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Pathogenic Effector T Cell Enrichment Overcomes Regulatory T Cell Control and Generates Autoimmune Gastritis

João P. Monteiro, Julia Farache, Ana Carolina Mercadante,* Julio A. Mignaco,† Martin Bonamino,* and Adriana Bonomo3*§

Regulatory T cells (Treg) deficiency leads to a severe, systemic, and lethal disease, as showed in immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome patients, and scurfy mouse. Postneonatal thymectomy autoimmune gastritis has also been attributed to the absence of Tregs. In this case however, disease is mild, organ-specific, and, more important, it is not an obligatory outcome. We addressed this paradox comparing T cell compartments in gastritis-susceptible and resistant animals. We found that neonatal thymectomy-induced gastritis is not caused by the absence of Tregs. Instead of this, it is the presence of gastritogenic T cell clones that determines susceptibility to disease. The expansion of such clones under lymphopenic conditions results in a reduced Treg:effector T cell ratio that is not enough to control gastritis development. Finally, the presence of gastritogenic clones is determined by the amount of gastric Ag expressed in the neonatal thymus, emphasizing the importance of effector repertoire variability, present even in genetically identical subjects, to organ-specific autoimmune disease susceptibility. The Journal of Immunology, 2008, 181: 5895–5903.

Foxp3+ regulatory T cell (Treg) activity is well-known as a key mechanism to control immune responses maintaining self-tolerance and immune homeostasis (1). Their absence, as exemplified in scurfy mice (2, 3) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome patients (4), leads to a severe, multiorgan-specific and lethal lymphoproliferative disease. Autoimmune disease after neonatal thymectomy (3dTx) also has been reported as the result of a lack of Tregs (1, 5). However, in contrast to scurfy mice, the disease that develops after 3dTx is mild and organ-specific (6, 7). Pathogenesis of such diseases involves recognition of organ-specific Ags by autoreactive CD4+ T cell clones in the draining lymph nodes, proliferation and migration of pathogenic clones, resulting in infiltration of mononuclear cells into the target organs, and production of specific autoantibodies (8, 9).

Although severe disease is observed in 100% of Treg-deficient mice (2, 3, 10), autoimmunity is not an obligatory outcome following 3dTx. The disease after 3dTx is highly variable among different mouse strains, with respect not only to susceptibility, but also to disease severity and specific target organ. Although some strains are highly susceptible to disease, such as BALB/c, A/J, and C3H/He, others are completely resistant, such as C57BL/6, AKR, DBA/2, and B10.A (11) and susceptibility is not linked to MHC alleles (11, 12). Finally, even in susceptible mouse strains, the incidence and pattern of disease is variable. Although thyroiditis, pancreatitis, and oophoritis/orchitis can be found in BALB/c mice, the predominant disease is gastritis, found in 60–70% of the individuals (5, 9, 13). In contrast, gastritis is uncommon in the A/J strain, which predominantly develops oophoritis (90%) (14). The reasons for this variability are unknown.

Several explanations for disease development after 3dTx have been proposed, including enrichment of the neonatal repertoire of pathogenic autoreactive T cell clones, as indicated by the escape of autoreactive immature T cell clones during the neonatal period (15–17). Also, the frequency of autoreactive clones in adult thymectomized mice is increased, and this is probably a consequence of the severe lymphopenic conditions found in 3dTx mice, which favor the expansion of self-reactive clones (15, 18, 19). However, lymphopenia does not seem to be the key event between susceptible and resistant mice. Both resistant and susceptible strains have similar cellularity (20), and susceptible mice do not develop diseases commonly related to long-lasting lymphopenic conditions such as inflammatory bowel disease (6, 11).

As regards the Treg depletion hypothesis, it is well established that at least part of the Treg repertoire is thymus-derived, and previous reports have suggested that thymus-derived CD25+ Tregs are absent during the first week of life (5). In addition, early transfer of CD4+CD25+ can prevent the development of disease in 3dTx mice (5). Altogether, these data had indicated that the primary event triggering autoimmunity in thymectomized mice is the lack of regulatory CD4+CD25+ T cells. However, CD4+CD25+ T cells have been found in both lymph nodes and spleens of adult 3dTx mice (18). Those cells do not seem exclusively to represent effector T cells (Teff), because they express Foxp3 mRNA, the most characteristic marker of Tregs, and are able to suppress autoimmune disease in vivo (21).

In this study, we asked whether susceptibility and resistance to 3dTx-induced autoimmunity are related to differences in the Treg
compartment. We chose autoimmune gastritis (AIG) as the model, because this is the best-characterized model of autoimmune post3dTx. In this disease model, the target Ag is well known (the H⁺K⁺ATPase of parietal cells) (22, 23). Resistance and susceptibility among a large number of mouse strains have been determined and the incidence of disease within a susceptible strain is not 100% (11). Our data show that susceptibility to AIG after 3dTx is not related to the numbers of Treg cells. Instead, disease is a consequence of increased numbers of gastrointestinal Teff, that are determined by the level of intrathymic expression of the gastric Ag, resulting in a low Treg:Teff ratio. The variation found in the intrathymic Ag expression rather than Treg frequency explains the differences in susceptibility to autoimmune diseases observed in genetically identical individuals within a susceptible background.

Materials and Methods

Animals

BALB/c (H-2d), C3H/He (H-2a), A/J (H-2a), DBA/2 (H-2a), B10.A (H-2a), C57BL/6 (H-2a), C.B17/SCID (H-2d) and BALB/cnu/nu (Nude, H-2d) mice were bred and maintained under the same conditions at the Instituto Nacional de Cancer animal breeding facilities (Rio de Janeiro, Brazil). The ages ranged from 3 days to 12 wk. All mice were used according to our institutional guidelines for animal well-being.

Neonatal thymectomy

Three-day-old mice were thymectomized as previously described (18). Sham-operated controls were subjected to a similar procedure, but not thymectomized. Complete thymectomy was confirmed in each adult animal by chest cavity exploration, and animals with incomplete thymectomy were discarded. For real-time PCR, thymuses obtained during thymectomy were collected individually, resuspended in TRIzol (Life Technologies) for RNA extraction and stored at −70°C.

Gastritis evaluation

Stomachs from adult 3dTx or 3dSham mice were harvested and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. Gastritis score was determined as previously described (24). The score was graded 0–2 (no gastritis), 2–4 (gastritis without parietal cell destruction), or 4–6 (severe gastritis with parietal cell destruction). A minimum of eight slices per stomach was evaluated and all analyses were blind. Alternatively, in some experiments gastritis was accessed by detection of anti-H⁺K⁺ATPase Abs in the serum by ELISA as previously described (13). Values to allow the discrimination of AIG⁺ and AIG⁻ mice were determined after analyzing anti-H⁺K⁺ATPase Abs titers in a population of 70 3dTx BALB/c mice. These results were correlated to the histopathologic gastritis score. The cutoff established to discriminate between AIG⁺ (histopathologic score >4) and AIG⁻ (histopathologic score <2) mice was the mean ± 2 SD.

Lymphoid organ analyses

Spleen and mesenteric or gastric lymph nodes (GLN) of adult (8–12-wk-old) 3dTx or 3dSham mice, and thymus, spleen, and pooled lymph nodes (axillaries, brachials, inguinals, popliteals, mesenterics, and gastric) of 3-day-old mice were harvested, the cells were dissociated in HBSS (Sigma-Aldrich) and used for further analyses. All animals were analyzed individually unless otherwise indicated.

Isolation of stomach cell infiltrates

Stomach cell infiltrates were isolated as previously described (24). The stomachs of BALB/c 3dTx or normal controls were harvested and the GLN was removed. The stomach was opened with an incision along its length, from the antrum to the fundus, the stomach contents were removed, and the organ was washed with cold PBS. Then, the stomachs were injected from the antrum to the fundus, the stomach contents were removed, and the organ was washed with cold PBS. The gastric mucosa was injected with 15–20 ml of cold PBS/5% FCS, using a 3-ml syringe with a 25-gauge needle. The cell suspension obtained was gently vortexed and passed through a 70-μm pore size nylon filter. Cells were washed in PBS/5%FCS and used for flow cytometry analyses.

Flow cytometry reagents

FITC anti-mouse CD4 (GK1.5), PE anti-mouse CD25 (7D4), FITC anti-mouse CD69 (3H.1.2F3), PE anti-mouse CTLA4 (CD152, clone UC10-4B9), PE anti-mouse GITR (DTA-1), PE-Cy5 anti-mouse CD25 (7D4), FITC anti-mouse BrdU (BRB-1), and allophycocyanin anti-rat/mouse Foxp3 staining kit (FJK-16) were all purchased from eBioscience.

Flow cytometry

For surface staining, cell suspensions were preincubated in 2% normal mouse serum HBSS (Sigma-Aldrich) to block FcγRIII/II receptors, washed, and incubated with appropriate Abs for 15 min. After a further wash, the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in HBSS or subjected to intracellular Foxp3 staining, standard protocol (eBioscience). All samples were acquired in a FACSCalibur (BD Biosciences). Acquisitions and analyses were performed using CellQuest software (BD Biosciences).

Assessment of in vivo T cell proliferation

Seven-day-old 3dTx mice were injected i.p. with 0.25 mg of BrdU (Sigma-Aldrich) in PBS. Eighteen hours later, the animals were sacrificed, the spleen or pooled lymph nodes were taken, and the cells were stained with anti-CD4 and anti-CD25 Abs. They were then fixed with 2% paraformaldehyde in PBS for 30 min at 37°C, permeabilized with cold 70% ethanol (Merck) for 30 min at −20°C, washed, and treated with 100 U/ml DNase (Sigma-Aldrich) in 0.15 M NaCl, 4.2 mM MgCl₂, 10 mM HCl (pH 5), for 40 min at 25°C. The cells were washed again and then stained with anti-BrdU and anti-Foxp3 (eBioscience) for 30 min at 37°C. BrdU incorporation was then assessed by flow cytometry.

Suppression assays

CD4⁺CD25⁺ T cells were purified from pooled lymph nodes of 8-wk-old 3dTx or 3dSham mice by positive selection on MACS, according to the manufacturer’s protocol (Miltenyi Biotec). CD4⁺CD25⁻ cells were >94% pure. Responder CD4⁺CD25⁺ cells were obtained from normal 8-wk-old BALB/c mouse mesenteric lymph nodes (MLN) by negative selection on MACS using anti-CD4/anti-B220/anti-CD25 magnetic beads (Miltenyi Biotec). Purity was >99%. For in vitro assay, purified CD4⁺CD25⁺ T cells were stained with 1 μM CFSE (Molecular Probes) and used as responders. Responder and 5 × 10⁵ CFSE-stained responder cells were cocultured at 1:1, 1:2, 1:5, 1:10, and 0.1 ratios in the presence of 5 × 10⁵ T cell-depleted splenocytes and 0.75 μg/ml anti-CD3 in DMEM with 10% FCS at 37°C for 72 h. CFSE dilution was analyzed by flow cytometry. For in vivo assay, 10⁵ purified CD4⁺CD25⁺ T cells were injected i.v. in Nude recipients, with or without 10⁵ CD4⁺CD25⁻ cells from 3dSham or 3dTx mice. Gastritis score was determined 6 wk after transfer.

Adoptive cell transfers

Adult 3dTx donors (60 days old) were sacrificed and mesenteric/GLN and the stomachs were taken individually. The stomachs were prepared for histopathological evaluation. Lymph node cells from each animal were subjected or not to regulatory T cell depletion using purified rat-anti-mouse CD25 (3C7) and rabbit serum as a source of complement. After lysis, the lymph node cells were resuspended in TRIzol (Life Technologies) for RNA extraction and stored at −70°C.

Analysis of H⁺K⁺ATPase β-chain expression by real-time PCR

Real-time PCR was performed on cDNA prepared from DNase-treated RNA obtained from the thymuses collected at the moment of thymectomy. cDNAs were run in triplicate, and the values were normalized to the endogenous reference, GAPDH. mRNA was measured by the Power SYBR Green System (Power SYBR Green PCR Master Mix, Applied Biosystems). The following primers were used for H⁺K⁺ATPase β-chain: forward: 5’ AGA GCT TGG CAG CTC AAA ACC 3’ (300 nM); reverse: 5’ CTT CCT CGA AGC AGC AGC TGG 3’ (300 nM) and for the constitutive GAPDH gene: forward: 5’ TGA AGT TGG TGT TGA ACG GAT TTG G 3’ (200 nM); reverse: 5’ ACG ACA TAC TCA GCA CCA GCA TCA C 3’ (200 nM). The PCR cycling conditions were used 95°C for 10 min and 50 cycles of 95°C for 15 s, 61°C for 1 min and 72°C for 1 s.
H⁺K⁺ATPase β-chain was detected on cycles 33–35. Values for H⁺K⁺ATPase β-chain expression in the neonatal thymus are expressed in arbitrary units where 1 unit is the level of ATPase expression for the animal with the highest gastritis score at adult age in each independent experiment. All analyses were performed at least twice for each sample with similar results.

Statistical analyses

Data are presented as mean ± SEM, except if described in the figure legend. Statistics were performed using one-way ANOVA with the Bonferroni posttest.

Results

Treg frequency is not affected by neonatal thymectomy and does not predict susceptibility to 3dTx-induced autoimmune disease

To investigate whether neonatal thymectomy results in the absence of Tregs in adult 3dTx mice, as previously suggested (5), 3-day-old mice from three AIG-susceptible (BALB/c, C3H/He, A/J) and three AIG-resistant (DBA/2, C57BL/6, B10.A) strains were thymectomized (3dTx) or sham-operated (3dSham). After 8 wk, the frequency of Tregs in spleen and MLN was determined by flow cytometry in MLN (A), spleen (B), or GLN (C). The results are presented as mean ± SEM of Treg frequency in the CD4⁺ population. White bars represent the 3dSham groups, gray bars the 3dTx groups of AIG-susceptible strains, and black bars the 3dTx of AIG-resistant strains. Data represent three independent experiments (n = 15–40 animals/group). *, p < 0.05.

The frequencies of Tregs in resistant and susceptible 3dTx mice (Fig. 1, A and B), clearly showing that susceptibility to thymectomy-induced autoimmunity did not result from the absence or reduced frequency of Tregs in peripheral lymphoid organs. If the Foxp3 population is separated into CD25⁺ and CD25⁻, the same results are observed (data not shown). Importantly, even when we compared absolute number of Tregs instead of frequency between susceptible and resistant strains, there was no sign of Treg depletion. Actually, their number was increased in the MLN of susceptible mice when compared with the resistant ones, while in the spleen no pattern was found (data not shown).

Treg frequencies in the GLN were also analyzed, because GLN are the main sites of activation of gastritogenic T cell clones (9) and are the places in which the gastric autoantigen is continuously being processed and presented by local dendritic cells (25). Fig. 1C shows that Tregs were present even in the GLN of AIG-susceptible mouse strains.

Tregs present in the periphery of susceptible 3dTx mice are fully functional

Although Tregs are present in the periphery of susceptible mice, they may not be functional. To tackle this issue, the phenotype and functional properties of 3dTx Tregs were studied. The results obtained using susceptible 3dTx BALB/c showed that their MLN and splenic Tregs have the usual Treg phenotype (1), expressing high levels of CD25, GITR, and CTLA-4 (Fig. 2A). Similar results were found in the other mouse strains (not depicted). In addition, purified 3dTx Tregs were as potent as 3dSham Tregs in inhibiting the
proliferation of purified effector CD4+ T cells in vitro (Fig. 2B) and gastritis development in vivo (Fig. 2C).

Tregs are present in the thymus and peripheral lymphoid organs of 3-day-old neonates

The relationship between Treg depletion and neonatal thymectomy was first proposed in reports that failed to find Tregs in the spleens of 3-day-old mice (5). Although we found functional Tregs in the periphery of adult 3dTx mice, these cells may not be present in the periphery of newborns. The absence of Tregs in such early period of life, associated with the lymphopenic condition that is intrinsic to the neonate and maintained after thymectomy (26), would create an environment that facilitates the expansion of pathogenic Teff clones. To investigate this hypothesis, we looked for Tregs in the thymus and peripheral lymphoid organs of 3-day-old neonates.

The low frequency of Tregs in neonatal thymuses (Fig. 3, A–C) was expected, because it was recently shown that thymic development of Tregs depends on medullary epithelial cells (27) and the thymic medulla is reduced at this age (28). However, it was completely unexpected that CD4+Foxp3+ Tregs were found in the periphery (spleen and pooled lymph nodes) of all mouse strains tested (Fig. 3, D and E). The Treg phenotype was the same as in the adults (Fig. 3, A and B). The frequencies were highly variable among different strains and once again, no pattern that could predict susceptibility to 3dTx-induced disease was found (Fig. 3, D and E).

Different degrees of lymphopenia could be associated with an increased susceptibility to post3dTx disease. However, the total number of total and CD4+ mesenteric lymph node cells in 4-day-old neonates do not statistically differ between susceptible and resistant strains (Fig. 3, F and G), showing that lymphopenia does not define susceptibility to disease.

Because Tregs are present at lower frequency in the periphery of neonates than of adults, it could be argued that there are insufficient numbers of them in 3dTx mice to suppress the emergence of pathogenic Teff. This possibility was investigated in the susceptible BALB/c strain by determining the behavior of the Treg population between postnatal day 3, when thymectomy is performed, and 3 mo, when disease is already established. The results show that the splenic Treg population expands more rapidly in 3dTx than in 3dSham mice (Fig. 4). The frequency of Foxp3+ within the

FIGURE 3. Tregs are present in thymus and periphery of newborn mice. Three-day-old mice from AIG-susceptible and resistant strains were tested for the presence of CD4+Foxp3+ regulatory T cells by flow cytometry. A and B, The top panel shows typical CD4/Foxp3 staining in the thymus, spleen and pooled lymph nodes of 3-day-old BALB/c (A) or DBA/2 mice (B). Lower panel, The phenotypic characterization of CD4+Foxp3+ (dark gray or black) and CD4+Foxp3− (light gray) lymph node cells from respective newborns. C–E, Frequency of CD4+Foxp3+ cells in thymus (C), lymph nodes (D), or spleen of 3-day-old mice (E). F and G, Analyses of individual MLN of 4-day-old newborns. F, Total cell count. G, Absolute number of CD4+ T cells. Gray bars represent AIG-susceptible strains while black bars represent AIG-resistant. n = 6–20 (A–E) or n = 5–10 (F and G).

FIGURE 4. Tregs expand earlier in 3dTx than in 3dSham BALB/c mice. The dynamics of the Treg population were evaluated by determining the frequency of Foxp3+ cells in the splenic CD4+ T cell population of BALB/c mice at different ages. Open circles (A) represent 3dSham BALB/c mice, and closed gray circles (B) are 3dTx BALB/c mice. B inset shows the frequency of proliferating Treg and Teff after an 18-h pulse of i.p.-injected BrdU in 7-day-old 3dTx BALB/c mice. Data represent two independent experiments (n = 3–15 animals/group).
CD4+ T cell population, rises abruptly immediately after thymectomy, during the first week of life, reaching the adult value on day 7, while in control mice this level is attained only on day 45 (Fig. 4, A and B). This abrupt rise in Treg frequency in 3dTx mice correlates with the high number of proliferating Tregs (~20%), as shown by BrdU incorporation in vivo (Fig. 4B, inset). Similar results are seen with mesenteric lymph node cells (data not shown).
Altered balance between effector and regulatory cells: a role for Teff in determining susceptibility to post3dTxAIG

The data obtained in adult 3dTx mice and 3-day-old neonates clearly show that lack of Treg is not a requirement for the development of autoimmune disease. Although Teff frequency is normal in 3dTx mice, it is not sufficient to prevent the expansion of pathogenic T clones. Therefore, the difference between susceptible and resistant mice could be due to the frequency of Tregs, but to the existence of effector CD4+ T cells clones that are able to trigger the disease. This possibility was investigated by studying the CD4+ T cell subpopulations in the GLN of AIG-susceptible BALB/c mice. We choose BALB/c because this strain is the prototype of this AIG model and all the available data on the pathophysiology of AIG were established using it. The frequency of post3dTxAIG in our BALB/c colony was 60% (n = 82) and this is consistent with the literature (5, 9, 13). Thus, we could compare animals with or without disease within the same genetic background.

Tregs, as already shown, are present in the GLN of adult 3dTx mice in higher numbers than in 3dSham (Fig. 5A). Regarding the activated effector population (Teff, CD4 A), the frequency of activated Teff and the Treg/Teff ratio found in the GLN was also found in the gastric mucosa infiltrate. Again, the frequency of Treg was around 30% in the cells obtained from the 3dTx AIG (Fig. 5B). The most important fact here is that while the Treg frequency has no relationship to the incidence/severity of AIG (Fig. 5D), the frequency of activated Teff and the Treg/Teff ratio are significantly higher in 3dTx AIG than in controls, resulting in lower Treg/Teff ratio (2.3 in AIG versus 9.7 in AIG−, Fig. 5H).

The reduced Treg/Teff ratio found in the GLN was also found in the gastric mucosa infiltrate. Again, the frequency of Treg was around 30% in the cells obtained from the 3dTxAIG+ animals, while it was half of this value in both 3dTxAIG− and normal BALB/c controls. However, the frequency of activated Teff was at least eight times higher in the 3dTxAIG+ than in the controls, resulting in lower Treg/Teff ratio (2.3 in AIG+ vs 9.7 in AIG−, Fig. 5H).

The results from BALB/c mice described above were confirmed for C3H/He, another post3dTxAIG susceptible strain. As shown for BALB/c, 3dTxC3H/He also show an increased frequency of activated Teff (Fig. 5f) and a decreased Treg/activated Teff ratio (Fig. 5f) compared with 3dSham mice. These disturbances in the Teff/Treg balance for 3dTx vs 3dTxAIG mice in susceptible strains were not found in the resistant strains DBA/2 and B10.A (Fig. 5, I and J), strongly suggesting an important role for the effector repertoire.

Teffs and not Tregs from 3dTxAIG BALB/c mice are the determinants of AIG susceptibility

Resistance to disease in genetically susceptible 3dTx mice could possibly be related to qualitative differences among Treg populations in mice with and without AIG. If this is true, we could predict that transfer of Treg-depleted lymph node cells from AIG-negative 3dTx BALB/c mice to syngeneic immunodeficient recipients would result in AIG because the gastritogenic T cell clones would be free of any Treg control. It is already clear from the literature that when immunodeficient hosts receive Treg-depleted lymph node cells from a nonselected pool of donors, 100% of the recipients develop AIG, demonstrating that all animals have the peripheral requirements to become ill (29). With all the above in mind we tested the hypothesis by transferring 107 total lymph node cells or CD25− cells from 8-wk-old 3dTx BALB/c donors individually to syngeneic SCID or BALB/c Nude mice so that the AIG scores could be compared between donor and recipient. Development of gastritis in the recipients was evaluated 6 wk after the transfer. We found that the recipients that developed AIG were those receiving cells from mice with AIG and this was independent of Treg depletion (Fig. 6) in the majority of the cases (39 in 41 immunodeficient hosts transferred). The absence of disease in recipients that received LN cells from AIG− mice was not due to an inefficient Treg depletion protocol, because depletion was confirmed by flow cytometry for each individual donor. Moreover, transfer of CD25-depleted LN cells from pooled normal or 3dTx BALB/c mice to immunodeficient recipients, using the same depletion protocol, resulted in gastritis induction in 100% of the recipients (Fig. 6B). It should be pointed out that all the recipients receiving CD25-depleted cells showed signs of lymphoproliferative disease, with enlarged spleen and lymph nodes, but this syndrome is independent of the development of gastritis (data not shown). However the incidence of severe gastritis is higher in the CD25− recipients than in the ones that receive total LN cells, indicating that Tregs play some role in the severity of disease, as long as the effectors are...
The levels of ectopically expressed thymic H⁺K⁺ ATPase β-chain in the neonatal thymus. Three-day-old newborns were thymectomized. The thymuses were recovered individually and the relative expression of H⁺K⁺ ATPase β-chain was determined by real-time PCR. After 8 wk, the adult 3dTx BALB/c were sacrificed and the AIG score was determined blindly. The AIG score obtained for each animal was then correlated with the level of H⁺K⁺ ATPase β-chain expressed in its thymus collected at day 3. The results show clear differences in the levels of expression of the same autoantigen in individuals with the same genetic background (Fig. 7). The results show unequivocally that the differences obtained are functionally relevant. The highest levels of H⁺K⁺ ATPase β-chain were found in the AIG-resistant BALB/c animals, and all AIG-susceptible mice of the same strain expressed very low levels of H⁺K⁺ ATPase β-chain in their thymuses (Fig. 7, A–D; each panel represents an independent experiment).

Because resistance to gastritis in susceptible BALB/c strain was intimately related to the level of H⁺K⁺ ATPase β-chain expression in the thymus, this could also be true for strains in which 100% of the individuals are resistant to post3dTx-AIG. We checked the level of expression of H⁺K⁺ ATPase β-chain in the thymuses of neonatal mice from the resistant DBA/2 strain. Surprisingly, the results were very similar to those obtained with the BALB/c strain. The levels of ectopically expressed H⁺K⁺ ATPase β-chain were highly variable, and the expected pattern, that high expression would be confined to resistant animals, was not observed (Fig. 7, E and F). These results imply that genetic susceptibility or resistance among different mouse strains depends on other mechanisms that are not determined within the thymus.

**Discussion**

The rebirth of suppressor cells as Tregs and their establishment as a major mechanism for controlling not only autoimmunity but also the amplitude of immune responses in general was based on the postneonatal thymectomy autoimmune disease model (1, 7). However, the phenotype observed in individuals genetically deficient in Tregs differs from the one observed after neonatal thymectomy. In the former case, a severe, multiorgan-specific lymphoproliferative lethal disease is observed in all the individuals (2, 3, 10). In the latter, the phenotype is of a mild, nonlethal organ-specific autoimmune disease (6, 7). Besides, autoimmunity is not an obligatory outcome following 3dTx. Several mouse strains are resistant to disease and even in susceptible strains not all individuals develop autoimmunity (11). There is no doubt that Tregs transferred to 3dTx mice during the first week after thymectomy reduce the incidence of AIG from 60% to virtually zero (5). Our data, however, suggest that the primary event triggering autoimmunity is not the lack of Tregs, but the existence of a population of gastritogenic T cells that take advantage of a genetically favorable background (11), a lymphopenic environment (6, 26), and a reduced T cell repertoire (19, 30) to expand and escape the surveillance of Tregs. In this situation, the frequency of Tregs is “normal”, but it would suffice to control autoimmunity only under normal conditions, i.e., a larger effector repertoire without lymphopenia. In the new steady state induced by thymectomy, the “indexation” between the Treg and Teff populations (31) is lost and a much higher Treg frequency is needed to avoid disease. This view explains why the transfer of Tregs after 3dTx inhibits the disease and why it only works during a short interval after thymectomy, i.e., before the effectors have expanded beyond Treg control.

We do not know whether the Treg population found in adult 3dTx mice is due to proliferation of thymic-derived Tregs or conversion of effector cells to the regulatory phenotype or both. The low frequency of Tregs in the neonatal thymus is consistent with recent data showing that the generation of thymic Tregs during this period is limited by a reduced thymic medulla (32). Nevertheless, we show that Treg can be found in spleen and MLN of 3-day-old neonates from all mouse strains analyzed. Previous reports found no Tregs in the spleen of 3-day-old neonates by flow cytometry.
using anti-CD3 and anti-CD25 Abs (5). This was probably due to the small number of events acquired, the use of anti-CD3 Ab rather than anti-CD4, and the unavailability of specific markers to identify the Treg population. Neonatal Tregs proliferate extensively after thymectomy, reaching a plateau after the first week of life. This early peak of Treg proliferation accompanies the proliferation kinetics of the Teff population. This association is expected, because proliferation of Tregs is IL-2-dependent and they are poor IL-2 producers, exploiting cytokine production by Teffs to proliferate (31, 33).

The frequency of Tregs achieved during the first week of life in 3dTx mice is maintained throughout adulthood, resulting in functional Tregs with the usual phenotype and suppressive properties both in vitro and in vivo. Our results confirm those published by Bandeira and colleagues (21) showing that CD4+CD25+ Tregs from 3dTx mice can inhibit inflammatory bowel disease, and more recently by Tung and colleagues (34) showing the presence of functional Tregs in a model of thymectomy-induced oophoritis.

In the gastritis model studied here, the frequency of Tregs even in the GLN does not correlate with the incidence or severity of post3dTx autoimmunity in susceptible BALB/c mice. Rather, the frequency of activated Teff distinguishes between mice with and without gastritis. In the GLN, this population is highly enriched in H+K+-ATPase-specific T clones (9). Moreover, there is a positive linear correlation between activated Teff frequency and gastritis severity, and the increase in activated Teff frequency at constant normal Treg frequency results in a decreased ratio between these two populations in 3dTx mice compared with controls. This ratio correlates negatively with disease severity, once more suggesting that the relative absence of Tregs in relation to the number of effectors is the key determinant of disease. It can be argued that analyzing the gastritogenic T cell frequency after the disease is established introduces a bias, and it does not mean that 3-day-old neonates that will or will not develop gastritis necessarily differ in the frequency of gastritogenic T cells. However, if the frequency of gastritogenic T cells in the periphery of resistant BALB/c is low, under the control of Tregs, we would expect that when those cells are transferred to a new lymphopenic host in the absence of Tregs they would expand and cause gastritis. Our results show that this is not the case: disease is transferred by gastritic donors and this does not depend on the absence of Treg in the inoculums.

These and the fact that there are no peripheral limitations to gastritis development (100% of immunodeficient BALB/c recipients become gastritic upon receiving gastritogenic T cells from Treg-depleted or 3dTx pooled donors; Ref. 24, 29), suggest that there are few if any gastritogenic clones in the repertoire of 3dTx BALB/c mice that do not develop disease. Although the presence of effector gastritogenic clones triggers disease, Treg appears to control disease progression and severity. Actually, all immunodeficient recipients that received CD25-depleted LN cells from gastritic donors showed signals of severe disease, while only 60% of recipients receiving total LN from gastritic donors showed gastritis with partial cell destruction. This viewpoint is supported by recent data showing that Treg depletion in adult B6AF1 3dTx mice results in a much severe oophoritis (34).

Because gastritogenic T cell clones seem to be responsible for susceptibility to AIG, we tried to identify the factors that enable those clones to be generated. Ectopic thymic expression of autoimmune antigens that are targets for organ-specific autoimmune diseases has been extensively demonstrated (35–39). As regards AIG, the major target Ag is parietal cell H+K+-ATPase, composed of two chains, α and β (22). Pathogenic T clones specific for both chains have already been described, although high-affinity anti-αH+K+-ATPase clones currently seem to be dominant (23, 40). Importantly, transgenic expression of H+K+-ATPase β-chain under the MHC class II promoter is related to the protection of BALB/c mice against post-3dTx gastritis (13). In anti-αH+K+-ATPase TCR transgenic mice, T cell clones directed against the α-chain are not deleted under normal conditions. However, if the β-chain is overexpressed in thymically, the anti-αH+K+-ATPase clones are eliminated, indicating that the endogenously expressed α-chain is enough to delete most of the transgenic clones. The above results indicate that the amount of β-chain expressed in the thymus is the limiting factor to induce tolerance to both α and β-chains (41). The α-chain mRNA is found in thymic dendritic and epithelial cells and its expression is autoimmune regulator independent (35, 42). However, thymic expression of the β-chain has been a controversial issue. Some reports failed to detect it (35, 42), while others detected it using more sensitive approaches (43, 44). Despite this controversy, unfractionated splenocytes from H+K+-ATPase β-deficient mice, but not H+K+-ATPase β-expressing mice, induce AIG after adoptive transfer to BALB/c nude mice (45). Altogether it is reasonable to propose that various levels of expression of the ectopically expressed Ag contribute to different post-3dTx outcomes. In fact, using real-time PCR, we were able to detect H+K+-ATPase β-chain mRNA in the neonatal thymus and we showed that its level determines whether a BALB/c mouse will develop gastritis or not. We favor the view that high H+K+-ATPase β-chain expression protects BALB/c mice against gastritis mainly by promoting negative selection of gastritogenic T cell clones. Positive selection of anti-H+K+-ATPase Treg clones seems unlikely because a Treg-depleted population from healthy thymectomized donors does not transfer disease to immunodeficient hosts. Moreover, polyclonal Tregs derived from both H+K+-ATPase α and β-chain knockout mice inhibit AIG as efficiently as Tregs derived from wild-type mice (46).

Although high thymic expression of the H+K+-ATPase β-chain confers resistance to mice that are genetically susceptible to AIG, this is not true for genetically resistant strains. Our results show that H+K+-ATPase β-chain expression is as variable in DBA/2 as it is in BALB/c thymuses. Altogether, these results suggest that the mechanisms that regulate H+K+-ATPase β-chain expression in the thymus are the same in both strains. Therefore, resistance to gastritis can be stratified on at least two levels. The first results from the genetic background of the strain, which has or lacks the potential to develop gastritis and is inherited, being the same for all individuals of the same strain. If the strain does not have this potential, gastritis will not develop even if gastritogenic clones are present. The second results from regulation of H+K+-ATPase β-chain expression in the thymus, which has no relationship to the animal background, is set individually and can overcome the genetic potential of the susceptible strains, turning susceptible mice into resistant ones.

Our results bring back the focus of organ-specific autoimmune diseases research to the effector repertoire and its variability present even in genetically identical individuals. Further studies will be necessary to understand the variables operating in genetically identical individuals and the differences between susceptible and resistant strains.

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