Regulatory potential and control of Foxp3 expression in newborn CD4+ T cells

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Thymectomy at day 3 after birth leads to autoimmune disease in some genetic backgrounds. This disease is thought to be caused by the lack/paucity of regulatory T cells. We show that 3-day-old mice already contain a significant compartment of Foxp3-expressing CD25+CD4+ splenocytes. Whereas, in adult spleen, the subsets of regulatory T cells (CD25+ and/or CD103+) express high amounts of Foxp3 mRNA, in 3-day-old mice, both thymic and splenic CD25+CD4+ T cell subsets express lower amounts of Foxp3 mRNA, and CD103+ cells are barely detected. In adult day 3-thymectomized mice, the CD25+CD4+ T cell subset is overrepresented (most of the cells being CD103+) and expresses high amounts of Foxp3 mRNA, independent of the development of autoimmune gastritis. These cells control inflammatory bowel disease and the homeostatic expansion of lymphocytes. This study demonstrates that the peripheral immune system of newborn mice is endowed of a remarkable regulatory potential, which develops considerably in the absence of thymic supply.

Regulatory T cells belonging to the natural pool of CD25+CD4+ T cells play a fundamental role in the maintenance of peripheral tolerance to self-antigens (1–3) and in the regulation of adaptive (4–7) and innate (8) immune responses against pathogens. Because of their capacity of efficiently inhibit T cell proliferation (9, 10), these cells are a key element in the systemic homeostatic mechanisms that control total lymphocyte numbers (10). The transcription factor Scurfin, encoded by the forkhead/winged helix (Foxp3) gene (11), is a specific marker for regulatory activity (12) and is essential for the development and function of regulatory CD25+CD4+ T cells (13, 14). Regulatory activity (4, 10, 15) and low amounts of Foxp3 mRNA (12) are also found in the CD25+CD4+ T cell population. The αβ integrin (CD103), expressed at high levels in T cells seeding mucosal tissues (16–18), identifies a small subset of splenic CD25+CD4+ T cells that displays regulatory activity (19). CD103 expression also distinguishes two types of regulatory CD25+CD4+ T cells secreting different cytokines and showing different regulatory capacities (19, 20). It has recently been shown that these three populations express comparable levels of Foxp3 mRNA (21). Their lineage relationship has not yet been determined.

Little is known on the ontogeny of the peripheral compartment of regulatory CD4+ T cells. It is now established that at least part of the regulatory CD25+CD4+ T cells are produced in the thymus (22, 23). However, it has been proposed that they are only significantly exported to the periphery after the third day of postnatal life (2). This hypothesis is based on the observation that mice thymectomized between days 2 and 4 after birth develop organ-specific autoimmune disorders, which are prevented by the early transfer of adult CD25+CD4+ T cells (2).

However, the pattern and the incidence/severity of these diseases vary among animals of the same strain and are highly dependent on their genetic background (24, 25). Indeed, in several strains, day 3 neonatal thymectomy does not lead to the development of autoimmune diseases despite inducing lymphopenia. Moreover, even in sensitive strains, thymectomized mice do not develop some types of inflammatory diseases, such as inflammatory bowel disease (IBD), and keep a long-lasting state of lymphopenia (2). All of these features are in sharp contrast with the syndrome resulting from the inactivation of the Foxp3 gene in the C57BL/6 mice, a strain resistant to neonatal thymectomy-induced autoimmune disease. In this case, the complete absence of regulatory CD25+CD4+ T cells leads to generalized lymphoproliferation and massive lymphoid infiltration of several tissues, with death occurring within four weeks after birth (11, 13).

A simple explanation for the differential regulatory deficit induced by Foxp3 inactivation versus neonatal thymic ablation is that peripheral regulatory CD4+ T cells are already present in significant numbers early after birth and that thymectomized animals will develop a significant compartment of regulatory CD25+CD4+ T cells (26).

To test this hypothesis, we searched for Foxp3-expressing CD4+ T cells in 3-day-old BALB/c mice, a strain sensitive to thymectomy-induced disease. The results demonstrate that the spleen of 3-day-old newborns (d3nb) already contains a well represented Foxp3-expressing CD25+CD4+ T cell population. However, in contrast to adult thymus and spleen, CD103+CD4+ cells are barely detected in 3-day-old mice, and the CD25+CD4+ subset displays lower amounts of Foxp3 mRNA. In the absence of further thymic supply, the regulatory potential of the CD4+ T cell compartment of newborns develops considerably. We show that adult day 3-thymectomized mice (d3Tx) contain an overrepresented Foxp3-expressing CD25+CD4+ T cell compartment, independently of the development of autoimmune gastritis. These cells are able to control IBD and CD4+ T cell expansions in vivo. Moreover, the levels of Foxp3 mRNA in this population are now as high as those in the correspondent normal adult population and most of the cells express CD103.

Materials and Methods

Mice. BALB/c mice were from Charles River Breeding Laboratories, and Rag2-deficient (Rag22) BALB/c mice were from Centre de Distribution, de Typages et d’Archivage Animal (CDTA, Orléans, France) and were bred in our animal facilities under specific pathogen-free conditions. All animal experiments were done in accordance with the guidelines of the Institut Pasteur, which are approved by the French Ministry of Agriculture.

Neonatal Thymectomy. The day of birth being considered as day 0, thymectomy was performed on day 3, unless otherwise specified. In brief, newborns were anesthetized on ice, their chests were opened, and the thymic lobes were removed by suction. Sham-thymectomized mice were anesthetized and opened in the same way. At death, the thorax was inspected and partially thymectomized mice were excluded from the experiment. In doubtful cases, the tissue was analyzed for the presence of CD4+CD8+ thymocytes.

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Abbreviations: d3nb, 3-day-old newborns; d3Tx mice, day 3-thymectomized mice; d3ShTx mice, day 3 sham-thymectomized mice; IBD, inflammatory bowel disease; PE, phycoerythrin; TCR, T cell receptor.

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Abs and Flow Cytometric Analysis. The following mAbs (Pharmin- 
gen) were used: anti-CD8-allophycocyanin (APC) or -biotin (Ly-2); 
anti-αβ-FITC (H57); anti-CD4-APC, -phycoerythrin (PE), or 
-FITC (LT3); anti-CD25-PE (PC61); anti-CD25-FITC or -biotin 
(7D4); anti-CD45RB-PE (16A); and anti-CD103-biotin (M290). 
Biotinylated Ab were revealed with streptavidin(Sav)-FITC, -PE, 
-APC, or -Cychrome. Cell suspensions were analyzed by using a 
FACSCalibur or a LSR flow cytometer and cellquest software 
version 3.3 (Becton Dickinson).

Cell Preparations. For cell transfers, spleen and lymph nodes cell 
suspensions were stained with anti-CD25-biotin Ab for 20 min at 
4°C in PBS/0.3% FCS, followed by staining with Sav-PE and final 
incubation with anti-PE MicroBeads (Miltenyi Biotec, Bergisch 
Gladbach, Germany) in PBS/0.5% FCS. The cells were passed 
through midiMACS separation column (Miltenyi Biotec). The 
positive fraction (CD25+) was passed a second time through 
another column to obtain a population enriched in CD25+CD4+ 
cells (these represented 60–80% of total cells and >95% of the 
CD4+ cells). The first negative fraction (CD25−) was stained with 
L3T4 MicroBeads and enriched for CD4+ cells following the same 
procedure. The cells were then incubated with anti-CD45RB-PE 
and anti-CD4-allophycocyanin Abs and the CD45RBhighCD4+ 
cells sorted on a MoFlo (Cytomation). The purity of the cells was 
always >95%.

For mRNA analysis, splenocytes were first enriched for CD4+ T 
cells by magnetic sorting using L3T4 MicroBeads, then incubated 
with the relevant Abs and the subsets of interest sorted on MoFlo 
directly into RNAPlus extraction solution (Obiogen, Montreal).

Cell suspensions of the colon were obtained as described (27). In 
brief, the colons were cut in pieces and incubated twice in pre-
warmed OptiMEM medium (GIBCO Life Technologies, Rockville, 
MD) containing 10% FCS and 20 units/ml collagenase (Sigma) for 
20 min at 37°C. After filtering through gauze, lymphoid cells were 
isolated on a 40% Percoll gradient and washed.

Cell Transfers. Rag2−/− mice were injected i.v. with 3 × 10^6 
CD45RB(high)CD4+ cells alone or with 6 × 10^5 CD25+CD4+ cells. 
The recipient’s weight was scored twice a week, and the animals 
were apparent. All of the other mice were analyzed 3 or 2 months 
after injection, respectively in the first and second experiment. The 
two independent experiments used donors and recipients that were 
kept in different animal houses. Day 3 and day 3/4 neonatally 
thytemocimized donors were used in the first and second 
experiments, respectively. Similar results were obtained for all groups in 
the two experiments.

Foxp3 Expression Analysis by Real-Time RT-PCR. RNA from sorted 
cells was extracted by using RNAPlus extraction solution (OBio-
gen) following the manufacturer’s instructions. Extracted RNA 
were then incubated at 70°C for 10 min with oliga(1T) (Amersham 
Pharmacia) and the first-strand cDNA were synthesized by using 
SuperScript II RNaseH− Reverse Transcriptase kit (Invitrogen) at 
37°C for 50 min. The reaction was stopped by incubation at 90°C 
for 5 min. The relative Foxp3 expression was determined as described 
(14). In brief, Foxp3 mRNA was measured by using an Applied 
Biosystems 7000 Sequence Detection System with the primers 
5′-GGCCCTTTCACAGGACA3′- and 5′-GCTGATCATG 
GCTGTTGTTG-3′ (300 nM) and the internal TaqMan probe 
5′-FAM-ACCTTCATGCTACGTCTCTCACTGTTGAT 
-TAMRA-3′ (100 nM). Dad1 was used as endogenous reference 
(28) and the mRNA was measured by using the primers 
5′-CCCTGTCGTGGGCTCTCTCTCTTTGTTG-3′ and 5′-CCGGAGAG 
AGATGCTGTGGAA-3′ (50 nM) and the internal TaqMan probe 
5′-FAM-AGCTTTCATTCAGGCTGTGGATG 
-TAMRA-3′ (100 nM). The TaqMan Universal Master Mix (Ap-
plied Biosystems) was used, and PCR cycling conditions were 95°C 
for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min. The 
reference cDNA sample used to make standard curves was ob-
tained from sorted αβ+CD25+CD4+ splenocytes. The relative 
expression of Foxp3 was obtained by dividing the relative quantity 
of Foxp3 mRNA by the relative quantity of Dad1 mRNA, and the 
result was multiplied by 100.

Statistical Analysis. Analyses were performed by using the unpaired 
t test. The data were considered significantly different when P 
values were <0.05.

Results

Foxp3-Expressing CD4+ T Cell Compartment in d3nb Spleens. Regu-
larly activity and high levels of Foxp3 mRNA have recently been 
found in a small subset of splenic CD25−CD4+ T cells expressing 
CD103 (21). The analysis of this marker, together with CD25, adds 
to a more complete description of the Foxp3-expressing regulatory 
CD4+ T cell compartment in normal individuals. Therefore, we 
analyzed the expression of these two markers in the peripheral 
compartment of BALB/c d3nb. On average, αβ+CD4+ T lympho-
cytes represent 1.3% of the splenocytes (Fig. 1a). We found that 
5.2% of these cells expressed the CD25 marker, correlated with 
discreetly lower levels of CD4, a feature typical of normal adult 
CD25−CD4+ T cells and not of naïve CD4+ T cells when activated 
in vivo (13, 14, 26, 29). In absolute numbers, the d3nb spleen harbors 
1.4 ± 0.9 × 10^6 αβ+CD4+ T cells, of which 7.6 ± 5.1 × 10^4 are 
CD25+. Consistent with previous results (19, 30, 31), CD103+ cells 
represented 15% of the adult CD25+CD4+ T cell population (Fig. 
1b). In sharp contrast, CD103+ cells were barely detectable in the 
equivalent newborn compartment. CD103+ cells were found at the 
same low frequency (<1%) in the CD25−CD4+ T cell pool of d3nb 
and adult, although at a lower fluorescence intensity in the former.

We compared Foxp3 expression in the αβ+CD4+ population of
adult versus d3nb and in the respective subsets defined by the CD25 marker (Fig. 2a). Substantial amounts of Foxp3 mRNA were detected in αβ⁺ CD4⁺ T cells from d3nb and, as in adult, preferentially in the CD25⁺ subset. However, the amount of Foxp3 mRNA in d3nb CD25⁺ cells was 2.6 ± 0.8-fold lower than in the equivalent adult population (P < 0.05). Low amounts of Foxp3 mRNA were detected in the d3nb CD25⁻ population, as in adults.

**Foxp3 Expression in Newborn and Adult Thymus Is Differentially Regulated.** The lower amounts of Foxp3 mRNA in the d3nb CD25⁻ CD4⁺ T cell compartment, as compared to the adult, could be due to a lower frequency of regulatory cells, a possibility that we cannot directly assess. Whereas in the peripheral lymphoid organs, part of the CD25⁺ CD4⁺ T cell population may represent recently activated cells, there is evidence supporting the notion that the mature CD25⁺ CD4⁺ CD8⁻ thymocyte population is essentially composed of regulatory cells (13, 22, 32). Therefore, we compared Foxp3 expression in the thymus and in the spleen of d3nb. The CD25⁺ CD4⁺ CD8⁻ thymocyte population displayed similar amounts of Foxp3 mRNA as their splenic equivalents (Fig. 2b). This result indicates that the peripheral pool of CD25⁺ CD4⁺ T cells in d3nb is mainly, if not entirely, composed of Foxp3-expressing regulatory T cells.

A major difference between the peripheral T cell compartment of newborns and adults is that, whereas in the former, essentially all T cells are recent thymic emigrants (RTEs), this population comprises only 1–2% of the T cell pool in the adult (33, 34). Lower levels of Foxp3 mRNA in the d3nb peripheral CD25⁺ CD4⁺ T cell pool could then reflect general characteristics of RTEs, independently of the age of the mouse. We thus compared Foxp3 expression in the thymus at both ages. CD25⁺ CD4⁺ CD8⁻ thymocyte populations had amounts of Foxp3 mRNA similar to those of their respective age-matched splenic equivalents (Fig. 2b). The quantities of Foxp3 mRNA in the d3nb populations were significantly lower with differences of 2- and 2.5-fold for the thymus (P < 0.05) and the spleen (P < 0.001), respectively. These results demonstrate that Foxp3 expression in mature CD25⁺ CD4⁺ thymocytes is differentially controlled in newborn and adult. This is also the case in respect to CD103 expression, because CD103⁺ cells are barely detected in d3nb CD25⁺ CD4⁺ mature thymocytes (<1%, data not shown).

**CD25 and CD103 Expression in the Lymphopenic CD4⁺ T Cell Pool of d3Tx.** The observation that d3nb already possess a significant peripheral compartment of Foxp3-expressing CD25⁺ CD4⁺ T cells favors the hypothesis that animals thymectomized at this age have the potential to develop a functional regulatory CD4⁺ T cell pool. To address this issue, we performed thymectomy of 3-day-old mice (d3Tx). As controls, day 3 sham-thymectomized mice (d3ShTx) were used. Reproducing previous observations (2), 2- to 3-month-old d3Tx were highly lymphopenic. The size of the splenic CD4⁺ T cell compartment was 8- to 10-fold smaller than the one of d3ShTx (Fig. 3a), mostly at the expense of the naïve CD45RBhigh cells (16-fold), which represented 17.4% of the CD4⁺ T cell pool. The pool of activated/memory (CD45RBlowCD25⁺) CD4⁺ T cells was 5- to 6-fold smaller than the one of d3ShTx. Within this compartment the cells with CD45RBlowCD25⁺ phenotype were 9-fold reduced. In contrast, as shown by others (2, 35), the CD25⁻ CD4⁺ T cell subset was only 3- to 4-fold reduced. Whereas CD25⁺ cells represented 14% of CD4⁺ T cells in d3ShTx (Fig. 3b), the average value scored was 34.3% in d3Tx. These CD25⁺ cells expressed the typical discrete low levels of CD4 (see legend of Fig. 3b). Moreover, whereas these cells represented 20% of the activated/memory compartment in d3ShTx, CD25⁺ cells represented 41% of the activated/memory compartment in d3Tx.

CD103⁺ cells represent >60% of all CD25⁺ CD4⁺ T cells in d3Tx spleens, in contrast to 15% in d3ShTx (Fig. 3c). The relative frequency of CD103⁺ CD25⁻ CD4⁺ T cells was 5-fold higher in...
correspond to the same animals studied in a corresponding 4 in d3ShTx spleens, the CD25 which were similar to those of their d3ShTx equivalents. Whereas, mRNA were found in the CD25-expressing subsets of d3Tx spleens, Foxp3 into BALB mice, and only slightly reduced in the CD103 d3Tx than in d3ShTx. In absolute numbers, the size of the CD103+CD25+CD4+ population was similar in both groups of mice, and only slightly reduced in the CD103+CD25- subset (Fig. 4b). In contrast, the number of CD103-CD25+CD4+ T cells was 7-fold decreased.

CD25+ and/or CD103+CD4+ T Cell Subsets of Adult d3Tx Spleens Express High Amounts of Foxp3 mRNA. We compared Foxp3 expression in the splenic CD4+ T cell subsets expressing CD25 and/or CD103 of d3Tx and d3ShTx (Fig. 5). High amounts of Foxp3 mRNA were found in the CD25-expressing subsets of d3Tx spleens, which were similar to those of their d3ShTx equivalents. Whereas, in d3ShTx spleens, the CD25+CD103+ population expressed half the amounts of Foxp3 mRNA as compared to the CD25+ subsets, in d3Tx, the three subsets displayed equivalent amounts of this transcription factor.

The variable incidence of thymectomy-induced autoimmune disease in BALB/c mice raised the question of whether high levels of Foxp3 mRNA would be detected in healthy as well as in sick animals. In agreement with previous results, the analysis of 7- to 8-month-old d3Tx show that gastritis developed in five of eight mice (63%), with variable severity, whereas all age-matched control mice had a normal gastric mucosa (Fig. 6 and Supporting Text, which are published as supporting information on the PNAS web site). As shown in Fig. 6, the quantities of Foxp3 mRNA in the splenic CD25+CD4+ T cell subset were similar in all animals.

Thus, adult d3Tx contained a highly significant compartment of Foxp3-expressing CD4+ T cells, independently of the development of autoimmune gastritis. The results also show that the newborn peripheral CD4+ T cell population can generate the two main subsets of Foxp3-expressing d3Tx cells and that the levels of Foxp3 mRNA can be substantially modified in the periphery.

CD25+CD4+ T Cells of d3Tx Display Regulatory Activity in Vivo. Finally, we evaluated the capacity of the CD25+CD4+ T cell population of nTx mice (day 3 or day 4 thymectomized mice) to protect Rag20 recipients of CD4+ T cell-mediated IBD. Sorted splenic CD45RBhigh (RBhigh) CD4+ T cells of normal BALB/c mice were intravenously injected into syngeneic Rag20 recipients either alone or in a mixed cohort with purified CD25+CD4+ T cells (ratio of 1:2) of normal or nTx animals. Another group of recipients received RBhighCD4+ T cells of nTx mice.

At the time of death, six of eight recipients of normal RBhighCD4+ cells injected alone had lost considerable weight (Fig. 7a). In all recipients, the colon was markedly enlarged, and >106 donor T cells were retrieved after enzymatic treatment (Fig. 7b). In contrast, practically all recipients of normal RBhighCD4+ T cells coinjected with normal (eight of eight) or nTx (seven of eight) CD25+CD4+ T cells gained weight and had no signs of wasting. In these two groups of mice, the colon was macroscopically normal and the number of infiltrating T cells was 10- to 50-fold lower than in the group injected with naïve T cells alone. Six of the eight recipients of nTx RBhighCD4+ T cells lost weight, and the colon also contained high numbers of infiltrating T cells. In these two aspects, no statistically significant difference was observed with the group injected with normal RBhighCD4+ T cells.

T cell expansion was assessed in all groups by scoring the number of donor cells in the spleen (Fig. 7c). Between 5 and 10 x 106 donor cells were recovered in the groups that received normal or nTx RBhighCD4+ T cells. In the groups of the mixed cohort with normal CD25+CD4+ T cells, the accumulation of donor T cells was strongly reduced in most animals. In the group coinjected with nTx CD25+CD4+ T cells, with the exception of two recipients that had a considerable number of donor cells, the distribution of T cells numbers was equivalent to that of animals that received normal CD25+CD4+ T cells.

In conclusion, CD25+CD4+ T cells from nTx or normal mice.
share the capacity to control T cell mediated wasting disease and to regulate homeostatic CD4+ T cell expansions. Moreover, nTx mice contain CD4+ T cells capable of inducing IBD and endowed of a high potential of expansion.

Discussion

Here we provide the demonstration that the immune system of 3-day-old BALB/c mice is endowed with a remarkable regulatory potential which develops considerably in the absence of further thymic supply. Indeed, ≈5.0% of the d3nb splenic αβ+ CD4+ T cells express CD25 and discretely lower density of CD4, a feature not found in recently activated nonregulatory CD4+ T cells (13, 14, 26, 29). This subset expresses high levels of Foxp3 mRNA. Analysis of adult d3Tx revealed substantial numbers of splenic CD25+ CD4+ T cells, globally displaying high amounts of Foxp3 mRNA, independently of the incidence of autoimmune disease (e.g., gastritis). Although it was previously shown that these cells could not control T cell proliferation in vitro (9), we show here that they have the capacity to protect against T cell-mediated IBD and to control CD4+ T cell expansions in vivo. Thus, the small (≈1.4 × 10^6 cells) peripheral CD4+ T cell compartment of d3nb BALB/c has the systemic properties required to react and adapt efficiently to lymphopenia. This regulatory system is also fully capable of controlling immune responses against gut-associated pathogens.

The distinct amounts of Foxp3 mRNA in peripheral CD25+ CD4+ cells in d3nb and adults could result from a lower frequency of regulatory T cells in newborns. However, this possibility is unlikely because this population displays similar amounts of Foxp3 mRNA and the same discretely lower density of CD4 as its thymic equivalent, the latter consisting mainly, if not entirely, of regulatory cells. This last contention is supported by two major observations, e.g., TdT activity (38) and cytokines (39, 40). Newborn and adult thymic stroma may also differ in the state of maturation (10), proliferate extensively and regulate their levels of Foxp3 and CD103 upon exposure to peripheral (endogenous and exogenous) antigens or cytokines (39, 40), all of them displaying high levels of Foxp3 mRNA (comparable to those of their splenic equivalents), and 8–15% of the cells express CD103 (ref. 20 and data not shown). In contrast, in d3nb thymus, besides the lower levels of Foxp3 mRNA, CD103+ CD25+ CD4+ thymocytes are barely detected. It was recently suggested that the strength of TCR signaling may be intimately linked to the levels of Foxp3 mRNA in regulatory CD4+ T cells, this relationship being central to the development of these cells (13). It is thus possible that the differential levels of Foxp3 mRNA between newborns and adults reflect a distinct average strength of TCR signaling in the selection process of regulatory CD4+ T cells. It is known that fetal and adult hematopoietic precursors have distinct developmental requirements, e.g., TdT activity (38) and cytokines (39, 40). Newborn and adult thymic stroma may also differ in the state of maturation and/or heterogeneity of cell types, resulting in distinct patterns of cytokines and distinct diversity of available antigens. Another possibility is that these microenvironmental differences, which, in the adult could be reinforced by external factors (e.g., circulating antigens or cytokines), may lead to postselection activation of a fraction of the regulatory CD4+ T cell population in the adult thymic medulla with consequent alteration of the levels of Foxp3 mRNA and the acquisition of CD103.

In the absence of thymic export, the d3nb CD4+ T cell population will generate the three subsets expressing CD25 and/or CD103, all of them displaying high levels of Foxp3 mRNA. Thus, the expression levels of Foxp3 and CD103 can also be regulated in the periphery. Further studies are needed to determine the origin of the Foxp3-expressing CD4 T cell population of adult d3Tx. Although some of the d3nb naive CD25+ CD4+ T cells may become regulatory cells, it is plausible that the peripheral CD25+ CD4+ T cells, which have a high potential of expansion in lymphopenic environments (10), proliferate extensively and regulate their levels of Foxp3 mRNA and CD103 upon exposure to peripheral (endogenous and environmental) antigens.

Finally, our present results provide an alternative explanation for a number of puzzling observations caused by thymic ablation early in life. The first aspect is the stable state of lymphopenia (occurring in any strain of mice), which, as confirmed here, is preceded by considerable T cell expansion (30- to 50-fold) during the first 3

Fig. 7. Regulatory capacity of CD25+ CD4+ T cells of neonatally thymectomized mice. Rag2−/− mice were injected with 3 × 10^6 CD45RBhigh CD4+ (RBh) cells sorted from nTx or age-matched normal mice. This later population was coinjected with 6 × 10^5 purified CD25+ CD4+ (25+) cells from nTx or age-matched normal mice. Recipients were analyzed between 5 and 14 weeks after injection. Results are from two independent experiments. (a) Percentage of initial body weight at the time of death. For the groups injected with RBh alone versus the two that received the mixed cohorts, P < 0.005. (b) For the groups injected with RBh alone versus the two that received the mixed cohorts, P < 0.005. Results for colon are shown. (c) For the groups that received RBh or RBh nTx versus RBh + 25+, P < 0.05. Results for spleen are shown.
weeks after thymectomy (2). This homeostatic expansion can be understood as a trend to “correct” cell numbers in particular those of activated/memory cells that represent >80% of the total CD4+ T cell compartment. A key role for regulatory CD25+CD4+ T cells in the homeostatic control of lymphocyte numbers is suggested by the observation that regulatory T cells are overrepresented in this pool. The steady-state in adult thymectomized mice is similar to the one reached in Rag2−/− recipients reconstituted with mixed cohorts of CD45RBhigh CD4+ and CD25+CD4+ T cells. Indeed, preceded by controlled expansion, lymphopenia persists in these recipients despite their capacity to sustain a much more extensive T cell proliferation when the transferred cohorts lack regulatory cells (10).

The second aspect is the absence of many inflammatory pathologies such as IBD in neonatal thymectomized mice, which can develop in a situation of lymphopenia (41). As shown here, d3Tx contain disease-inducing CD4+ T cells but also the correspondent protective regulatory CD25+CD4+ T cells. It has recently been shown that, within the splenic CD4+CD4+ T cell population, CD103+ cells are the most efficient in the protection of CD4+ T cell-induced IBD (19) and in the regulation of lymphocyte numbers (20). The observation that this subset is overrepresented in the CD4+ compartment of adult d3Tx further reinforces, but does not prove, the potential role of regulatory CD25+CD4+ T cells in maintaining lymphopenia and protecting from IBD in these mice.

The third aspect is the relatively restricted pattern and variable incidence of the autoimmune disorders, which are highly dependent on the genetic background of the strain (25). The presence of an important compartment of Foxp3-expressing CD25+CD4+ T cells early in life readily explains why many strains are resistant to disease induced by neonatal thymectomy despite their severe lymphopenia. In the case of sensitive strains, there is the additional aspect that the incidence/severity of a given disease is variable among individuals. We propose that this variable regulatory deficit is the result of “discrete holes” in an otherwise diverse TCR repertoire of regulatory CD4+ T cells. These holes would be the consequence of small variations in the number of peripheral cells at the time of thymectomy, causing the absence of particular TCR specificities in some but not other individuals. The absence/presence of certain TCR specificities in the repertoire of newborn regulatory CD4+ T cells may also depend on individual or strain variations in the expression time of tissue-specific antigens by the thymic stroma (42, 43). In this context, it should be noted that some of the candidate genes implicated in the genetic susceptibility to disease are inhibitors of the cathepsin S, which play an important role in antigen processing in the thymic medulla (44). The repertoire deficit in thymectomized mice would not be restricted to the regulatory compartment but would also affect the repertoire of naïve T cells. In other words, the fact that, in BALB/c mice, 40% of the thymectomized mice do not develop gastritis could also be explained by the absence of the respective disease-inducing cells. However, it has recently been shown that the incidence of gastritis is near 100% in neonatally thymectomized mice that were injected several times with poly(I:C) early after thymic ablation (45). This observation strongly indicates that healthy animals do contain gastritis-inducing cells, and it is compatible with the idea that tolerance in these mice is caused by the presence of the respective regulatory CD25+CD4+ T cells.

The final aspect worth discussing is why neonatal thymectomy affects regulatory cells controlling autoimmune syndromes but not those controlling IBD. A simple hypothesis is that a higher frequency of regulatory cells control IBD. Although, to our knowledge, there is no quantitative data on this issue, in transfer experiments, the numbers of cells necessary to induce or protect against IBD are one or even two orders of magnitude lower than those necessary to induce or protect from gastritis. Alternatively, it could be argued that IBD is more sensitive to suppression than autoimmune diseases. Available evidence actually supports the opposite, namely that the levels of inflammation in IBD are much higher than in, for example, gastritis, and therefore require a much stronger regulatory activity (46).

In summary, our results advance the understanding of the genesis of the peripheral compartment of Foxp3-expressing CD25+CD4+ T cells, and provide a better comprehension of the lymphopenic state caused by thymic ablation early in life.

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