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Running title: Thymic selection of effector and regulatory CD4$^+$ T lymphocytes

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Abstract

Natural $CD4^+CD25^+$ regulatory T lymphocytes are key protagonists in induction and maintenance of peripheral T cell tolerance. Their thymic origin and biased repertoire continue to raise important questions on the signals that mediate their development. We validated analysis of MHC class II-capture by developing thymocytes from thymic stroma as a tool to study quantitative and qualitative aspects of the cellular interactions involved in thymic T cell development, and used it to analyze regulatory T cell differentiation in wild type mice. Our data indicate that antigen-presenting cells of bone-marrow origin, but surprisingly and importantly not thymic epithelial cells, induce significant negative selection among the very autoreactive regulatory T cell precursors. This fundamental difference between thymic development of regulatory and effector T lymphocytes leads to development of a regulatory T cell repertoire enriched in cells specific for a selected subpopulation of self-antigens, i.e. those specifically expressed by thymic epithelial cells.

Introduction

Thymic deletion and anergy induction play an essential role in tolerizing the developing T lymphocytes repertoire to self-antigens. Some potentially autoreactive T lymphocytes, however, escape to the periphery where they are kept under control by multiple mechanisms, e.g. induction of anergy, deletion, and suppression by regulatory T cells (T_{reg}). The best-characterized T_{reg} population is composed of $CD4^+CD25^+$ T lymphocytes (1, 2). $CD4^+CD25^+$ T_{reg} have been shown not only to prevent autoimmunity (3-5), but also to mediate transplantation tolerance (6-9), to suppress maternal immune aggression against the fetus (10), and to regulate immunity to viral and parasite infections (11, 12). Their critical role in the generation and maintenance of peripheral tolerance renders them ideal candidates for cell therapy in autoimmunity and transplantation and has sparked interest in how these cells develop. Although recent reports suggest that $CD4^+CD25^+$ lymphocytes can also differentiate in the periphery (13-16), probably the majority of $CD4^+CD25^+$ T_{reg} is generated in the thymus from $CD4^-CD8^-$ precursors (17). In the thymus, $CD4^+CD25^+$ T_{reg} appear to be positively selected by cortical epithelial cells (as are conventional effector $CD4^+$ lymphocytes) (18), and have been reported to be susceptible to deletion (18-20). The TCR-ligand interaction involved in $CD4^+CD25^+$ T_{reg} differentiation has been postulated to be of relative high affinity, to allow peripheral re-activation by self-antigen. Consistent with this hypothesis, increased percentages of $CD4^+CD25^+$ T_{reg} have been found in different TCR transgenic mice expressing agonist ligand (21-24). However, it has recently been shown that in mice doubly transgenic for another TCR/ligand pair, agonist ligand does not promote $CD4^+CD25^+$ T_{reg} differentiation. Rather, the authors concluded that T_{reg} are more resistant to deletion than are conventional $CD4^+$ thymocytes (25). Combined, these data indicate that the

nature of the ligand involved in positive selection of T_{reg} remains unknown and that negative selection of Treg precursors remains incompletely understood.

Several years ago, it was shown that mouse T cells do not express I-A determinants (26, 27) but that developing murine thymocytes acquire MHC class II molecules from the thymic microenvironment (28). Transfer of APC-determinants to T cells is a process that has recently been investigated in detail (reviewed in ref. (29) and has been called trogocytosis (30). This process may be involved in T-cell affinity maturation (31) and immunoregulation (32, 33). T cells are known to capture membrane fragments from APC (34), thus acquiring a variety of surface molecules (35). Trogocytosis requires activation of the T cell, but not only the MHC/peptide complexes directly interacting with the TCR are transferred. Thus, in F1 animals, developing T cells acquire MHC class II molecules of both haplotypes (28). However, in chimeric thymi thymocytes acquire mainly one type of MHC class II (36). Combined, these data show that MHC class II molecules are acquired only from the stromal cells that are involved in the activation of the thymocyte. It has been shown that the level of MHC and membrane capture is proportional to the dose of peptide presented by the APC and that it reflects T cell reactivity against APC (34, 35, 37). Therefore, we reasoned that analysis of MHC class II display by different thymocyte subpopulations should shed light on the nature of the cellular interactions involved in the selection of $CD25^-$ and $CD25^+$ CD4SP thymocytes. We here report how this approach allowed us to follow the evolution of the T_{reg}-repertoire during the distinct stages of its development, and to evaluate the contribution of the different thymic cellular compartments in this process.

Materials and Methods

Mice

C57Bl/6, DBA/2, BALB/c and DO11.10 TCR transgenic BALB/c mice (38) mice were purchased from the Centre de Recherche et d'Elevage Janvier (Le Genest St. Isle, France). C57Bl/6 mice deficient in β2-microglobulin (39), IA_{β}^{b} (40) and TCRα (41) were obtained from the 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 (Regional approval N° 31-13).

Antibodies

The following antibodies (abs) and secondary reagents were used for phenotypic analysis: FITC, PE and APC-labeled anti-CD4 (GK1.5), FITC and APC-labeled anti-CD8 (53.6.7), PE-labeled anti-CD25 (PC61), FITC-labeled anti-HSA (M1/69), FITC-labeled anti-TCR (H57), FITC-labeled anti-CD69 (H1.2F3), FITC-labeled anti-CD11c (N418), FITC-labeled anti B220 (RA3-6B2), PE-Cy5.5-labeled Streptavidin (eBioscience, San Diego, CA); FITC-labeled anti-CD44 (IM7), biotin-labeled anti-I-A_{β}^{b} (AF6-120.1), biotin-labeled anti-Ly-51 (6C3/BP-1), MTS10, FITC-labeled anti-Vβ3 (KJ25), anti-Vβ4 (KT4), anti-Vβ6 (RR4-7), anti-Vβ8 (MR5-2), anti-Vβ12 (MR11-1), anti-Vβ14 (14-2) TCR and biotin-labeled isotype-matched controls (BD PharMingen, Heidelberg, Germany), biotin-labeled anti-I-A/I-E (M5/114).

Flow cytometry

Thymi and lymph nodes were homogenized, washed once in medium, and resuspended in 2.4G2 (anti-FcγR mAb, (42)) hybridoma supernatant. After an incubation of 30 min on ice, saturating concentrations of abs were added. 20 minutes later, cells were washed three times in

PBS, 2.5% FCS and 0.02% NaN3 and incubated with the appropriate secondary reagent. Labeled cells were analyzed using a FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). Doublets and dead cells were excluded using appropriate FSC/SSC gates.

In vitro MHC class II decay studies

Thymocytes depleted of $CD8^+$ cells using anti-CD8 mAb (31.M, (43)) and complement (Saxon Europe, Suffolk, U.K.) were cultured in RPMI supplemented with 10% fetal calf serum, 1mM non essential amino acids, 1mM sodium pyruvate, 1mM HEPES, and antibiotics. At different time points cells were stained with anti-CD4, anti-CD8, anti-CD25 and anti-IA^b mAbs.

Bone marrow chimeras

Irradiation bone marrow chimeras were generated by lethally irradiating (8.5 Gy gamma) C57Bl/6 hosts using a ^{137}Cs source (7 Gy/min). The next day, irradiated mice were reconstituted by i.v. injection of $10x10^6$ bone marrow cells depleted of T cells using anti-Thy 1 mAb (AT83, (44)) and complement (Saxon Europe, Suffolk, U.K.). Chimeras were kept on antibiotic containing water (0.2% Bactrim, Roche, Basel, Switzerland) for the complete duration of the experiment (4 weeks).

Statistical analysis

The relative MHC class II expression was calculated as follows: MFI of I-A ÷ MFI of isotype matched control of all the thymic and lymph node subpopulations analyzed. Statistically significant differences between subpopulations were assessed using Student's *t* test and are indicated as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$, **** $p<0.0001$.

Results

Thymocytes capture MHC class II molecules from the thymic microenvironment.

It has previously been reported that thymocytes do not express endogenous MHC class II molecules but that they can acquire it from the thymic environment (26-28). We verified these findings in mixed bone marrow chimeras generated by lethally irradiating wild type (wt) C57BL/6 animals and reconstituting them with a mixture of MHC class II deficient (Thy1.2) and wt (Thy1.1) bone-marrow (MHC II°+wt→wt). We used mixed bone marrow chimeras to allow developing thymocytes to acquire MHC class II from the radioresistant and radiosensitive cellular elements normally involved in thymic selection. In MHC II°+wt→wt chimeras, CD4SP CD25^{high}, CD4SP CD25^{-} and CD8SP thymocytes generated from MHC II° *vs*. wt precursors harbored comparable amounts of I-A, confirming that these molecules are acquired from the thymic microenvironment and not expressed endogenously (Fig. 1).

MHC class II levels on thymocytes vary according to developmental stage and lineage.

We next analyzed if the level of MHC class II found on the surface of distinct thymocyte subsets varied during development. As shown in figures 2A and 2B, we found that the percentage of thymocytes displaying acquired MHC class II molecules increased during differentiation of DP to SP cells. We reproducibly observed a bimodal distribution of MHC class II display, potentially reflecting interaction with limited numbers of selecting niches (45). Interestingly, the amount of I-A displayed by all thymocytes increases with development, as shown by the shift of the total I-A curve. Therefore, to compare the amount of I-A captured by the distinct thymic subpopulations we calculated the relative MHC class II display on the entire population (=MFI I-A ÷ MFI isotype-matched control). As shown in figures 2A and B, some immature CD4^{+}CD8^{+}

("DP") thymocytes harbor detectable levels of I-A. $CD4^+CD8^{int(ermediate)}$ thymocytes, which only develop in animals expressing at least one class of MHC and therefore have undergone TCR-mediated selection events (46, 47), and mature $CD4^+CD8^-$ ("CD4SP") thymocytes carry significantly more I-A than their DP precursors. Finally, mature $CD4^-CD8^+$ ("CD8SP") thymocytes display significantly less I-A than CD4SP cells. Therefore, the level of I-A found on thymocytes depends on their developmental stage and lineage.

It has recently been shown that during their interaction with APC, T cells capture membrane fragments, thus conveying a subset of membrane proteins from the presenting cells (34, 35). We therefore tested if, in addition to I-A, other stroma- or APC-specific proteins could be found on the surface of thymocytes. As shown in figure 2A, we only detected very limited amounts of a cortical epithelium specific antigen (6C3/BP-1) on the different populations of thymocytes. Similar results were obtained using antibodies specific for a medullar antigen (MTS10) or for dendritic cells (CD11c) (data not shown).

Upon TCR-engagement with MHC class I or II, DP thymocytes rapidly upregulate CD69 expression (48). A higher percentage of $CD69^+$ DP thymocytes carry I-A than $CD69^-$ cells, suggesting that MHC class II acquisition is an activation-mediated process (Fig. 3A). The absolute level of I-A displayed by $I\text{-}A^+$ cells does not change between these two populations (Fig. 1A). To test if MHC class II acquisition is TCR-dependent, we analyzed I-A levels on TCRα-deficient DP thymocytes. As shown in figure 3B, few of these cells harbored limited levels of I-A. These data indicate that MHC class II acquisition by developing T cells heavily depends on TCR-mediated activation. To evaluate if higher avidity interactions result in more MHC class II acquisition during thymocyte development (as previously shown *in vitro*, (37)), we compared MHC class II display by thymocytes from C57Bl/6 and DBA/2 mice. DBA/2 mice express

endogenous superantigens encoded by mammary tumor viruses 1, 6, 7, 8, 11, and 13. These superantigens are high affinity ligands for Vβ3, Vβ6 and Vβ12, while they do not interact with Vβ4, Vβ8 and Vβ14. As a consequence, $V\beta3^+$, $V\beta6^+$, and $V\beta12^+$ thymocytes are deleted during thymic development. C57BL/6 mice do not present these superantigens (49). Since superantigen mediated deletion in DBA/2 mice is already visible at the $CD4^+CD8^{int}$ stage, we analyzed I-A display by the $CD4^{low}CD8^{low}$ ("double dull") population, which is enriched in cells that have undergone TCR-ligand interactions (50). Interestingly, superantigen-reactive $V\beta3^+$, $V\beta6^+$, and $V\beta12^+$ double dull thymocytes display higher levels of MHC class II in DBA/2 than in C57BL/6 mice, while $V\beta4^+$, $V\beta8^+$ and $V\beta14^+$ cells carry comparable levels of I-A (Fig. 3C). MHC class II amounts were analyzed in both strains with the mAb M5/114 previously shown to have similar affinity for the $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes (51). These data indicate that the level of I-A acquired during thymocyte development reflects the avidity of the TCR-ligand interaction involved.

We next investigated if thymic negative selection (by induction of anergy or apoptosis) is reflected by a gradual decrease in the acquisition of MHC class II molecules during progression through T cell development. We have not observed any reduction in MHC class II display in CD4SP vs. $CD4^+CD8^{int}$ thymocytes. However, CD4SP cells express approximately 1,5-fold higher levels of TCR than their $CD4^+CD8^{int}$ precursors (Fig. 4B and C), rendering interpretation of this observation difficult. In contrast, all CD4SP thymocytes express the same high level of TCR. These cells complete their intrathymic differentiation by downmodulating HSA expression (52). It has been shown that HSA^{high}, but not HSA^{low} thymocytes are sensitive to deletion (53). We therefore analyzed I-A levels on CD4SP HSA^{high} and HSA^{low} thymocytes. As shown in figure 3D, HSA^{high} CD4SP thymocytes display higher I-A levels than HSA^{low} cells. These results demonstrate *in vivo* late negative selection of thymocytes expressing high avidity TCR. To

corroborate these findings, we analyzed MHC class II display on HSA^{high} and HSA^{low} CD4SP thymocyes in TCR transgenic mice in which negative selection does not take place. DO11.10 TCR-transgenic CD4SP HSA^{high} and CD4SP HSA^{low} thymocytes display comparable amounts of MHC class II molecules (Fig. 3E), supporting our conclusions.

$CD25^{high}$ thymocyte populations display significantly higher levels of MHC class II than $CD25^{-}$ cells.

Peripheral CD4SP $CD25^{+}$ T cells with regulatory function express high levels of CD25 at their surface and all $CD4^{+}CD25^{high}$ T cells express Foxp3 (54, 55). FACS analysis using anti-Foxp3 antibody showed that also in the thymus all $CD4^{+}CD25^{high}$ cells express Foxp3 (PR, data not shown). The majority of T cells expressing intermediate levels of CD25 do not have immunomodulatory capacity, do not express Foxp3, and (at least in the periphery) do not appear to have the potential to become Foxp3 expressing $CD25^{high}$ cells (54, 55). We therefore limited our analysis to $CD25^{high}$ thymocytes. This population is readily detectable in the thymus (Fig. 4A). Distinguishable regulatory $TCR^{+}CD25^{high}$ T cell-precursors start to appear in the $CD4^{+}CD8^{int}$ population (24, 55). We therefore analyzed MHC class II levels on $CD25^{high}$ regulatory thymocyte-precursors at the intermediate and SP stage and compared them to those on $CD25^{-}$ cells with equivalent CD4/CD8 phenotypes. Interestingly, $CD4^{+}CD8^{int}CD25^{high}$ cells display significantly higher levels of MHC class II than $CD4^{+}CD8^{int}CD25^{-}$ thymocytes (Figs. 4A and B). At the SP stage this level decreases, but still remains significantly higher than on $CD25^{-}$ cells (Figs. 4A and B).

$CD4^{+}CD8^{int}$ and CD4SP, $CD25^{-}$ and $CD25^{high}$ cells do not express identical TCR-levels (Fig. 4C and Fig. 4B, lower panel). We observed that $CD25^{-}$ $CD4^{+}CD8^{int}$ cells expressed lower TCR-

levels than CD25^{high} cells. CD25^{-} CD4SP cells expressed approximately 1,5-fold higher TCR-levels than CD25^{high} cells. CD25^{high} CD4^{+}CD8^{int} and CD4SP cells expressed similar TCR-levels. These differences in TCR-levels might contribute somewhat to the differences in MHC class II capture by CD4^{+}CD8^{int} CD25^{-} and CD25^{high} thymocytes we observed. In contrast, they can only emphasize the differences in MHC class II-capture by CD4SP CD25^{-} and CD25^{high} cells.

Higher levels of I-A on CD4^{+}CD25^{high} cells could result from a longer half life of these molecules on the cell surface of CD25^{high} or from increased capture. To distinguish between these two possibilities, we analyzed the kinetics of MHC class II decay on CD25^{high} cells cultured in the absence of APC. As shown in figure 4D, I-A on CD4^{+}CD25^{high} and CD4^{+}CD25^{-} thymocytes decays with similar kinetics. Taken together, these results indicate that TCR^{+}CD25^{high} thymocytes capture significantly more MHC class II molecules than CD25^{-} cells. Furthermore, these data show that the presence of captured MHC class II at the surface of thymocytes is transient and that the *in vivo* I-A display we observed requires continuous interaction with stromal thymic cells (56). Moreover, the level of MHC class II display reflects avidity of recent interactions with thymic stromal cells.

MHC class II harbored by thymocytes is acquired from thymic epithelium as well as from cells of hematopoietic origin.

To evaluate if the variation in MHC class II levels found between CD25^{-} and CD25^{high} cells is due to differences in acquisition from (radioresistant) thymic epithelium and/or from (radiosensitive) antigen-presenting cells of hematopoietic origin (APC), we next generated bone-marrow chimeras by lethally irradiating wild type C57BL/6 animals and reconstituting them with MHC class II deficient bone-marrow (MHC II°→wt). DP thymocytes from MHC II°→wt

animals harbored I-A levels comparable to those on DP thymocytes isolated from control wt→wt chimeras, indicating that at this developmental stage thymocytes mainly interact with radioresistant thymic (cortical) epithelium (Figs. 5A and B). I-A levels on the more mature $CD4^+CD8^{int}$ and CD4SP thymic populations were significantly lower in MHC II°→wt than in wt→wt chimeras (Figs. 5A and B). These results indicate that a significant portion of the MHC class II found on more mature thymocytes is acquired from APC. Therefore, levels of MHC class II displayed by thymocyte-populations developing in the different chimeras cannot be compared.

Because of negative selection, fully mature HSA^{low} CD4SP thymocytes harbor lower levels of MHC class II than their HSA^{high} precursors (that express identical levels of TCR, Figs. 3D and E). To evaluate the contribution of epithelial cells in this negative selection, we analyzed I-A levels on HSA^{high} and HSA^{low} CD4SP thymocytes from MHC II°→wt chimeras. As shown in figure 5C, despite absence of negative selection by APC in these chimeras, HSA^{low} cells displayed significantly less I-A than their HSA^{high} precursors. This result is consistent with the known role of medullary epithelial cells in anergy induction and deletion of autoreactive cells (57-59).

$CD25^{high}$ regulatory T cell-precursors are negatively selected by APC but not by thymic epithelial cells

We then analyzed the contribution of epithelial cells and APC to negative selection of $CD25^{high}$ thymocytes. Since all $CD4^+CD25^{high}$ thymocytes display a mature phenotype, expressing low levels of HSA (ref. 17, and data not shown), the two last identifiable stages of T_{reg} development are the $CD4^+CD8^{int}$ and $CD4^+CD8^-$ stages. Importantly, $CD25^{high}$ $CD4^+CD8^{int}$ and $CD4^+CD8^-$ cells express identical TCR-levels (Fig. 4C). We therefore analyzed MHC class II display by these two $CD25^{high}$ thymocyte subsets (Fig. 5D). In MHC II°→wt chimeras, $CD25^{high}$ CD4SP thymocytes

and their $CD4^+CD8^{int}$ precursors harbored similar levels of MHC class II. This result indicates that thymic epithelial cells do not induce significant negative selection of regulatory T cell precursors. In contrast, in wt→wt chimeras, in which APC express MHC class II, $CD25^{high}$ CD4SP thymocytes display lower levels of MHC class II than $CD4^+CD8^{int}$ (Fig. 5D). Combined, these data show that regulatory T cell precursors are sensitive to negative selection induced by APC.

To corroborate this conclusion we compared MHC class II acquisition by fully mature $CD25^-$ and $CD25^{high}$ thymocytes from thymic epithelium in MHC II° → wt chimeras. Fully mature CD4SP $CD25^{high}$ thymocytes (that are all HSA^{low}) acquired significantly more MHC class II from thymic epithelium than fully mature CD4SP HSA^{low} (mainly $CD25^-$) cells (compare hatched bars in Figs. 5C and D, $p<0.01$).

Peripheral $CD4^+CD25^{high}$ regulatory T lymphocytes display more I-A than $CD4^+CD25^-$ cells.

Peripheral T lymphocytes from TCR transgenic mice have previously been shown to display MHC class II molecules (32). To determine if this was also detectable in wildtype mice, we analyzed I-A levels on $CD25^{high}$ and $CD25^-$ $CD4^+$ T lymphocytes isolated from lymph nodes. $CD4^+CD25^{high}$ lymphocytes harbor significantly higher levels of MHC class II than $CD4^+CD25^-$ cells (Fig. 6A).

The lower I-A level observed on peripheral T lymphocytes as compared to thymocytes (compare Figs. 6A and 4A/B) could be due either to TCR tuning (60) or to less frequent encounters with antigen (61). To distinguish between these two possibilities, we analyzed the MHC class II levels displayed by activated and resting $CD4^+$ lymph node cells. As shown in figure 6B, activated $CD69^{high}$ T lymphocytes displayed higher MHC class II levels than the

corresponding resting populations ($CD69^{low}$), indicating that the relatively low I-A level on peripheral T cells is mainly due to rare antigen-encounter. Finally, both resting ($CD69^{low}$) and recently activated ($CD69^{high}$) $CD25^{high}$ cells carry significantly higher amounts of I-A than corresponding $CD25^{-}$ lymphocytes (Fig. 6B, right). Furthermore, they support the view that the T_{reg} repertoire, enriched during thymic selection in cells bearing TCR with high avidity for self-ligand, is maintained in the periphery.

Discussion

In this report we show that the level of MHC class II display by developing thymocytes correlates with the avidity of their recent interactions with thymic stromal cells, and can therefore be used to monitor quantitative and qualitative aspects of cellular interactions involved in T cell development. Using this molecular signature of thymic selection events, we show that precursors of $CD25^{-}$ effector and $CD25^{high}$ regulatory T lymphocytes are both efficiently negatively selected by APC of bone-marrow origin. In contrast, while thymic epithelial cells efficiently negatively select effector T cell precursors, they only marginally (if at all) induce negative selection of regulatory T cell precursors.

In vitro capture of MHC II molecules by T cell clones is an activation-mediated process (34, 35, 37). In the thymus, a larger fraction of newly positively selected DP $CD69^{+}$ than $CD69^{-}$ cells carry MHC class II. Moreover, only few DP thymocytes from TCRα-deficient mice display I-A on their surface and in significantly less quantity than wt cells. Taken together, these data indicate that also MHC class II acquisition by developing thymocytes is predominantly a TCR-mediated activation-dependent process. Since acquired MHC class II was rapidly lost in absence of MHC class II expressing APC, the level of MHC class II displayed by thymocytes reflects the level of active acquisition of these molecules. Moreover, superantigen-reactive (but not yet deleted) thymocytes acquired higher levels of MHC class II, indicating that the avidity of the developing thymocytes for thymic stromal cells determines the level of MHC class II acquisition.

MHC class II acquisition depends on the developmental stage and lineage of thymocytes, with CD4SP T cells harboring significantly higher amounts of MHC class II than DP and CD8SP cells. On the latter cells, I-A has probably been “passively” acquired *via* trogocytosis during cellular contacts mediated by TCR/MHC class I interaction. Alternatively, but less likely, it may

be the result of a direct TCR/MHC class II interaction-mediated process, consistently with the observation that class II-restricted CD8 T cells arise in CD4° mice (62-64).

Bone marrow chimeras expressing MHC II/peptide complexes on radioresistant (epithelial) cells but not on radiosensitive (bone-marrow-derived) cells revealed that developing thymocytes can capture MHC class II molecules from both thymic epithelium and bone-marrow derived APC. $CD4^+CD8^+$ DP thymocytes appear to capture MHC class II exclusively from the thymic epithelium, while the more mature $CD4^+CD8^{int}$ and $CD4^+CD8^-$ thymocyte-populations acquire I-A from epithelial cells and from APC. These results are in agreement with spatial and temporal aspects of thymocyte selection. Based on TCR affinity for self-peptide/MHC complexes, DP thymocytes localized in the cortex can undergo positive selection. Positively selected cells then migrate to the medulla where, *via* interactions with medullary epithelium and APC of bone-marrow origin, potentially autoreactive cells undergo negative selection.

Previous reports showing that thymocytes derived from F1 into parent (F1→P) bone marrow chimeras mainly carry MHC determinants of host origin (28) are not at odds with our present findings. Thymocytes are positively selected only if their TCR are of sufficiently high affinity for self-ligands expressed by radioresistant cortical epithelial cells (65, 66). Consequently, thymocytes that have been positively selected express TCR specific for host-type MHC, and therefore mainly interact with host type APC and capture host-type MHC determinants in bone marrow chimeras. In line with this, it has also been shown that in MHC haplotype-mosaic stromal environments individual thymocytes preferentially acquire only one of the two MHC determinants (36). Importantly, MHC class II acquisition is not limited to the MHC/peptide complexes with which the thymocyte's TCR interact. On the other hand, thymocytes will only acquire MHC class II from stromal cells for which they have sufficiently high avidity.

Mature thymic regulatory CD4SP CD25^{high} cells display higher amounts of MHC class II molecules than conventional CD4SP CD25^{-} thymocytes. Since the stability of acquired MHC class II on these two subsets is comparable, the higher expression is due to increased acquisition from thymic stromal cells. As discussed before, the level of MHC class II acquisition reflects the avidity of the developing thymocytes for thymic stroma. Our results therefore indicate that the normally diverse CD4SP CD25^{high} regulatory thymocyte population selected on naturally expressed ligands, has higher avidity for self than CD4SP CD25^{-} cells. The greater avidity of Treg may be caused by higher avidity (despite lower TCR-expression levels) and/or affinity TCR, by differences in expression of adhesion molecules, and/or by higher intrinsic reactivity.

Also immature CD4^{+}CD8^{int}CD25^{high} thymocytes capture much higher amounts of MHC class II than the corresponding CD25^{-} population. This result is consistent with a potential role for high-affinity ligands in the selection of regulatory T cells, as previously proposed (21-25). Interestingly, mature CD4SP CD25^{high} thymocytes acquire significantly less MHC class II molecules than their more immature precursors. This reduction in self-reactivity was mainly due to interactions with bone-marrow derived cells and to a much lower extend (if at all) to interactions with epithelial cells. These observations indicate that a significant part of the regulatory T cell precursors is negatively selected after interaction with APC. In contrast, direct interactions with thymic epithelial cells hardly reduced the self-reactivity (by deletion or anergy-induction) of regulatory T cell precursors. These data are consistent with earlier reports on induction of CD4^{+} T cell-mediated dominant tolerance by grafting thymic epithelium (67). Therefore, our data provide an explanation for apparently contradictory reports in the literature showing sensitivity as well as resistance to deletion of thymic regulatory T cell precursors (19, 20, 25). They also show for the first time in one single system and for an unmanipulated TCR-

repertoire that regulatory T cell precursors are sensitive to negative selection induced by APC but hardly (if at all) by thymic epithelial cells. Although thymic APC are known to present some medullary epithelium-derived antigens (59), and may therefore delete some tissue-specific antigen-reactive $CD25^{high}$ precursors, this is unlikely to be the case for all antigens expressed by mTEC (25, 68). The observation that Treg precursors are sensitive to deletion by APC but not by (m)TEC therefore indicates that the mature Treg repertoire is pruned of cells specific for e.g. household antigens and thus relatively enriched in cells recognizing the tissue-specific antigens expressed by mTEC.

Several reports have documented the important role of MHC molecules in homeostasis of the peripheral pool of T lymphocytes (reviewed by (69). The MHC class II display by peripheral $CD4^+$ T cells we observed is consistent with such a role. We also document that resting and activated peripheral regulatory $CD4^+CD25^{high}$ T cells display higher levels of MHC class II than the corresponding $CD25^-$ populations. These results support our previous findings indicating that the T_{reg} repertoire in enriched in autoreactive T cells (19), and more recent data documenting the role of self-antigen in the peripheral expansion of regulatory T cells (70-72).

In conclusion, using MHC class II-transfer as a tool to analyze the nature of cellular interactions involved in T lymphocyte-selection, we have shown that an autoreactive regulatory T cell repertoire is selected already early during thymic development. Negative selection subsequently significantly prunes this repertoire of cells overly reactive to antigens specifically presented by APC but not by epithelial cells, thus generating a fully mature regulatory T cell population with biased specificity for a subpopulation of autoantigens, among which “tissue-specific” antigens expressed by thymic medullary epithelium (73).

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Abbreviations used in this paper: T_{reg}, regulatory T lymphocyte; MFI, mean fluorescence intensity; DP, double positive; SP, single positive; mTEC, medullary thymic epithelial cell.

Legends to the figures

Figure 1. Thymocytes acquire MHC class II molecules from the thymic environment.

Lethally irradiated C57Bl/6 hosts were reconstituted with a mixture of bone marrow cells from C57Bl/6 (Thy1.1$^+$) and C57BL/6 MHC II° (Thy1.2$^+$) mice at a 1:1 ratio. Four weeks later, thymocytes were analyzed by four-color flow-cytometry using anti-CD4, anti-CD8, anti-CD25 and anti-IA^b. To analyze I-A surface levels on Thy1.1$^+$CD4SP cells, FITC-labeled anti-CD11c, anti-B220 mAbs and FITC-labeled anti-Thy1.2 mAbs were used in addition to FITC-labeled anti-CD8 in the exclusion gate. To analyze I-A surface levels on Thy1.2$^+$CD4SP cells, FITC-labeled anti-Thy1.1 mAb was used in the antibody mixture. A reciprocal staining was performed to analyze I-A surface levels on CD8SP thymocytes. Solid lines indicate I-A staining, dotted lines isotype-matched controls. Bar graphs represent relative I-A expression level on different thymocyte subsets. Error bars indicate SD, n=4 from 2 independent experiments.

Figure 2. Displayed MHC class II levels depend on maturation-stage and CD4/CD8 lineage

A: Total thymocytes from C57Bl/6 mice were analyzed by four-color flow cytometry using anti-CD4, anti-CD8, anti-CD25 and anti-I-A^b (bold line) or isotype-matched control (thin line) abs. To analyze I-A surface levels, FITC-labeled anti-CD11c and anti-B220 mAbs were used in the exclusion gate as described in figure 1. Distinct thymocyte subsets were electronically gated based on CD4/CD8 expression and the respective I-A and 6C3/BP-1 levels were plotted as histograms. **B:** Bar graphs representing % of I-A positive cells in distinct thymocyte subpopulations (upper panel) and relative I-A expression level on different thymocyte subsets from C57Bl/6 mice (lower panel). All graphic representations of I-A levels were compiled from four independent experiments. Error bars indicate SD, n=10 mice.

Figure 3. MHC class II acquisition is an avidity-dependent, TCR- activation mediated process

FACS-histograms and bar graphs represent relative I-A expression level on different thymocyte subsets from the following mice **A:** C57Bl/6 mice: DP thymocytes were electronically gated according to their CD69 expression level. **B:** wt and TCRα° C57BL/6 mice: DP thymocytes, in the histogram the two dotted lines depict the isotype matched controls. **C:** C57Bl/6 and DBA/2 mice: $CD4^{low}CD8^{low}$ thymocytes were electronically gated. The bar graph (upper panel) represents relative MHC class II display on double dull thymocytes expressing different TCR Vβ, in bold Vβ reactive to Mmtv-encoded superantigens presented in DBA/2 mice, in normal characters the ones that are not. Lower panels: examples of FACS-histograms of I-A display by $Vβ12^+$ and $Vβ8^+$ thymocytes. **D:** C57Bl/6 mice: Thymocytes were electronically gated as indicated and I-A display analyzed by FACS. Lower panel: FACS histograms of CD4SP thymocytes gated on HSA expression as indicated. Upper panel: Relative I-A display by the distinct thymocyte subpopulations. **E:** DO11.10 TCR transgenic mice (analyzed as in D). In panels A, B, and D the anti-MHC class II mAb used was AF6-120.1, M5/114 in panels C and E. In all graphic representations error bars indicate SD, n=4 mice.

Figure 4. $CD4^+CD25^{high}$ Regulatory T cell precursors display higher levels of I-A on their surface than $CD4^+CD25^-$ cells.

A: Total thymocytes from C57Bl/6 mice were analyzed by four-color flow cytometry using anti-CD4, anti-CD8, anti-CD25 and anti-$I\text{-}A^b$ mAbs, and distinct thymocyte subsets were electronically gated as described in the legend to figure 2. **B:** Bar graphs representing relative I-A and TCRβ levels on $CD4^+CD8^{int}$ and $CD4^+CD8^-$ thymocytes, electronically gated based on CD25

expression. Error bars indicate SD, n=8. **C:** $CD4^+CD8^{int}$ and $CD4^+CD8^-$ thymocytes were electronically gated based on CD25 expression and the respective TCR levels were displayed as histograms. Thick lines indicate TCR expression by $CD4^+CD8^{int}$ cells, thin lines that of $CD4^+CD8^-$ cells. **D:** CD8-depleted thymocytes were cultured *in vitro* and, at different time points, I-A levels on $CD25^-$ and $CD25^{high}$ CD4SP thymocytes were analyzed. Error bars indicate SD, n=3 mice. Similar results were obtained in two independent experiments.

Figure 5. Regulatory T cell precursors are negatively selected by APC but not by thymic epithelial cells

A: Lethally irradiated C57Bl/6 hosts were reconstituted with bone marrow cells from either MHC II° or wt C57Bl/6 mice. I-A levels on distinct thymocyte subsets were analyzed by flow-cytometry as described in the legend to figure 2. **B/C/D:** I-A levels on distinct thymocyte subsets from MHC II°→wt and wt→wt chimeras are displayed in bar graphs. Error bars indicate SD, n=5. Results from one same experiment are shown; two more independent experiments gave similar results.

Figure 6. Resting and activated peripheral regulatory $CD4^+CD25^{high}$ lymphocytes display higher I-A levels than $CD4^+CD25^-$ cells.

A: I-A surface levels were analyzed on $CD25^-$ and $CD25^{high}$ $CD4^+CD8^-CD11c^-B220^-$ lymphocytes isolated from lymph nodes of C57Bl/6 mice. The data are displayed as FACS-histograms (Left panel) and data from multiple experiments shown as bar graphs (middle panel). Right panel: MHC class II expression by lymph node $B220^+$ cells. error bars represent SD, n=5). **B:** I-A levels on resting ($CD69^{low}$) and recently activated ($CD69^{hi}$) $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T

lymphocyte subsets from lymph nodes of C57Bl/6 mice. Right panel: Relative I-A expression levels are depicted for 5 individual mice, indicated with distinctive symbols.

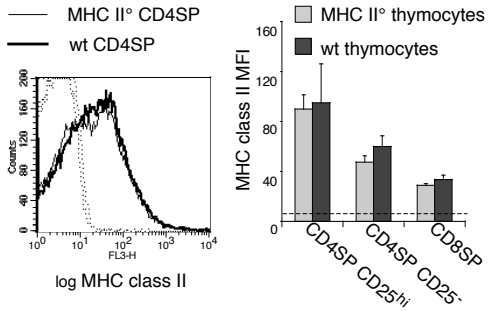

Figure 1

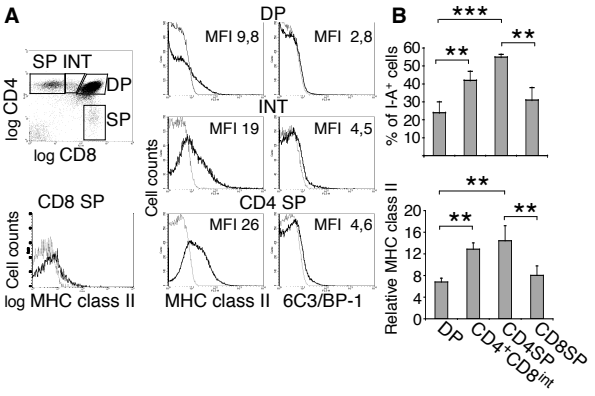

Figure 2

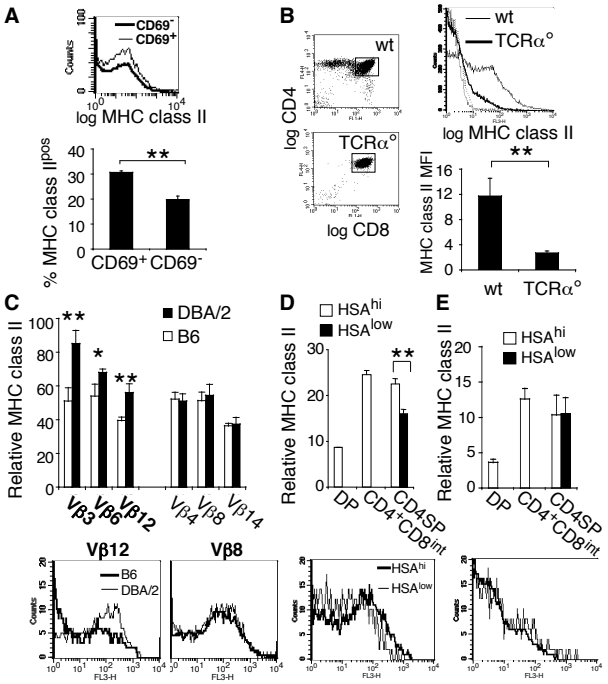

Figure 3

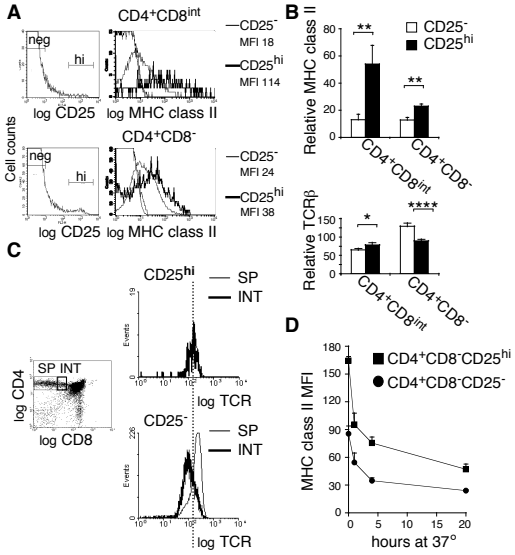

Figure 4

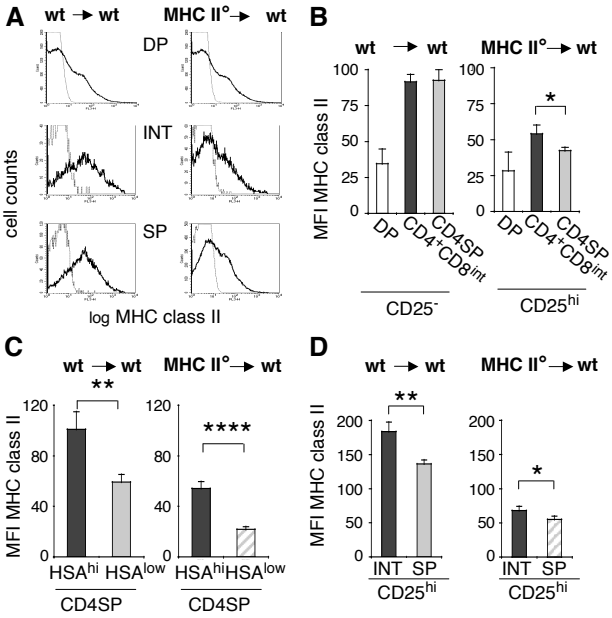

Figure 5

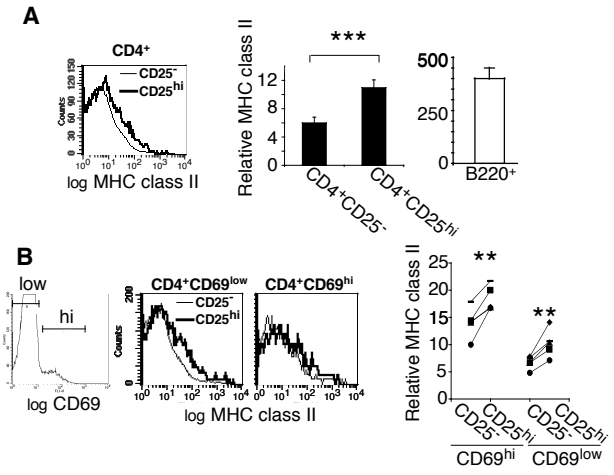

Figure 6