Thymic Stromal Lymphopoietin and Thymic Stromal Lymphopoietin–Conditioned Dendritic Cells Induce Regulatory T-Cell Differentiation and Protection of NOD Mice Against Diabetes

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OBJECTIVE—Autoimmune diabetes in the nonobese diabetic (NOD) mouse model results from a breakdown of T-cell tolerance caused by impaired tolerogenic dendritic cell development and regulatory T-cell (Treg) differentiation. Re-establishment of the Treg pool has been shown to confer T-cell tolerance and protection against diabetes. Here, we have investigated whether murine thymic stromal lymphopoietin (TSLP) re-established tolerogenic function of dendritic cells and induced differentiation and/or expansion of Tregs in NOD mice and protection against diabetes.

RESEARCH DESIGN AND METHODS—We examined the phenotype of TSLP-conditioned bone marrow dendritic cells (TSLP-DCs) of NOD mice and their functions to induce noninflammatory Th2 response and differentiation of Tregs. The functional relevance of TSLP and TSLP-DCs to development of diabetes was also tested.

RESULTS—Our results showed that bone marrow dendritic cells of NOD mice cultured in the presence of TSLP acquired signatures of tolerogenic dendritic cells, such as an absence of production of pro-inflammatory cytokines and a decreased expression of dendritic cell costimulatory molecules (CD80, CD86, and major histocompatibility complex class II) compared with LPS-treated dendritic cells. Furthermore, TSLP-DCs promoted noninflammatory Th2 response and induced the conversion of naïve T-cells into functional $CD4^+CD25^+Foxp3^+$ Tregs. We further showed that subcutaneous injections of TSLP for 6 days or a single intravenous injection of TSLP-DCs protected NOD mice against diabetes.

CONCLUSIONS—Our study demonstrates that TSLP re-established a tolerogenic immune response in NOD mice and protects from diabetes, suggesting that TSLP may have a therapeutic potential for the treatment of type 1 diabetes. ***Diabetes*** **57: 2107–2117, 2008**

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Dendritic cells are professional antigen-presenting cells (APCs) that have the potential to induce immune response and T-cell tolerance (1). Immature or semimature tolerogenic dendritic cells have been shown to induce and maintain peripheral T-cell tolerance, whereas terminally differentiated mature dendritic cells induce the development of effector T-cells (1). Tolerogenic dendritic cells (tDCs) produce interleukin (IL)-10 and have impaired abilities to synthesize IL-12p70 and indolamine 2,3-dioxygenase and to activate T-cells in vitro (2). Conditioning dendritic cells with granulocyte macrophage–colony-stimulating factor (GM-CSF) (3), IL-10, and/or transforming growth factor-β (TGF-β) (4,5) as well as 1,25-dihydroxyvitamin D3 (6) has been shown to promote tDCs that induce Th2 response and/or differentiation of $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs). When injected in mice, tDCs were able to suppress acute graft-versus-host disease (7) and autoimmunity (8). Recently, we and others have shown that injections of GM-CSF prevented the development of autoimmune diseases by increasing the number of semimature tDCs and by inducing Treg differentiation (9–11).

Tregs arise during the normal process of T-cell maturation in the thymus, and their differentiation can be induced in the periphery by conversion of $CD4^+CD25^-Foxp3^-$ into $CD4^+CD25^+Foxp3^+$ Tregs (12–14). Tregs are crucial for suppressing autoimmune responses and maintaining peripheral immunological tolerance (15). The influence of Tregs in maintaining T-cell tolerance is strongly supported by the observations of the development of autoimmune syndromes in mice lacking Tregs and by the findings that defects in Foxp3 gene expression in humans and mice lead to autoimmune syndromes in early life (16,17). In agreement with these observations, prevention of rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes has been achieved by reconstitution of autoimmune-prone mice with Tregs (18).

Autoimmune diabetes in the nonobese diabetic (NOD) mouse model results from a breakdown of T-cell tolerance due to impaired development of tDCs and Treg differentiation (19,20). In addition, bone marrow–derived dendritic cells (BM-DCs) of NOD mice were shown to express abnormal levels of costimulatory molecules under pro-inflammatory conditions and increased capacity to secrete IL-12p70 and to stimulate $CD4^+$ and $CD8^+$ T-cells (21–23). Consequently, the capacity of dendritic cells in NOD mice to sustain the pool and suppressive function of Tregs is altered, which leads to progression of type 1 diabetes (24,25).

Thymic stromal lymphopoietin (TSLP) was first identified in conditioned medium supernatants of the mouse thymic stromal cell line Z210R.1 (26). TSLP, a member of the IL-7 cytokine family, is preferentially expressed by epithelial cells mainly in the lung, skin, and gut (27,28). Recently, clues for a function of TSLP in humans came from two observations. TSLP was found to be selectively expressed by thymic epithelial cells of Hassall's corpuscles, and TSLP-activated dendritic cells (TSLP-DCs) induced differentiation of $CD4^+Foxp3^-$ thymocytes into $CD4^+Foxp3^+$ Tregs (29). Recently, Jiang et al. (30) have reported that TSLP produced by mouse medullary thymic epithelial cells contribute to $Foxp3^+$ expression and Treg maturation. In addition, Lee et al. (31) have shown that TSLP triggered the conversion of thymic $Foxp3^-CD4^+$ T-cells into $Foxp3^+$ T-cells in a dendritic cell–independent manner.

Here, we have investigated whether murine TSLP-DCs and TSLP induce differentiation and/or expansion of Tregs in the NOD mouse model and protection against diabetes. We found that TSLP-DCs acquire signatures of tDCs and induce the conversion of naïve T-cells into functional Tregs. We have further shown that subcutaneous injections of TSLP or a single intravenous injection of TSLP-DCs protects NOD mice against diabetes. Our data are the first to report that TSLP induces a tolerogenic immune response and protects against diabetes in NOD mice.

RESEARCH DESIGN AND METHODS

NOD/Ltj mice were from The Jackson Laboratories (Bar Harbor, ME). 8.3-NOD mice obtained from Dr. P. Santamaria (Microbiology and Infectious Diseases, University of Calgary, Alberta, Canada) have been described previously (32). The mice were housed under pathogen-free conditions, in accordance with the guidelines of the local institutional animal care committee.

Antibodies and reagents. Anti–CD8α-PE (clone 53–6.7), anti–CD4-fluorescein isothiocyanate (FITC)/biotin/APC (clone GK1.5), anti–CD25-FITC (clone 7D4), anti–CD11b-FITC (clone M1/70), anti–CD11c-FITC/biotin (clone HL3), anti–CD80-biotin (clone 16-10A1), anti–CD86-biotin (clone GL1), and anti–$I\text{-}A^{g7}$-biotin (clone 10-3.6) antibodies, and streptavidin-PerCP were from Becton Dickinson (San Jose, CA). Anti–Foxp3-FITC/PE (FJK-16s) and anti-Rat IgG2a (eBR2a) antibodies were from eBiosciences (San Diego, CA). Anti-CD3 antibody (clone 145-2C11) was from Dr. P. Santamaria. The NRP-A7 peptide, a mimotope of the endogenous IGRP peptide that is recognized by the TCR of 8.3 $CD8^+$ T-cells, and tumor-derived negative control peptide (TUM) were from C. Servis (Biochemistry Institute, Lausanne University, Switzerland). Murine recombinant TSLP (lot no. ELR0307011) was from R&D Systems (Minneapolis, MN).

Treatment and dendritic cells transfer. Female NOD/Ltj mice were injected subcutaneously with recombinant mouse TSLP (500 ng · 200 μl^{-1} · $mouse^{-1}$ · day^{-1}) or PBS for 6 days. In dendritic cell transfer experiments, 3-week-old female NOD/Ltj mice received one intravenous injection of TSLP-DCs or LPS-DCs (5×10^6 cells/mouse). Diabetes was monitored by a urine glucose test using Uristix (Bayer, Minneapolis, MN), and blood glucose was measured with an Advantage Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN). The animals were considered diabetic after two positive Uristix readings or when blood glucose was >15 mmol/l.

T-cell isolation. $CD4^+$ T-cell subpopulations and $CD8^+$ T-cells were purified using antibody-coated magnetic beads from Miltenyi Biotec (Bergish Gladbach, Germany).

Generation of BM-DCs. BM-DCs were generated with GM-CSF and IL-4 as previously described (33). On day 7, dendritic cells were left unstimulated (immature DCs [iDCs]) or exposed (48 h) to 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) or 20 ng/ml TSLP (R&D Systems).

Proliferation assays and cytokine quantification. $CD8^+$ T-cells (2×10^4 lymphocytes/well) were incubated with 1 μg/ml NRP-A7 peptide– or 1 μg/ml TUM peptide–pulsed irradiated dendritic cells (5×10^3 cells/well) for 3 days at 37°C. $CD4^+$ T-cells (2×10^4 lymphocytes/well) were incubated with a combination of 5 μg/ml anti-CD3 and 20 units/ml IL-2 in the presence of irradiated dendritic cells (5×10^3 cells/well) under similar conditions. Supernatants were collected 48 h later for cytokine quantification using ELISA kits (R&D Systems). Cultures were pulsed with 1 μCi [^{3}H]thymidine/well during the last 18 h and radioactivity was counted.

Foxp3 expression. Foxp3 staining was assessed by intracellular staining using FITC–anti-mouse/rat staining kit (eBiosciences, San Diego, CA) (11). Cells were analyzed using the CellQuest (BD Biosciences) or the FCS express V3 software (De Novo Software, Los Angeles, CA).

CFSE-based killing assay. Killing assays were adapted from the technique of Jedema et al. (34). Briefly, RMAS-K^d cells (preincubated at 26°C overnight) were labeled with CFSE, washed, and resuspended (5×10^4 cells/ml) in lymphocyte complete medium. Carboxy fluorescein diacetate succinimidyl ester (CFSE)-labeled RMAS-K^d cells (5×10^3 cells · 100 μl^{-1} · $well^{-1}$) were pulsed with NRP-A7 or TUM (1 μg/ml) and used as target cells. Effector 8.3-$CD8^+$ T-cells were added at 1:2, 1:4, and 1:10 target:effector ratios. The plates were incubated at 37°C for 6 h and analyzed by fluorescence-activated cell sorter (FACS). The percentage of survival was calculated as follows: % survival = [number of viable $CFSE^+$ target cells ($t = 6$ h)]/[number viable $CFSE^+$ target cells ($t = 0$)] × 100.

T-cell and BM-DC co-cultures. BM-DCs were cultured for 48 h in the presence of LPS or TSLP, extensively washed, and resuspended in fresh medium. Dendritic cells were then co-cultured with total splenic T-cells, purified $CD4^+CD25^-$ T-cells, or purified $CD4^+CD25^+$ T-cells in round-bottom anti-CD3–coated 96-well culture plates in LCM containing 20 units/ml IL-2. Cultures were done in triplicate at a 1:4 dendritic cell:T-cell ratio.

Suppression assays. Purified $CD4^+CD25^-$ T-cells cultured in the presence of iDCs, LPS-DCs, or TSLP-DCs for 3 or 7 days were co-cultured with purified 8.3-$CD8^+$ T-cells at a 1:1 ratio in the presence of 1 μg/ml peptide-pulsed APCs (10^5 irradiated splenocytes/well) for 3 days at 37°C as described previously (11). Cells were pulsed with 1 μCi [^{3}H]thymidine/well during the last 18 h and radioactivity was counted.

Histopathology. Pancreata were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Islet insulitis was scored as described previously (11).

Statistical analyses. Student's t test and χ^2 tests were used to determine the statistical significance, which was set at the 95% confidence level.

RESULTS

TSLP-DCs display a tolerogenic phenotype. We first examined the effect of TSLP on the phenotype of BM-DCs generated with GM-CSF and IL-4. As expected, nonstimulated BM-DCs expressed low levels of CD80, CD86, and major histocompatibility complex (MHC) class II molecules, a characteristic of iDCs (Fig. 1*A*), and further stimulation with LPS (LPS-DCs) increased levels of CD80, CD86, and MHC class II (Fig. 1*A*), a characteristic of fully mature dendritic cells. Interestingly, iDCs exposed to TSLP (TSLP-DCs) expressed levels of CD80, CD86, and MHC II intermediate between those expressed by iDCs and fully mature LPS-DCs (Fig. 1*A* and *B*). The phenotype observed in the case of TSLP-DCs was characteristic of semimature dendritic cells. Furthermore, TSLP-DCs expressed low levels of OX40L (CD134) than LPS-DCs (Fig. 1*A* and *B*). Together, these results suggested that TSLP-DCs acquired a semimature phenotype.

We next quantified tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-12p70 in the supernatants of iDCs, LPS-iDCs, and TSLP-DCs. Consistent with previous studies (21,35), we found that LPS-DCs of NOD mice produced high amounts of TNF-α, IFN-γ, and IL-12p70, whereas TSLP-DCs produced low or barely detectable amounts of IFN-γ, TNF-α, and IL-12p70 (Fig. 1*C*). These data suggested that, in contrast to fully mature LPS-DCs of NOD mice, TSLP-DCs adopt a semimature phenotype and halt their production of inflammatory cytokines.

TSLP-DCs induce antigen-specific $CD8^+$ T-cells to proliferate and to differentiate into cytotoxic T-cells. The capacity of TSLP-DCs to activate and differentiate NRP-A7–reactive 8.3-$CD8^+$ T-cells into cytotoxic T-cells was compared with iDCs and LPS-DCs. Data showed no differences in the antigen-specific proliferation of 8.3-$CD8^+$ T-cells in the presence of iDCs, LPS-DCs, or

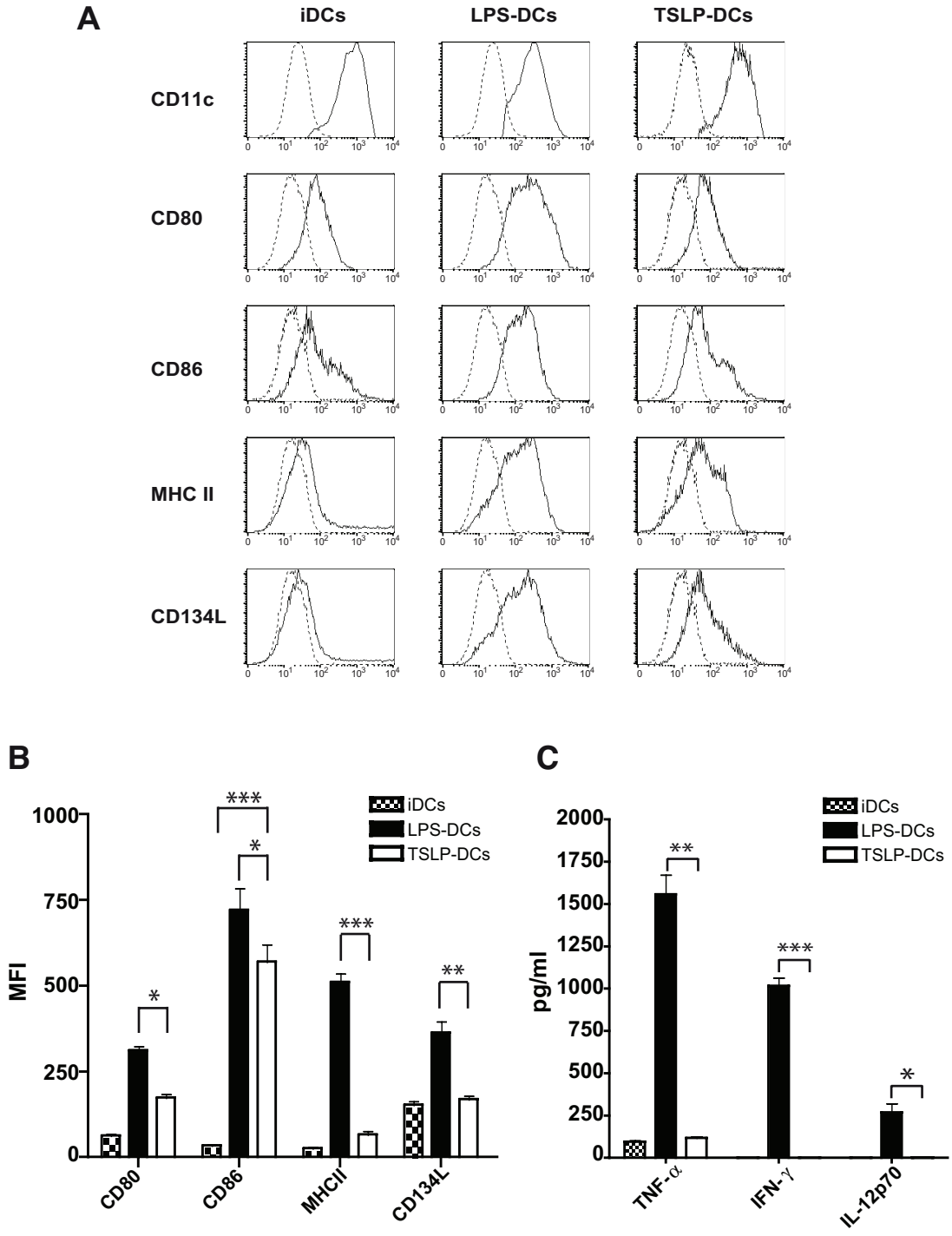

FIG. 1. TSLP-DCs display tolerogenic properties. *A*: BM-DCs (1×10^5 cells/well) were cultured for 2 days in the absence of stimulus (iDCs) or in the presence of 1 μg/ml LPS (LPS-DCs) or 20 ng/ml TSLP (TSLP-DCs). Dendritic cells were stained with isotype control antibodies (dashed line) or with specific antibodies against CD11c, CD80, CD86, I-A^{g7}, and CD134L (bold line) and analyzed by FACS. *B*: Mean fluorescence intensities (MFI) were quantified in each case. *C*: Quantification of TNF-α, IFN-γ, and IL-12p70 in the supernatants of BM-DCs cultured in absence of stimulus or in the presence of LPS or TSLP. Data are shown as the average ± SD of four independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

TSLP-DCs (Fig. 2*A*). To determine whether TSLP-DCs induced naïve 8.3-$CD8^+$ T-cells to produce Tc1 or Tc2 cytokines, we quantified IL-2, IFN-γ, and IL-4. There were no differences in the amounts of IL-2 and IFN-γ produced by 8.3-$CD8^+$ T-cells primed with iDCs, LPS-DCs, or TSLP-DCs (Fig. 2*B* and *C*). In addition, no IL-4 was detected under all experimental conditions (data not shown). To investigate whether TSLP-DCs affected differentiation of naïve 8.3-$CD8^+$ T-cells into cytotoxic T-cells, we tested the capacity of 8.3-$CD8^+$ T-cells cultured with iDCs or LPS-DCs or TSLP-DCs to kill RMAS-K^d cells (34). Similar levels of cytotoxic activity were detected in 8.3-$CD8^+$ T-cells primed with NRP-A7–pulsed iDCs, LPS-DCs, or TSLP-DCs (cytolytic activities were 41.0 ± 7.45, 50.0 ± 7.04, and 50.74 ± 3.45%, respectively) (Fig. 2*D*).

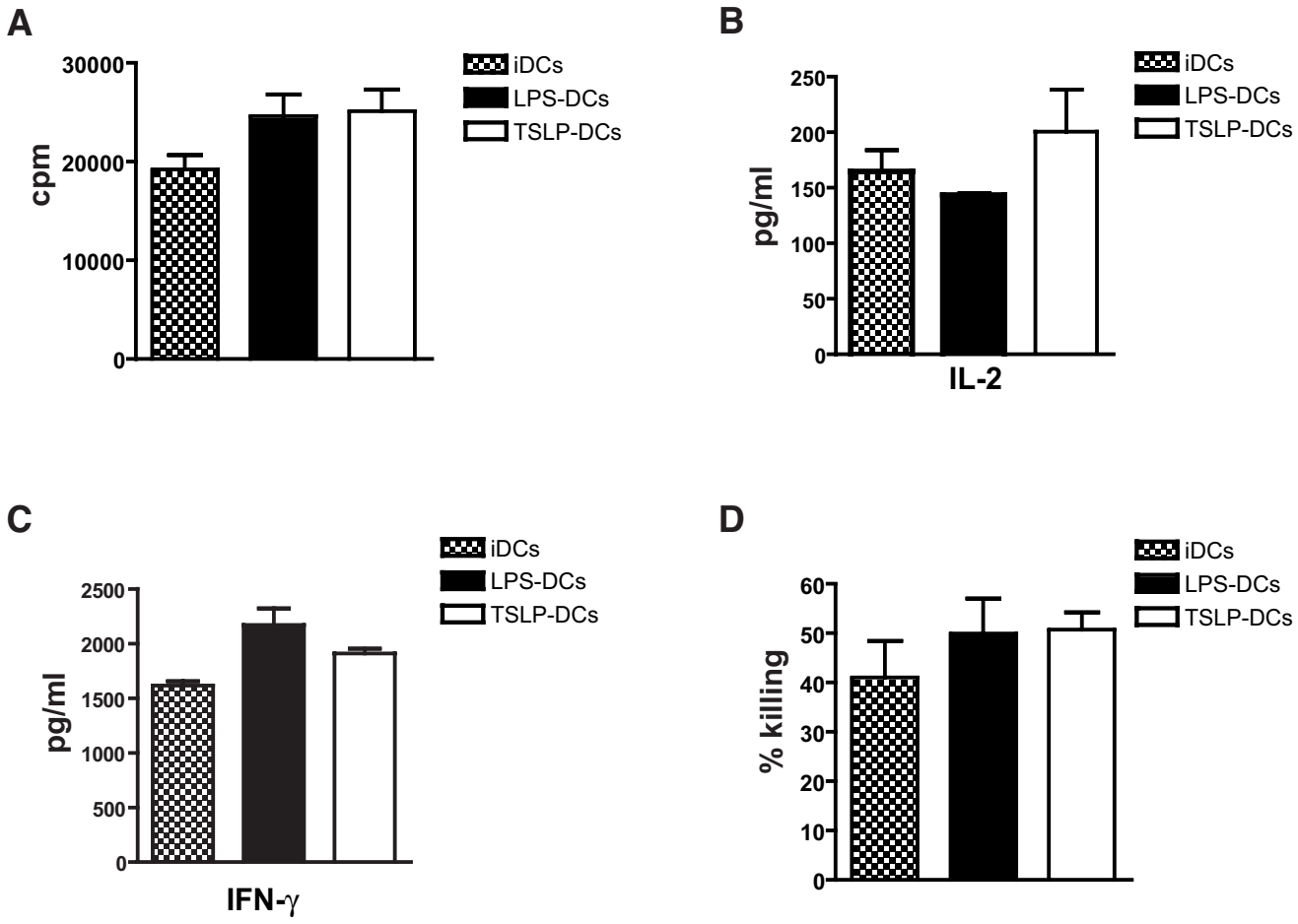

FIG. 2. TSLP-DCs induce antigen-specific CD8$^+$ T-cell differentiation. *A*: Proliferative response of naïve 8.3-CD8$^+$ T-cells (2 × 10^4 lymphocytes/well) to 1 μg/ml NRP-A7 peptide–pulsed iDCs or LPS- or TSLP-irradiated dendritic cells (5 × 10^3 cells/well), as indicated. Background responses against the negative control peptide TUM were subtracted. Quantification of IL-2 (*B*) and IFN-γ (*C*) secreted by naïve 8.3-CD8$^+$ T-cells cultured under these conditions. *D*: Cytotoxic activity of BM-DC–activated 8.3-CD8$^+$ T-cells against 1 μg/ml NRP-A7– or 1 μg/ml TUM-pulsed CFSE-labeled RAMS-K^d target cells. The percentage of killing was determined by subtracting nonspecific (TUM) from specific (NRP-A7) killing. Data are representative of two to three independent experiments and are the average ± SD.

TSLP-DCs differentiate CD4$^+$ T-cells into noninflammatory Th2 cells. Human TSLP-DCs induce a robust expansion of CD4$^+$ T-cells that can differentiate into noninflammatory or inflammatory Th2 cells (36–38). Therefore, we examined the ability of TSLP-DCs to polarize CD4$^+$ T-cells into Th2 cytokine-producing cells. Proliferation and cytokine production by splenic CD4$^+$ T-cells were determined by incubating naïve CD4$^+$ T-cells with anti-CD3 and IL-2 in the absence or presence of allogeneic iDCs, LPS-DCs, or TSLP-DCs following the protocol of Watanabe et al. (29). Whereas, anti-CD3 and IL-2 induced moderated proliferation of CD4$^+$ T-cells, a combination of anti-CD3 and IL-2 with iDCs, LPS-DCs, or TSLP-DCs induced 4.1-, 3.8-, and 4.5-fold increases of CD4$^+$ T-cell proliferation, respectively (Fig. 3*A*). We next examined cytokine production and found that CD4$^+$ T-cells cultured with TSLP-DCs produced significantly high amounts of Th2 cytokines, such as IL-4, and low amounts of IFN-γ, as reported previously (38). Furthermore, these cells produced more TGF-β and IL-10 than naïve CD4$^+$ T-cells cultured with iDCs or LPS-DCs (Fig. 3*B*).

CD4$^+$ T-cells expanded with TSLP-DCs are enriched in Tregs. We next determined whether the increased production of IL-10 and TGF-β was due to an increased pool of Tregs within the population of CD4$^+$ T-cells cultured in the presence of TSLP-DCs. The percentage of CD4$^+$CD25$^+$Foxp3$^+$ T-cells present in CD4$^+$ T-cells expanded with a combination of anti-CD3 and IL-2, in the absence or presence of iDCs, LPS-DCs, or TSLP-DCs, was analyzed by FACS (Fig. 4). Results showed that after 7 days of culture, CD4$^+$ T-cells activated in the absence of dendritic cells or in the presence of iDCs or LPS-DCs contained 6.12 ± 0.55, 6.68 ± 0.73, and 6.86 ± 0.23% of CD4$^+$CD25$^+$Foxp3$^+$ T-cells, respectively (Fig. 4*A* and *B*). Of significance, CD4$^+$ T-cells co-activated in the presence TSLP-DCs contained nearly twice as much Foxp3$^+$CD4$^+$ T-cells (11.25 ± 1.33%, $P < 0.05$) as CD4$^+$ T-cells co-activated in the presence of iDCs or LPS-DCs (Fig. 4*A* and *B*).

TSLP-DCs induced expansion and differentiation of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in vitro. We next investigated whether the increased number of Tregs in splenic CD4$^+$ T-cells co-activated in the presence of TSLP-DCs resulted from an expansion of Tregs and/or the Treg differentiation. Purified CD4$^+$CD25$^+$ (Foxp3$^+$) and CD4$^+$CD25$^-$ (Foxp3$^-$) were cultured with anti-CD3 and IL-2 in the absence of dendritic cells or presence of iDCs, LPS-DCs, or TSLP-DCs. The expansion of CD4$^+$CD25$^+$Foxp3$^+$ T-cells was determined by [^{3}H]thymidine incorporation assay. Stimulation with anti-CD3 and IL-2 induced marginal proliferation of CD4$^+$CD25$^+$ T-cells, whereas co-activation with iDCs or LPS-DCs weakly increased

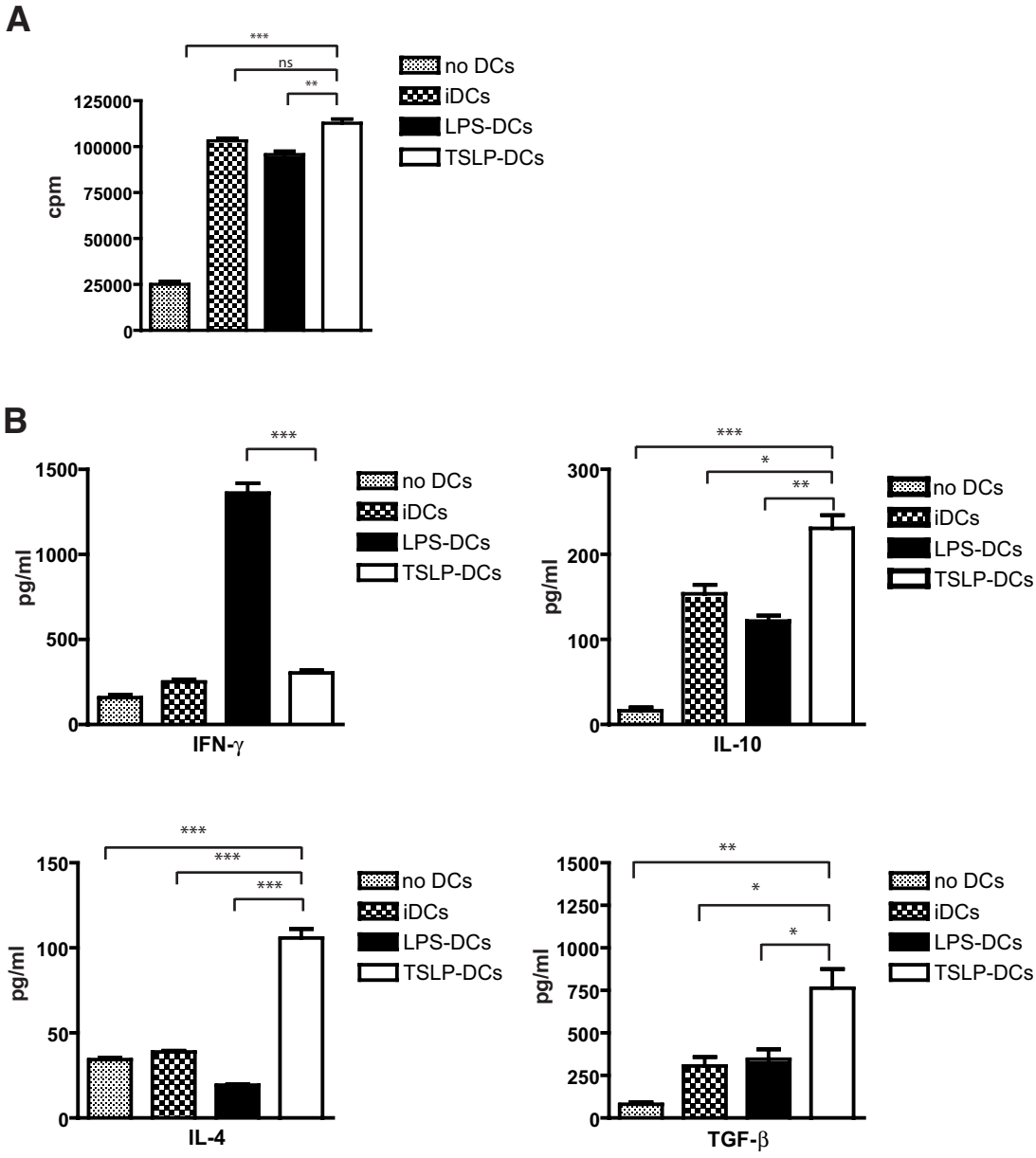

FIG. 3. TSLP-DCs induce CD4$^+$ T-cells to produce immunoregulatory cytokines. *A*: Proliferation of naïve CD4$^+$ T-cells (2×10^4 lymphocytes/well) activated by a combination of 5 μg/ml anti-CD3α and 20 units/ml IL-2 in the absence of BM-DCs or in the presence of irradiated BM-DCs (5×10^3 cells/well) that had been left unstimulated (iDCs) or that that had been exposed to 1 μg/ml LPS (LPS-DCs) or 20 ng/ml TSLP (TSLP-DCs) for 48 h. *B*: Quantification of IFN-γ, IL-10, IL-4, and TGF-β released by CD4$^+$ T-cells cultured under the same conditions. Data are the average ± SD and are representative of four independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

proliferation (Fig. 5*A*). In contrast, TSLP-DCs induced robust proliferation (Fig. 5*A*), as previously reported (29).

The role of TSLP-DCs in de novo differentiation of CD4$^+$CD25$^+$Foxp3$^+$ Tregs was investigated in the same allogeneic assay using purified naïve CD4$^+$CD25$^-$ T-cells of NOD mice. The combination of anti-CD3 and IL-2 did not induce expression of Foxp3$^+$ (data not shown). At day 3, TSLP-DCs induced a moderate (3.32 ± 0.18%) but significant ($P < 0.01$) higher percentage of Foxp3$^+$CD4$^+$ T-cells compared with cells cultured in the presence of iDCs or LPS-DCs (1.80 ± 0.11 and 1.38 ± 0.09%, respectively) (Fig. 5*B*). Of importance, the percentage of Foxp3$^+$ CD4$^+$ T-cells further increased in the presence of TSLP-DCs (13.78 ± 1.84%) in comparison with cultures performed in the presence of iDCs (8.23 ± 0.38%) or LPS-DCs (4.54 ± 0.91%), after 7 days of culture (Fig. 5*B–D*). Foxp3$^+$-differentiated CD4$^+$ T-cells expressed high levels of CD25, CTLA4, GITR, and CD62L (data not shown).

To confirm that CD4$^+$CD25$^+$Foxp3$^+$ Tregs induced by TSLP-DCs were functional Tregs, we investigated the capacity of these cells to inhibit the proliferation and IFN-γ production of diabetogenic 8.3-CD8$^+$ T-cells in vitro (11). Results showed that CD4$^+$CD25$^-$Foxp3$^-$ T-cells activated with anti-CD3 and IL-2 in the absence of dendritic cells or in the presence of iDCs or LPS-DCs did not suppress the proliferation of 8.3-CD8$^+$ T-cells (Fig. 5*E*). In marked contrast, CD4$^+$CD25$^-$Foxp3$^-$ T-cells activated with anti-CD3 and IL-2 in the presence of TSLP-DCs decreased the proliferation of 8.3-CD8$^+$ T-cells by 50% (Fig. 5*E*). The suppressive effect of converted cells was also observed on IFN-γ release (Fig. 5*F*). These results indicated that TSLP-DCs acquired the capacity to induce

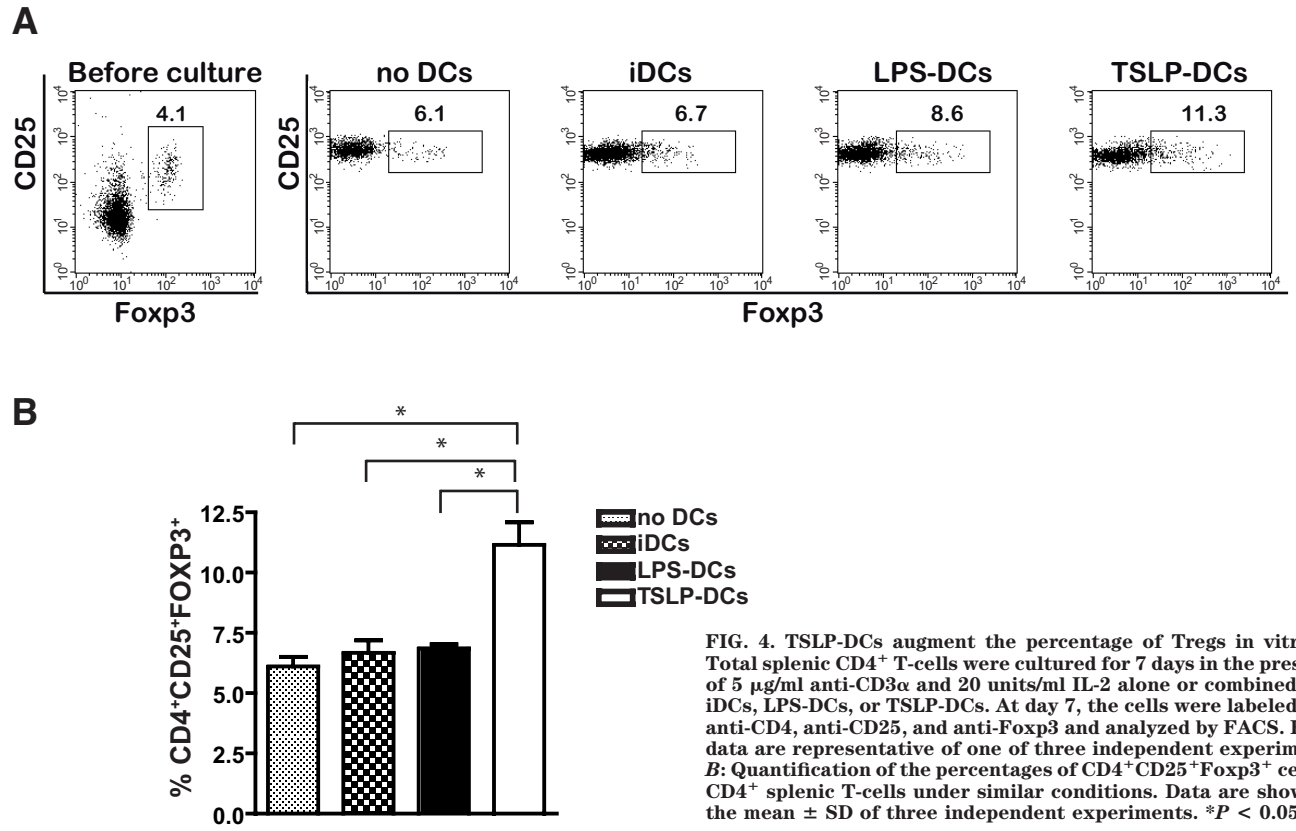

FIG. 4. TSLP-DCs augment the percentage of Tregs in vitro. *A*: Total splenic $CD4^+$ T-cells were cultured for 7 days in the presence of 5 μg/ml anti-CD3α and 20 units/ml IL-2 alone or combined with iDCs, LPS-DCs, or TSLP-DCs. At day 7, the cells were labeled with anti-CD4, anti-CD25, and anti-Foxp3 and analyzed by FACS. FACS data are representative of one of three independent experiments. *B*: Quantification of the percentages of $CD4^+CD25^+Foxp3^+$ cells of $CD4^+$ splenic T-cells under similar conditions. Data are shown as the mean ± SD of three independent experiments. *$P < 0.05$.

the expansion of Tregs and de novo differentiation of $CD4^+CD25^+Foxp3^+$ Tregs by conversion of $CD4^+CD25^-Foxp3^-$ T-cells and/or expansion of $CD4^+CD25^-Foxp3^+$ T-cells.

TSLP increases the number of Tregs in NOD mice. The findings that TSLP instructed dendritic cells to acquire tolerogenic properties in vitro prompted us to investigate the effect of TSLP on the development of Tregs in NOD mice. TSLP (500 ng · $mouse^{-1}$ · day^{-1}) was injected subcutaneously in the nucchal area for 6 days. The frequency of thymic and peripheral $CD4^+CD25^+Foxp3^+$ T-cells was determined 24 h and 7 days after the last injection. Results showed increased percentages of $CD4^+CD25^+Foxp3^+$ T-cells of TSLP-treated mice compared with control in the thymus (0.43 ± 0.03 vs. 0.27 ± 0.03%) and the spleen (3.92 ± 0.13 vs. 2.34 ± 0.12%) at 24 h (Fig. 6*A* and *C*). There were no changes in lymph nodes. At day 7, the percentage of $CD4^+CD25^+Foxp3^+$ T-cells in the thymus of TSLP-injected NOD mice decreased to the levels of control mice (0.31 ± 0.03 and 0.27 ± 0.03%, respectively). The percentage of Tregs was significantly increased in lymph nodes (4.17 ± 0.14%) and spleen (4.19 ± 0.23%) compared with control (2.66 ± 0.11 and 2.30 ± 0.16%, respectively) (Fig. 6*B* and *C*), indicating that TSLP promoted the pool of thymic and peripheral Tregs in NOD mice.

TSLP-DCs prevent diabetes development in NOD mice. The in vitro and in vivo studies described above led us to assess first the capacity of TSLP-DCs to prevent diabetes development in NOD mice. Therefore, 3-week-old NOD mice received a single injection of LPS-DCs (control) or TSLP-DCs and were monitored for diabetes. Diabetes occurred in 87% of control mice at 12 weeks, whereas only 25% of TSLP-DC–injected mice developed diabetes starting at 18 weeks (Fig. 7*A*). The TSLP-DC–induced protection was maintained up to 45 weeks ($P < 0.001$; Fig. 7*A*).

TSLP treatment inhibits diabetes development in NOD mice. To investigate the capacity of TSLP to protect against diabetes, 3-week-old NOD mice were treated each day with a single subcutaneous injection of PBS (control) or TSLP (500 ng/mouse) for 6 days and followed for diabetes development. Results showed that at 35 weeks, almost all control animals had developed diabetes, whereas 75% of TSLP-treated mice were diabetes free (blood glucose 5–7 mmol/l) for at least 50 weeks ($P < 0.001$; Fig. 7*A*), did not show any signs of side effects, and were fertile. Histopathological analysis of pancreata of these animals showed that 93.7% of islets lacked lymphocytic infiltration (Fig. 7*B*). A representative field from diabetes-free TSLP-treated NOD mice is shown (Fig. 7*C*). In contrast, the majority of pancreata from control NOD mice were devoid of islets (data not shown).

DISCUSSION

We report the ability of murine TSLP to activate dendritic cells of NOD mice and to blunt their pro-inflammatory potential. TSLP-DCs induced the conversion of naïve T-cells of NOD mice into Tregs in vivo and protected NOD mice from diabetes. We showed for the first time that injection of TSLP into NOD mice led to an increased number of Tregs in the thymus followed by increased peripheral Tregs numbers and conferred a significant protection against diabetes.

In humans and mice, stimuli such as CD40L or TLR ligands, LPS, and poly I:C strongly upregulate the expression of CMH II, CD80, CD86, and CD40 in dendritic cells. These stimuli induce the maturation of dendritic cells,

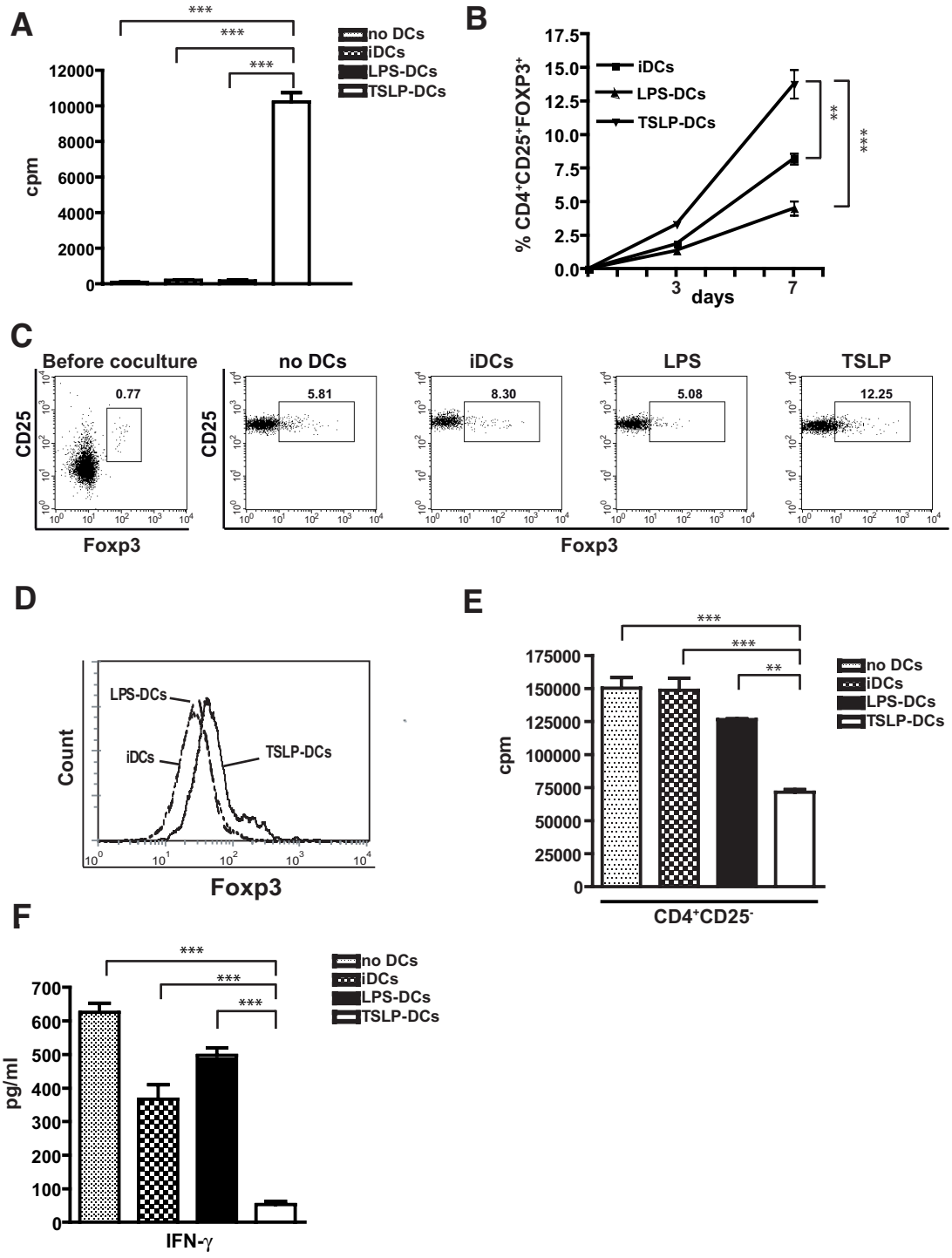

FIG. 5. TSLP-DCs induce the expansion of Tregs and the conversion of naïve $CD4^+CD25^-$ T-cells into $CD4^+CD25^+Foxp3^+$ T-cells. *A*: Proliferative response of purified $CD4^+CD25^+$ T-cells (2×10^4 lymphocytes/well) exposed to a combination of 5 μg/ml anti-CD3α and 20 units/ml IL-2 and irradiated iDCs or LPS- or TSLP-DCs (5×10^3 cells/well). *B*: Purified $CD4^+CD25^-$ T-cells were incubated with a combination of 5 μg/ml anti-CD3α and 20 units/ml IL-2 and iDCs or LPS- or TSLP-DCs at a 1:4 ratio of T-cells:BM-DCs. The percentages of $CD4^+CD25^+Foxp3^+$ T-cells were determined at days 3 and 7. Data represent the mean ± SD of two to three independent experiments. *C* and *D*: Representative FACS analysis of the percentages of splenic $CD4^+CD25^+Foxp3^+$ T-cells and Foxp3 expression in splenic $CD4^+CD25^-$ T-cells after 7 days of cultures under similar conditions. *E* and *F*: Splenic $CD4^+CD25^-$ T-cells (1×10^5 cells/well) were first cultured in the presence of anti-CD3α, IL-2, and conditioned BM-DCs. On day 7, the cells were collected, washed, and added to 8.3-$CD8^+$ T-cells (2×10^4 lymphocytes/well) exposed to 1 μg/ml NRP-A7– or 1 μg/ml TUM-pulsed irradiated splenic APCs (10^5 cells/well). The proliferative response of 8.3-$CD8^+$ T-cells was determined by [^{3}H]thymidine incorporation, and the production of IFN-γ was quantified by ELISA. $^{**}P < 0.01$ and $^{***}P < 0.001$.

which produce large amounts of inflammatory cytokines, such as IL-12, TNF-α, IL-1β, and IL-6, which induce Th1 differentiation. However, unlike CD40L and TLR ligands, human TSLP induces the upregulation of dendritic cells maturation markers without stimulating the production of inflammatory cytokines (39). Studies in mice have indicated that TSLP acts directly on early B- and T-cell development (40,41) and on dendritic cell maturation (42). Using BM-DCs of NOD mice, we show here that TSLP promoted a phenotype and a cytokine profile consistent

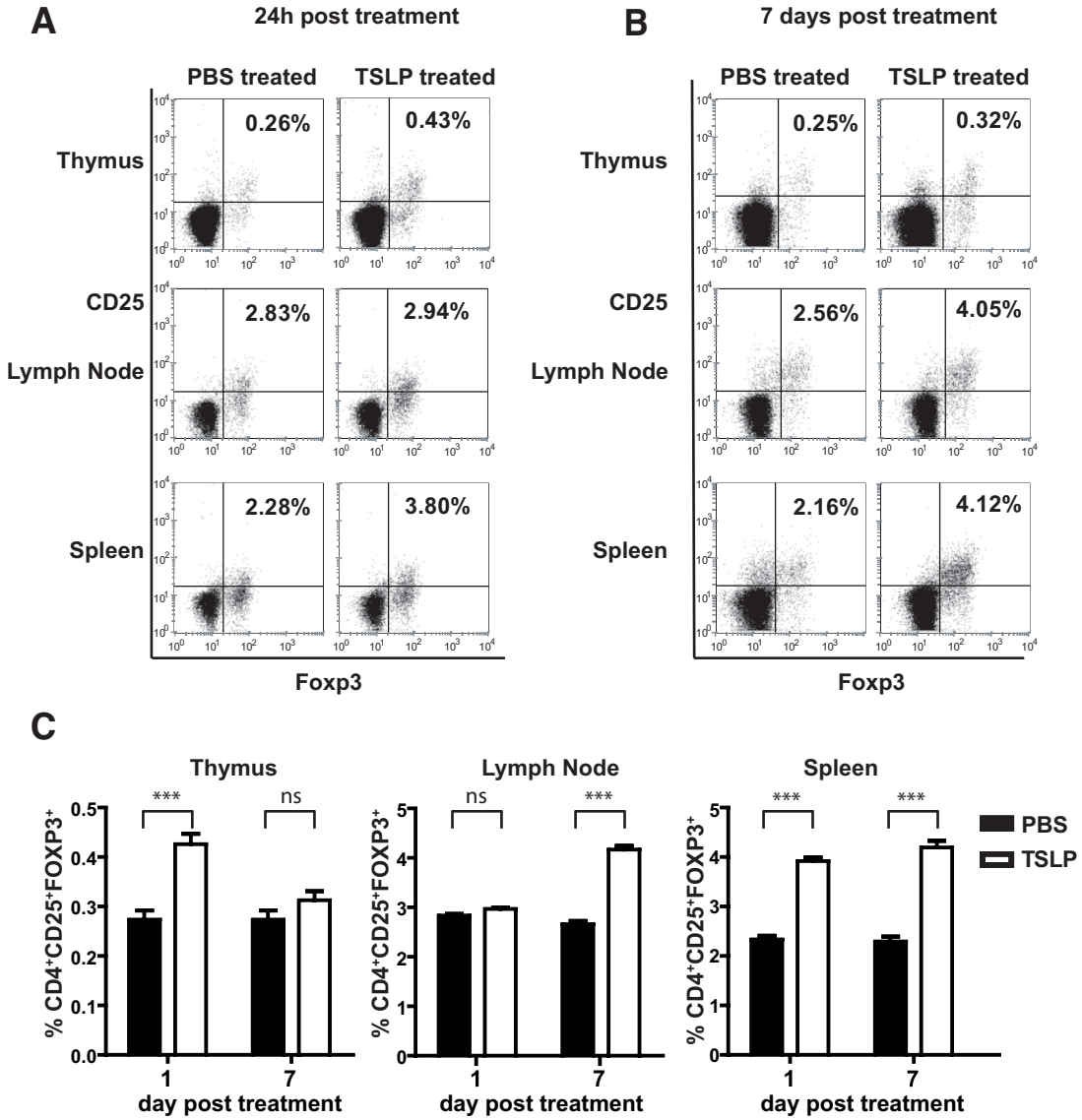

FIG. 6. TSLP increases the number of Tregs in NOD mice. *A* and *B*: NOD mice were injected subcutaneously with PBS (control) or TSLP (500 ng/animal) for 6 consecutive days. Thymus, pooled lymph nodes (mesenteric lymph node, pancreatic lymph node, brachial, inguinal, and axillary lymph nodes), and spleen were analyzed for the presence of Tregs 24 h and 7 days after treatment, as indicated. Cells were gated on $CD4^+$ T-cells, and percentages indicate the proportion of cells that were $CD4^+CD25^+Foxp3^+$. Data are representative of one of five independent experiments. *C*: Percentages of $CD4^+CD25^+Foxp3^+$ T-cells in different organs in PBS- or TSLP-treated mice (five mice in each group). Data represent the average ± SD. ***$P < 0.001$.

with the signature of tolerogenic semimature dendritic cells reported to be involved in initiating and maintaining T-cell tolerance (43). Unlike LPS-DCs, TSLP-DCs of NOD mice displayed less mature phenotypes and switched off their production of inflammatory cytokines (IL-12p70, TNF-α, and IFN-γ) known to induce a Th1 response. Consistent with our results, TSLP blunted the production of IL-12p70, TNF-α, IFN-γ, or mRNA encoding IL-12 by human dendritic cells and type 1 IFN family members that induce Th1 differentiation (29,44,45). Recent studies have shown that TSLP-DCs induced the expansion of $CD4^+$ and $CD8^+$ T-cells and the differentiation of naïve $CD4^+$ T-cells toward Th2 cells that produced IL-4, IL-13, and TNF-α but not IL-10 and IFN-γ (38,45). In addition, TSLP-DCs have been reported to prime $CD8^+$ T-cells into IL-5– and IL-13–producing effector cells exhibiting poor cytotoxic activity (46). Here, TSLP-DCs of NOD mice induced antigen-specific $CD8^+$ T-cell proliferation and differentiation into cytotoxic T-cells. In addition, TSLP-DCs primed $CD4^+$ T-cells to proliferate and to differentiate into noninflammatory Th2 cells that produced large amounts of IL-4 and IL-10, as reported previously (37). Interestingly, TSLP-DCs promoted $CD4^+$ T-cells to produce large amounts of TGF-β, a cytokine required for Foxp3 gene expression during Treg development (47). These data suggested that TSLP-DC–primed $CD4^+$ T-cells contained a high proportion of $Foxp3^+$ Tregs. When splenic $CD4^+$ T-cells were cultured in the presence of TSLP-DCs, the percentage of $Foxp3^+$ Tregs was significantly increased compared with $CD4^+$ T-cells primed with LPS-DCs or iDCs. The increase in the pool of Tregs was not only due to the expansion of existing Tregs but also to the conversion of $CD4^+Foxp3^-$ T-cells into Tregs. Our data were in agreement with a previous study that showed the capacity of TSLP-DCs to induce differentiation of $Foxp3^-$ T-cells into Tregs and their expansion using a similar allogeneic culture assay (29). Moreover, newly formed Tregs displayed characteristics of naturally occurring Tregs (15), such as expression

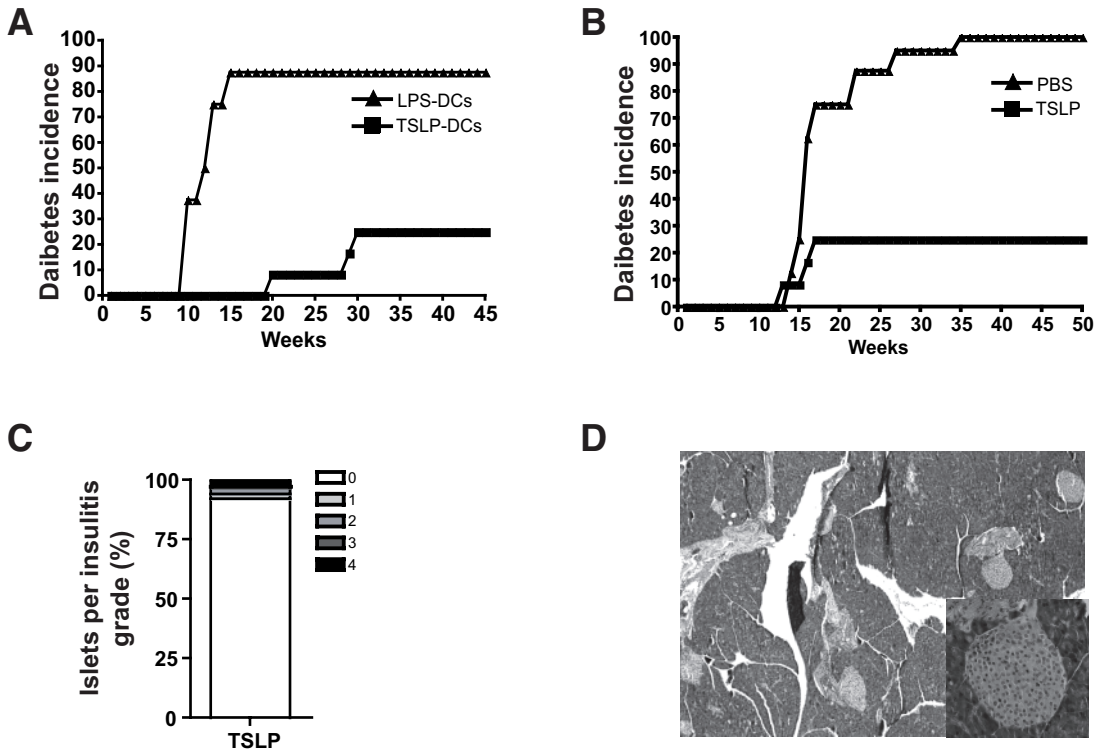

FIG. 7. TSLP and TSLP-DCs prevent diabetes development in NOD mice. *A*: NOD mice (12 animals/group) were injected intravenously at 3 weeks of age with LPS-DCs (5×10^6 cells/animal) or TSLP-DCs (5×10^6 cells/animal) and monitored for diabetes development. *B*: NOD mice (12 animals/group) were treated with PBS or TSLP (500 ng/animal) for 6 consecutive days and monitored for diabetes development. *C*: Pancreata of TSLP-treated NOD mice ($n = 6$, 40–50 islets/mouse) were scored for insulitis. *D*: Representative microphotographs of hematoxylin-eosin–stained pancreatic sections of TSLP-treated NOD mice.

of high levels of CD62L, CTLA-4, and GITR and suppression of proliferation of $CD8^+$ T-cells and IFN-γ production.

In NOD mice, dendritic cell development from myeloid progenitors is impaired and is associated with abnormal levels of expression of costimulatory molecules and increased capacity to secrete IL-12p70 and to stimulate $CD4^+$ and $CD8^+$ T-cells (21–23). The findings that TSLP restored tolerogenic functions of BM-DCs of NOD mice were further extended in in vivo experiments. Results showed that a single injection of TSLP-DCs in young NOD mice was sufficient to induce a significant protection against diabetes, whereas LPS-DC–treated NOD mice developed accelerated diabetes. This protection resulted from an increased pool of Tregs that exhibited increased suppressive functions and contained high proportion of $Foxp3^{high}$ T-cells (G.B., S.G., G.D., A.A., unpublished data).

Recently, OX40 engagement of $Foxp3^-$ T-cells has been shown to suppress the induction of Foxp3 driven by antigen or exogenous TGF-β (48). Here, the conversion of $Foxp3^-$ to $Foxp3^+$ $CD4^+$ T-cells could be explained by a reduced expression of OX40L on TSLP-DCs. This possibility was supported by the findings of enhanced induction of $Foxp3^+$ T-cells when the OX40L/OX40 signaling pathway was blocked (G.B., S.G., G.D., A.A., unpublished data). Moreover, the involvement of the OX40/OX40L pathway in diabetes was consistent with previous reports of diabetes protection in NOD mice treated with anti-OX40L antibodies and in $OX40L^{-/-}$ NOD mice (49,50). These data suggested the important role of OX40/OX40L interaction in the differentiation of Tregs and the protection against diabetes observed here in TSLP-DCs transferred NOD mice.

Naturally occurring Tregs arise from thymus and are exported to the peripheral lymphoid organs to contribute to peripheral tolerance. In NOD mice, the breakdown of T-cell tolerance is associated with quantitative and qualitative decreases in the pool of $CD4^+CD25^+$ Tregs (24,25). In view of these observations, several studies have shown that adoptive transfer of Tregs (51) or reestablishment of Treg pool using anti-CD3 or GM-CSF treatment (11,52) were effective in the restoration of T-cell tolerance and consequent protection from diabetes. Here, subcutaneous injections of TSLP in NOD mice led to increased number of Tregs in the thymus and, subsequently, in the peripheral organs (spleen and lymph node), confirming the capacity of TSLP to promote Treg differentiation in vivo. Although TSLP appears to act on dendritic cells in the human system and on dendritic cells and T-cells in the murine system (39), increased Treg pool in the thymus may result from a direct effect of TSLP on T-cells or on dendritic cells. The underlying mechanisms of increased Tregs have not been fully elucidated. The involvement of dendritic cells with tolerogenic properties in TSLP-protected NOD mice is supported by in vitro data. Furthermore, FITC skin painting experiments clearly showed that TSLP injection mobilized skin dendritic cells to the thymus, suggesting involvement of such dendritic cells in thymic Treg differentiation (data not shown). Whereas Tregs were increased in the spleen and lymph nodes 7 days after injection, the pool of thymic Tregs decreased to the levels observed in control mice. These data suggested that positively selected thymic Tregs were exported to the periphery and contributed to the Treg pool required for efficient induction and maintenance of T-cell tolerance in NOD mice. However, induction of Treg differentiation in the periphery by immi-

grant TSLP-conditioned skin dendritic cells cannot be excluded. This possibility is under current investigations in our laboratories. The induction of tolerogenic function of dendritic cells and increased Tregs in TSLP-treated mice could unveil potential therapeutic treatments of autoimmune diabetes.

In conclusion, our study showed for the first time that the existing default of tolerance in autoimmune diabetes in NOD mice could be restored by TSLP through induction of tolerogenic dendritic cells, resulting in Treg differentiation and promotion of noninflammatory IL-10–producing Th2 cells that are a hallmark immune response involved in the prevention of autoimmune diabetes.

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