

## On the Edge of Autoimmunity

# T-Cell Stimulation by Steady-State Dendritic Cells Prevents Autoimmune Diabetes

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**Targeting of antigens to immature dendritic cells has been shown to result in antigen-specific T-cell tolerance in vivo. In the INS-HA/TCR-HA transgenic mouse model for type 1 diabetes, we tested the potential of the dendritic cell-specific monoclonal antibody DEC-205 conjugated to the hemagglutinin (HA) antigen (DEC-HA) to prevent disease onset. Whereas untreated INS-HA/TCR-HA mice all develop insulinitis, and ~40% of these mice become diabetic, repeated injection of newborn mice with DEC-HA protected almost all mice from disease development. Histological examination of the pancreata revealed significant reduction of peri-islet infiltrations in DEC-HA-treated mice, and the islet structure remained intact. Moreover, HA-specific CD4<sup>+</sup> T-cells from anti-DEC-HA-treated INS-HA/TCR-HA mice exhibited increased expression of Foxp3, cytotoxic T-lymphocyte-associated antigen-4, and the immunosuppressive cytokines interleukin-10 and transforming growth factor- $\beta$ . The findings indicate that targeting of the HA antigen to immature dendritic cells in vivo leads to a relative increase of antigen-specific Foxp3<sup>+</sup> regulatory T-cells that suppress the development of type 1 diabetes. Our results provide a basis for the development of novel strategies focusing on prevention rather than treatment of autoimmune diseases. *Diabetes* 54:3395–3401, 2005**

**T**ype 1, or insulin-dependent, diabetes is regarded as an immune-mediated disease in which  $\beta$ -cells of the pancreatic islets of Langerhans are destroyed as a consequence of inflammatory stimuli (1). Several observations demonstrated a pathological role of T-cells specific for  $\beta$ -cell-associated antigens in

this process, i.e., pancreata from diabetic mice are infiltrated with T-cells (2), and crossing of diabetes-prone NOD mice on the SCID (3) background interferes with development of diabetes. Moreover, the disease can be induced in animals by transfer of autoreactive T-cells (4), and neonatal thymectomy prevents the disease (5).

In the INS-HA/TCR-HA transgenic mouse model, which is based on the concomitant expression of hemagglutinin (HA) in the  $\beta$ -cells of the pancreas and an HA-specific major histocompatibility complex class II-restricted transgenic T-cell receptor (TCR) specific for this antigen, one observes 2–3 weeks after birth a rather strong accumulation of lymphocytes around the pancreatic islets. This infiltrate can persist for long periods of time, but the  $\beta$ -cells undergo destruction in only one-third of the mice, and diabetes develops (6). In this particular model, the diabetes incidence decreases in INS-HA/TCR-HA mice on the RAG<sup>-/-</sup> genetic background. Furthermore, HA-specific CD4<sup>+</sup> T-cells isolated from double-transgenic mice exhibit a reduced proliferative capacity (7). These observations suggest the possibility that immunoregulation by CD4<sup>+</sup> T-cells contributes to the control of  $\beta$ -cell destruction. This assumption is consistent with results showing that the development of diabetes can be provoked by maneuvers that bypass immunoregulation, such as treatment with cyclophosphamide or transfer of HA-specific T-cells into lymphopenic mice expressing HA in the pancreas (7). Likewise, in the NOD mouse model for type 1 diabetes, an imbalance between diabetogenic effectors and immunoregulatory T-cell subsets has been described (8,9).

The induction of antigen-specific tolerance is a tempting approach to prevent type 1 diabetes, particularly because some autoantigens triggering the disease are currently being defined (10,11). In this context, the in vivo induction of antigen-specific regulatory T-cells that may prevent autoimmunity is of particular interest. Recently, it has been shown that it is possible to artificially generate or expand suppressor T-cells inside (12,13) and outside (14) the thymus, resulting in tolerance in the absence of immunity. Another approach uses the immunomodulatory properties of immature dendritic cells to induce T-cell tolerance rather than immunity. This attempt is based on the use of a monoclonal antibody directed against the endocytosis receptor DEC-205 that targets antigens in vivo nearly exclusively to dendritic cells (15). In contrast to other methods using in vitro-generated, i.e., mature and/or activated, dendritic cells, targeting of antigens to DEC-205 allows efficient loading and antigen presentation

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CTLA, cytotoxic T-lymphocyte-associated antigen; DEC-HA, DEC-205 conjugated to the hemagglutinin antigen; HA, hemagglutinin; IL, interleukin; TCR, T-cell receptor; TGF, transforming growth factor; TRP2, tyrosinase-related protein-2.

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by immature, so-called steady-state dendritic cells. Results obtained in adoptive transfer models suggested that presentation of antigens by steady-state dendritic cells leads to short-term proliferation of antigen-specific T-cells followed by deletion (15,16). Such targeting of antigen can also lead to an increase in regulatory T-cells either by expansion of already existing cells or by conversion of nonregulatory into regulatory T-cells (14,17).

In this report, we show that repeated treatment of INS-HA/TCR-HA mice with anti-DEC-205 conjugated to the HA antigen (DEC-HA) conjugates protected mice from developing spontaneous diabetes. In untreated or DEC-205-treated controls, accumulation of lymphocytes around the pancreatic islets resulted in progression to diabetes in 40% of mice, whereas in DEC-HA-treated mice, there was an increase of Foxp3<sup>+</sup> T-cells and of the immunosuppressive cytokines interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$ , which efficiently prevented the onset of diabetes. Thus, our results emphasize the potential of targeting to tolerogenic dendritic cells as a tool to prevent autoimmunity in predisposed individuals.

## RESEARCH DESIGN AND METHODS

BALB/c mice were obtained from Harlan (Borchen, Germany). TCR-HA transgenic mice expressing a TCR- $\alpha\beta$  specific for peptide 110–120 from influenza HA presented by I-E<sup>H</sup> have been described previously (18). TCR-HA mice were crossed to INS-HA mice expressing the HA protein from the same virus in pancreatic  $\beta$ -cells under the control of the rat insulin promoter (19) to generate INS-HA/TCR-HA mice. The incidence of spontaneous diabetes in double-transgenic mice was 40%. Mice aged 12–16 weeks were used for the experiments and were housed under specific pathogen-free conditions. All animal experiments were performed in accordance with institutional, state, and federal guidelines.

**Conjugation of the HA110-120 peptide to the DEC-205-specific antibody.** Purified anti-DEC-205 (NLDC-145 HB 290) was conjugated to activated HA<sub>110-120</sub> peptide as described before (17), using the heterobifunctional cross-linker SMCC [succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate] (Pierce) according to the manufacturer's protocol. Purification and coupling of recombinant tyrosinase-related protein-2 (TRP2) to DEC-205 has been described elsewhere (20). To test for successful conjugation, the DEC-HA conjugate was subjected to SDS-PAGE, followed by blotting on a nylon membrane and incubation with the HA-specific antibody CMI1.2. Nonconjugated anti-DEC-205 antibody served as negative control, and a lysate of control bacteria expressing this particular HA epitope served as positive control.

**Isolation of dendritic cells and in vitro T-cell stimulation.** Popliteal, inguinal, and axillary lymph nodes were isolated from BALB/c mice and carefully minced on ice, followed by 1 h of digestion at 37°C with collagenase. Cells were passed through a 70- $\mu$ m filter, and single-cell suspension was incubated with anti-mouse CD11c MACS microbeads (Miltenyi) following the manufacturer's instructions. CD11c<sup>+</sup> cells were isolated using an AutoMACS (Miltenyi), and  $5 \times 10^5$  cells were incubated overnight in 48-well plates with 10  $\mu$ g/ml DEC-HA conjugate or DEC-TRP2 as negative control. Cells were then harvested, washed, and used to stimulate  $2 \times 10^5$  CD4<sup>+</sup> T-cells isolated from the spleen of TCR-HA mice using the CD4<sup>+</sup> isolation kit from Miltenyi. Alternatively, BALB/c mice were injected intra-foot pad with 0.5  $\mu$ g DEC-HA or DEC-TRP2 conjugate. Then, 1 day later, popliteal, inguinal, and axillary lymph nodes were isolated, followed by CD11c<sup>+</sup> enrichment (see above), and  $5 \times 10^4$  of these cells were used to stimulate  $2 \times 10^5$  HA-specific T-cells isolated from the spleen of TCR-HA mice. Assays were performed in Iscove's modified Dulbecco's media medium containing 10% FCS in 96-well plates. After 48 h, 1  $\mu$ Ci per well of [<sup>3</sup>H]thymidine was added for the last 8 h of the experiment, and thymidine incorporation was measured by scintillation counting. Assays were performed in triplicate, and the SD never exceeded 10%.

**Treatment of INS-HA/TCR-HA mice with the DEC-HA conjugate.** We injected 3-day-old INS-HA/TCR-HA mice with 0.5  $\mu$ g i.p. DEC-HA conjugate or DEC-205 antibody twice a week until 4 weeks of age, and thereafter they were injected once a week until 12–16 weeks of age.

**Diabetes monitoring.** Blood glucose concentrations in mice were monitored using a Haemo Glukotest 200-800R (Roche). Mice were considered diabetic when glycemia was >200 mg/dl for two consecutive measurements.

**Histology.** Pancreata were fixed with neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Staining for intracellular insulin was performed with a guinea pig anti-human insulin antibody from Dako and revealed with a horseradish peroxidase-conjugated goat anti-guinea pig conjugate. Histological details were quantified morphometrically using a Colorview II digital camera on a BX 41 Olympus light microscope and SIS image analysis software (Munster, Germany). Immunohistochemical stains for insulin were used to measure the size (area) of islets containing insulin-producing  $\beta$ -cells (controls) or, where appropriate, the area of the largest fragments of disrupted islets (INS-HA/TCR-HA). Areas were given (in  $\mu$ m<sup>2</sup>) as the means  $\pm$  SD,  $n = 5$ .

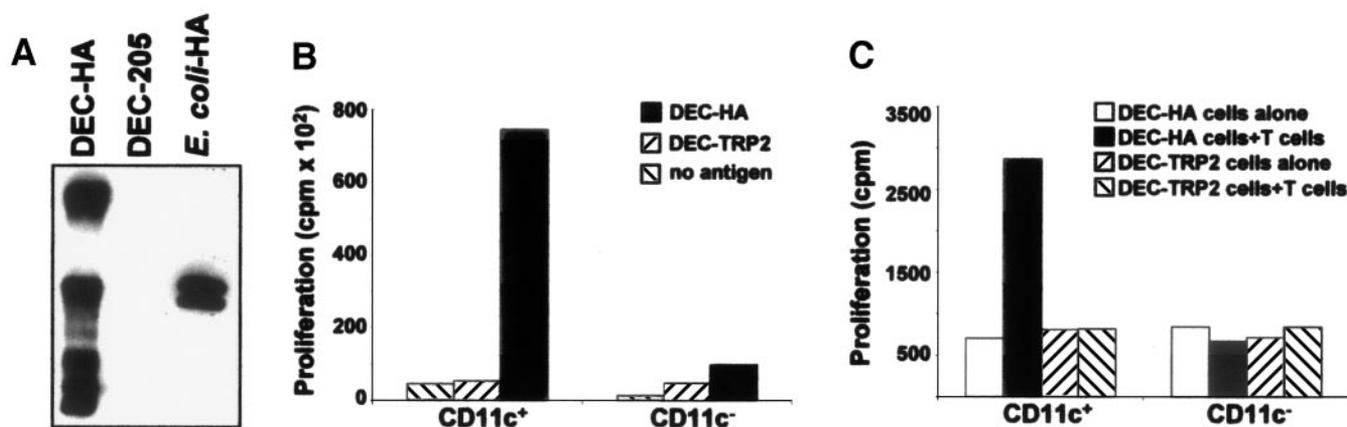
**Antibodies and flow cytometry.** The monoclonal antibodies 6.5 (anti-TCR-HA) and CD62L (Mel-14) were purified from hybridoma supernatants by protein G affinity chromatography. All other antibodies were obtained from BD Biosciences (San Jose, CA). Two- and three-color flow cytometry was performed on a FACSCalibur (BD Biosciences). For quantitative real-time RT-PCR, CD4<sup>+</sup>6.5<sup>+</sup> T-cells were sorted with a MoFlow cell sorter (Cytomation, Fort Collins, CO), and purity was >97%.

**Real-time RT-PCR.** Total RNA was prepared from  $2 \times 10^5$  sorted T-cells using an RNeasy kit (Qiagen, Hilden, Germany). cDNA synthesis was performed using Superscript II reverse transcriptase, oligo dT, and random hexamer primers (Invitrogen) according to the manufacturer's recommendations. Quantitative real-time RT-PCR was performed in an ABI PRISM cyclor (Applied Biosystems), using a SYBR Green PCR kit from Stratagene and specific primers optimized to amplify 90- to 250-bp fragments from the different genes analyzed. A threshold was set in the linear part of the amplification curve, and the number of cycles needed to reach it was calculated for every gene. Relative mRNA level were determined, using included standard curves for each individual gene and further normalization to ribosomal protein S9. Melting curves established the purity of the amplified band. Primer sequences are: Foxp3 (5'-CTG GCG AAG GGC TCG GTA GTC CT-3', 5'-CTC CCA GAG CCC ATG GCA GAA GT-3'), cytotoxic T-lymphocyte-associated antigen (CTLA-4; 5'-GGG CTG GGT CTT TAC ACT TTT-3', 5'-CTT CCT GTG GCA TTA ACT TTG TGT-3'), IL-10 (5'-CTG GAC AAC ATA CTG CTA ACC GAC TC-3', 5'-ATT TCT GGG CCA TGC TTC TCT GC-3'), TGF- $\beta$  (5'-ACC TGG GTT GGA AGT GGA T-3', 5'-GAA GCG CCC GGG TTG TGT TGG TT-3'), and ribosomal protein S9 (5'-CTG GAC GAG GGC AAG ATG AAG-C-3', 5'-TGA CGT TGG CGG ATG AGC ACA-3').

## RESULTS

**DEC-HA treatment prevents the onset of diabetes in INS-HA/TCR-HA mice.** To target the HA antigen to immature dendritic cells, the HA<sub>110-120</sub> peptide was conjugated to the monoclonal antibody NLDC-145 specific for the endocytic receptor DEC-205. Successful conjugation was verified by Western blot analysis (Fig. 1A). It has been shown before that anti-DEC-205 targets antigen to lymph nodes in vivo, where it specifically binds to CD11c<sup>+</sup> dendritic cells, resulting in uptake and efficient presentation of the particular antigen (15,17). Consistent with these results, we confirmed the capacity of the DEC-HA conjugate to target dendritic cells in vitro and in vivo and to stimulate HA-specific T-cells (Fig. 1B and C).

To study the influence of antigen presentation by dendritic cells in the steady state on the outcome of CD4<sup>+</sup> T-cell-driven autoimmune diabetes, we used the INS-HA/TCR-HA double-transgenic mouse model, in which all mice develop insulinitis, and diabetes occurs in about one-third of these mice (6). Newborn mice were injected with 0.5  $\mu$ g i.p. DEC-HA or DEC-205, respectively, twice a week for 4 weeks and then once a week until 12–16 weeks of age, and diabetes was monitored by blood glucose measurement. We found that 40% of untreated and 57% of DEC-205-treated mice developed diabetes within 10 weeks of life, whereas only 6% (1 of 16) mice treated with the DEC-HA conjugate became diabetic (Table 1). Histological examination of the pancreata revealed that untreated nondiabetic and diabetic INS-HA/TCR-HA mice developed massive intra-islet infiltration and widespread fragmentation of pancreatic islets. This was accompanied by massive disruption of the islet architecture and reduced



**FIG. 1.** Conjugation of the HA peptide to DEC-205 and targeting of CD11c<sup>+</sup> dendritic cells. **A:** Immunoblotting with anti-HA<sub>110-120</sub> antibody. The antigenic peptide HA<sub>110-120</sub> was conjugated to the monoclonal antibody NLDC-145 (anti-DEC-205) and subsequently analyzed by Western blot analysis. Unconjugated anti-DEC-205 antibody served as negative control, and a lysate generated from *E. coli* bacteria expressing the HA<sub>110-120</sub> epitope was included as positive control. **B and C:** DEC-HA targets CD11c<sup>+</sup> dendritic cells in vitro and in vivo and induces HA-specific T-cell proliferation. **B:** Lymph nodes were isolated from BALB/c mice, digested with collagenase, and separated in CD11c<sup>+</sup> and CD11c<sup>-</sup> cells. Cells were incubated overnight with the DEC-HA conjugate or DEC-TRP2 as irrelevant antigen control, washed, and used as antigen-presenting cells to stimulate HA-specific CD4<sup>+</sup> T-cells. **C:** Alternatively, the DEC-HA or DEC-TRP2 conjugate was injected into the foot pads of BALB/c mice, and 24 h later, popliteal, inguinal, and axillary lymph nodes were harvested and CD11c<sup>+</sup> and CD11c<sup>-</sup> lymph node cells used as in vivo-loaded antigen-presenting cells for in vitro stimulation of HA-specific T-cells. T-cell proliferation was assessed by [<sup>3</sup>H]thymidine incorporation. Assays were performed in triplicate, and the SD did not exceed 10%.

numbers of insulin-staining  $\beta$ -cells. In contrast, in DEC-HA-treated mice, the severity of insulinitis was clearly reduced, and the islet structure remained intact (Fig. 2). Thus, our results indicate that repeated treatment of diabetes-prone mice with DEC-HA, leading to targeting of the respective HA antigens to steady-state dendritic cells, efficiently prevents the onset of CD4<sup>+</sup> T-cell-mediated autoimmune diabetes.

**Phenotype of HA-specific CD4<sup>+</sup> T-cells.** We next addressed the question of whether injection of INS-HA/TCR-HA mice with the DEC-HA conjugate results in deletion of HA-specific CD4<sup>+</sup> T-cells, thus preventing autoimmune  $\beta$ -cell destruction. Although the percentage of HA-specific T-cells was lower in treated versus untreated mice, we did not observe extensive clonal deletion in DEC-HA-injected mice. Moreover, no alteration in the TCR expression level was observed (Fig. 3A). To examine the proliferative capacity of HA-specific CD4<sup>+</sup> T-cells in mice treated with multiple doses of the DEC-HA conjugate, HA-specific CD4<sup>+</sup> T-cells were sorted from the spleen of DEC-HA-injected, DEC-205-injected, and untreated INS-HA/TCR-HA mice, as well as from TCR-HA single-transgenic mice. Cells were stimulated in vitro with the HA peptide. Compared with naïve T-cells isolated from TCR-HA mice, the proliferative response of splenic T-cells

from DEC-HA-treated mice was strongly reduced (Fig. 3B), suggesting that T-cell anergy may play a role in controlling the progression from peri-islet infiltration and  $\beta$ -cell destruction. However, as already observed previously, CD4<sup>+</sup> T-cell responses were also poor in untreated normoglycemic INS-HA/TCR-HA mice (7,21) (Fig. 3B), a fact that might reflect the existence of peripheral tolerance mechanisms contributing to the protection of some of these untreated mice from progression toward diabetes. Further characterization by flow cytometric analysis revealed that HA-specific T-cells from INS-HA/TCR-HA mice repeatedly injected with the DEC-HA conjugate exhibit increased expression of CD25 and CD69 compared with naïve T-cells from TCR-HA mice (Fig. 4). But when compared with normoglycemic INS-HA/TCR-HA mice that were not treated with DEC-HA, we recorded lower levels of the activation markers CD25 and CD69 as well as comparably high expression of CD45RB and the lymph node homing receptor CD62L.

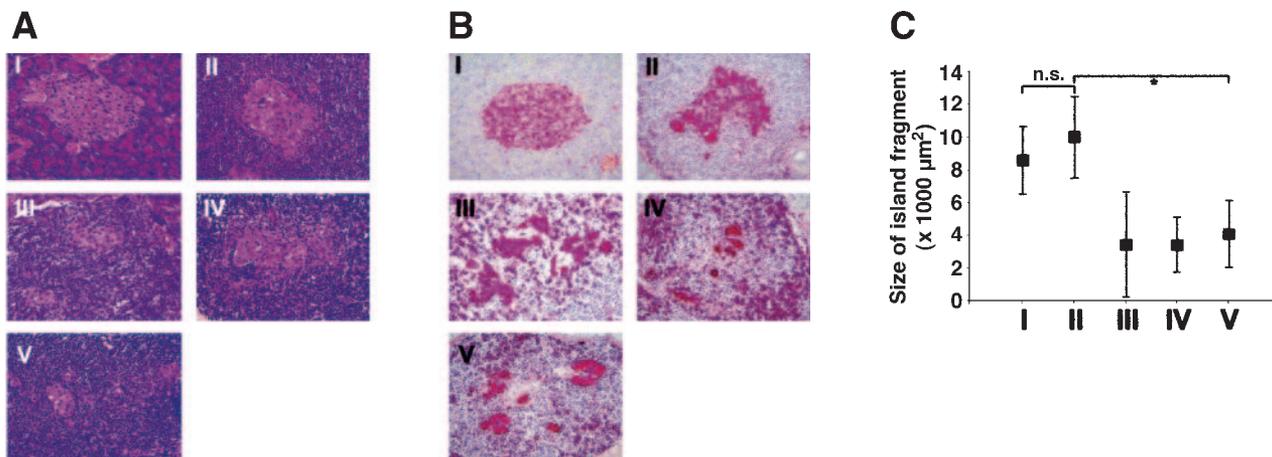
**Injection of DEC-HA leads to the expansion of antigen-specific Foxp3<sup>+</sup> regulatory T-cells.** In the INS-HA/TCR-HA model, 60% of the mice never become diabetic, despite a very extensive accumulation of lymphocytes around the islets, and HA-specific CD4<sup>+</sup> T-cells from secondary lymphatic organs have impaired proliferative capacity. These observations led to the proposition of peripheral tolerance mechanisms counterbalancing disease progression (7). Assuming that repetitive stimulation of naïve CD4<sup>+</sup> T-cells with immature dendritic cells results in expansion of regulatory T-cells, perhaps accompanied by deletion of nonregulatory T-cells or even de novo generation of regulatory T-cells in the absence of proliferation (14), one would expect that HA-loaded immature dendritic cells would further shift the balance toward prevention of diabetes. Consistent with this hypothesis, DEC-HA-treated INS-HA/TCR-HA mice maintained an intact islet structure and generally resisted the onset of the disease (Fig. 2 and Table 1), despite obvious lymphocytic infiltrations surrounding the pancreatic islets.

To evaluate whether prevention of autoimmune disease

**TABLE 1**  
DEC-HA treatment prevents the onset of diabetes

	Total (n = 52)	Diabetic (>200 $\mu$ g/dl)*	Nondiabetic ( $\leq$ 200 $\mu$ g/dl)
INS-HA/TCR-HA			
DEC-HA treated†	16	1 (6)	15 (94)‡
DEC-205 treated†	21	12 (57)	9 (43)§
Untreated	15	6 (40)	9 (60)

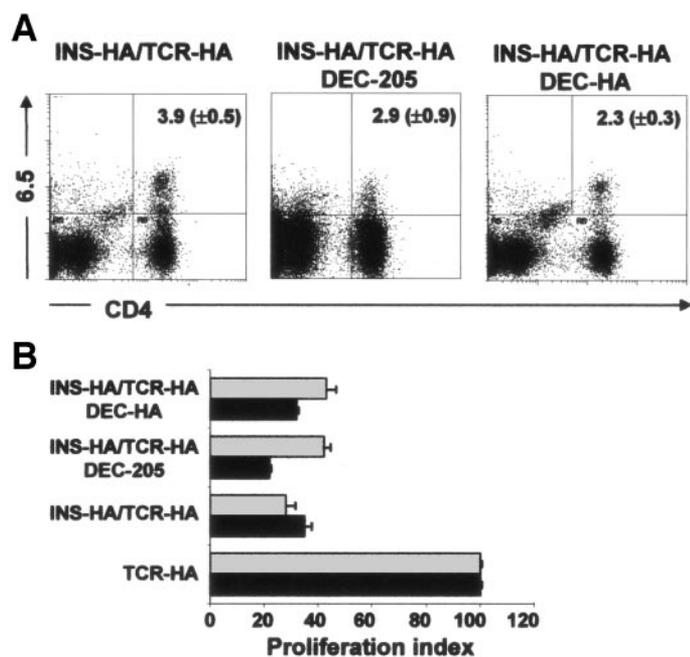
Data are n or n (%). \*Diabetes was monitored by blood glucose measurement. †INS-HA/TCR-HA mice were injected with 0.5  $\mu$ g DEC-HA conjugate or unconjugated DEC-205 antibody twice a week for 4 weeks, followed by injections once a week until the age of 12–16 weeks. ‡ $P \leq 0.025$ , DEC-HA-treated vs. untreated mice; § $P \leq 0.01$ , DEC-HA-treated vs. DEC-205-treated mice; ||DEC-205-treated vs. untreated mice.



**FIG. 2.** Injection of DEC-HA prevents the onset of diabetes in INS-HA/TCR-HA mice. Shown are histological examinations of pancreatic islets from INS-HA mice (I) and DEC-HA-treated (II), DEC-205-treated nondiabetic (III), untreated nondiabetic (IV), and diabetic (V) INS-HA/TCR-HA mice. Pancreatic sections were stained with hematoxylin and eosin (A) and with guinea pig anti-insulin followed by horseradish peroxidase-conjugated goat anti-guinea pig (B). Insulin-producing  $\beta$ -cells are depicted in red. C: Histological results were corroborated morphometrically by measuring the area of pancreatic islets (controls) or islet fragments (INS-HA/TCR-HA) to quantify the degree of islet disruption. Areas are given in  $1,000 \mu\text{m}^2$  for each group ( $n = 5$ ; means  $\pm$  SD). \*Statistically significant difference (unpaired  $t$  test,  $P \leq 0.05$ ). n.s., no statistical difference.

in DEC-HA-treated INS-HA/TCR-HA mice is associated with an increase of antigen-specific regulatory T-cells, quantitative gene expression analysis for selected regula-

tory T-cell markers was performed on HA-specific  $\text{CD4}^+$  T-cells isolated from TCR-HA, diabetic INS-HA/TCR-HA, nondiabetic-INS-HA/TCR-HA, as well as DEC-205- and DEC-HA-treated nondiabetic INS-HA/TCR-HA mice. As shown in Fig. 5, elevated expression of CTLA-4, IL-10, and TGF- $\beta$  in peripheral HA-specific  $\text{CD4}^+$  T-cells from nondiabetic INS-HA/TCR-HA mice was apparent compared with diabetic control mice. Moreover, expression of these markers further increased in T-cells derived from DEC-HA-treated mice. The expression of the transcription factor Foxp3, which was recently described as a key molecule required for the development and function of regulatory T-cells (22), followed a similar pattern, with stepwise increased expression levels in the antigen-specific  $\text{CD4}^+$  T-cell pool from diabetic and nondiabetic INS-HA/TCR-HA mice and highest expression in HA-specific T-cells from DEC-HA-injected mice (Fig. 5). Thus, DEC-HA treatment resulted in elevated expression of regulatory factors (such as immunosuppressive cytokines) within the HA-specific T-cell population as well as molecules indicative of regulatory T-cells (i.e., CTLA-4 and Foxp3). This shift in balance probably accounts for the observed block in diabetes development in INS-HA/TCR-HA mice.



**FIG. 3.** Proliferative capacity and frequency of HA-specific  $\text{CD4}^+$  T-cells in DEC-HA-treated INS-HA/TCR-HA mice. A: Frequency of islet-specific  $\text{CD4}^+$  T-cells in the spleen of DEC-HA-injected INS-HA/TCR-HA mice. Splenocytes from nondiabetic untreated and DEC-HA- or DEC-205-treated mice were isolated, and the percentage of  $\text{CD4}^+\text{6.5}^+$  T-cells was determined by fluorescence-activated cell sorting analysis. Data were obtained from a pool of  $n \geq 3$  individual mice, and mean values of two independent experiments ( $\pm$ SD) are indicated. B: Proliferative capacity of islet-specific  $\text{CD4}^+$  T-cells in INS-HA/TCR-HA mice.  $\text{CD4}^+\text{6.5}^+$  T-cells from the spleen of TCR-HA mice and untreated and DEC-HA-treated INS-HA/TCR-HA mice ( $n \geq 3$ ) were isolated by fluorescence-activated cell sorting and stimulated in vitro in the presence of irradiated antigen-presenting cells with  $10 \mu\text{g/ml}$  HA<sub>110-120</sub> peptide. Proliferation was measured by [ $^3\text{H}$ ]thymidine incorporation, and the proliferation index indicates percent proliferation referred to the maximal proliferation value obtained for T-cells from TCR-HA mice, which was arbitrarily set to 100%. Assays were performed in triplicate. ■ and □, results obtained from two independent experiments.

**DISCUSSION**

The mechanisms acting on peripheral tolerance are not entirely clear yet, but they can be divided into those directly affecting the responding T-cells, such as inactivation or deletion of specific T-cells (recessive), and those that act through additional cells or factors, such as regulatory T-cells or suppressive cytokines (dominant). Although there are several possible mechanisms that underlie the induction of peripheral T-cell tolerance by steady-state dendritic cells, our results suggest that targeting of antigens to immature dendritic cells might prevent the onset of type 1 diabetes in mice by the combined action of both T-cell-recessive and -dominant mechanisms. We have shown that partial deletion of HA-specific  $\text{CD4}^+$  T-cells may occur in DEC-HA-treated INS-HA/TCR-HA mice and that remaining T-cells exhibit reduced

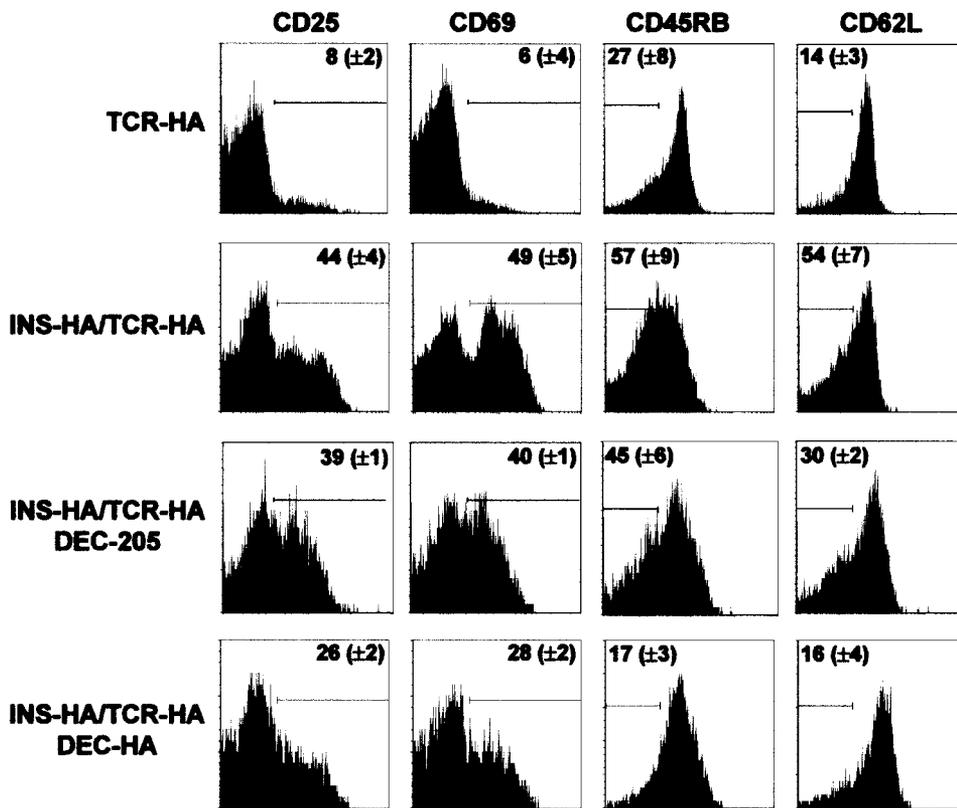


FIG. 4. Phenotype of HA-specific CD4<sup>+</sup> T-cells in mice injected with the DEC-HA conjugate. CD4<sup>+</sup>6.5<sup>+</sup> splenocytes from either TCR-HA mice or DEC-HA-treated, DEC-205-treated, or untreated INS-HA/TCR-HA mice ( $n \geq 3$ ) were analyzed by fluorescence-activated cell sorting for the expression of CD25, CD69, CD45RB, and CD62L. Numbers in the diagrams display the mean percentages of islet-specific CD4<sup>+</sup>6.5<sup>+</sup> T-cells expressing the particular surface marker from two independent experiments ( $\pm$ SD).

proliferative capacity compared with naïve CD4<sup>+</sup> T-cells from TCR-HA mice. Moreover, HA-specific CD4<sup>+</sup> T-cells from DEC-HA-treated mice showed elevated levels of CTLA-4, a coinhibitory molecule of the CD28 family, which has been shown to be essential for the maintenance of self-tolerance (23,24). This is consistent with previous studies showing that stimulation of T-cells by immature dendritic cells results in increased CTLA-4 expression and induction of peripheral T-cell tolerance (17).

Besides these recessive tolerance mechanisms, we found that repeated injection of diabetes-prone mice with the DEC-HA conjugates leads to the induction of dominant tolerance mechanisms. We observed in these mice an increase of HA-specific T-cells expressing Foxp3, a molecule whose stable expression is linked with regulatory T-cell function (22). This increase in Foxp3 expression among HA-specific T-cells may come about by expansion of regulatory T-cells and simultaneous deletion of effector cells (because there is no increase in absolute numbers

when compared with noninjected mice) or by the conversion of nonregulatory into regulatory T-cells in the absence of significant expansion (7,15). Both scenarios would account for the increase in Foxp3 expression in the absence of significant expansion. Recently, it was demonstrated that islet antigen-specific, Foxp3-transduced T-cells have the potential to stabilize and even revert disease with recent-onset diabetes in mice (25) and that Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells control diabetogenic T-cells in a rat adoptive transfer system (26), corroborating the role of Foxp3-expressing  $\beta$ -cell-specific T-cells in the prevention and therapy of autoimmune diabetes. Furthermore, autoreactive T-cells from DEC-HA-treated INS-HA/TCR-HA mice exhibit high expression of the immunosuppressive cytokines IL-10 and TGF- $\beta$ , both of which are secreted by type 1 regulatory T-cells, i.e., regulatory T-cells that are believed to be generated from CD4<sup>+</sup>CD25<sup>-</sup> peripheral precursors (13,27). This was not unexpected because dendritic cells and in particular im-

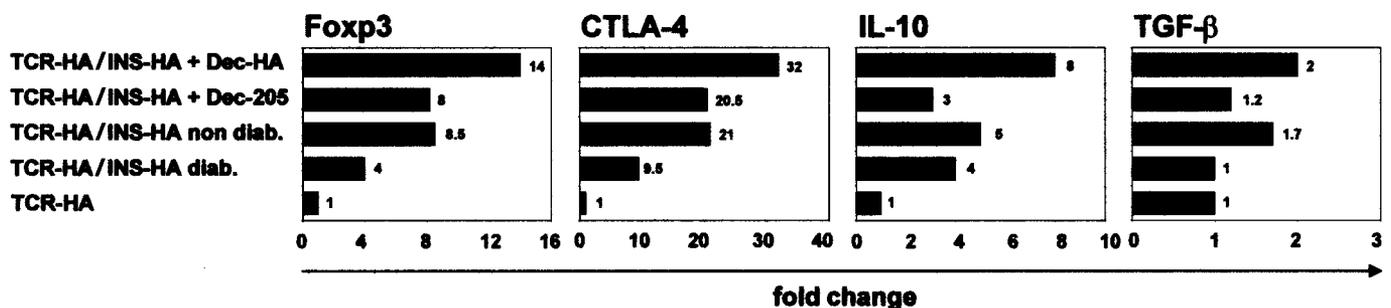


FIG. 5. Gene expression pattern of islet-specific CD4<sup>+</sup>6.5<sup>+</sup> T-cells from INS-HA/TCR-HA mice. CD4<sup>+</sup>6.5<sup>+</sup> T-cells were sorted from TCR-HA and from diabetic and nondiabetic INS-HA/TCR-HA as well as DEC-205- and DEC-HA-injected INS-HA/TCR-HA mice. Total RNA was prepared and reverse transcribed, and mRNA expression levels were determined by real-time RT-PCR. The mean relative regulation is indicated. Results are from pooled individual mice ( $n = 4$ ). Ribosomal protein S9 mRNA expression served as a housekeeping gene control. Shown are the values obtained from one of two performed experiments with similar results.

mature or IL-10-secreting dendritic cell subpopulations can contribute to the induction/expansion of IL-10-secreting type 1 regulatory T-cells (28–30). Although the effects of IL-10 on the outcome of autoimmune diabetes are disputable (31–34), it has recently been shown that IL-10-secreting type 1 regulatory T-cells suppressed diabetogenic T-cells via IL-10 secretion and lost their diabetogenic potential (35).

Characterization of  $\beta$ -cell-specific CD4<sup>+</sup> T-cell by flow cytometry revealed that in contrast to untreated nondiabetic INS-HA/TCR-HA mice, cells isolated from DEC-HA-treated mice exhibit a less activated phenotype, as indicated by reduced expression of CD25, CD69, and elevated expression of CD62L and CD45RB. This might be the consequence of inadequate T-cell stimulation by immature dendritic cells lacking important costimulatory function. Impaired T-cell activation could also be the consequence of an expansion of regulatory T-cells within the HA-specific T-cell pool secreting immunosuppressive cytokines such as IL-10. IL-10 is known to act as a central modulator or effector molecule of tolerance because it downregulates costimulation by antigen-presenting cells (36), T-cell responses to antigen through the inhibition of IL-2 production, and IL-2R  $\alpha$ -chain expression (37,38), and it is known to play a complex role in autoimmunity (39). Alternatively, a part of recently activated T-cells might be deleted because results obtained in adoptive transfer systems suggest that stimulation of T-cells by steady-state dendritic cells only leads to short-term proliferation and expansion of antigen-specific T-cells before a fraction of these cells undergoes clonal deletion (15). This explanation would also be consistent with the overall reduced proportion of CD4<sup>+</sup>CD6.5<sup>+</sup> T-cells in DEC-HA-treated mice.

Quantitative RT-PCR analysis revealed elevated expression of genes discussed in the context of regulatory T-cells. We currently cannot precisely distinguish whether increased expression levels of Foxp3, IL-10, TGF- $\beta$ , and CTLA-4 in potentially diabetogenic T-cells from DEC-HA-treated mice is the consequence of 1) de novo generation of regulatory T-cells from naive precursors, 2) upregulated expression of these genes by preexisting regulatory T-cells, or 3) the expansion of  $\beta$ -cell islet-specific regulatory T-cells caused by repeated stimulation by immature dendritic cell. However, we favor the hypothesis that antigen-specific regulatory T-cells already present in INS-HA/TCR-HA mice do expand and thus accumulate in DEC-HA-treated INS-HA/TCR-HA mice, perhaps at the expense of effector cells, which are deleted. Certainly, further experiments are needed to shed more light on the mechanisms underlying this immature dendritic cell-based strategy to prevent T-cell-mediated autoimmune reactions. Moreover, after establishing the conditions required for preventing diabetes onset, we will now continue improving the experimental protocol with respect to frequency and duration of DEC-HA application.

In conclusion, our results demonstrate that in vivo targeting of antigen to immature dendritic cells is sufficient to prevent the onset of organ-specific autoimmune disease and that this most likely involves the expansion of IL-10-secreting Foxp3<sup>+</sup> regulatory T-cells. Our approach, which is based on the in situ induction of peripheral tolerance, may represent a safe strategy focusing on prevention rather than therapy of autoimmune disorders.

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