pronounced. Importantly, superantigen-specific CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> thymocytes were depleted to the same extent as CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> cells. These results indicate that precursors of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells are normally susceptible to superantigen-induced deletion.

#### Thymic APCs induce deletion of self Ag-specific CD4<sup>+</sup>CD25<sup>+</sup> thymocytes

The majority, if not all, of thymic deletion depends on MHC/peptide complexes expressed by thymic APCs, in particular by dendritic cells (1, 37). In bone marrow chimeras expressing MHC/peptide complexes on radioresistant cells but not on cells of hematopoietic origin, a 2- to 3-fold increased generation of mature thymocytes was observed (2). To analyze whether precursors of regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells are susceptible to deletion induced by self peptide/MHC ligands expressed by thymic APCs, we analyzed their generation in bone marrow chimeras.



**FIGURE 3.** Normal thymic deletion of self-MHC/peptide complex-specific regulatory T cell precursors. **A**, Reconstitution of CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> populations after sublethal gamma irradiation. Adult C57BL/6 mice were irradiated at 600 rad gamma, and their thymocytes were analyzed 9–15 days later by flow cytometry. Error bars indicate SD ( $n = 3$ ). **B**, Increased generation of CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>high</sup>CD25<sup>-</sup> ( $p < 0.005$ , Student's  $t$  test) and CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>high</sup>CD25<sup>+</sup> ( $p < 0.02$ ) thymocytes in MHC<sup>0</sup>→C57BL/6 compared with C57BL/6→C57BL/6 bone marrow chimeras. Bone marrow chimeras were sublethally irradiated 4 wk post-bone marrow reconstitution, and their thymi were analyzed by flow cytometry 15 days later. Error bars indicate SD (B6→B6,  $n = 9$ ; MHC<sup>0</sup>→B6,  $n = 5$ ).

Regulatory T cells are known to develop later than normal CD4<sup>+</sup> cells during ontogeny. Therefore, we first compared kinetics of the development of regulatory vs normal mature CD4<sup>+</sup> thymocytes in sublethally irradiated adult C57BL/6 animals. As shown in Fig. 3A, normal mature CD4<sup>+</sup> thymocytes accumulated over a 1-wk period, from days 9 to 15 postirradiation. Surprisingly, CD4<sup>+</sup>CD25<sup>+</sup> thymocytes developed simultaneously and with similar kinetics.

To analyze the susceptibility of regulatory T cell precursors to thymic deletion by APCs of hematopoietic origin, we lethally irradiated wild-type (wt) C57BL/6 hosts and reconstituted them with MHC<sup>o</sup> (or control wt) bone marrow cells (MHC<sup>o</sup>→wt and wt→wt chimeras, respectively). Four weeks after reconstitution, the chimeras were sublethally irradiated and the development of normal and regulatory mature CD4SP thymocytes was analyzed at day 15 postirradiation (Fig. 3B). Approximately 2-fold more normal mature CD4<sup>+</sup> thymocytes had developed in MHC<sup>o</sup>→wt than in wt→wt chimeras, consistent with our previous results (2). Similarly, in absence of thymic deletion by bone marrow-derived APCs in MHC<sup>o</sup>→wt chimeras, more mature CD4<sup>+</sup>CD25<sup>+</sup> thymocytes developed as well. Therefore, at least a measurable fraction of self peptide/MHC complex-specific regulatory T cell precursors are deleted in the thymus upon recognition of self MHC/peptide ligands expressed by thymic APCs.

## Discussion

In this report, we show that the frequency of autospecific regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells is higher than that of cells specific for allogeneic ligands. However, precursors of regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells are susceptible to thymic deletion by professional APCs. Importantly, this result is valid for a large panel of superantigens deleting several different TCR Vβ-expressing thymocytes, as well as for self peptide/MHC complexes. Therefore, in contrast to earlier suggestions, autospecific regulatory T cells preferentially develop despite susceptibility to thymic deletion.

The kinetics of development of regulatory and normal CD4<sup>+</sup> T cells (in sublethally irradiated mice) were similar. This result is in contrast to the situation in neonates, in which CD4<sup>+</sup>CD25<sup>+</sup> T cells develop at least 3 days later than normal CD4<sup>+</sup> cells (17, 24). Therefore, precursors of regulatory T cells may arrive in the thymus later than precursors of normal T cells, but once in the thymus these two populations develop with similar kinetics. Alternatively, thymic stroma may generate specific conditions required for regulatory T cell differentiation starting at day 3 postbirth. In this respect, it would be of interest to combine adult precursor populations with fetal thymic stroma, e.g., in reaggregate cultures (38).

Our results indicating that normal and regulatory T cells develop with similar kinetics in sublethally irradiated animals may also appear to be in contrast to the results of Jordan et al. (24), who analyzed development of regulatory T cells in HA-specific TCR and HA doubly transgenic animals using BrdU incorporation. In adult doubly transgenic mice, BrdU<sup>+</sup> regulatory T cells appeared with ~50% slower kinetics than normal mature CD4<sup>+</sup> thymocytes. The reasons for the discrepancy between those and our results are not clear, but it would suggest that normal and regulatory T cells are derived from different precursor populations in the thymus and that regulatory T cell precursors divide less frequently than normal thymocytes.

We show that regulatory T cell precursors specific for a large panel of endogenous superantigens are normally deleted in the thymus. These data are in contrast to the increased accumulation of Vβ6-expressing CD4<sup>+</sup>CD25<sup>+</sup> thymocytes in mouse mammary tumor virus SW-infected BALB/c (as compared with uninfected) mice reported by Papiernik et al. (23). This discrepancy may be

due to differences in the experimental systems used (endogenous vs exogenous superantigens) and/or in the superantigen expression pattern.

In TCR-transgenic mice, CD25<sup>+</sup> regulatory T cells express an endogenous TCRα chain in addition to the transgenic one (39, 40). Therefore, even if regulatory T cells are equally sensitive to deletion induced by superantigens (which almost exclusively interact with TCRβ chains (36)), they might not be sensitive to MHC-peptide complex-mediated deletion (41, 42). However, our data show that significantly more regulatory T cells develop in absence than in presence of MHC/peptide complexes expressed by APCs of hematopoietic origin. These data confirm that regulatory T cell precursors are susceptible to thymic deletion. Increased (rather than decreased) differentiation of CD4<sup>+</sup>CD25<sup>+</sup> thymocytes has been observed in doubly transgenic mice expressing an influenza HA S1 peptide/I-E<sup>d</sup>-specific TCR as well as its agonist ligand (24), which would suggest their resistance to deletion. However, the low level of HA-transgene expression in the thymus has been reported to be limited to the cortical region (43), which is devoid of cells capable of inducing deletion *in vivo* (3, 4, 6). The transgenic HA expression patterns and/or levels in three other HA-transgenic mouse lines induced deletion rather than development of transgenic S1/I-E<sup>d</sup> TCR-expressing thymocytes (24). Whatever the precise explanation, our data clearly indicate for a large panel of superantigens as well as for the normal "repertoire" of self peptide/MHC complexes that autospecific regulatory T cell precursors are deleted in the thymus. Our data agree with and significantly extend recently published results concerning regulatory T cells developing in transgenic mice expressing MHC class II molecules exclusively on cortical epithelium. These cells appeared to be significantly more autoreactive than CD4<sup>+</sup>CD25<sup>+</sup> cells developing in wt mice, indicating that medullary epithelium and/or APCs of bone marrow origin induce anergy and/or deletion-mediated self-tolerance in this T cell subset (44). It will be interesting to assess the relative contributions of medullary epithelium and APCs to the induction of regulatory T cell tolerance to self MHC/peptide ligands.

Despite normal thymic deletion of endogenous superantigen or self peptide/MHC-specific regulatory T cell precursors, a high frequency of self-specific CD4<sup>+</sup>CD25<sup>+</sup> cells nevertheless leaves the thymus and populates the periphery. In our limiting dilution assay, more autoreactive than alloreactive regulatory T cells were reproducibly found. Therefore, the repertoire of regulatory T lymphocytes appears to be biased toward cells specific for self Ags, which could be explained by the recently reported positive selection of TCR-transgenic regulatory T cells in presence of agonist ligand (24). If confirmed, this could explain how regulatory T cells mainly inhibit autoimmunity, allowing useful immune responses to develop. Importantly, because only a part of self Ags is expressed by splenic APCs, the real frequency of autospecific regulatory T cells is probably even higher. Because thymic deletion by APCs normally applies to regulatory T cell precursors, the observed high frequency of autoreactivity reflects the level of autospecific precursors that either recognize their MHC/peptide ligand at the surface of cells incapable of apoptosis induction (e.g., thymic cortical (4, 6) and probably also medullary epithelium (3)) or do not encounter it at all in the thymus (e.g., tissue-specific Ags). If a normal precursor recognizes its ligand at the surface of medullary epithelium, it will be rendered anergic and this unresponsive state can only inefficiently be reversed by IL-2 (3). In contrast, the naturally anergic state of regulatory T cells can readily be reversed by IL-2 (23, 34). This probably explains how a high frequency of autospecific regulatory T cells can develop, despite normal susceptibility to thymic deletion.

Whatever the precise explanation, our data firmly establish that the repertoire of regulatory T lymphocytes is biased toward auto-specific cells. However, in contrast to earlier proposed explanations for the proposed autospecificity, we showed in this study that regulatory T cell precursors are as susceptible to induction of deletion as are their normal counterparts. Combined with the earlier reported increased generation of autospecific regulatory T cells in presence of agonist ligand (24), our data indicate that thymic generation of these cells depends on recognition of an MHC/peptide ligand exclusively at the surface of thymic elements incapable of induction of deletion, i.e., thymic cortical and/or medullary epithelial cells (4, 6). Finally, our observation that significantly more regulatory T cells recognize self than non-self ligands would explain how these cells mainly inhibit autoimmunity, leaving useful non-self-specific immune responses to freely develop.

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