Insulin B-Chain Reactive CD4$^+$ Regulatory T-Cells Induced by Oral Insulin Treatment Protect From Type 1 Diabetes by Blocking the Cytokine Secretion and Pancreatic Infiltration of Diabetogenic Effector T-Cells

Isabelle Bergerot, Guillermo A. Arreaza, Mark J. Cameron, Marie D. Burdick, Robert M. Strieter, Stephen W. Chensue, Subrata Chakrabarti, and Terry L. Delovitch

The mechanism of protection from type 1 diabetes conferred by regulatory T-cells induced by oral insulin treatment of NOD mice is not well understood. We demonstrate that oral insulin feeding of NOD mice induces the function of insulin B-chain reactive CD4$^+$ regulatory T-cells, which compete with diabetogenic effector T-cells for the recognition of insulin in NOD.*Scid* recipient mice. These effector T-cells become deprived of interleukin (IL)-2 and interferon (IFN)-γ and are unable to expand and migrate to the pancreas. Type 1 diabetes–protective splenic regulatory T-cells secrete relatively little transforming growth factor (TGF)-β1, suggesting that TGF-β may not contribute to the inactivation of effector T-cells in NOD.*Scid* recipients. The observed preferential infiltration of insulin-reactive regulatory T-cells rather than effector T-cells in the pancreas results in a nondestructive insulitis that correlates with an increased intrapancreatic expression of macrophage inflammatory protein-1β. Thus, oral insulin therapy overcomes a deficiency in regulatory T-cells and protects against type 1 diabetes by inducing insulin B-chain reactive regulatory T-cells to block cytokine secretion and migration of diabetogenic effector T-cells to the pancreas. Our data emphasize that continuous oral insulin feeding over a prolonged period is required to prevent type 1 diabetes. *Diabetes* 48:1720–1729, 1999

From the Autoimmunity/Diabetes Group (I.B., G.A.A., M.J.C., T.L.D.), The John P. Robarts Research Institute; the Departments of Microbiology and Immunology (M.J.C., T.L.D.), Pathology (S.C.), and Medicine (S.C., T.L.D.), University of Western Ontario, London, Ontario, Canada; and the Division of Pulmonary and Critical Care Medicine (M.D.B., R.M.S.), University of Michigan Medical Center, and the Department of Pathology and Laboratory of Medicine (S.W.C.), Veterans Administration Medical Center, University of Michigan, Ann Arbor, Michigan.

Address correspondence and reprint requests to Dr. Terry L. Delovitch, Autoimmunity/Diabetes Group, The John P. Robarts Research Institute, 1400 Western Road, London, Ontario N6G 2V4, Canada. E-mail: del@rri.on.ca.

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CFA, complete Freund's adjuvant; EAE, experimental allergic encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MACS, magnetic cell sorting; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MLN, mesenteric lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; PLN, pancreatic lymph node; TGF, transforming growth factor; Th, T-helper; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling.

Immunological tolerance prevents T-cell reactivity to self-antigens that culminates in autoimmunity. Although central tolerance is mediated by the deletion of self-reactive T-cells in the thymus, peripheral tolerance may involve other mechanisms, including anergy (1) and active suppression (2). A failure in one or more of these mechanisms of tolerance induction may result in autoimmune disease. In particular, a lack of T-cell tolerance to pancreatic islet β-cell antigens plays a critical role in the pathogenesis of type 1 diabetes (3,4) and may lead to a T-helper (Th)1-cell–mediated destruction of islet β-cells (5). Defects in T-cell function, such as T-cell proliferative hyporesponsiveness and reduced interleukin (IL)-2 and IL-4 secretion in response to T-cell receptor stimulation, have been detected (6) in the NOD mouse model of type 1 diabetes (7,8). Recombinant IL-4 reverses this hyporesponsiveness, favors the expansion of regulatory CD4$^+$ Th2-cells, and prevents both destructive pancreatic lymphocyte infiltration (insulitis) and progression to type 1 diabetes (9). Early events in T-cell differentiation, such as the defective maturation of natural killer (NK)-like T-cells, which may result from insufficient IL-7 bioavailability (10), contribute to reduced IL-4 production in NOD mice (11) and may interfere with normal Th2-cell development. Other deficiencies in peripheral T-cell function, e.g., decreased proliferation in a mixed lymphocyte reaction (12) and defective suppression of an allogeneic mixed lymphocyte reaction response (13), precede the onset of overt type 1 diabetes. A deficiency in regulatory T-cell activity due to an abnormality in cytokine secretion may reveal the activity of otherwise functionally silent (14) islet-specific autoreactive Th1-cells and lead to a breakdown of self-tolerance and type 1 diabetes (5,6,8,15).

Oral antigen administration is a current focus of attention in clinical trials for preventive therapy of several T-cell–mediated autoimmune diseases (16,17). Treatment with low doses of an oral antigen induces systemic T-cell hyporesponsiveness to this antigen and can suppress several experimental autoimmune diseases, including type 1 diabetes and experimental allergic encephalomyelitis (EAE) (16,17), experimental autoimmune uveoretinitis (18), and collagen-induced arthritis (19). Oral insulin treatment prevents type 1 diabetes in NOD mice (20–22) by inducing peripheral CD4$^+$

regulatory T-cells that can adoptively transfer protection from disease (23). These insulin-induced regulatory T-cells secrete several anti-inflammatory cytokines, including IL-4 (24), IL-10, and transforming growth factor (TGF)-β (25,26) in the pancreases of protected mice, suggesting, albeit indirectly, that immune deviation from a Th1 to a Th2 phenotype occurs at the local site of inflammation. However, the mechanism by which oral insulin-induced regulatory T-cells restore peripheral tolerance and afford protection from type 1 diabetes is poorly understood.

It is possible that oral insulin–induced regulatory T-cells are functional primarily at a site(s) upstream of the pancreas. We tested this possibility and demonstrate here that oral insulin therapy protects against type 1 diabetes in NOD mice by inducing insulin-reactive regulatory T-cells in the spleen, which deprive pathogenic effector T-cells of IL-2 and prevent their infiltration of the pancreas. The insulin-reactive regulatory T-cells preferentially migrate to the pancreas, and this correlates with both a nondestructive insulitis and a significant increase in the intrapancreatic expression of the macrophage inflammatory protein (MIP)-1β C-C chemokine. Taken together with our previous finding that insulin-induced regulatory $CD4^+$ T-cells block the adoptive transfer of type 1 diabetes (23), our observations indicate that oral antigen-induced tolerance is mediated by $CD4^+$ T-cell–mediated immunoregulatory mechanisms that occur upstream of the target tissue of inflammation.

RESEARCH DESIGN AND METHODS

Mice. NOD, NOD.NON–Thy-1.1, and NOD.*Scid* congenic mice were bred under specific pathogen-free conditions in our animal facility. In our NOD mouse colony, insulitis is detectable at 4–5 weeks of age, and the incidence of type 1 diabetes is 80–90% in females and 10–20% in males at 30 weeks of age. Congenic NOD.NON–Thy-1.1 mice (27) and NOD.*Scid* mice were generously provided by Drs. E. Leiter and L. Shultz (The Jackson Laboratory, Bar Harbor, ME), respectively, and were bred in our animal facility. Blood glucose levels were monitored weekly with a Glucometer Encore (Miles/Bayer, Toronto, Canada), and mice with a blood glucose level >11.1 mmol/l (200 mg/dl) for 2 consecutive days were considered diabetic.

Oral antigen administration. NOD or NOD.NON female mice (5–6 weeks old) were fed through an 18-gauge stainless steel needle by gastric intubation with either 0.8 mg of human insulin (Elli Lilly, Indianapolis, IN) or ovalbumin (OVA) (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). Mice were fed three times a week for 2 or 4 weeks.

T-cell isolation. Spleen and mesenteric lymph node (MLN) and pancreatic lymph node (PLN) T-cells were isolated by filtration of lymphocytes through murine T-cell enrichment columns (R&D Systems, Minneapolis, MN) and were shown by flow cytometry to consist of ≥90% $CD3^+$ T-cells. Enriched $CD4^+$ T-cells obtained by treatment (15 min, 23°C) of lymphocytes with an anti-CD4 monoclonal antibody (mAb) and filtration through a T-cell subset column (R&D Systems) were demonstrated by cytofluorometry using a fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 mAb (clone H129.19; Pharmingen, San Diego, CA) to consist of ≥90% $CD4^+$ T-cells. For the separation of Thy-1.2^+ and Thy-1.1^+ T-cells, splenic T-cells were treated (15 min, 4°C) with magnetic cell sorting (MACS) microbeads conjugated with a rat anti-mouse Thy-1.2 mAb (clone 30H12; Miltenyi Biotec, Sunnyvale, CA), loaded onto a MiniMACS column and magnetically separated using a MiniMACS separation unit (Miltenyi Biotec). Cytofluorometry showed that the positive fraction consisted of ≥90% Thy-1.2^+ T-cells while the negative fraction contained ≥70% Thy-1.1^+ T-cells.

Antigen specificity of regulatory T-cells. Splenic or MLN $CD4^+$ T-cells (5×10^5) from antigen-fed mice were cultured in triplicate in 96-well plates with irradiated (3,000 R) splenocytes (5×10^5) in the presence of human insulin, bovine insulin A-chain or bovine insulin B-chain (10 μg/ml; all purchased from Sigma), or OVA (10 μg/ml) in complete RPMI 1640. Cultures were incubated for 4 days at 37°C, pulsed with [^{3}H]thymidine (1 μCi) during the final 18 h of culture and collected using a 96-well cell harvester (TOMTEC, Orange, CT). Incorporation of [^{3}H]thymidine was quantified using a 1450 Microbeta Plus counter (Wallac, Turku, Finland). Data are expressed as the mean cpm ± SE.

Recall T-cell response to insulin. Eight-week-old NOD female mice were fed daily for 3 days with either human insulin or OVA (0.8 mg in PBS). One day after the last feeding, mice were immunized intra-footpad with insulin or OVA (50 μg) in 100 μl of complete Freund's adjuvant (CFA) (Sigma). Eight days after immunization, mice were killed and T-cells (5×10^5) from the spleen or draining lymph nodes (popliteal and inguinal) were cultured for 3 days at 37°C in triplicate in 96-well plates with irradiated (3,000 R) splenocytes (5×10^5) in the presence of 10 μg/ml of insulin or OVA. Cultures were pulsed with 1 μCi of [^{3}H]thymidine 18 h before cell harvest. Data are expressed as the mean cpm ± SE.

Adoptive T-cell transfer. Adult 6- to 8-week-old NOD.*Scid* female mice were injected intraperitoneally with 5×10^6 T-cells from oral antigen–fed (insulin or OVA) nondiabetic female NOD mice either alone or together with 5×10^6 T-cells from diabetic NOD females. The fate of injected T-cells was evaluated by cytofluorometry using a FITC-labeled anti-mouse CD4 or FITC-labeled anti-mouse CD8α mAb. To track the migration of diabetogenic T-cells, congenic NOD.NON–Thy-1.1 females were used as oral antigen–treated donors. T-cells from NOD.*Scid* recipients were stained with either an FITC-labeled anti-mouse Thy-1.2 (clone 30H12, Sigma) or anti-mouse Thy-1.1 (clone HIS51, Pharmingen) mAb that does not cross-react with mouse Thy-1.2.

Analysis of DNA fragmentation by flow cytometry. DNA fragmentation in lymphocytes was analyzed after staining apoptotic cells by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) technique (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's recommendations. To assay the apoptosis of T-cells, lymphocytes were initially stained with a phycoerythrin (PE)-labeled anti-mouse CD3 mAb (clone 29B, Sigma). Broken ends of double-stranded DNA were then labeled with FITC-conjugated dUTP using the terminal deoxynucleotidyl transferase (dTd) enzyme.

Histopathology. Pancreases were excised and either snap frozen in liquid nitrogen for cytokine content determination or immersed in 10% normal buffered formalin before paraffin embedding. Paraffin sections (5 μm) were stained with hematoxylin and eosin. The severity of insulitis was scored in at least 20 islets per mouse as follows: 0, no visible sign of islet infiltration; 1, peri-islet infiltrate; 2, <50% infiltration; and 3, >50% islet infiltration. The insulitis score was calculated as follows: [(% islets with a score of 1) × 1 + (% islets with a score of 2) × 2 + (% islets with a score of 3) × 3] × 100^{-1}.

Cytokine and chemokine assay. Splenic T-cells (2×10^5) were cultured in 96-well plates coated with an anti-CD3ε mAb (clone 145-2C11 ascites, 1/500 dilution), and after 48 h, culture supernatants were assayed for their concentrations of IL-2, IL-4, IL-10, and interferon (IFN)-γ by enzyme-linked immunosorbent assay (ELISA) (9). Briefly, 96-well microtiter plates were coated (16 h, 4°C) with an anti-cytokine mAb (1 μg/ml in 0.1mol/l $NaHCO_3$, 50 μl/well), and nonspecific binding sites were blocked (2 h, 23°C) with 3% bovine serum albumin in PBS. Standards (20–20,000 pg/ml; 100 μl/well) or samples were added, let stand (16 h, 4°C), and then incubated (45 min, 23°C) with a biotinylated anti-cytokine mAb (1 μg/ml; 50 μl/well). A streptavidin-peroxidase conjugate (0.2 U/ml, Boehringer Mannheim) and chromogen substrate (p-nitrophenyl phosphate; 1 mg/ml, Sigma) in diethanolamine buffer were added to develop the reaction, and the absorbance at 405 nm was determined using an automated microplate reader (Bio-Rad, Hercules, CA). The rat anti-mouse cytokine mAb pairs (Pharmingen) used and their clone designations were as follows: JES6-1A12 and biotinylated JES6-5H4 for IL-2, 11B11 and biotinylated BVD6-24G2 for IL-4, JES5-2A5 and biotinylated SXC-1 for IL-10, and R4-6A2 and biotinylated XMG1.2 for IFN-γ. TGF-β1 was measured by ELISA using a detection kit (Quantikine; R&D Systems) according to the manufacturer's instructions.

Alternatively, frozen tissues were homogenized, sonicated in an anti-protease buffer, and filtered (1.2-μm filters). Filtrates were analyzed for total protein and both cytokine (IL-4, IL-10, and IFN-γ) and chemokine (monocyte chemotactic protein [MCP]-1, MIP-1α, MIP-1β) contents by ELISA (9).

Statistical analysis. Results of T-cell proliferation and cytokine levels were compared using the Student's *t* test for unpaired samples. The efficacy of diabetes transfer was compared between animal groups using a Wilcoxon's rank test.

RESULTS

Oral insulin treatment generates insulin B-chain reactive $CD4^+$ T-cells. Preliminary experiments confirmed our previous report (23) that oral insulin treatment induces regulatory $CD4^+$ T-cells in NOD mice that can block the adoptive transfer of type 1 diabetes. We assessed the antigenic specificity of these insulin-induced $CD4^+$ T-cells by measuring the proliferative responses of splenic and MLN $CD4^+$ T-cells from OVA- (control) or insulin-fed NOD mice to insulin, insulin A-chain, or insulin B-chain. Spleen- and MLN-derived $CD4^+$ T-cells from OVA-fed mice responded preferentially to OVA compared with insulin, whereas $CD4^+$ T-cells from insulin-fed

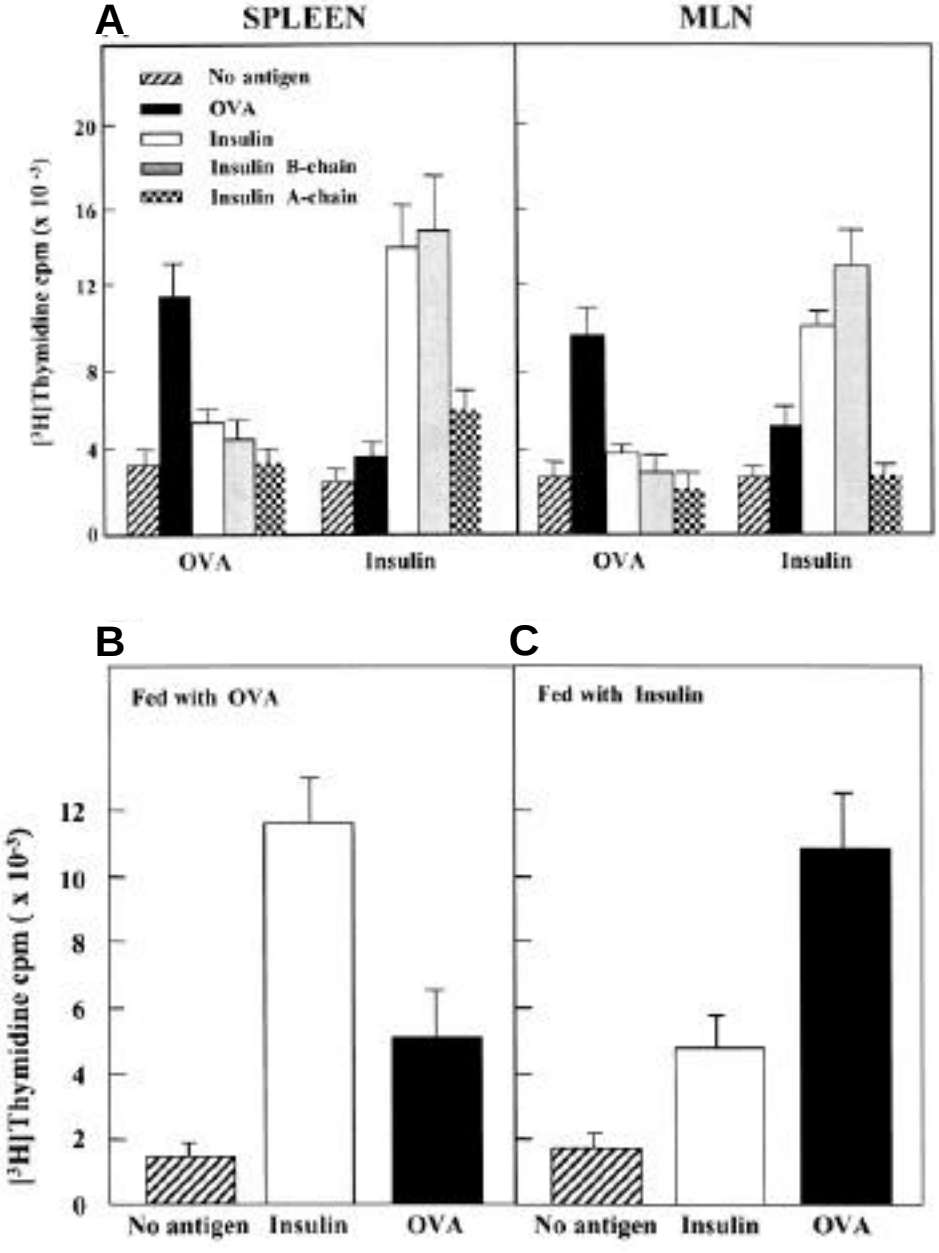

FIG. 1. ***A*****: Oral insulin treatment stimulates T-cells responsive to insulin B-chain. Six-week-old NOD female mice were fed for 4 weeks with either OVA or insulin. CD4⁺ T-cells were isolated from the spleen or MLN of treated mice (*n* = 5 per group) and cultured for 4 days in the absence (No antigen) or presence of 10 µg/ml of OVA, insulin, insulin A-chain, or insulin B-chain. T-cell proliferation data from one of three representative experiments are expressed as the mean cpm ± SE for triplicate samples.** ***B*** **and** ***C*****: Orally administered antigen specifically modulates T-cell responses to this antigen. NOD mice were fed once a day for 3 consecutive days with either OVA or insulin (0.8 mg) and were then immunized with 50 µg of insulin in CFA. Responses of splenic T-cells isolated 8 days after immunization and stimulated in vitro with OVA or insulin (10 µg/ml) were determined after 3 days of culture. Data from one of three representative experiments are expressed as in** ***A*****.**

mice responded preferentially to insulin and insulin B-chain compared with OVA and insulin A-chain (Fig. 1*A*). These results indicate that oral administration of insulin specifically induces insulin-specific CD4⁺ T-cells that react to an insulin B-chain epitope(s).

The antigenic specificity of T-cells from insulin- and OVA-fed mice was further analyzed after intra-footpad immunization of these mice with insulin in CFA and analysis of the in vitro recall response of these antigen-primed T-cells. Control mice fed with OVA displayed a strong splenic T-cell recall response to insulin and a much weaker response to OVA (Fig. 1*B*). In contrast, mice fed with insulin displayed a vigorous splenic T-cell response to OVA and a poor response to insulin (Fig. 1*C*). Similar results were obtained in recall responses of T-cells from the draining popliteal lymph node (data not shown). Moreover, when insulin-fed mice were immunized with OVA and their T-cells were restimulated with OVA in vitro, T-cell reactivity to OVA was not altered (data not shown). These findings demonstrate that oral administration of insulin specifically modulates T-cell responses to insulin and not to a control antigen.

Oral insulin treatment blocks the migration of peripheral T-cells to pancreatic islets. The effect of oral insulin treatment on the modulation of regulatory T-cell function in NOD mice was determined by assaying the relative capacity of T-cells from 10-week-old OVA- or insulin-treated mice to migrate to the pancreas of NOD.*Scid* mouse recipients. NOD.*Scid* mice are homozygous for the recessive *Scid* mutation, lack functional T- and B-cells, and do not develop either insulitis or type 1 diabetes (27). Type 1 diabetes is adoptively transferred relatively rapidly by splenocytes from diabetic NOD females into NOD.*Scid* recipients (28). At 1 week post-transfer (intraperitoneally) of splenic T-cells (5×10^6) from either OVA- or insulin-fed NOD mice, about 5- to 10-fold more T-cells localized to the spleens than to the MLN in NOD.*Scid* mice (Fig. 2*A*). Repopulation of the spleen and

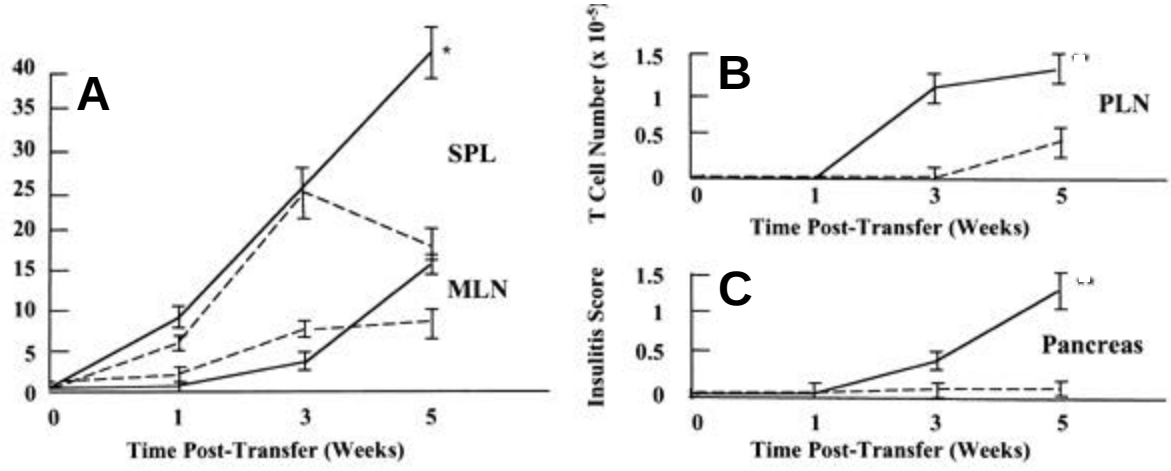

FIG. 2. Migration of adoptively transferred splenic T-cells from OVA- and insulin-treated mice in NOD.*Scid* recipients. On day 0, NOD.*Scid* female mice were injected intraperitoneally with 5 × 10^6 T-cells from either OVA- (——) or insulin-fed (– – – –) NOD mice. The migration of injected T-cells into the spleen, MLN, PLN, and pancreas of NOD.*Scid* recipients was determined at 1, 3, and 5 weeks post-transfer. A total of 9–16 mice per group were analyzed at each time point. A CD4:CD8 ratio of 5:1 was observed in both insulin- and OVA-fed mice. Data from one of three representative experiments are expressed as means ± SE. *A*: T-cell number in the spleen (SPL) and MLN of NOD.*Scid* recipients. *B*: T-cell number in the PLN of NOD.*Scid* recipients. *C*: Severity of insulitis in the pancreas of NOD.*Scid* recipients. *Statistically significant difference from insulin-treated mice, $P < 0.01$.

MLN of NOD.*Scid* mice injected with splenic T-cells from insulin-fed NOD mice peaked at ~3 weeks after transfer. In contrast, the spleen and MLN of NOD.*Scid* mice injected with splenic T-cells from OVA-fed NOD mice continued to be repopulated between 3 and 5 weeks post-transfer. By 5 weeks post-transfer, about threefold more T-cells from OVA-fed donor NOD mice than T-cells from insulin-fed NOD mice infiltrated the PLN of NOD.*Scid* mice (Fig. 2*B*). This difference in T-cell infiltration into the PLN was paralleled by a significant increase in lymphocyte infiltration into the pancreas in NOD.*Scid* recipients of T-cells from OVA-fed NOD mice, as demonstrated by the increased insulitis score (~1.4) at 5 weeks post-transfer (Fig. 2*C*). This relative insulitis score reflects the presence of both intact and destroyed islets at this time. In addition, most (≥95%) NOD.*Scid* recipients of T-cells from OVA-fed mice were overtly diabetic 2 weeks later at 7 weeks post-transfer, whereas only ~20% of recipients of T-cells from insulin-fed mice developed type 1 diabetes (data not shown). The latter result is consistent with the finding that T-cells from insulin-fed NOD mice remained in the spleen and MLN and did not migrate appreciably into either the PLN or pancreas of NOD.*Scid* recipients at 5 weeks post-transfer. Thus, oral insulin feeding preferentially blocks the migration of T-cells from peripheral lymphoid tissues to the PLN and pancreas.

Oral insulin treatment induces regulatory T-cells that inhibit the ability of diabetogenic T-cells to adoptively transfer type 1 diabetes. The failure of peripheral T-cells from insulin-fed NOD mice to migrate to the pancreas of NOD.*Scid* recipients suggests that orally administered insulin induces otherwise pathogenic T-cells to become tolerant to pancreatic islet cell autoantigens. To test the hypothesis that this tolerance is mediated by insulin-induced regulatory T-cells, we analyzed the capacity of T-cells from insulin- or OVA-fed NOD mice to inhibit the adoptive transfer of type 1 diabetes into NOD.*Scid* recipients. The co-transfer of splenic T-cells from insulin-fed NOD mice with diabetogenic T-cells from newly diagnosed diabetic NOD mice significantly delayed the onset and reduced the incidence of type 1 diabetes ($P < 0.01$) in NOD.*Scid* recipients at 8 weeks post-transfer, as revealed by the development of type 1 diabetes in only 6/16 (38%) of NOD.*Scid* mice (Fig. 3). By comparison, 14/16 (88%) of NOD.*Scid* recipients of splenic T-cells from OVA-fed NOD mice plus diabetogenic NOD T-cells developed type 1 diabetes. All (16/16 [100%]) NOD.*Scid* recipients of diabetogenic T-cells alone developed type 1 diabetes.

To distinguish between the tissue distribution of donor regulatory T-cells from insulin-fed NOD mice and donor effector (diabetogenic) T-cells from diabetic NOD mice after transfer into NOD.*Scid* recipients, regulatory T-cells were derived from insulin-fed congenic NOD.NON–Thy-1.1 female mice (27), and effector T-cells were obtained from newly diabetic Thy-1.2 female NOD mice. At 5 weeks after cell transfer, the numbers of Thy-1.1^+ and Thy-1.2^+ T-cells present in different tissues of recipient NOD.*Scid* mice were enumerated. Table 1 shows that in the presence of insulin-induced Thy-1.1^+ regulatory T-cells, the migration to and/or expansion of effector Thy-1.2^+ T-cells of the spleen was reduced about twofold. The number of regulatory Thy-1.1^+ T-cells in the spleen of insulin-

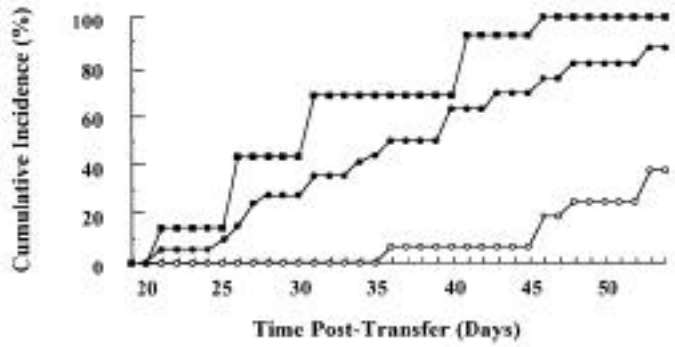

FIG. 3. Cumulative incidence of type 1 diabetes adoptively transferred into NOD.*Scid* mice. NOD.*Scid* recipients (n = 16 per group) were injected intraperitoneally with 5 × 10^6 diabetogenic T-cells alone (■) or mixed with 5 × 10^6 splenic T-cells from either OVA- (●) or insulin-fed (○) mice. Mice with blood glucose values >200 mg/dl on 2 consecutive days were considered diabetic. Data from one of two representative experiments are shown.

TABLE 1
Distribution of diabetogenic Thy-1.2$^+$ T-cells and regulatory Thy-1.1$^+$ T-cells from OVA- or insulin-fed mice at 5 weeks post-transfer into NOD.*Scid* recipients

Organ	Thy-1.1$^+$ donor mice fed with OVA or insulin	Diabetogenic Thy-1.2$^+$ T-cells ($\times 10^{-5}$)	Regulatory Thy-1.1$^+$ T-cells ($\times 10^{-5}$)
Spleen	OVA	19.4	18.9
	Insulin	10.0*	12.3
MLN	OVA	8.1	22.0
	Insulin	8.6	28.5
PLN	OVA	3.4	2.4
	Insulin	0.7†	4.3†

Data from one of three representative experiments (n = 4–6 mice per group) are shown. NOD.*Scid* recipients received 5×10^6 diabetogenic Thy-1.2$^+$ T-cells plus 5×10^6 Thy-1.1$^+$ T-cells from either OVA- or insulin-fed NOD.NON–Thy-1.1$^+$ female mice. Five weeks post-transfer, recipient mice were killed, and the relative distribution of Thy-1.1$^+$ and Thy-1.2$^+$ T-cells in the spleen, MLN, and PLN was determined by cytofluorometry. *$P < 0.05$, as determined using the Student's t test for unpaired samples. †$P < 0.01$, as determined using the Student's t test for unpaired samples.

fed mice was also reduced, but this reduction was not statistically significant. The number of Thy-1.2$^+$ T-cells that homed to the PLN was decreased approximately fourfold, while the number of Thy-1.1$^+$ T-cells found in the PLN was increased about twofold in NOD.*Scid* recipients of insulin-induced Thy-1.1$^+$ T-cells. The numbers of Thy-1.1$^+$ and Thy-1.2$^+$ T-cells localized to the MLN were similar in NOD.*Scid* recipients of insulin- or OVA-induced T-cells. Thus, insulin-induced Thy-1.1$^+$ T-cells interfere with the expansion and/or migration of diabetogenic Thy-1.2$^+$ T-cells, particularly to the PLN. PLNs are the primary organs that harbor diabetogenic T-cells before islet infiltration and may play a role in T-cell activation during the early stages of type 1 diabetes onset (29). Our results support the idea that the accumulation of diabetogenic Thy-1.2$^+$ T-cells in the PLN is a critical step that precedes pancreatic infiltration and the onset of type 1 diabetes in NOD mice.

Insulin-induced T-cells do not enhance the apoptosis of diabetogenic effector T-cells. The reduced number of effector Thy-1.2$^+$ T-cells in the spleen and PLN of recipients co-transferred with insulin-induced Thy-1.1$^+$ regulatory T-cells may result from their increased susceptibility to apoptosis. To examine this possibility, splenic CD3$^+$ T-cells from co-transferred NOD.*Scid* mice were gated by flow cytometry (Fig. 4*A*), and their levels of apoptosis were measured. The time period of 5 weeks post-transfer was chosen for analysis, since at earlier times after transfer, it generally proved difficult to quantify DNA fragmentation by the TUNEL assay because of the low number of T-cells localized to the spleens of NOD.*Scid* recipient mice. At 5 weeks post-transfer, when a significant difference was noted between the incidence of type 1 diabetes transferred by OVA-induced T-cells (50% incidence) and insulin-induced T-cells (5% incidence), the percentage of apoptotic CD3$^+$ T-cells was similar in recipients of OVA-induced (12.7 ± 1.15%, Fig.4*B*) versus insulin-induced (14.5 ± 1.7%, Fig. 4*C*) T-cells. Similar results were obtained when the levels of CD3$^+$ T-cell apoptosis were assayed in NOD.*Scid* recipients at 4 and 7 weeks after T-cell transfer using either the TUNEL method or an FITC-conjugated annexin/propidium iodide double staining technique (data not shown). Thus, while our data suggest that an increase in the amount of apoptosis of effector T-cells may not account for the lower number of effector Thy-1.2$^+$ T-cells observed in the presence of insulin-induced Thy-1.1$^+$ T-cells, we cannot rule out the possibility that effector Thy-1.2$^+$ T-cells are eliminated by apoptosis at a time point(s) earlier than 4 weeks post-transfer.

Oral insulin treatment induces regulatory T-cells to block cytokine secretion by diabetogenic T-cells. Splenic T-cells from type 1 diabetes–protected NOD.*Scid* recipient mice were stimulated in vitro with an anti-CD3 mAb

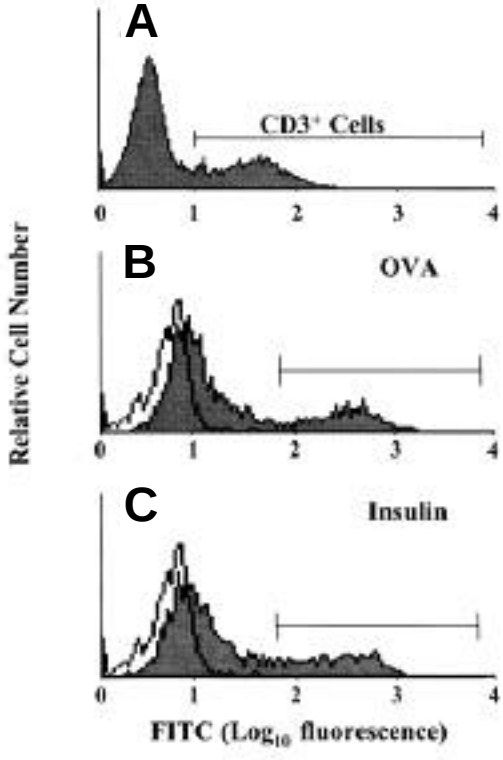

FIG. 4. Analysis of the levels of apoptosis in splenic CD3$^+$ T-cells from co-transferred NOD.*Scid* mice. Splenic cells were obtained from NOD.*Scid* mice (n = 8) 5 weeks after the co-transfer of 5×10^6 diabetogenic T-cells plus 5×10^6 splenic T-cells from mice previously fed with either OVA (*B*) or insulin (*C*). Freshly isolated splenic T-cells were gated (CD3$^+$) according to their level of CD3 surface expression (*A*). CD3$^+$ T-cells stained with FITC-conjugated dUTP were assayed by the TUNEL method for their relative amounts of fragmented DNA, and their relative levels of apoptosis were calculated (*B* and *C*). Data are plotted as the relative cell number versus the Log_{10}fluorescence. Results from one of three representative experiments are shown.

TABLE 2
Cytokine secretion by anti-CD3–stimulated splenocytes from NOD.*Scid* recipients of adoptively transferred T-cells

Recipients of splenic T-cells from NOD mice fed with OVA or insulin	Cytokine secretion (pg/ml)				
	IL-2	IL-4	IL-10	IFN-γ	TGF-β1
OVA	2,629	85	1,239	51,647	<10
Insulin	826*	183	2,607	15,667*	53

Data from one of three representative experiments are shown and expressed as mean values. NOD.*Scid* recipients received 5×10^6 diabetogenic T-cells plus 5×10^6 T-cells from either OVA- or insulin-fed NOD female mice. At day 52 post-transfer, NOD.*Scid* mice were killed, and splenic T-cells were assayed for their level of anti-CD3–stimulated cytokine secretion (n = 16 mice per group). The SE of these values was ≤10%. *$P < 0.01$, as determined using the Student's t test for unpaired samples.

to facilitate detection of their secreted cytokines. The amount of IL-2 secreted by these T-cells was diminished about three- to fourfold (Table 2), while the levels of secretion of IL-4 and IL-10 did not change significantly. Anti-CD3–stimulated splenic T-cells from NOD.*Scid* recipients of insulin-induced T-cells secreted a three- to fourfold lower level of IFN-γ. Although only relatively low levels of T-cell secretion of TGF-β1 were observed, note that the level of TGF-β1 secreted by splenic T-cells from NOD.*Scid* recipients of insulin-induced T-cells (53 pg/ml) was about five- to sixfold greater than that produced by splenic T-cells from control NOD.*Scid* recipients of OVA-induced T-cells (≤10 pg/ml).

The functional activity of the co-transferred effector diabetogenic T-cells was evaluated after the recovery of splenic Thy-1.2$^+$ T-cells from NOD.*Scid* recipient mice. Thy-1.2$^+$ T-cells from recipients of Thy-1.1$^+$ T-cells from insulin-fed NOD mice secreted lower but not significantly different amounts of IL-2 and IFN-γ in response to anti-CD3 stimulation than did Thy-1.2$^+$ T-cells from recipients of Thy-1.1$^+$ T-cells from OVA-fed NOD mice (Table 3). The co-transferred Thy-1.1$^+$ T-cells originating from insulin-fed mice and recovered from the spleens of NOD.*Scid* mice displayed a threefold increase in anti-CD3–stimulated IL-4 and IL-10 secretion compared with that of the recovered Thy-1.1$^+$ T-cells derived from OVA-fed mice. The latter finding may explain the discrepancy between the amounts of IL-2 and IFN-γ production by splenic T-cells (Table 2) compared with that detected here for enriched Thy-1.2$^+$ T-cells, since the presence of IL-4– and IL-10–secreting Thy-1.1$^+$ regulatory T-cells is required to block the function of diabetogenic T-cells. Thus, despite the presence of normal amounts of IFN-γ secreted by Th1-cells in the Thy-1.1$^+$ population from insulin-fed mice, IL-4–secreting Th2-cells within this population are still able to transfer protection from type 1 diabetes. The latter result is consistent with our previous findings of unaltered levels of IFN-γ secretion and increased levels of IL-4 secretion in the pancreas of female NOD mice protected from type 1 diabetes by treatment with either IL-4 (9) or anti-CD28 mAb (30).

Prevention of type 1 diabetes transfer by insulin-induced T-cells is associated with the increased intra-pancreatic expression of MIP-1β. The differential migration of Thy-1.1$^+$ and Thy-1.2$^+$ T-cells to the pancreas and PLN of NOD.*Scid* recipients may arise from the local tissue-specific expression of distinct types of chemokines. C-C chemokines, such as MIP-1α, MIP-1β, and MCP-1, are T-cell chemoattractant cytokines that can regulate the migration of specific subsets of lymphocytes to sites of inflammation (31). To determine whether these chemokines mediate the patterns of homing of T-cells to the pancreas observed in our T-cell co-transfer experiments, we quantified the intra-pancreatic chemokine content in NOD.*Scid* recipient mice before and after T-cell transfer. In naive 7- to 8-week-old NOD.*Scid* mice that did not receive T-cells, the levels of expression of these chemokines were either extremely low (MCP-1) or virtually undetectable (MIP-1α, MIP-1β) (Table 4). These low intra-pancreatic levels of MCP-1, MIP-1α, and MIP-1β remained essentially constant from 2–20 weeks of age in NOD.*Scid* mice (32,33).

The relative content of MIP-1β was significantly enhanced in pancreases of mice that received insulin-induced regulatory T-cells compared with that observed in the pancreases of mice that received T-cells from OVA-fed mice (Table 4). In con-

TABLE 3
Cytokine production by anti-CD3–stimulated diabetogenic Thy-1.2$^+$ T-cells or regulatory Thy-1.1$^+$ T-cells from NOD.*Scid* recipients

Cytokine secretion (pg/ml)	Recipients of regulatory T-cells from NOD mice fed with OVA or insulin			
	Thy-1.2$^+$ T-cells		Thy-1.1$^+$ T-cells	
	OVA	Insulin	OVA	Insulin
IL-2	2,493	1,602	403	263
IL-4	<40	<40	247	601*
IL-10	2,611	3,214	869	2,342*
IFN-γ	45,161	27,436	33,799	34,920

Data from one of three representative experiments (n = 5 mice per group) are shown and are expressed as mean values with an SE of <20%. NOD.*Scid* recipients received 5×10^6 diabetogenic Thy-1.2$^+$ T-cells plus 5×10^6 Thy-1.1$^+$ T-cells from OVA- or insulin-fed NOD.NON–Thy-1.1$^+$ female mice. Five weeks post-transfer, fractions of diabetogenic Thy-1.2$^+$ T-cells or Thy-1.1$^+$ T-cells were isolated and stimulated for 48 h with an anti-CD3 mAb. Culture supernatants were processed for cytokine quantification by ELISA (n = 13 mice per group). *$P < 0.01$, as determined using the Student's t test for unpaired samples.

TABLE 4
Intra-pancreatic chemokine content in NOD.*Scid* mice before and after adoptive T-cell transfer

NOD.*Scid* mice	Chemokine content (ng/mg of protein)		
	MCP-1	MIP-1α	MIP-1β
Nontransferred	0.2 ± 0.03	0.02	0.01
Transferred OVA	3.6 ± 1.0	0.9 ± 0.1	51.2 ± 2.8
Transferred insulin	4.3 ± 1.1	0.9 ± 0.1	170.4 ± 4.3*

Data from one of two representative experiments (n = 8 mice per group) are shown and are means ± SE. NOD.*Scid* mice either did not (Nontransferred) or did receive 5×10^6 diabetogenic T-cells plus 5×10^6 T-cells from OVA-fed (Transferred OVA) or insulin-fed (Transferred insulin) NOD female mice. At day 52 post-transfer, both nontransferred and transferred NOD.*Scid* mice were killed and pancreases were immediately snap-frozen for chemokine content determination. *$P < 0.01$, as determined using the Student's t test for unpaired samples.

trast, the intra-pancreatic contents of MIP-1α and MCP-1 were similar in both groups of mice. Note that the intra-pancreatic contents of MCP-1 and MIP-1α were elevated by ~20- and 50-fold, respectively, and that of MIP-1β by ~10,000-fold in NOD.*Scid* recipients of transferred T-cells compared with nontransferred NOD.*Scid* mice. These findings indicate that the transferred T-cells are a major source of the intra-pancreatic chemokines detected in these mice. Together, the data suggest that MIP-1β may play a role in the recruitment of regulatory insulin-reactive T-cells to the pancreas of insulin-fed mice that are protected from type 1 diabetes.

DISCUSSION

In this study, we further investigated how regulatory T-cells induced by oral insulin therapy protect from type 1 diabetes. Our data demonstrate that oral insulin administration generates splenic insulin B-chain reactive regulatory CD4$^+$ T-cells that prevent the adoptive transfer of type 1 diabetes by blocking the function of diabetogenic effector T-cells and their migration to the pancreas. Thus, oral antigen–induced tolerance may be mediated by a T-cell–dependent immunoregulatory mechanism(s) that occurs upstream of the target tissue of inflammation.

Previously, we observed that peripheral CD4$^+$ regulatory T-cells are functionally impaired in NOD female mice and that this deficiency can be overcome by the treatment of NOD mice with oral insulin (3). This result raises the possibility that the CD4$^+$ regulatory T-cells induced by oral insulin administration in female NOD mice function in a similar manner to the type 1 diabetes–protective CD4$^+$ regulatory T-cells found in NOD male mice with low type 1 diabetes susceptibility (34). Several immunosuppressive NOD CD4$^+$ T-cell clones that recognize various islet autoantigens were recently isolated. These include islet-reactive Th1-like clones obtained from the spleens of unprimed 4-week-old NOD mice that suppress the adoptive transfer of type 1 diabetes via unidentified soluble factors (35,36) and a NOD PLN-derived insulin-reactive Th1-like clone that inhibits the development of spontaneous type 1 diabetes as well as the adoptive transfer of type 1 diabetes in irradiated young NOD recipient mice (37). Interestingly, the functional characteristics of the latter insulin-reactive Th1-like clone are similar to those of the CD4$^+$ regulatory T-cells induced by oral insulin in our studies, since this clone also blocks the migration of diabetogenic effector T-cells into the pancreas in co-transfer experiments. The high level of secretion of TGF-β by this Th1-like clone is believed to contribute to its type 1 diabetes–protective effect because its ability to inhibit the proliferation and IL-2 secretion of diabetogenic T-cells in response to islets in vitro can be reversed in the presence of an anti–TGF-β mAb (37). However, note that the phenotype of the immunosuppressive Th1 T-cell clones described above differs from the oral insulin-induced CD4$^+$ regulatory T-cells we have investigated, since oral insulin treatment seems to induce Th2-like cells (3,16,17). In addition, our results demonstrating the relatively low production of TGF-β1 by oral insulin-induced CD4$^+$ regulatory T-cells suggest that TGF-β may not play a central role in blocking the function of diabetogenic effector T-cells.

The reduced pancreatic infiltration and islet β-cell destruction observed in type 1 diabetes–protected NOD.*Scid* recipients of splenic T-cells from insulin-fed NOD mice shows that type 1 diabetes effector T-cell function is inhibited in the peripheral lymphoid organs of these mice. The decrease in the number of diabetogenic T-cells in the spleen and PLN reflects that these T-cells are limited in their capacity to migrate to and/or expand in these organs in the presence of insulin-induced CD4$^+$ regulatory T-cells. The spleen and PLN represent early sites of localization of effector T-cells after the adoptive transfer of type 1 diabetes, and T-cell activation occurs in the PLN during the early stages of development of type 1 diabetes (29,38). Recent studies have shown that preferential proliferation and reduced apoptosis rather than preferential immigration may be responsible for the accumulation of activated T-cells in a given tissue of origin (39). By comparison, we did not find any detectable increase in the number of apoptotic T-cells in the spleen of type 1 diabetes–protected NOD.*Scid* recipients. In addition, the levels of T-cell surface expression of various adhesion molecules, such as L-selectin, lymphocyte functional antigen (LFA)-1, and intercellular adhesion molecule (ICAM)-1, which mediate T-cell homing to the pancreas (40), were not altered in insulin-fed NOD mice compared with that observed in OVA-fed or unfed control NOD mice (I.B., G.A.A., unpublished observations). Thus, insulin-induced CD4$^+$ regulatory T-cells may preferentially block the activation and subsequent proliferation of type 1 diabetes effector T-cells. According to this scenario, the observed loss in function (i.e., reduction in IFN-γ and IL-2 secretion) of effector T-cells may lead to their diminished capacity to migrate to the pancreas. Transfer of insulin-induced CD4$^+$ regulatory T-cells 7 days after the transfer of diabetogenic T-cells neither reduces the severity of insulitis nor delays the onset of type 1 diabetes in irradiated NOD recipients (41). Thus, interactions between effector and regulatory T-cells and

their simultaneous trafficking to the pancreas seem to be required for active T-cell–mediated suppression.

The differential migration of type 1 diabetes effector T-cells and insulin-induced $CD4^+$ regulatory T-cells to the pancreas may be under the control of C-C chemokines. We observed that the intra-pancreatic expression of MIP-1β is significantly increased in NOD.*Scid* recipients of splenic T-cells from insulin-fed versus OVA-fed NOD female mice. Different C-C chemokines can each attract distinct populations of lymphocytes (42), and hence C-C chemokines may mediate various types of inflammatory reactions (43). For example, MCP-1 mediates monocyte/macrophage infiltration of a target tissue (44,45), whereas MIP-1α and MIP-1β are associated with the recruitment of different T-cell subsets. In NOD mice, we have found that an enhanced intra-pancreatic expression of MIP-1α correlates with the development of a destructive insulitis, dominant Th1-like cytokine profile, and onset of type 1 diabetes (32,33). In contrast, an increased expression of MIP-1β in the pancreas is associated with the presence of nondestructive insulitis and a predominant Th2-like cytokine profile in NOD mice protected from type 1 diabetes by treatment with IL-4 or an anti-CD28 mAb (32,33). By analogy, our observed increase in expression of MIP-1β in the pancreas of NOD.*Scid* recipients of splenic T-cells from oral insulin–fed NOD mice (Table 4) may lead to the preferential recruitment of insulin B-chain reactive $CD4^+$ regulatory T-cells to the pancreatic islets of these NOD.*Scid* mice. This may explain the two- to threefold increase in insulin-induced Thy-1.1^+ T-cells in the PLN (Fig. 2) and the nondestructive insulitis and low incidence of type 1 diabetes (Fig. 3) observed in recipients of T-cells from insulin-fed NOD mice. Alternatively, insulin B-chain reactive $CD4^+$ regulatory T-cells may secrete MIP-1β preferentially, or MIP-1β may play a role in the inhibition of migration of effector T-cells.

How do oral insulin-induced $CD4^+$ regulatory T-cells impair the function of effector T-cells in NOD mice? $CD4^+$ regulatory T-cell–derived cytokines, such as IL-4, IL-10, and TGF-β, may functionally inactivate effector T-cells (46). Oral insulin treatment of NOD mice has been reported to induce an immune deviation from a Th1- to a Th2-cell profile in islet infiltrates, favoring an increase in the intra-pancreatic expression of IL-4, IL-10, and TGF-β (25). Although we were unable to detect either the induced expression of IL-4 or enhanced expression of IL-10 in the pancreas of oral insulin–fed NOD mice in this study, we did find that oral insulin therapy induces IL-4– and IL-10–secreting regulatory Th2-like cells to suppress diabetogenic effector Th1-cell function. Alternatively, it is possible that diabetogenic effector Th1-cells are inactivated by competition for antigen recognition (47). This competition may occur if effector and regulatory T-cells possess the same antigen specificity. We found that oral insulin–induced regulatory $CD4^+$ T-cells are reactive with insulin B-chain determinants. Insulin is a dominant islet β-cell–derived autoantigen that may also be recognized by diabetogenic T-cells in NOD mice (48). However, despite the local high concentration of insulin in pancreatic islets, the presentation of insulin peptides to T-cells may occur in tissues other than the pancreas. Indeed, we showed that oral insulin feeding suppresses the recall response to insulin by T-cells obtained from the spleen and PLN. By comparison, oral administration of a control antigen, such as OVA, does not alter the recall peripheral T-cell response to insulin, demonstrating the antigen specificity of this response. Thus, oral insulin–induced T-cells may specifically inhibit the recognition of insulin and accompanying IL-2 secretion by diabetogenic T-cells in peripheral lymphoid tissues of NOD mice. Our results support the idea that the inactivation of type 1 diabetes effector T-cells in NOD mice occurs via the competition for antigen by $CD4^+$ regulatory T-cells induced by oral antigen administration.

In lymphoid organs drained by non-mucosal sites, the bioavailability of IL-2 plays a central role in T-cell proliferation and function (49) and is critical for the clonal expansion of autoreactive T-cells during progression to type 1 diabetes (50). We observed that T-cell–mediated protection from type 1 diabetes is accompanied by a significant decrease in IL-2 production by effector T-cells and that the presence of regulatory T-cells is necessary for the downregulation of IL-2 secretion by diabetogenic T-cells. This relative decrease in IL-2 secretion may elicit the loss of function of effector T-cells as revealed by the noted downregulation of IFN-γ secretion. By analogy, in EAE-susceptible mice, the feeding of low doses of the myelin basic protein autoantigen induces protection from EAE via a mechanism of active T-cell suppression that is reversible by treatment with IL-2 (51). Moreover, protection from collagen-induced arthritis by oral administration of an immunodominant human collagen peptide correlates with the inhibition of IL-2 production by effector T-cells in draining lymph nodes (19). Thus, the generation of T-cell–mediated tolerance by orally administered autoantigens involves IL-2–dependent mechanisms in several experimental models of autoimmune disease.

In summary, our studies indicate that the loss of function of a peripheral regulatory T-cell population in NOD mice may disrupt the Th1/Th2 balance and favor the expansion of autoreactive effector Th1-cells that selectively target and mediate the destruction of islet β-cells. This conclusion is supported by our demonstration that oral insulin administration restores the function of peripheral $CD4^+$ regulatory T-cells, which prevent type 1 diabetes by inhibiting the expansion of pathogenic effector Th1 T-cells and their infiltration of the pancreas. Active suppression induced by oral autoantigen feeding therefore appears to play an important role in the maintenance of self tolerance by the correction of a deficiency in $CD4^+$ regulatory T-cell activity. These findings may have important implications for oral insulin clinical trials being conducted to treat first-degree relatives of type 1 diabetes patients at risk for the disease.

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