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Abstract
Summary

Among the several mechanisms known to be involved in the establishment and maintenance of immunological tolerance, the activity of $CD4^+CD25^+$ regulatory T lymphocytes has recently incited most interest because of its critical role in inhibition of autoimmunity and anti-tumor immunity. Surprisingly, very little is known about potential genetic modulation of intrathymic regulatory T lymphocyte-development. We show that distinct proportions of $CD4^+CD25^+FoxP3^+$ regulatory T cells are found in thymi of common laboratory mouse strains. We demonstrate that distinct levels of phenotypically identical regulatory T cells develop with similar kinetics in the studied mice. Our experimental data on congenic mouse-strains indicate that differences are not caused by the distinct MHC haplotypes of the inbred mouse strains. Moreover, the responsible loci act in a thymocyte intrinsic manner, confirming the latter conclusion. We have not found any correlation between thymic and peripheral levels of regulatory T cells, consistent with known homeostatic expansion and/or retraction of the peripheral regulatory T cell pool. Our data indicate that polymorphic genes modulate differentiation of regulatory T cells. Identification of responsible genes may reveal novel clinical targets and still elusive regulatory T cell-specific markers. Importantly, these genes may also modulate susceptibility to autoimmune-disease.

MESH Keywords Animals ; Cell Differentiation ; genetics ; immunology ; Female ; Forkhead Transcription Factors ; biosynthesis ; Immune Tolerance ; genetics ; Immunophenotyping ; Lymphocyte Count ; Mice ; Mice, Inbred BALB C ; Mice, Inbred C57BL ; Mice, Inbred DBA ; Receptors, Interleukin-2 ; biosynthesis ; Species Specificity ; T-Lymphocytes, Regulatory ; cytology ; immunology ; metabolism ; Thymus Gland ; cytology ; growth & development ; immunology

Author Keywords immunomodulation ; thymus ; regulatory T lymphocyte ; development

Introduction

Immune-tolerance is established by a variety of mechanisms acting in primary lymphoid organs during lymphopoiesis and in so-called "peripheral" lymphoid organs during the activation or differentiation-phase of mature lymphocytes [1]. Among the several mechanisms known to be involved, active tolerance (i.e. mediated by regulatory or suppressor lymphocytes) has incited great interest because of its potential in treatment of diseases as varied as autoimmune-disorders and cancer, as well as in transplantation [2–4]. The best studied regulatory T cell subset is of $CD4^+CD25^+$ phenotype. These cells were discovered because of their crucial role in the inhibition of multiorgan autoimmune-disorder induced by thymectomy of mice at day three of life [5]. Later, these cells were shown to inhibit inflammatory bowel disease, to fine-regulate immunity to pathogens, to inhibit anti-tumor immunity, and to protect the fetus from maternal immune aggression [6–9]. Because of their crucial role in vivo, $CD4^+CD25^+$ regulatory T lymphocytes are very good candidates as therapeutic agents for the regulation of transplantation tolerance and inhibition of autoimmunity. It has recently been shown that these cells can inhibit Graft-versus-Host disease [10–14], rejection of transplanted tissue [2, 15], and autoimmune disease in experimental settings [16–18].

Probably the majority of (but not all) $CD4^+CD25^+$ regulatory T lymphocytes develop in the thymus [19–23]. In this organ, $CD25^+$ regulatory and $CD25^-$ effector T lymphocytes appear to have common $CD4^-CD8^-$ [24] and $CD4^+CD8^+$ (our unpublished data) precursors. Similar to effector T cells, regulatory T cells are positively selected via interaction with thymic cortical epithelial cells [25]. Expression of high-affinity ligands by thymic epithelial cells has been reported to favor development of regulatory T cells [19, 26, 27]. A recent report suggests that this may be due to deletion of $CD4^+CD25^-$ but not $CD4^+CD25^+$ precursors upon recognition of their cognate ligand expressed by thymic epithelial cells [28]. Interestingly, interaction with high affinity/avidity ligands expressed by thymic antigen-presenting cells of bone-marrow origin can lead to deletion of regulatory T cell-precursors [29, 30]. Consistent with these observations, we and others have previously shown that the peripheral repertoire of regulatory T lymphocytes is enriched in auto-specific cells [29, 31, 32].

Surprisingly, despite the generally appreciated crucial importance of active tolerance, little is known about genetic control of regulatory T cell development and function. Such potential genetic variations might modulate susceptibility to a large panel of pathologies.

Moreover, they would help in providing information concerning fundamental issues as lineage choice and selection of regulatory T cell-precursors in the thymus and functioning of these cells in the periphery.

Only one very rare genetic polymorphism is known to modulate differentiation of regulatory T lymphocytes. The forkhead/winged-helix transcription factor FoxP3 is preferentially (but not exclusively) expressed by regulatory T lymphocytes [33–37]. Transfection of effector T cells with constructs encoding this transcription factor causes these cells to exert potent suppressor effector functions [33–36]. Mice carrying a natural mutation in the gene encoding FoxP3 ("scurfy") lack regulatory T lymphocytes and die after a few weeks of life [33, 34]. In humans, a natural mutation in FOXP3 causes the rare lethal autoimmune disorder IPEX [38, 39]. To our knowledge this is the only genetic polymorphism known to modulate regulatory T lymphocyte development.

We here present data indicating the existence of genetic polymorphisms causing quantitative differences in regulatory T lymphocyte development in common laboratory mouse-strains. We show that genes outside the MHC and acting in a thymocyte intrinsic manner modulate intrathymic differentiation of regulatory T lymphocytes. Ultimate identification of the responsible loci should prove important for the analysis of thymic regulatory T cell lineage choice and selection, may allow for identification of still elusive regulatory T cell-specific markers, and may yield more insight in mechanisms modulating susceptibility to autoimmune-disease.

Results

Distinct proportions of CD25$^+$ regulatory T cells in thymus of different inbred-mouse strains

We analyzed the proportion of CD25$^+$ regulatory cells among CD4$^+$CD8$^-$ (CD4SP) TCR^{high} thymocytes and peripheral blood lymphocytes in the inbred mouse strains B6, B10, BALB/c, DBA/2, DBA/1, and SJL (Fig. 1A). Since regulatory CD4SP T cells express high levels of CD25, while cells expressing intermediate levels of CD25 proliferate and produce IL-2 [40, 41], we only considered thymocytes of CD25^{high} phenotype. In the thymus, statistically significant different percentages of CD25^{high} cells were observed between B6 and B10 mice on one hand, and DBA/2, BALB/c, DBA/1, and SJL strains on the other (Fig. 1B). These differences reached, in the strains analyzed, up to 1.7 fold (DBA/1 vs. B6). The quantitative differences might be caused by distinct CD25$^-$ effector (rather than CD25$^+$ regulatory) T cell percentages. To evaluate this possibility, we analyzed the ratio of mature CD4SP TCR^{high}CD25^{high} regulatory T cells to their CD4$^+$CD8$^+$ precursors in B6, DBA/2, and SJL mice. This ratio was significantly higher in DBA/2 and SJL mice than in B6 animals (Fig. 1C top). On the other hand, the ratio of CD4SP CD25$^-$ to CD4$^+$CD8$^+$ thymocytes was similar in all three mouse-strains (Fig. 1C bottom). These data indicate that the increased proportions of CD25$^+$ regulatory T cells among CD4SP TCR^{high} cells correspond to increased production from immature precursors.

To evaluate if the CD4SP CD25^{high} thymocytes found in the different mouse strains belong to the same regulatory T cell population, we assessed their surface phenotype (Fig. 1D). All CD4SP CD25^{high} thymocytes were TCR^{high} in all mouse strains studied. Interestingly, CD25^{high} cells expressed relatively low HSA (CD24) and CD69 levels, clearly distinguishing them from their CD25$^-$ and CD25$^{int(ermediate)}$ counterparts. Moreover, in all mouse strains all CD25^{high} cells expressed very high levels of the Glucocortocoid Induced TNF-like Receptor (GITR), characteristic for regulatory T cells. Most importantly, all CD4SP CD25^{high} thymocytes expressed FoxP3. These data indicate that the CD25^{high} cells found in the different mouse strains all belong to the same regulatory T lymphocyte lineage.

It has previously been shown that regulatory CD4$^+$CD25$^+$ T lymphocytes can develop in the periphery from CD25$^-$ precursors [22, 23]. Recent data suggests that CD25$^-$ precursors for CD25$^+$ regulatory cells express Foxp3 [42]. Moreover, CD4$^+$CD25$^-$FoxP3$^+$ T cells inhibit T cell activation in vitro [41]. We therefore analyzed the percentage of Foxp3$^+$ cells among CD4SP thymocytes by flow-cytometry. As shown in figure 1E, substantially higher percentages of FoxP3-expressing cells were observed in DBA/2 and SJL mice than in B6 animals, confirming and extending our data on CD25^{high} thymocytes.

We also analyzed levels of regulatory T cells in the periphery. As shown in figure 1F, we failed to observe a direct correlation of CD4$^+$CD25$^+$ percentages in thymus versus PBMC. Similar data were obtained for secondary lymphoid organs (not shown).

Distinct proportions of thymic regulatory T cells are caused by differences in their differentiation

Distinct proportions of regulatory CD25$^+$ cells among CD4SP thymocytes may be due to differences in their development or in thymic retention of mature thymocytes. To study the former possibility, we analyzed the kinetics of regulatory T cell development by measuring the appearance of BrdU$^+$ cells in mice continuously fed with this nucleotide analog in their drinking water. As shown in figure 2, more CD4$^+$CD25$^+$ regulatory T lymphocytes differentiated from their dividing precursors in SJL mice than in B6 thymi. This result establishes that significant differences in thymic differentiation of these cells exist between these two mouse strains. However, it formally does not exclude the possibility that differences in thymic retention of regulatory T cells may also exist.

Distinct regulatory T cell proportions are not caused by differences in MHC haplotype

We next studied if the MHC haplotypes of the distinct mouse strains analyzed were responsible for the quantitative differences in thymic regulatory T cell generation. SJL mice have significantly higher percentages of $CD25^+$ CD4SP thymocytes than B10 mice (Fig. 3A). Congenic B10.S mice (which carry the H-2^s locus from SJL mice on a B10 genetic background) have a similar proportion of thymic regulatory T cells as B10 mice. Similarly, B10.D2 mice (carrying the DBA/2-derived H-2^d locus) have $CD4^+CD25^+$ percentages similar to those in B10 mice (Fig. 3B). Therefore, the distinct MHC class I and II haplotypes of these mouse strains are not responsible for the different proportions of thymic regulatory T cells, and the genetic loci involved are not linked to the MHC.

Thymocyte-intrinsic factors determine the distinct proportions of regulatory T cells

Differences in the development of regulatory T cells may be due to thymocyte-intrinsic factors or to variations in the thymic microenvironment. To distinguish between these two possibilities we generated mixed bone marrow chimeras in which thymocytes derived from the different donor mouse-strains differentiate simultaneously in the same thymic microenvironment. (B6 × DBA/2)F_1 (B6D2F1) hosts were lethally irradiated and reconstituted with a 1:1 mixture of B6 and DBA/2 bone-marrow cells (B6 + DBA/2 → B6D2F1 chimeras). Six weeks later the thymi of these chimeras were analyzed by flow-cytometry. As shown in figures 4A and C, among B6-derived cells from these mixed chimeras the same (lower) proportion of thymic regulatory T cells was found as in the parent strain. Among DBA/2-derived thymocytes the (higher) percentage of $CD25^+$ cells in the CD4SP population was similar to that found in the DBA/2 parent strain. In B6 + SJL → B6SJLF1 mixed bone-marrow chimeras we observed a proportion of B6-derived regulatory T cells similar to that observed in the parent strain (Figs. 4B and C). Interestingly, among SJL-derived thymocytes significantly more regulatory T cells were observed than among B6-derived cells but also than in the SJL parent strain (compare figs. 4C and 1B). The exceptionally high percentage of SJL-derived regulatory thymocytes was also observed in SJL → F1 chimeras (not shown). While we currently do not have a satisfactory explanation for the high levels of SJL regulatory T cells in bone marrow chimeras, this result suggests that thymocyte extrinsic (i.e. environmental) factors can also modulate $CD4^+CD25^+$ regulatory T cell development. Whatever the precise explanation, these results indicate that the different levels of regulatory T cells in the distinct mouse strains studied are caused by thymocyte intrinsic factors.

Discussion

The data presented in this paper demonstrate that polymorphic genetic factors quantitatively control intrathymic generation of $CD4^+$ $CD25^+FoxP3^+$ regulatory T lymphocytes. In the mouse strains studied the distinct regulatory T cell levels are caused by differences in their thymic differentiation from immature precursors. Moreover, we show that thymocyte intrinsic factors modulate regulatory T cell development. Finally, we report that the genes responsible for modulation of regulatory T cell development in the studied mouse-strains are located outside the MHC locus.

Several hypotheses may explain our observation that thymocyte-instrinsic genetic factors cause quantitative differences in regulatory T cell differentiation. They may be caused by quantitative differences in commitment to the regulatory T cell lineage. The gene encoding FoxP3, located on the X-chromosome (http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000039521), would therefore be among the candidate genes (see introduction). Another candidate gene would be Notch3. Transgenic expression of a constitutively active form of Notch-3 also leads to strongly increased thymic generation of regulatory T cells [43]. However, Notch3 is closely linked to the MHC locus (http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000038146) and is therefore unlikely to be involved in the differences in regulatory T cell development in the inbred mouse strains reported here.

Alternatively, differences in regulatory T cell positive and/or negative selection may be responsible. While initial reports suggested that thymic CD4 vs. CD8 lineage-commitment is independent of TCR-specificity, more recently it has become clear that selection mechanisms are responsible, and the two processes therefore actually seem to be very closely linked [44]. In a still unresolved manner, TCR-mediated signals appear to control expression of Th-POK, a zinc-finger transcription factor, as well as of the chromatin remodeling protein Runx, recently identified as binary switches regulating CD4 vs. CD8 lineage commitment, respectively [45–47]. Also the distinct proportions of regulatory cells among mature $CD4^+$ thymocytes may be a consequence of differences in thymic selection and/or lineage commitment. Since thymocyte-intrinsic factors determine quantitative variations in regulatory T cell development, adhesion or signaling molecules may be involved. These molecules would probably also play important roles in function of peripheral regulatory T cells. Thus, one of the many candidate-regions is the diabetes-susceptibility locus Idd5. Within this locus three genes are located that encode proteins expressed by regulatory T cells: CD28, CTLA-4, and ICOS. CD28 is known to play a crucial role in regulatory T cell development and homeostasis [48–50]. Very closely linked is the gene encoding CTLA-4, which is critically involved in regulatory T cell function [51]. Moreover, Ctla-4 is a diabetes susceptibility gene in humans [52], and has been reported to modulate thymic negative selection of effector T cells in mice [53]. A third gene within the Idd5 locus, ICOS, is also known to play an important role in regulatory T cell function [18, 54] . However, none of these genes has thus far been shown to modulate regulatory T cell development.

Identification of the responsible gene(s) may also reveal entirely novel factors critically involved in regulatory T cell development and function, and thus allow for better understanding of these processes. Such factors may also constitute unique markers for regulatory T cells, which have thus far proven elusive, and become clinical targets. Whatever the precise explanation for genetic modulation of

regulatory T cell development, it may have important consequences for regulatory T cell repertoire and/or function and thus modulate susceptibility to e.g. autoimmune diseases. It would therefore be important to assess regulatory T cell differentiation in autoimmune-prone animals, and to identify responsible genetic loci.

Materials and Methods

Mice

C57BL/6 (B6), SJL, DBA/2, DBA/1, BALB/c, (B6xDBA/2)F1 (B6D2F1) and (B6xSJL)F1 (B6SJLF1) females of 5 to 7 weeks of age were purchased from Janvier (Le Genest St Isle, France). C57BL/10 (B10) mice were purchased from Charles River (Les Oncins, France). B10.D2, B10.S, and B10.D1 (B10.Q) mice were bred in our facilities. All experiments involving animals were performed in compliance with the relevant laws and institutional guidelines (INSERM; approval # 31-13, ethical review # MP/02/32/10/03).

Antibodies

The following antibodies and secondary reagents were used for phenotypic analysis: PE-Cy7 or APC-labeled anti-CD4 (GK1.5), FITC or APC-labeled anti-CD8 (53.6.7), APC or PE-labeled anti-CD25 (PC61), FITC-labeled anti-HSA (M1/69), FITC-labeled anti-CD69 (H1.2F3), FITC-labeled anti-TCRβ, FITC-labeled anti-CD45.1, PE-labeled anti-Foxp3, FITC-labeled anti-CD5.1, PE-Cy5.5-labeled streptavidine (eBioscience, San Diego, CA). Biotin-labeled anti-GITR was purchased from R&D, Lille, France.

Bone-marrow chimeras

Bone-marrow from femurs and tibias was collected in DMEM medium supplemented with 10% FCS. $Thy1^+$ cells were eliminated using AT83 hybridoma supernatant and rabbit complement (Saxon Europe, Suffolk, UK). Cells from each donor were injected intravenously into lethally γ-irradiated hosts (8.5 Gy; 137 Cs source, 6.3 Gy/min) that were kept on antibiotic-containing water (0.2% of Bactrim; Roche) for the complete duration of the experiment (6 weeks).

Flow cytometry

Thymocytes or peripheral blood mononuclear cells were incubated 30 min on ice in 2.4G2 (anti-FcγR mAb) hybridoma supernatant. Cells were then incubated 20 min with saturating concentrations of Abs. Intracellular Foxp3-staining on CD8-depleted thymocytes (using anti-CD8 mAb 31M and complement) was performed according to the instructions of the manufacturer. Labeled cells were analyzed using a FACSCalibur cytometer and CellQuest software (BD Biosciences, San Diego, CA).

BrdU incorporation studies

Mice were continuously exposed to the thymidine analogue bromodeoxyuridine (0.8mg/mL) in their drinking water. Extracellular staining of thymocytes with mAbs against CD4, CD8 and CD25 was performed as described above. Cells were fixed, permeabilized and stained with FITC-labelled anti-BrdU using the BrdU Flow Kit (BD Pharmingen, Heidelberg, Germany).

Statistical analysis

Statistical significance of the data was analyzed using Student's t test (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

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Abbreviations

B6: C57BL/6

B10: C57BL/10

CD4SP: $CD4^+CD8^-$

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Figure 1

Distinct proportions of CD25$^+$ regulatory thymocytes in various inbred mouse strains

(A) Freshly isolated thymocytes were analyzed by 4-color flow cytometry for expression of CD4, CD8, CD25 and TCR. The histograms depict TCR and CD25 levels of cells electronically gated on CD4 and CD8 as indicated. (B) The percentage of CD25$^+$ among CD4$^+$CD8$^-$ TCR^{hi} thymocytes in several inbred strains of mice (n≥10 per strain) was calculated using gates indicated in A. Mean values (± SD) are shown. Statistical significance between B6 and other strains is indicated (***p<0.001, NS, not significant, Student's t-test). (C) CD25$^+$TCR^{hi} CD4$^+$CD8$^-$ ("Treg")/CD4$^+$CD8$^+$ ("DP")(upper panel) and CD25$^-$TCR^{hi}CD4$^+$CD8$^-$ ("Teff")/CD4$^+$CD8$^+$ ("DP") ratios (lower panel) were determined for B6, SJL, and DBA/2 mice. Depicted are mean values ± SD (n≥4)(**p<0.01, ***p<0.001, Student's t-test). (D) Thymocytes from DBA/2, SJL and B6 mice were labeled with Abs specific for CD4, CD8, CD25 and either TCR, HSA, CD69, GITR, or Foxp3. CD4$^+$CD8$^-$ cells were electronically gated and analyzed for expression of indicated surface markers. (E) Percentage of FoxP3 expressing thymocytes among CD4SP thymocytes in the indicated mouse-strains. Mean values (± SD) are shown (n=4). Statistical significance is indicated (*** p<0.001, *p<0.05, Student's t-test). (F) PBMC and thymocytes from different inbred strains were stained with anti-TCR, anti-CD4, anti-CD8 and anti-CD25 antibodies and analyzed by flow cytometry. The percentage of CD25$^+$ cells among CD4$^+$CD8$^-$TCR^{hi} cells was calculated. Mean values (± SD) are shown.

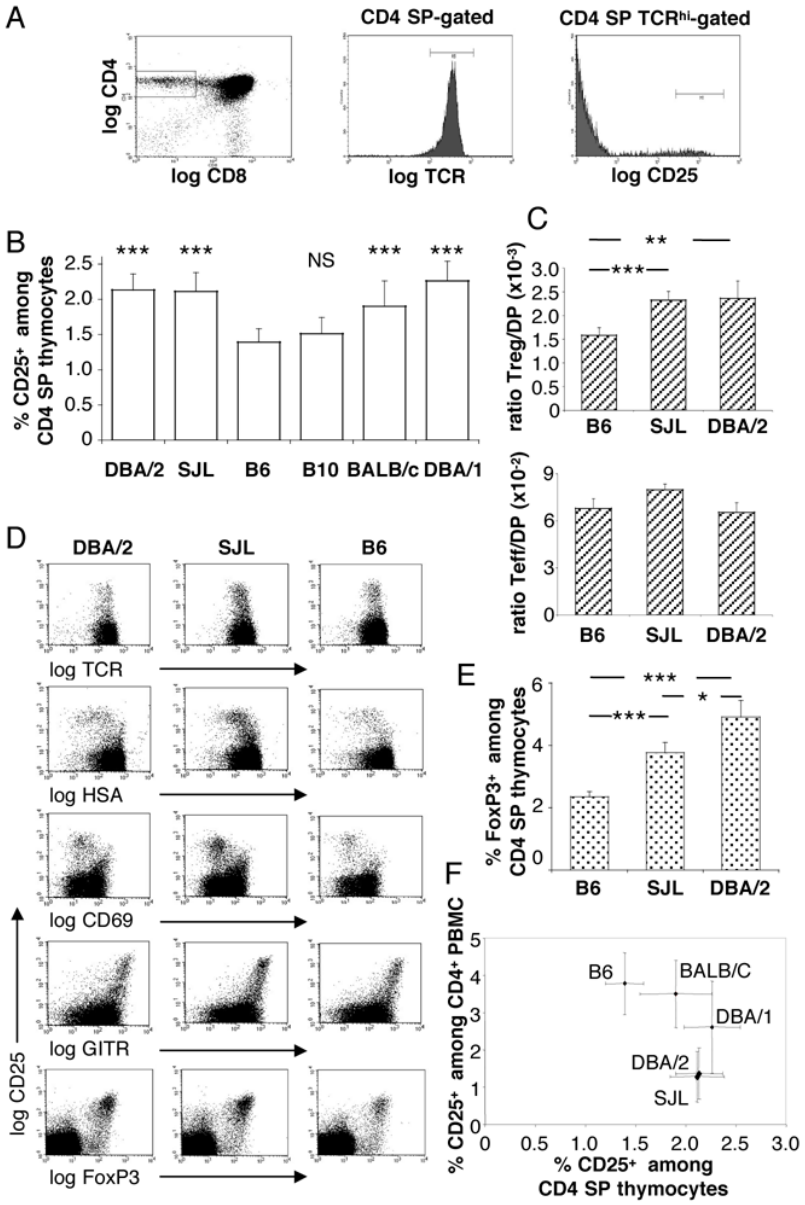

Figure 2

Quantitative difference in differentiation of mature $CD25^+$ regulatory thymocytes in B6 vs. SJL mice

BrdU was continuously administrated to mice in their drinking water. At indicated timepoints, thymocytes were analyzed by 4-color flow cytometry using anti-CD4, anti-CD8, anti- CD25 and anti-BrdU Abs. The percentage of $BrdU^+CD25^+$ among $CD4^+CD8^-$ ("CD4SP") cells was calculated. Each point represents one mouse. Statistical significance of the difference between the two mouse strains was calculated for each day using Student's t-test (**p<0.01, ***p<0.001).

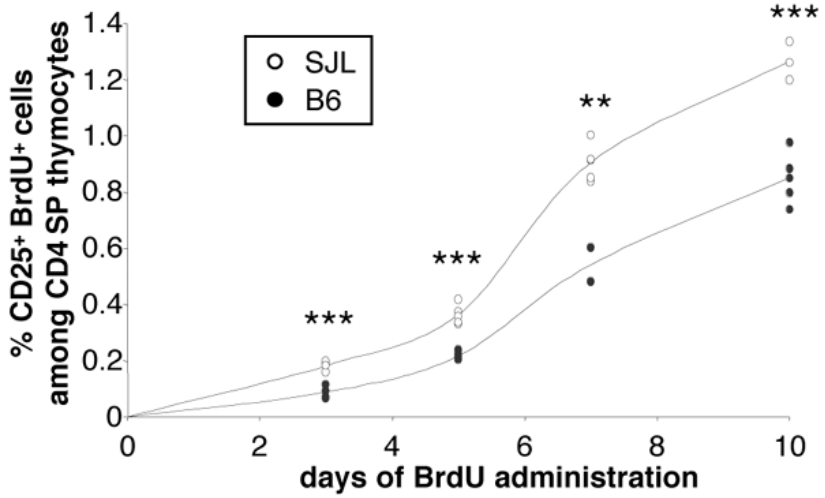

Figure 3

Genes modulating CD25$^+$ regulatory T cell differentiation are located outside the MHC

Thymocytes from indicated mouse strains were stained with anti-CD4, anti-CD8, anti-CD25 and anti-TCR Abs and analyzed as described in the legend to figure 1. Mean values (± SD) are shown (n≥5). Statistical significance was calculated using Student's t-test (**p<0.01, *** p<0.001).

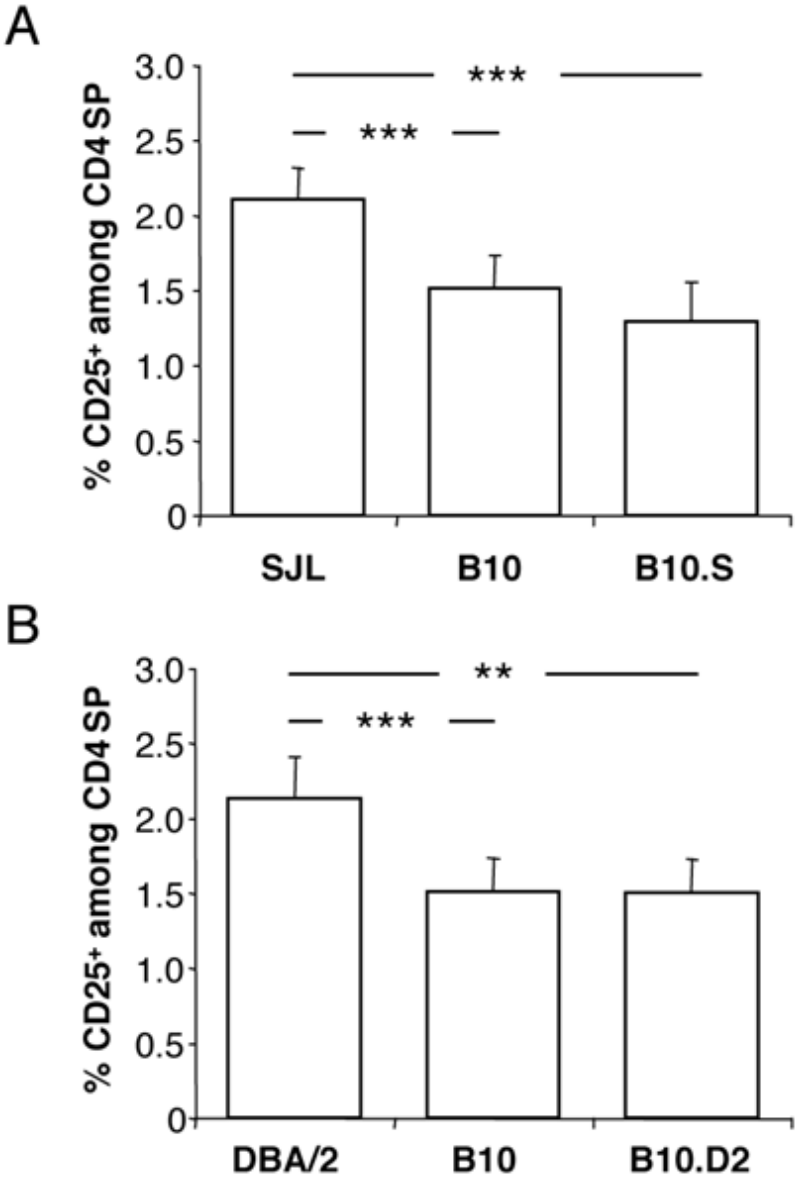

Figure 4

Distinct proportions of CD25$^+$ regulatory T cells are determined by a thymocyte-intrinsic mechanism

(A) Lethally irradiated B6D2F1 hosts were reconstituted with bone marrow cells from B6 (CD5.2) and DBA/2 (CD5.1) at a 1:1 ratio. Six weeks later, thymocytes were analyzed by 4- color flow cytometry for expression of CD4, CD8, CD25 and CD5.1, using indicated electronic gates. (B) Similar experiments were performed using B6SJLF1 recipients injected with B6 (CD45.2) and SJL (CD45.1) bone marrow cells. (C) Quantitative analysis of CD25$^+$ thymocyte development in mixed chimeras. Bar graphs depict mean values (± SD), n≥5. Statistical significance was calculated using Student's t-test (**p<0.01, ***p<0.001).