The Journal of Immunology

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J Immunol 2003; 171:4504-4511; ;
doi: 10.4049/jimmunol.171.9.4504
http://www.jimmunol.org/content/171/9/4504

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Expression of Activated Notch3 in Transgenic Mice Enhances Generation of T Regulatory Cells and Protects against Experimental Autoimmune Diabetes[1]

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Thymic-derived dysregulated tolerance has been suggested to occur in type 1 diabetes via impaired generation of $CD4^+CD25^+$ T regulatory cells, leading to autoimmune β cell destruction. In this study, we demonstrate that Notch3 expression is a characteristic feature of $CD4^+CD25^+$ cells. Furthermore, streptozotocin-induced autoimmune diabetes fails to develop in transgenic mice carrying the constitutively active intracellular domain of Notch3 in thymocytes and T cells. The failure to develop the disease is associated with an increase of $CD4^+CD25^+$ T regulatory cells, accumulating in lymphoid organs, in pancreas infiltrates and paralleled by increased expression of IL-4 and IL-10. Accordingly, $CD4^+$ T cells from Notch3-transgenic mice inhibit the development of hyperglycemia and insulitis when injected into streptozotocin-treated wild-type mice and display in vitro suppressive activity. These observations, therefore, suggest that Notch3-mediated events regulate the expansion and function of T regulatory cells, leading to protection from experimental autoimmune diabetes and identify the Notch pathway as a potential target for therapeutic intervention in type 1 diabetes. *The Journal of Immunology,* **2003, 171: 4504–4511.**

Type 1 diabetes is a chronic autoimmune disease that results from the destruction of insulin-producing β cells (reviewed in Ref. 1). β cell destruction appears to be mediated by Th1 $CD4^+$ and cytotoxic $CD8^+$ lymphocytes and by macrophages via the action of IFN-γ, TNF-α, and IL-1 proinflammatory cytokines, Fas-FasL interactions, perforins, and granzymes (1). Moreover, type 1 diabetes is characterized by autoantibodies generated against islet cell Ags (2, 3).

Thymic-derived dysregulated tolerance, leading to an imbalance between β cell Ag self-reactive T cells and regulating factors, is a key event in autoimmune diabetes (4, 5). The autoantigens glutamate decarboxylase 65, IA-2, tyrosine phosphatase, and insulin in thymic stromal cells in addition to high-affinity glutamate decarboxylase 65-reactive T cells have been reported to be associated with diabetes (6–8), suggesting that these proteins combined with MHC expression on APC in the thymus are physiologically sufficient for the negative selection of autoreactive T cells and the establishment of immune tolerance. Studies of organ-specific autoimmune disease, in a number of experimental models, provide convincing evidence that specialized thymic-derived regulatory T cells (T-reg)[4] are capable of controlling autoimmunity and are an integral part of the T cell repertoire (9, 10). More specifically, development of autoimmune diabetes is inhibited by naturally occurring thymic-derived $CD4^+CD25^+$ T-reg cells, suggesting that their impaired generation might be involved in disease pathogenesis (10–14).

Regulated CD25 expression is a hallmark of intrathymic T-reg cell generation (15) and it has been recently reported that $CD25^+CD4^+$ thymocytes develop at the late $CD8^+CD4^+$ double-positive stage from a precursor pool, progressing from the $CD8^{low}CD4^+CD25^+$ to the $CD4^+CD25^+$ developmental stage (16).

A number of recent reports have indicated that the Notch pathway represents a major regulatory network underlying intrathymic T cell differentiation. The Notch pathway includes a conserved family of transmembrane receptors (Notch 1–4) that interact with a number of specific ligands (Delta-like family members, Jagged/Serrate 1 and 2) to regulate cell fate specification, cell growth, and differentiation in a variety of vertebrate and invertebrate cell lineages (17).

The role of members of the Notch receptor family in lymphoid cell development has been addressed in transgenic and/or knockout mouse models, which demonstrate that Notch1 regulates T cell vs B cell outcome (reviewed in Ref. 18), whereas Notch3 controls pre-TCR-dependent $CD4^-CD8^-$ double-negative $CD25^+$ to double-positive $CD25^-$ transition (19, 20). A striking feature of Notch3-I-intracellular domain (IC)/transgenic (Tg) mice is CD25 up-regulation in thymocytes and T cells (20). Therefore, dysregulated CD25 expression may underlie Notch3-specific events that lead to T-reg cell generation.

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Received for publication February 21, 2003. Accepted for publication August 27, 2003.

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[1] This work was supported by the Associazione Italiana per la Ricerca sul Cancro, the Ministero dell'Istruzione, dell'Università e della Ricerca, and the Ministero della Salute and the Molecular Biology and Medicine Center of Excellence.

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[4] Abbreviations used in this paper: T-reg, T regulatory; ld-STZ, low-dose streptozotocin; Tg, transgenic; GITR, glucocorticoid-induced TNFR; wt, wild type; IC, intracellular domain; FCA, flow cytometry analysis.

0022-1767/03/$02.00

These observations have prompted us to study the role of dysregulated Notch3 signaling in T lymphocytes involved in the immune response that underlies the development of autoimmune diabetes. To this purpose, previously generated transgenic mice expressing the constitutively active Notch3 intracellular domain (Notch3-IC) in thymocytes and T cells (20) were examined for the generation of multiple low-dose streptozotocin (ld-STZ)-induced experimental diabetes. The development of hyperglycemia, the presence and grade of insulitis, the presence of T-reg cells in peripheral lymphoid organs and pancreas, their expression of regulatory cytokines, as well as their in vivo and in vitro regulatory functions, were evaluated to understand the specific role for dysregulated Notch3-mediated events.

Materials and Methods

Mice

The generation, genetic background, and typing of Notch3-IC-transgenic (Notch3-IC Tg) mice have been described elsewhere (20). Notch3-IC Tg mice and wild-type (wt) littermates were used in this study. Notch3-IC Tg mice have been previously described to develop aggressive T cell lymphomas (20). To minimize the possibility that tumor development interferes with the effect of Notch3 on T-reg cell differentiation and functions, we used Notch3-IC Tg mice derived from the no. 31 line, characterized by a late onset (10 wk of age) of the Notch3-IC-associated T cell lymphoma while preserving all of the previously described effects of Notch3-dysregulated expression on T cell differentiation (20). Notch3-IC Tg and wt male mice (age 4 wk) received i.p. injections of 40 mg/kg STZ (Sigma-Aldrich, St. Louis, MO) dissolved in citrate buffer for 5 consecutive days. Nonfasting blood was obtained from the tail via a capillary tube and used for glucose levels determination. Mice were followed up for 22 days and were sacrificed at day 9 and at day 22 from the beginning of the STZ treatment. Two untreated animals were included at each time point in each experiment. We defined as representing diabetes nonfasting glucose levels $\geq$250 mg/dl.

Mice were bred and maintained in the Department of Experimental Medicine and Pathology Animal Facility according to the authorization of the Italian Ministry of Health and the experimental procedures were conducted following the regulations established by the Italian animal care laws (D.L. 116, dated January 27, 1992).

Flow cytometry analysis (FCA)

Freshly isolated cells from thymi, spleens, and lymph nodes were prepared and stained as previously described (20) and analyzed on FACScan (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences). Forward and side scatter gating were used to exclude dead cells from the analysis. Cells were stained with FITC-, PE-, or biotin-conjugated mAbs against: CD4 (H-129.19), CD8a (53-6.7), and CD25 (IL-2Rα chain 7D4; BD PharMingen, San Diego, CA). For intracellular staining with anti-CTLA-4, anti-Notch3 and anti-IL-10 cells were treated as previously described (21, 19) and stained with PE-conjugated hamster anti-mouse CD152 (CTLA-4, UC10-4F10-11), PE-conjugated rat anti-mouse IL-10 (JES5-16E3; both from BD PharMingen), or rabbit polyclonal Ab against the intracellular domain of Notch3 protein (sc-7424; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. FITC-conjugated affinity-purified goat anti-rabbit IgG (F-1262; Sigma-Aldrich) was used as secondary Ab to detect unconjugated Notch3. PE- and FITC-conjugated rat and hamster IgG (BD PharMingen) were used as controls.

RNA analysis

Total RNA was prepared from pancreas and lymphocytes in guanidine isothiocyanate and further processed for RT-PCR as previously described (19). PCR were performed at the annealing temperature of 61°C using the following mouse primers: IL-4: 5′ (5′-ATGGGTCTCAACCCCCAGCTAGT-3′) and 3′ (5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′); IL-10: 5′ (5′-TCAAACAAAGGACCAGCTGGACAACATACTGC-3′) and 3′ (5′-CTGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′); TGF-β: 5′ (5′-CGGGGCGACCTGGGCACCATCCATGAC-3′) and 3′ (5′-CTGCTCCACCTTGGGCTTGCGACCCAC-3′); Jagged2: 5′ (5′-GTCCTTCCCACATGGGAGTT-3′) and 3′ (5′-GTTTCCACCTTGACCTCGGT-3′); Notch1: 5′ (5′-GTGGATGCTGACTGCATGGATGTC-3′) and 3′ (5′-ATGCAAAGCCGACTTGCCTAGGTC-3′); HES-1: 5′ (5′- ATGCCAGCTGATATAATGGAG-3′) and 3′ (5′-CACGCTCGGGTCTGTGCTGAG3′); glucocorticoid-induced TNFR (GITR): 5′ (5′-CCATGCTGTATGGAGTCTCG-3′) and 3′ (5′-TTGCAGATC TTGCACTGAGG-3′); Deltex: 5′ (5′-CACTGGCCCTGTCCACCCAGCCTTGGCAG-3′) and 3′ (5′-ATGCGAATTCGGGAAGGCGGGCAACTCAG-3′); and β-actin: 5′ (5′-GTGGGCCGCTCTAGGCACCAA-3′) and 3′ (5′-CTCTTTGATGTCACGCACGATTTC-3′). To quantitate expression levels of the transcripts, PCR were conducted in the linear exponential phase of amplification throughout 15–35 cycles and samples loading was monitored by a β-actin transcript that was subjected to the same treatment.

Histology

Pancreata were removed, snap frozen in liquid nitrogen, and stored at −80°C. Serial cryostatic sections (5-μm thick) were cut from each pancreas: H&E-stained slides were used to study the presence of insulitis.

Affected islets were assorted in scoring categories as follows: grade 0, no significant lymphoid infiltration; grade 1, with lymphocytes present as surrounding the islet; grade 2, with lymphocytes present also inside the islet, without altering its normal architecture; and grade 3, with lymphocytes massively infiltrating the islet and altering its normal architecture.

To evaluate insulin-positive cells, acetone-fixed cryostatic sections were stained for insulin by using indirect immunofluorescence and studied by laser confocal microscopy (Zeiss LSM 310; Zeiss, Oberkochen, Germany). Guinea pig anti-insulin polyclonal Ab was used as primary Ab (A 0654; DAKO, Glostrup, Denmark). The secondary Ab was FITC-conjugated rabbit anti-guinea pig IgG (DAKO).

To analyze the phenotype of lymphocytes infiltrating the pancreas and the pancreatic expression of IL-10, tissue sections were immunolabeled with anti-mouse CD 152 (CTLA-4, UC10-4F10-11; BD PharMingen) and IL-10 (JES052A5; R&D Systems, Minneapolis, MN) mAbs, followed by FITC-conjugated secondary Abs and examined by confocal microscopy.

Cell purification

Unfractionated thymocytes were incubated with anti-CD8 microbeads (Miltenyi Biotec, Auburn, CA) and separated on LD depletion columns (Miltenyi Biotec) to deplete the CD8-expressing cells, then the obtained $CD8^-$ cells were positively selected from the flow-through by anti-CD4 microbeads staining and separation by LS separation columns (Miltenyi Biotec) according to the suggested protocol (Miltenyi Biotec). Total T lymphocytes and $CD4^+$ subsets were purified from spleen lymphocytes by positive separation after incubation with anti-CD90 (Thy 1.2)- and anti-CD4-coated microbeads, respectively, as described above. In the proliferation assay, magnetically T cell-depleted spleen cells (3000 rad) from wt mice were used as APCs. Sorted populations were >95% pure, as assessed by FACS analysis.

In vitro proliferation assay

Along with APCs (10×10^4) and $Thy1.2^+$ T splenocytes (2.5×10^4), both obtained from wt mice, Tg, or wt $CD4^+$ thymocytes or splenocytes (2.5×10^3), sorted as described above, were cultured for 72 h in 96-well round-bottom plates (Costar, Cambridge, MA) in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and anti-CD3 mAb (145-2C11; BD PharMingen) at a final concentration of 10 μg/ml. Cultures were pulsed with 1 μCi/well [^{3}H]TdR for the last 6 h of culture and [^{3}H]TdR incorporation by proliferating lymphocytes was measured and expressed as cpm. In another set of experiments, 2.5×10^4 wt $CD4^+$ splenocytes, along with wt APCs (10×10^4), were incubated as responder cells with graded numbers of Tg $CD4^+$ splenocytes as suppressor cells.

In vivo adoptive cell transfer

$CD4^+$ thymocytes (6×10^6/mouse) from wt or Tg mice, selected as described above, were resuspended in 200 μl of PBS and injected in the tail vein of wt male recipient mice (age 4 wk) 4 days before the ld-STZ treatment induction, described above. The progression to diabetes was monitored throughout 25 days after drug induction.

Results

Expansion of T-reg cells in lymphoid organs of Notch3-IC Tg mice

We previously generated Notch3-IC Tg mice in which a hemagglutinin epitope-tagged intracellular domain of the Notch3 receptor was driven by the lck proximal promoter, resulting in constitutive expression of activated Notch3 in thymocytes and T cells (20). Although no obvious impairment of the thymocyte subset distribution with respect to $CD4^+$ and/or $CD8^+$ cells was observed in the thymus from Tg animals, there was a reproducible increase

in total thymocyte yield and in the CD25-expressing cells in Tg vs wt littermates (20). To investigate whether Notch3-IC Tg mice display an imbalance toward T-reg cell development, we studied the distribution of the $CD8^{low}CD4^+CD25^+$ subset in the thymus and of the $CD4^+CD25^+$ subset in thymus, spleen, and lymph nodes of Notch3-IC Tg with respect to wt mice. Fig. 1 shows a significant increase of $CD8^{low}CD4^+CD25^+$ thymocytes and $CD4^+CD25^+$ cells in thymus (Fig. 1*A*) and spleen (Fig. 1*B*) of Tg vs wt mice.

In addition to CD25, T-reg cells carry additional markers including CTLA-4, which plays a role in mediating regulatory function (21). CTLA-4 expression by lymphocytes obtained from thymus and spleen of Notch3-IC Tg mice exhibited a significant increase, when compared with wt littermates, as indicated by increased total numbers of $CTLA\text{-}4^+CD25^+$ lymphocytes (3.2×10^6 vs 1×10^6 and 2.7×10^6 vs 0.8×10^6 for thymocytes and splenic T cells, respectively; Fig. 1*F* and not shown). As for the $CD4^+CD25^+$ cells in thymus and spleen, the expansion of $CTLA\text{-}4^+CD25^+$ thymocytes not only paralleled the overall increase of thymocyte yield, but also represented a larger percentage among thymocytes in Notch3-IC Tg when compared with wt thymus (1.62 vs 0.26%; Fig. 1*F*, *right panel*).

Several studies have provided strong evidence for a role of cytokines in the effector function of T-reg cells in vivo. In particular, IL-4 and IL-10 have been suggested to protect mice from autoimmune diseases, including type 1 diabetes (reviewed in Ref. 22). Fig. 1*C* displays significantly increased levels of IL-4 and IL-10 mRNAs in lymphocytes from thymus, spleen, and lymph nodes of Notch3-IC Tg when compared with wt mice. To directly address which cells IL-4 and IL-10 regulatory cytokines originate from, we performed a flow cytometry three-color analysis of CD4 vs CD25 vs intracellular IL-4 or IL-10 in splenocyte suspensions from Notch3-IC Tg mice. Fig. 1*D* shows that a significantly higher percentage of $CD4^+CD25^+$ splenocytes expresses intracellular staining for IL-10 when compared with $CD4^+CD25^-$ cells (72.3 vs 13.4%, respectively), suggesting that the increased levels of this cytokine originate from $CD4^+CD25^+$ cells. Although IL-4 expression is higher in overall Notch3 Tg with respect to wt T cell populations (Fig. 1*C*), no significant difference was observed in the expression of this cytokine by $CD4^+CD25^+$ and $CD4^+CD25^-$ cells (data not shown).

A role in regulating $CD4^+CD25^+$ T-reg suppressive function was recently suggested for TGF-β as well as for the GITR (reviewed in Ref. 23). However, TGF-β and GITR mRNA expression were not modulated in T lymphocytes from either thymus or spleen of Notch3-IC Tg, when compared with wt mice, as assessed by a semiquantitative RT-PCR assay (Fig. 1*E*).

Together, these results show that Notch3-IC Tg mice exhibit an imbalance of $CD4^+$ subset distribution, characterized by an expansion of $CD4^+CD25^+$ lymphocytes which may represent a T-reg subset, as for their expression of CTLA-4 and regulatory cytokines.

To demonstrate the specific role of Notch3 in sustaining the generation and the expansion of T-reg in Notch3-IC Tg mice, we analyzed the mRNA expression of other Notch receptors and cognate ligands. Namely, we considered the expression of Notch1 that has previously been described to have a crucial role in T cell commitment and in thymocyte differentiation (reviewed in Ref. 18) and of the Jagged2 ligand that we previously showed to be readily detectable in the lymphoid compartment of murine thymus (19).

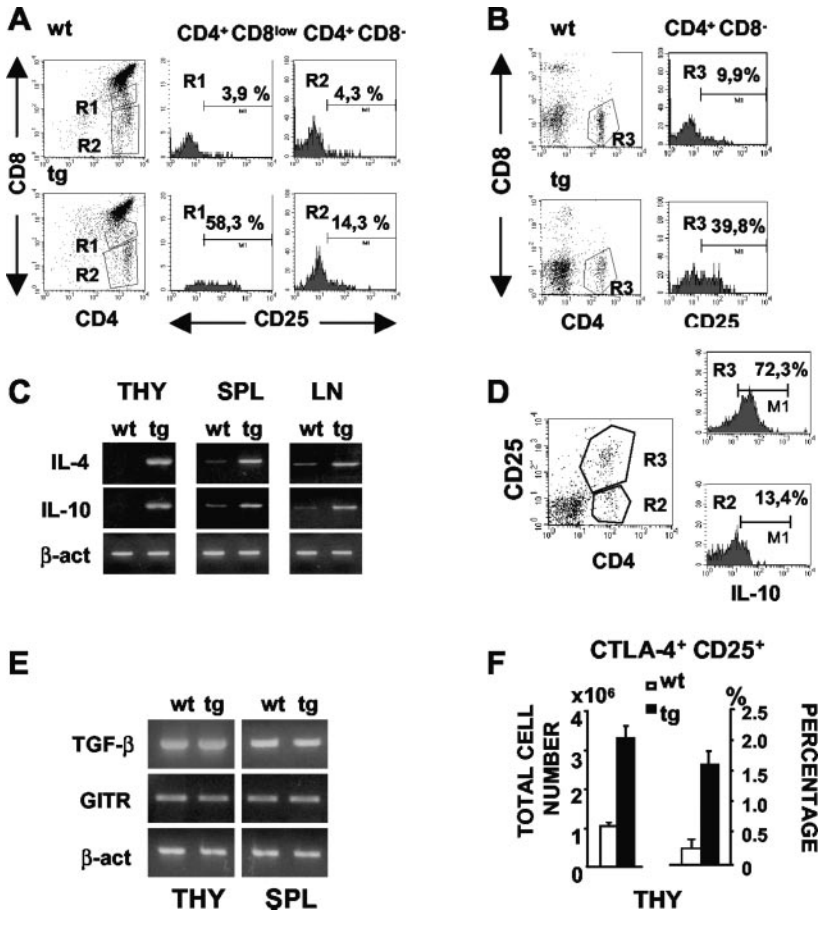

FIGURE 1. T-reg cell distribution and characterization in lymphoid organs of 4-wk-old Notch3-IC Tg mice compared with wt littermates. Three-color analysis of CD25 vs CD4 vs CD8 expression in thymocytes: $CD4^+CD8^{low}$ (R1 = 5.8 and 4.4% in Tg and wt, respectively) and $CD4^+CD8^-$ (R2 = 10.9 and 10.5% in Tg and wt, respectively, *A*) and in splenocytes: $CD4^+CD8^-$ (R3 = 26.4 and 36.2% in Tg and wt, respectively, *B*). Numbers indicate the percentages of $CD25^+$ cells. *Left panels*, The T lymphocyte subset distribution. *C*, Expression of IL-4 and IL-10 mRNAs assessed by RT-PCR in lymphocytes from thymus (THY), spleen (SPL), and mesenteric lymph node (LN) of wt and Tg animals. Cytokine expression was monitored along the exponential phase of the amplification and normalized to β-actin (β-act) expression. The results are representative of three independent experiments. *D*, Three-color analysis of CD4 vs CD25 vs intracellular IL-10 expression in Tg T splenocytes. Numbers indicate percentages of $IL\text{-}10^+$ cells. *Left panel*, The distribution of $CD4^+$ splenocytes (R2 = $CD4^+CD25^-$; R3 = $CD4^+CD25^+$). *E*, Expression of TGF-β and GITR mRNAs assessed by RT-PCR in lymphocytes from thymus (THY) and splenic T cells (SPL) of wt and Tg animals. mRNAs expression was monitored along the exponential phase of the amplification and normalized to β-actin (β-act) expression. The results are representative of three independent experiments. *F*, Total cell number (*left panel*) and percentage (*right panel*) of $CTLA\text{-}4^+CD25^+$ T lymphocytes from thymus (THY) of wt (□) and Tg (■) mice (detected by CD25 vs intracellular CTLA-4 two-color FCA).

Semiquantitative RT-PCR analysis of both thymocyte and splenocyte mRNAs from 4-wk-old mice revealed no significant differences in the expression levels of Notch1 and Jagged2 either in thymocytes or in T splenic lymphocytes from Notch3-IC Tg mice, when compared with the wt littermates (Fig. 2*A*). In contrast, the Tg expression of a constitutively active Notch3 induces the activation of specific Notch target genes, such as *Hes1* and *Deltex*, in Tg T lymphocytes. Indeed, as previously described (20), we observed an increase in the expression levels of both of these genes in thymocyte RNA as well as of the *Deltex* transcript in splenic T cell RNA of Tg animals (Fig. 2*A*).

Finally, to investigate a possible role of the Notch3 receptor in T-reg subsets under physiological condition, we analyzed Notch3 expression by peripheral $CD4^+$ T lymphocytes of wt mice. According to our hypothesis, $CD4^+CD25^+$ splenocytes display a consistently higher proportion of $Notch3^+$ cells than $CD4^+CD25^-$ cells (69.6 vs 7.1%; Fig. 2*B*).

Protection from ld-STZ-induced diabetes in Notch3 Tg mice

The ld-STZ model of autoimmune diabetes has been shown to be useful for the design of strategies aimed to protect β cells from destruction and/or to understand the immune phenomena involved (24, 25).

To study the role of dysregulated Notch3 signaling in T lymphocytes during autoimmune diabetes development, we investigated the effects of ld-STZ treatment on diabetes induction in Notch3-IC Tg mice compared with wt littermates. Under these experimental conditions, control mice have the same genetic background of Notch3-IC Tg, thus ruling out the previously reported possibility that different mouse strains may exhibit different susceptibility to the effect of ld-STZ treatment (26).

The overexpression of the Notch3 intracellular domain in thymocytes and T cells of Tg mice invariably leads to the development of T cell leukemia/lymphoma (20). To minimize any possible interference of Notch3-induced tumors in the experimental plan, Notch3-IC Tg mice derived from a line with a mild phenotype (see *Materials and Methods*) were used and all the experiments were conducted before the appearance of neoplasia. By multiple criteria, the analysis of lymphoid organs in all of the sacrificed animals revealed no evident signs of the histological abnormalities previously described in Notch3-IC Tg mice (Ref. 20 and data not shown).

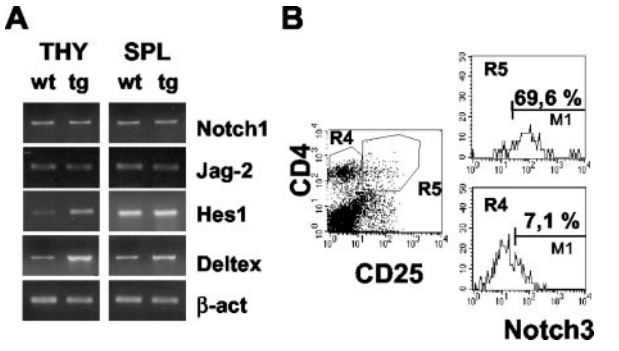

FIGURE 2. Expression of Notch signaling components in T cell compartments of 4-wk-old Notch3-IC Tg mice and wt littermates. *A*, RT-PCR analysis of Notch1, Jagged2, HES-1, and Deltex mRNA expression in lymphocytes from thymus (THY) and spleen (SPL) of wt and Tg animals, as monitored along the exponential phase of the amplification and normalized to β-actin (β-act) expression. *B*, Three-color analysis of Notch3 vs CD4 vs CD25 expression in T splenic lymphocytes of wt mice: $CD4^+CD25^-$ (R4: 15,8%) and $CD4^+CD25^+$ (R5: 4,8%). Numbers indicate percentages of $Notch3^+$ cells. *Left panel*, The T lymphocyte subset distribution.

ld-STZ treatment of 4 wk-old wt mice induced a significant increase in glycemia levels within 15 days after drug administration (Fig. 3*A*). A further increasing hyperglycemia, reaching levels >350 mg/dl, was detected thereafter (Fig. 3*A*).

In contrast, hyperglycemia was not detected in ld-STZ-treated Notch3-IC Tg mice throughout the 22-day time course (blood glucose levels = 139 ± 20 mg/dl; Fig. 3*A*), indicating that experimental diabetes does not develop in the presence of constitutive expression of activated Notch3 in T lymphocytes.

Histological analysis revealed that high glucose levels in ld-STZ-treated wt mice were associated with significant pancreatic lymphoid infiltration at the islet periphery by 9 days after drug administration (Fig. 3*Da*) and with a frank insulitis detected 22 days after drug administration (Fig. 3*Db*). In contrast, low glucose levels in ld-STZ-treated Notch3-IC Tg mice were associated with only a modest insulitis (Fig. 3*D*, *d* and *e*). Pancreatic sections collected from 9-day ld-STZ-treated Notch3-IC Tg mice exhibited no significant lymphoid infiltration (insulitis grade 0) *in* 78.5% of islets investigated, whereas in 9-day ld-STZ-treated wt mice 54% of the islets showed mild to severe insulitis (grades 1- 3). At day 22, mild insulitis (grades 1 and 2) was present in 30% of the islets in pancreatic sections obtained from ld-STZ-treated Notch3-IC Tg mice, whereas mild to severe insulitis (grades 1 and 3) was detected in 73.5% of the islets examined in the pancreatic sections obtained from ld-STZ-treated wt mice (Fig. 3, *D* and *C*).

Immunostaining with anti-insulin Ab confirmed a preserved function of islet β cells in pancreata from ld-STZ-treated Notch3-IC Tg mice (Fig. 3*D f*) when compared with islets in pancreata from ld-STZ-treated wt mice that exhibited impaired insulin expression (Fig. 3*Dc*).

Similar results were obtained in the protection against induced autoimmune diabetes by using different Notch3-IC Tg lines (data not shown).

T-reg cells accumulate in lymphoid organs and pancreata of Notch3-IC Tg mice after ld-STZ treatment

Although significantly reduced with respect to wt animals, lymphocyte infiltration was observed in a number of pancreatic islets of Notch3-IC Tg animals after ld-STZ treatment (Fig. 3*B* and 3*D*, *d* and *e*). To elucidate whether a constitutive imbalance of T-reg cell distribution, characterized by the expansion of $CD4^+CD25^+$ and $CTLA\text{-}4^+$ lymphocytes, observed in lymphoid organs of Notch3-IC Tg may also influence the T cell population infiltrating the pancreas and, because of their peculiar function, sustain the resistance to ld-STZ-induced diabetes, we performed CTLA-4 staining of pancreatic sections from wt and Tg mice after ld-STZ treatment. Immunohistochemical analysis of the pancreata of treated animals, at different times postinduction with ld-STZ, revealed that $CTLA\text{-}4^+$ lymphocytes preferentially infiltrated the pancreas of Notch3-IC Tg compared with wt mice (Fig. 4*A*). The analysis of CD4 and CD25 expression by splenic lymphocytes revealed that the $CD4^+CD25^+$ cell percentage, significantly higher in untreated Notch3-IC Tg with respect to wt mice (Fig. 1*B*), was further increased after ld-STZ-treatment (Fig. 4*B*). Moreover, the $CTLA\text{-}4^+CD25^+$ lymphocyte absolute number was also significantly higher in spleen and mesenteric lymph nodes of ld-STZ-treated Notch3-IC Tg when compared with wt mice (Fig. 4*C*), further suggesting the expansion of the T-reg cell population in peripheral lymphoid organs.

Together, these results suggest that protection from type 1 diabetes in Notch3-IC Tg mice is associated with expansion and peripheral recruitment of T-reg cells.

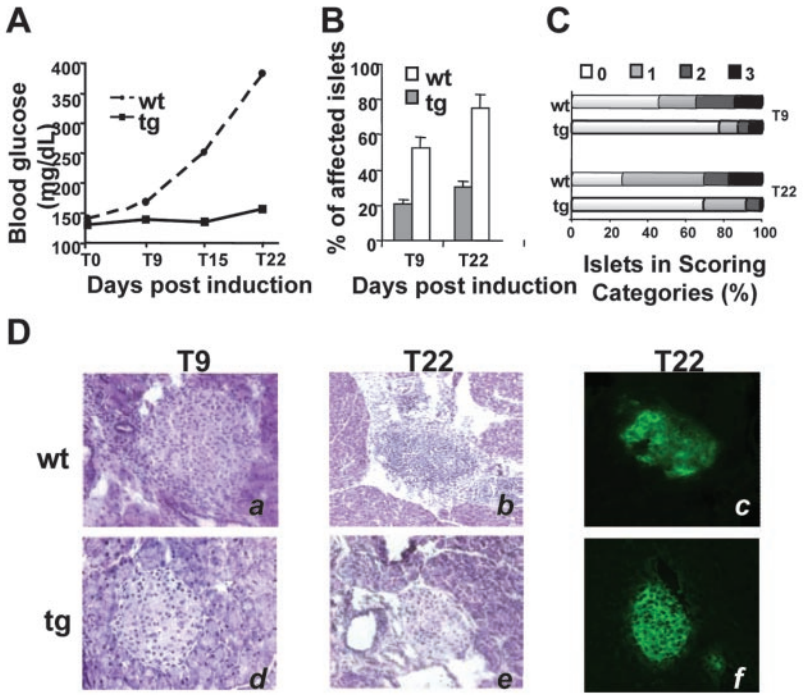

FIGURE 3. Protection from STZ-induced diabetes in Notch3-IC Tg mice. *A*, Blood glucose levels (milligrams per deciliter) determined at 0, 9, 15, and 22 days after STZ administration (T0, T9, T15, and T22, respectively) in wt and Notch3-IC Tg mice. Values are reported as mean of three independent experiments. *B*, Percentages of islets affected by insulitis in pancreata from wt (□) and Notch3-IC Tg mice (▨) at 9 (T9) and 22 (T22) days after drug treatment, evaluated as described in *Materials and Methods*. *C*, The same affected islets determined in *B* were assorted in scoring categories as described in *Materials and Methods*. *D*, Representative islets stained with H&E from wt mouse at 9 and 22 days after induction of diabetes (*a* and *b*, respectively) and from Notch3-IC Tg animal at the same time (*d* and *e*, respectively) are shown. The function of β cells was evaluated with the confocal microscope by immunostaining with an anti-insulin Ab in pancreata from wt and Tg mice at 22 days after STZ treatment (*c* and *f*, respectively).

IL-4 and IL-10 mRNA expression is increased in Notch3-IC Tg mice after ld-STZ treatment

The role of $CD4^+CD25^+$ T-reg cells in the control of self-reactive T cell responses in vivo is mediated by a number of cytokines (reviewed in Ref. 27) and, in particular, IL-4 and IL-10 have been suggested to protect mice from type 1 diabetes (reviewed in Ref. 22). Since Notch3-IC Tg mice fail to develop hyperglycemia and insulitis while displaying expanded T-reg cell population in response to ld-STZ, we investigated IL-4 and IL-10 mRNA expression in splenic T lymphocytes (Fig. 5*A*) and in pancreata (Fig. 5*B*) of ld-STZ-treated animals by semiquantitative RT-PCR. IL-4 and IL-10 mRNAs were not detectable in wt animals under basal conditions, with only low levels of IL-10 mRNA expression detected in splenic lymphocytes 9 days posttreatment (Fig. 5*A*). In contrast, a significantly higher expression of both IL-4 and IL-10 was observed in splenic T lymphocytes from Notch3-IC Tg mice, which progressively increased following STZ treatment (Fig. 5*A*). Moreover, significantly higher expression of both cytokine mRNAs was observed in pancreata of Notch3-IC Tg mice with a progressive increase of IL-10 mRNA expression following ld-STZ-treatment when compared with wt mice (Fig. 5*B*). The immunostaining of pancreatic sections of ld-STZ-treated animals with anti-IL-10 Ab confirmed an increased IL-10 expression in the pancreas of Notch3-IC Tg mice (Fig. 5*C*, *lower panel*).

$CD4^+$ T cells from Notch3-IC Tg mice display enhanced regulatory functions

The above results all together suggest that Notch3-IC Tg mice are protected against the development of autoimmune diabetes through the constitutive expansion of $CD4^+CD25^+$ lymphocytes with putative regulatory function. To directly test the regulatory function, we designed in vivo adoptive cell transfer and in vitro experiments to demonstrate the enhanced suppressive activity of $CD4^+$ T cells obtained from Tg mice. $CD4^+CD25^+$ thymocytes have been shown to prevent diabetes on transfer into prediabetic recipients (12); therefore, we isolated $CD4^+$ thymocytes from Tg and wt mice and injected them (6×10^6 cells/mouse) in wt littermates 4 days before the ld-STZ treatment induction. The progression to diabetes was monitored throughout 25 days after drug induction. Fig. 6 shows that mice receiving Tg $CD4^+$ cell population, including a larger percentage of $CD4^+CD25^+$ cells with respect to wt animals (Fig. 1*A*), appear to be fully protected against diabetes development since their blood glucose levels remained below 200 mg/dl (Fig. 6*A*) and they did not develop high-grade insulitis (Fig. 6, *B* and *C*). In contrast, all of the mice receiving wt $CD4^+$ cells or vehicle alone progressed to diabetes (Fig. 6*A*). Consistently, high-grade insulitis was evident 25 days after drug induction in mice receiving wt $CD4^+$ cells as well as in control mice receiving vehicle alone (Fig. 6, *B* and *C*). It is noteworthy that mice receiving wt $CD4^+$ cells progressed to diabetes with a delayed kinetic when compared with control mice injected with vehicle alone (Fig. 6*A*), possibly depending on the presence of the low percentage of $CD4^+CD25^+$ T-reg cells.

Enhanced suppressive activity by $CD4^+$ Tg cells was confirmed in in vitro experiments. Indeed, $CD4^+$ cells obtained from thymus and spleen of Notch3-IC Tg display an enhanced regulatory activity, as indicated by the suppression of CD3-dependent proliferation of splenic T cells cultured in the presence of APCs, when compared with the activity of $CD4^+$ lymphocytes obtained from thymus and spleen of wt littermates (Fig. 6*D*). In another set of in vitro experiments, we used graded numbers of $CD4^+$ Tg splenocytes at different ratios with $CD4^+$ wt splenocytes (Fig. 6*E*). The results indicate that $CD4^+$ Tg splenocytes inhibit the proliferation of $CD4^+$ wt splenocytes in a dose-dependent manner and suggest that the suppression activity correlates with the number of $CD4^+CD25^+$. Indeed, $CD4^+CD25^+$ cells are clearly expanded in $CD4^+$ lymphocytes from Notch3-IC Tg with respect to wt littermates (39.8 and 9.9%, respectively, as shown in Fig. 1*B*).

Discussion

We report that Tg expression of the intracellular domain of Notch3 in T cells, resulting in the constitutive activation of the receptor signaling, leads to resistance against ld-STZ-induced autoimmune diabetes in mice. Such resistance is associated with an expansion,

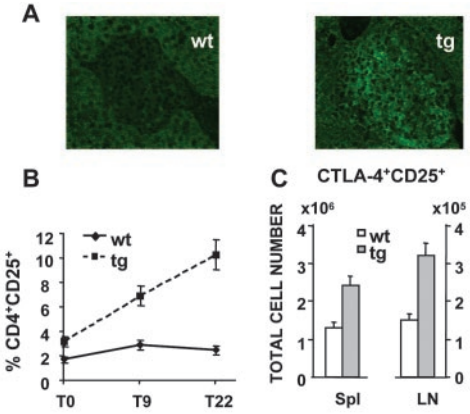

FIGURE 4. Recruitment of T-reg cells in lymphoid organs and pancreata of ld-STZ-treated Notch3-IC Tg mice. *A,* Islets immunostained with anti-CTLA-4 from pancreata of wt and Tg mice at 22 days after diabetes induction. *B,* Percentages of $CD25^+CD4^+$ cells in splenic lymphocytes from wt and Notch3-IC Tg at 0 (T0), 9 (T9), and 22 (T22) days after drug administration, as assessed by two-color FCA (data represent the mean of three independent experiments). *C,* Total cell number of $CTLA\text{-}4^+CD25^+$ lymphocytes in spleen (Spl) and mesenteric lymph node (LN) of wt (□) and Tg (▨) mice at 22 days after STZ injection (values, calculated by FCA, are the mean of three independent experiments).

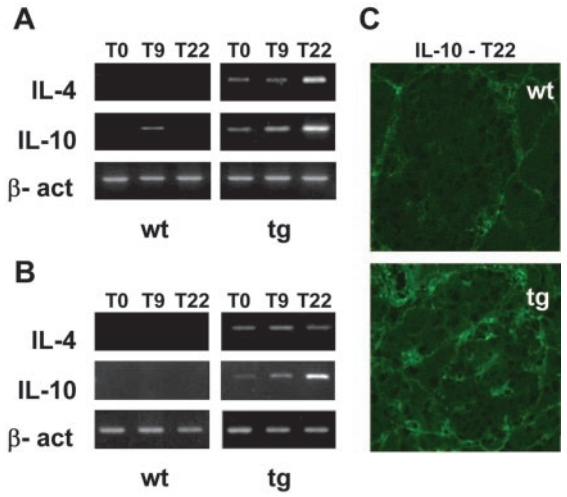

FIGURE 5. Enhanced expression of IL-4 and IL-10 regulatory cytokines in lymphoid organs and pancreata of Notch3-IC Tg ld-STZ-treated mice compared with wt littermates. *A,* Expression of IL-4 and IL-10 mRNA in splenic lymphocytes, analyzed by semiquantitative RT-PCR in representative cases of untreated (T0) and 9 and 22 days postinduction (T9 and T22, respectively) Notch3-IC Tg compared with wt animals. mRNA of β-actin (β-act) was also investigated as a control. *B,* RT-PCR analysis of IL-4 and IL-10 expression in total RNA from pancreata of the same animals as in *A. C,* Anti-IL-10 immunostaining of pancreatic section from wt (*upper panel*) and Tg (*lower panel*) mice at 22 days after drug administration. The results are representative of three independent experiments.

in the thymus and peripheral lymphoid organs, of $CD4^+CD25^+$ cells that exhibited a constitutive expression of CTLA-4 and suppressor cytokines (i.e., IL-4 and IL-10), as well as a suppressive activity upon CD3-dependent proliferation of wt splenic T cells cultured in the presence of APCs, consistent with a T-reg phenotype. Such an increase of T-reg cells could be responsible for protection against the development of experimental diabetes since $CD4^+CD25^+$ lymphocytes progressively increase in spleen, lymph nodes, and pancreas of Notch3-IC Tg mice following ld-STZ treatment. Accordingly, adoptive transfer of $CD4^+$ thymocytes from Notch3-IC Tg into wt mice inhibited the development of ld-STZ-induced diabetes.

The expansion of $CD8^{low}CD4^+CD25^+$ thymocytes, possibly representing an intrathymic pool of T-reg cell precursors (16), and of $CD4^+CD25^+$ cells observed in the thymus and peripheral lymphoid organs of Notch3-IC Tg mice is consistent with the previously described dysregulation of intrathymic T cell differentiation, characterized by a persistent increase in CD25 expression (20). Interestingly, the T-reg cell expansion in lymphoid organs was also associated with their peripheral recruitment, at the level of the pancreas, following ld-STZ treatment.

Together, these observations suggest that the Notch3 pathway may be implicated in the intrathymic generation of T-reg cells and that the dysregulated T cell development following constitutive Notch3 activation in thymocytes and progeny T cells facilitates the expansion and peripheral recruitment of T-reg cells in response to autoimmune injury. In addition, the absence of any modulation in the expression of other Notch receptors and ligands considered sustains the Notch3 specificity in the determination of the functional phenotype of Notch3-IC Tg mice.

Finally, our observation that $CD4^+CD25^+$ splenocytes from wt mice include a higher proportion of Notch3-expressing cells with respect to $CD4^+CD25^-$ splenocytes further supports a specific physiological role for the Notch3 receptor in T-reg cells.

The constitutive high expression of IL-4 and IL-10 inhibitory cytokine mRNAs in thymocytes of Notch3-IC Tg mice and their progressive increase in splenic T lymphocytes and pancreas of Tg mice, following ld-STZ-treatment, suggest a possible double role for such cytokines in protecting against generation of type 1 diabetes in Notch3-IC Tg mice. On one hand, they could exert an inhibitory function directly on the T cells involved in the self-reactive response in the pancreas (22). On the other hand, they could promote the generation of T-reg cells. Thus suggesting that Notch3 may also sustain intrathymic T-reg cell precursor generation through increasing the production of IL-4 and IL-10. Indeed, both of these cytokines promote the generation of T cells with regulatory function in vitro (28, 29).

A direct effect on peripheral generation of T-reg cells may also be mediated by activation of the Notch3-dependent signaling pathway. Indeed, it has been previously shown that activation of Notch signaling, by overexpressing the specific Notch ligand *Jagged*/*Serrate1* in APCs, in peripheral murine T cells results in the differentiation of Ag-specific $CD4^+$ lymphocytes into T-reg cells, which can transfer tolerance to naive mice (30). Conversely, Ag-dependent T cell activation has been shown to trigger the activation of the Notch signaling pathway, since stimulation of purified murine $CD4^+$ T cells with anti-CD3 and anti-CD28 Abs has been shown to induce a transient increase in Notch ligand and receptor expression and the concomitant addition of IL-10 further increases the transcription of Notch ligand genes (31). More recently, Notch signaling, including the expression of the *Hes1* target gene, has also been shown to be enhanced in human peripheral $CD4^+CD25^+$ cells upon in vitro stimulation with anti-CD3 and anti-CD28 Abs, while *Deltex*, a positive regulator of the Notch signaling pathway, is highly up-regulated in resting $CD4^+CD25^+$ cells compared with $CD4^+CD25^-$ cells (32). Overall, these data support a key role for the Notch signaling pathway in sustaining the differentiation and possibly the function of T-reg cells. The presence of an increased percentage of Notch3-expressing cells in $CD4^+CD25^+$ wt splenocytes we observed represents to our knowledge the first observation that a member of the Notch receptor family is specifically expressed in resting T-reg cells.

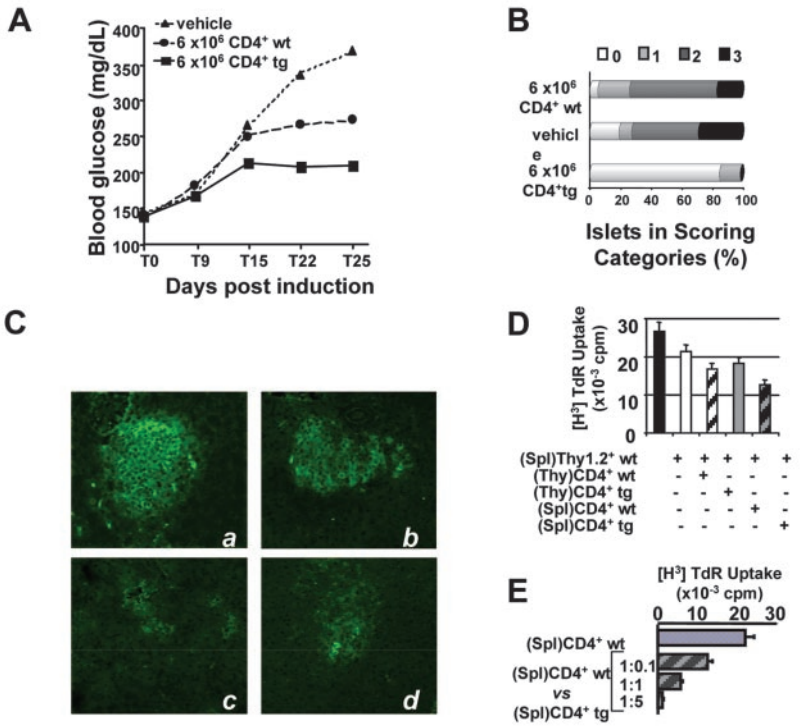

FIGURE 6. Enhanced regulatory functions of $CD4^+$ T cells from Notch3-IC Tg mice. *A*, Blood glucose levels (milligrams per deciliter) determined at 0, 9, 15, 22, and 25 days after ld-STZ treatment (T0, T9, T15, T22, and T25, respectively) in wt mice injected with 6×10^6 $CD4^+$ thymocytes from wt (●) or Tg (■) mice or with vehicle alone (▲) 4 days before the drug induction. Values are reported as the mean of three independent experiments. *B*, Percentage of insulitis-affected islets, assorted in scoring categories, as described in *Materials and Methods*, in pancreata from the animals receiving the same treatment as in *A* sacrificed at 25 days. *C*, Representative islets immunostained with anti-insulin Ab in a wt-untreated mouse (*a*) and in wt mice at 25 days after induction of diabetes and previously injected, as indicated in *A*, with 6×10^6 $CD4^+$ thymocytes from wt or Tg mice (*d* and *b* respectively) or with vehicle alone (*c*). *D*, Suppression activity of anti-CD3-dependent in vitro proliferation of wt splenic T (Spl $Thy1.2^+$ wt) cells cultured in the presence of APCs by $CD4^+$ cells isolated from thymus (Thy) or spleen (Spl) of wt or Tg mice. *E*, wt $CD4^+$ splenocytes (Spl $CD4^+$ wt) in the presence of APCs were mixed as responder cells at various ratios with Tg $CD4^+$ splenocytes (Spl $CD4^+$ tg) as suppressor cells. The means of three experiments are shown and vertical bars denote SD.

Moreover, the increased production of regulatory cytokines (i.e., IL-10 and IL-4) by either resting splenic T cells or after induction of experimental diabetes in Notch3 Tg mice suggests that activation of the Notch3 signaling pathway in T lymphocytes may contribute to enhance the peripheral T-reg cell function and could, consequently, play a role in the protection against autoimmune diseases.

The significant increase of $CD4^+CD25^+$ T-reg cells in either thymocytes or peripheral T cells of Notch3 Tg mice we have observed is occurring independently of any specific antigenic stimulation. In addition, the suppressive properties of Tg $CD4^+$ cells are present before STZ treatment, as demonstrated by in vitro suppression experiments, suggesting that the protection from experimental autoimmune diabetes is primarily linked to Notch3-induced expansion of a "naturally occurring" T-reg population displaying a bystander effect.

Together, these observations are in keeping with the described regulatory function of $CD4^+CD25^+$ cells in autoimmune diseases and suggest that a role for $CD4^+CD25^-$ in Notch3-dependent inhibition of experimental diabetes we describe here might be ruled out. However, because of the reported putative regulatory role for $CD4^+CD25^-$ cells that is primarily revealed under particular experimental conditions (as reviewed in Ref. 33), this issue needs to be specifically further investigated.

It remains to be addressed whether the expansion of $CD4^+CD25^+$ cells may additionally affect tumor immunity in Notch3-IC Tg mice that have been shown to spontaneously develop T cell lymphomas (20). Indeed, a role for $CD4^+CD25^+$ T-reg cells in regulating the development of tumor immunity has been suggested. Indeed, removal of T-reg cells has been shown to evoke immune response to syngeneic tumors by spontaneous generation of protective NK cells (34).

In conclusion, our observations suggest that the activation of the Notch pathway could protect against autoimmune diseases by favoring the establishment of tolerance via the enhancement of both central generation and peripheral recruitment and function of T-reg cells. Therefore, using a Tg mouse model, we propose that the Notch3 pathway may represent a novel regulator of immune response, the imbalance of which may result in impaired tolerance and development of autoimmune diseases such as type 1 diabetes. The rescue of tolerance impairment by modulating Notch signaling might represent a novel opportunity for therapeutic intervention in autoimmunity.

Acknowledgments

We thank B. Milana and P. Birarelli for FCA.

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