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Short Communication

Suppression of Insulitis in Non-Obese Diabetic (NOD) Mice by Oral Insulin Administration Is Associated with Selective Expression of Interleukin-4 and -10, Transforming Growth Factor-β, and Prostaglandin-E

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Oral administration of autoantigens suppresses development of autoimmunity in several animal models, and is being tested in clinical trials in patients with autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. Non-obese diabetic (NOD) mice spontaneously develop insulin-dependent diabetes mellitus at 15 to 20 weeks of age, after mononuclear cell (MNC) infiltration of the pancreatic islets of Langerhans and destruction of insulin-producing β cells. We have previously shown that oral administration of insulin suppresses insulitis and development of diabetes in the NOD mouse. Oral insulin has no metabolic effect on blood glucose. Oral insulin mediates its effect through a T cell-dependent mechanism as shown by adoptive transfer and T cell depletion experiments, but the mechanisms responsible have not been fully explored. We now report a serial analysis of the cells and cytokines associated with development of diabetes in NOD mice, and contrast this with the findings in animals fed equine insulin or a control protein (ovalbumin). Animals were fed 1 mg twice a week for 5 weeks, beginning at 5 weeks of age. Marked insulitis in naive or ovalbumin-fed NOD mice occurred at 10 weeks, at which time a dense peri-islet and intra-islet MNC infiltration was observed. Immunohistological studies using monoclonal antibodies showed that infiltrating MNC consisted mainly of CD4$^+$ T cells (>75% of leukocytes) plus smaller numbers of macrophages and CD8$^+$ T cells. These cells displayed evidence of immune activation with expression of receptors for interleukin-2 (IL-2R) plus Th1 cytokines; dense labeling for IFN-γ and tumor necrosis factor-α, plus lesser amounts of IL-2, was observed. MNC lacked labeling for IL-4, IL-10, prostaglandin-E, or transforming growth factor-β. By contrast, at 10 weeks, pancreatic tissues from NOD mice fed insulin showed considerably less insulitis, and the residual MNC, although still largely CD4$^+$ T cells plus macrophages, showed dense labeling for IL-4, IL-10, prostaglandin-E, and transforming growth factor-β and an absence of IL-2, IFN-γ or tumor necrosis factor-α. Taken together with our previous findings, these data indicate that oral administration of insulin affects the development of diabetes in NOD mice through the generation of cells that elaborate immunoregulatory cytokines within the target organ and shift the balance from a Th1 to a Th2 pattern of cytokine expression. (Am J Pathol 1995, 147:1193–1199)

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Exposure of the immune system to antigens via the gut commonly results in a form of antigen-specific unresponsiveness that is termed oral tolerance. As recently reviewed,[1] induction of this form of peripheral tolerance has proven effective in the treatment of several autoimmune diseases in animal models and has shown initial positive effects in humans. Non-obese diabetic (NOD) mice spontaneously develop insulin-dependent diabetes mellitus (IDDM) from 13 to 25 weeks of age, after mononuclear cell (MNC) infiltration of their islets of Langerhans and destruction of insulin-producing β cells, and provide an excellent model for testing new approaches to immunotherapy for IDDM. We and others have shown that oral administration of insulin suppresses the spontaneous development of diabetes in NOD mice.[2,3] Based on these results, trials of oral insulin for the prevention of IDDM are currently being undertaken in first degree relatives at risk for development of IDDM. This study describes the key pathological features associated with development of insulitis in the NOD mouse model, and shows the selective modulation of cellular activation and cytokine expression associated with oral antigen exposure.

Materials and Methods

Animals

Female NOD (K^d, $I\text{-}A^{g7}$, D^b) mice (4 weeks of age) were obtained from Taconic Farms (Germantown, NY), maintained in our virus antibody-free animal facility and fed Agway Rolab 3500 Chow.

Experimental Design

We have shown, by weekly monitoring of urinary glucose for up to 1 year, that NOD mice begin to become glycosuric from 13 to 25 weeks of age[2]; diabetes was confirmed by showing hyperglycemia (>13.8 mmol/L) for 2 consecutive weeks and reached >80% by 25 weeks of age. In preliminary studies, insulitis and cytokine expression were evaluated in NOD mice that were fed porcine insulin dissolved in phosphate-buffered saline (PBS) as described.[2] This led to the current study in which various groups of mice were fed different proteins and monitored at serial intervals. To analyze the intrapancreatic events occurring before the onset of diabetes, tissues (pancreas, liver, and kidney) from NOD mice sacrificed by cervical dislocation were collected for immunopathology at 5, 8, or 10 weeks (3 animals/group/time point). Liver and kidney samples were used to assess the extent to which nonpancreatic organ infiltration by cytokine-producing MNC might occur. The effects of oral insulin administration were determined by comparison with a control protein, ovalbumin.

Oral Antigen Administration

Equine insulin or ovalbumin (each from Sigma Chemical Co., St. Louis, MO) were dissolved (5 mg/ml) in neutral PBS and given twice weekly via gavage tube (0.2 ml/feed) to NOD mice, beginning at week 5, for a total of 5 weeks (n = 10 feeds).

Antibodies

Isotype-matched control monoclonal antibodies (mAbs) (immunoglobulin (Ig) G1, IgG2a, IgG2b) and rat mAbs to mouse cell surface and cytokine antigens were purchased from Pharmingen (San Francisco, CA), unless specified; mAbs were directed against all mouse T cells (CD5, 53–7.3) or T cell subsets (CD4, H129.19; CD8a, 53–6.7); B cells (CD45R/B220, RA3–6B2); mononuclear phagocytes (CD11b, M1/70; Mac-3, M3/84); and granulocytes (Gr-1, RB6–8C5). MNC activation was assessed using an MAb to interleukin (IL)-2R (CD25, 3C7); and by labeling for the cytokines IL-2 (S4B6); IL-4 (11B11), interferon (IFN)-γ (R4–6A2); tumor necrosis factor-α (TNF-α) (MP6-XT22), IL-10 (JES5–2A5), and transforming growth factor-β (TGF-β, neutralizing polyclonal rabbit antibody from R&D (Minneapolis, MN), catalog AB-100-NA), plus prostaglandin-E (PGE, neutralizing polyclonal rabbit antibody (Sigma Chemical Co.), which detects PGE1 and PGE2 but not other prostaglandins). Secondary antibodies, consisting of mouse Ig-absorbed goat anti-rat Ig (Sigma Chemical Co.), rabbit anti-goat Ig, swine anti-rabbit Ig, goat peroxidase-antiperoxidase (PAP) and rabbit PAP, and control rabbit Ig, were purchased from DAKO (Carpinteria, CA).

Immunohistology and Sample Evaluation

Tissues were quick frozen in liquid nitrogen and stored at −70°C until sectioning. Cryostat sections were fixed (10′, 4°C) in paraformaldehyde-lysine-periodate for demonstration of leukocyte and activation antigens, or in acetone for localization of cytokines.[4] Tissues were stained by a four-layer PAP method involving overnight incubation with MAb (4°C), followed by goat anti-rat Ig (5 μg/ml, 30′), methanol/hydrogen peroxide block (10′), rabbit anti-goat Ig (1:50, 30′) and goat PAP complexes (1:50, 30′), and diaminobenzidine substrate. In the case of primary rabbit antibodies (TGF-β, PGE), sections

Table 1. *Histology of Cells and Cytokines within Pancreas from Untreated NOD Mice**

Feature	Week 5	Week 8	Week 10
Histology	Normal in 2/3 mice; 1/3 with a mild peri-islet MNC infiltrate	Normal	Dense peri- and intra-islet MNC infiltration
T cells	Few cells/section except in 1/3 in which >75% MNC + ve	Few cells/section	>75% MNC + ve
$CD4^+$ subset	As for T cells	Few cells/section	Dense, as for T cells
$CD8^+$ subset	Few cells/section	Few cells/section	Few peri-islet cells
Macrophages	Few adventitial and peri-islet cells	Few cells/section	5 to 10% of MNC (intra- and peri-islet cells)
IL-2R	Few cells/section except for 1/3 with 5 to 10% MNC + ve	1/3 with a few perivascular MNCs	5 to 10% MNC + ve
IL-2	Negative	Negative	5 to 10% MNC + ve
IFN-γ	Negative except for 1/3 with small numbers of peri-islet MNC	1/3 showed focal peri-islet MNC	>50% intra- and peri-islet MNCs
TNF-α	Negative	1/3 had perivascular and peri-islet MNCs	>50% intra- and peri-islet MNCs
IL-4	Negative	<5% peri-islet MNCs	Negative
IL-10	Negative	<5% peri-islet MNCs	Negative
TGF-β	Negative	Negative	Negative
PGE	Negative	Negative	Negative

*n = 3 animals/group.

were incubated with primary antibody overnight, followed by methanol block, swine anti-rabbit Ig (1:50, 30′), rabbit PAP (1:50, 30′), and substrate. Sections were then washed, counterstained in hematoxylin, and mounted.

Groups were assigned a random number, and were processed and evaluated in a blinded fashion. Each specimen was evaluated at two to three different levels of sectioning, allowing examination of cross sections of at least 20 islets, and labeled cells within cross sections of islets were assessed with the aid of an ocular grid micrometer. Insulitis was scored as mild, moderate, or dense based on whether only small numbers of leukocytes (<10 cells) were detected adjacent to or within islets (mild); >10 leukocytes present, but with less than one-third of the islet cross section involved (moderate); or >100 leukocytes and/or greater than one-third of the islet infiltrated by leukocytes (dense).

Cytokine expression was scored semiquantitatively, and the specificity of labeling was assessed in three ways. First, isotype-matched mAbs or purified Ig, and a control for endogenous peroxidase activity, were included in each experiment. Second, studies were undertaken to confirm the lack of labeling of normal pancreas, but preservation of labeling of mouse cardiac allograft specimens.[4] Third, overnight MAb absorption with cytokines (IL-2, IL-4, IL-10, TNF-α, and IFN-γ, obtained from Pharmingen, and TGF-β from R&D) was undertaken using antigen-coated enzyme-linked immunosorbent assay wells (4°C, 1 to 5 μg/ml) before immunohistological labeling.

Results

Development of Insulitis in NOD Mice

Histological features and the results of semiquantitative analysis of cells and cytokines within pancreatic tissues from control NOD mice are summarized in Table 1. At week 5, MNC infiltration, in association with expression of IL-2R by $CD4^+$ T cells, was seen in a third of animals. By week 10, all islets in all samples showed dense accumulation of $CD4^+$ T cells plus lesser numbers of macrophages and $CD8^+$ T cells (5 to 10% of MNC). MNCs were associated with dense expression of IFN-γ and TNF-α, as well as focal IL-2 and IL-2R expression, but lacked labeling for the related cytokines IL-4, IL-10 and TGF-β, or PGE (Figure 1). The specificity of labeling was demonstrated by the absence of labeling after cytokine absorption controls (not shown) and by the contrasting labeling after oral insulin administration (see next section).

Effects of Oral Insulin Administration on Insulitis

In contrast to control NOD mice (Table 1) and to those fed ovalbumin (Table 2), oral equine insulin administration markedly altered the extent of insulitis and type of cytokine expression at week 10 (Table 2). Oral insulin not only decreased the overall incidence of insulitis, but suppressed to background levels the extent of IL-2R expression, and effectively eliminated production of IL-2, IFN-γ, or TNF-α. How-

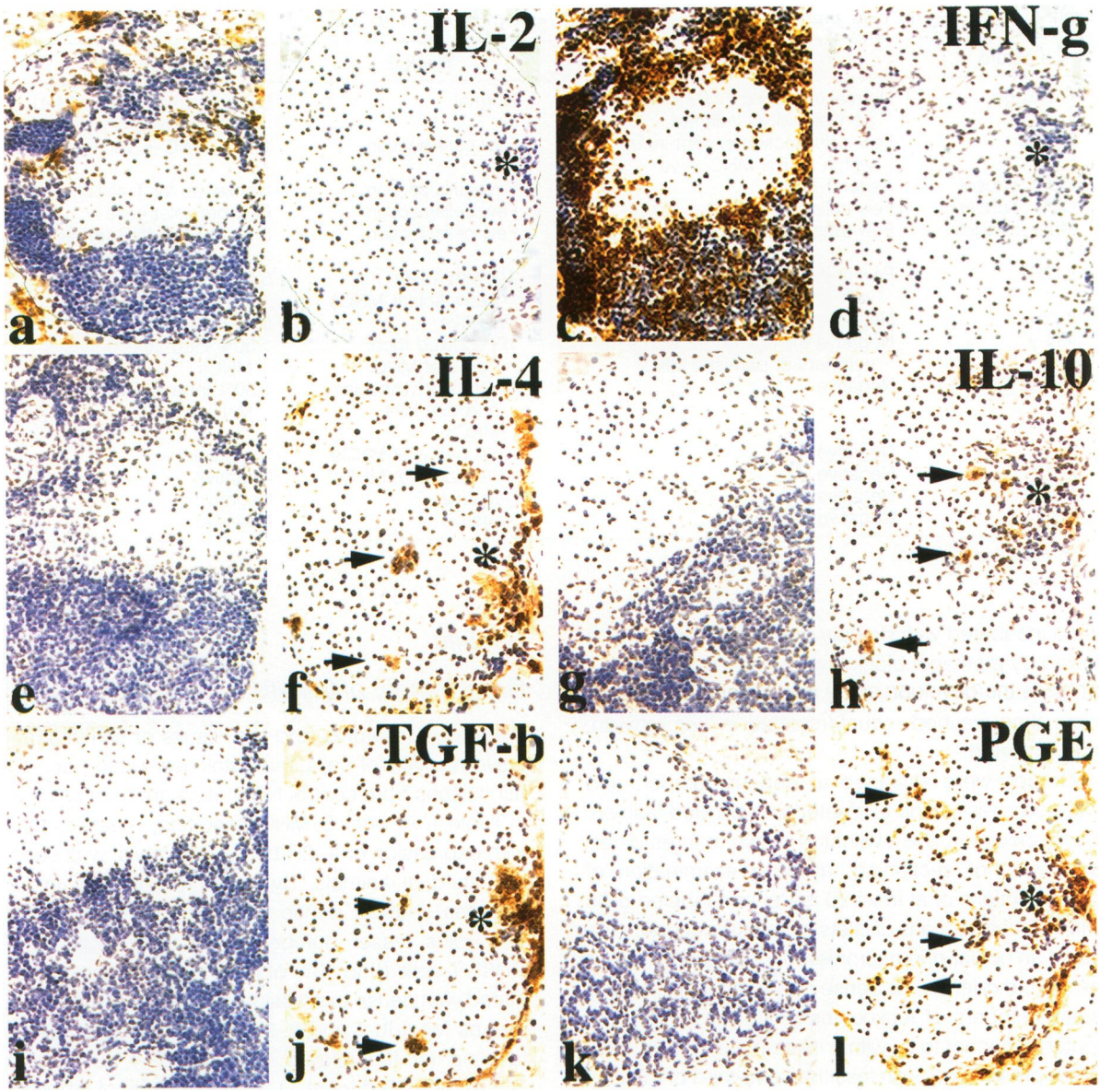

Figure 1. *Immunoperoxidase staining of pancreatic sections from NOD mice fed ovalbumin or insulin (week 10). Representative photomicrographs of the contrasting patterns of labeling for key cytokines and PGE in these animals are shown; for each antigen, the left panel is from an animal fed ovalbumin, and the paired right panel shows the effects of insulin feeding. At the magnification used here to adequately document discrete cell labeling, an entire islet largely fills the field; the outline of each islet, shown in subsequent serial sections, is indicated by the fine line in* a *and* b. (a *and* b) *IL-2-labeling of small numbers of intra-islet MNC was detected in the ovalbumin group, whereas residual MNC (*) associated with the mild isletitis of the insulin-fed group were unstained. Similarly, labeling for IFN-γ (*c *and* d) *was confined to the ovalbumin-fed group; diffuse staining over MNC infiltrating an islet is seen. No labeling of residual MNC in the insulin group (*) was observed. In contrast to the results for IL-2 and IFN-γ, labeling for IL-4 (*e *and* f), *IL-10 (*g *and* h), *TGF-β (*i, j), *and PGE (*k *and* l) *was essentially confined to scattered intra-islet MNC (*arrows) *plus the residual collection of MNC (*) within the axial stalk of the islet in the insulin-fed group (*right), *whereas these proteins were absent from the albumin-fed group (*left). (*Cryostat sections, hematoxylin counterstain; all magnifications ×250).*

ever, the effects of oral insulin were not exclusively inhibitory in nature; NOD mice fed insulin showed considerable labeling for the cytokines IL-4, IL-10 and TGF-β, and PGE (Figure 1). Sections of liver or kidney from each mouse were normal, lacking cytokine expression or more than basal numbers of interstitial macrophages and rare T cells. Furthermore, a pilot experiment in NOD mice fed porcine insulin in PBS showed analogous changes in cytokine expression and decreased insulitis, although TGF-β and PGE expression were not examined.

Discussion

Oral administration of autoantigen suppresses a number of organ-specific autoimmune diseases, in-

Table 2. *Histology of Cells and Cytokines at Week 10 in Pancreas of NOD Mice Fed Insulin or Ovalbumin**

Feature	Insulin-fed	Ovalbumin-fed
Histology	Mild to moderate peri-islet MNC	Dense peri- and intra-islet MNC infiltrates
T cells	>75% MNC + ve	>75% MNC + ve
$CD4^+$ subset	>75% MNC + ve	>75% MNC + ve
$CD8^+$ subset	Few cells/section	~5% peri-islet cells
Macrophages	Few adventitial and peri-islet MNC	5 to 10% of MNC (intra- and peri-islet cells)
IL-2R	Few cells/section	5 to 10% MNC + ve
IL-2	Negative	5 to 10% MNC + ve
IFN-γ	Negative	>50% intra- and peri-islet MNC
TNF-α	Negative	>50% intra- and peri-islet MNC
IL-4	5 to 10% intra- and peri-islet MNC	Negative
IL-10	5 to 10% intra- and peri-islet MNC	Negative
TGF-β	10 to 20% intra- and peri-islet MNC	Negative
PGE	10 to 20% intra- and peri-islet MNC	Negative

**n = 3 animals/group.*

cluding experimental autoimmune encephalomyelitis, collagen and adjuvant arthritis, uveitis, experimental autoimmune myasthenia gravis, immune complex nephritis, and diabetes.[1] We have previously shown that oral administration of insulin suppresses the development of insulitis and diabetes in the NOD mouse by a T cell-dependent mechanism, as shown by adoptive transfer and the effects of T cell depletion.[2] These findings were recently confirmed by Bergerot et al,[3] who identified $CD4^+$ T cells as the primary cells mediating the effect. Other investigators have also described $CD4^+$ regulatory cells in the NOD mouse.[5] The current study presents an analysis of the cell types and cytokine expression associated with development of diabetes in NOD mice, and contrasts this with the findings in animals fed insulin or a control protein, ovalbumin.

Marked insulitis in naive or ovalbumin-fed NOD mice occurred at 10 weeks, wherein dense peri-islet and less extensive intra-islet MNC infiltration was observed. Immunohistological studies using mAbs showed that infiltrating cells consisted predominantly of $CD4^+$ T cells (>75% of leukocytes) plus smaller numbers of macrophages and $CD8^+$ T cells. A subset of these MNCs had undergone immune activation, as demonstrated by their expression of receptors for IL-2R, and demonstrated a Th1 pattern of cytokine expression, with dense labeling for IFN-γ and TNF-α, plus lesser amounts of IL-2, whereas cells lacked labeling for the Th2 cytokines, IL-4 and IL-10, or for PGE or TGF-β. These data are consistent with studies in NOD mice in which an important role for $CD4^+$ T cells in the development of diabetes was shown.[6] By contrast, at 10 weeks, pancreatic tissues from NOD mice fed insulin showed considerably less insulitis; the MNCs present, primarily $CD4^+$ T cells plus macrophages, showed labeling for IL-4, IL-10, PGE, and TGF-β but lacked IL-2, IFN-γ, or TNF-α.

IL-4[7] and IL-10[8] are each known to prevent development of diabetes in NOD mice, presumably by downregulating pathogenic Th1 responses. Treatment of NOD mice with adjuvant also inhibits diabetes in association with increased IL-4-producing cells and decreased IFN-γ-producing cells.[9] Moreover, treatment of young NOD mice with an anti-TNF-α antibody prevents development of diabetes.[10] Hence, it appears that modulation of the immune response toward a Th2-type pattern has an ameliorating effect on diabetes in the NOD mouse. In this regard, orally administered antigens preferentially generate Th2 type responses most probably because IL-4 serves as the primary growth factor for gut-associated lymphoid tissues.[1] In addition, we have shown that cells that secrete TGF-β can suppress cell-mediated autoimmune diseases such as experimental autoimmune encephalomyelitis and are induced by orally administered antigens.[11,12] The preferential generation of a Th2 and a TGF-β response in the gut may be related to the observation that IL-4 is the primary growth factor produced by cells from the mucosal immune system[13] and that TGF-β serves as a switch factor for IgA responses in the gut.[14]

The current findings support the concept[1,15] that administration of an islet cell autoantigen such as insulin results in the activation of Th2-type T cells that produce IL-4 and IL-10, as well as the generation of PGE- and TGF-β-producing cells, and in the subsequent suppression of Th1-mediated autoimmune responses involving IL-2, IFN-γ and TNF-α production. These Th2 cells are antigen-specific and preferentially localize to the islets where insulin is expressed,

because neither Th2 cells or Th2-related cytokines were detected in other tissues. The data complement our findings in animals with experimental autoimmune encephalomyelitis in which feeding of myelin basic protein is associated with downregulation of inflammatory cytokines but upregulation of TGF-β, IL-4, and PGE expression in the brain.[16] Our findings are also consistent with the recently documented effects of PGE on switching of T cell responses toward a Th2-predominant state with selective expression of regulatory cytokines such as IL-4, IL-10, and TGF-β.[17]

In summary, these findings suggest that after oral absorption of insulin, a T cell response is induced in gut-associated lymphoid tissues, leading to selective expansion of Th2 and TGF-β-producing cells that localize to the pancreatic β-cells. Because these cells secrete antigen-nonspecific cytokines such as IL-4 and TGF-β, after antigen-specific triggering in response to the fed antigen they mediate what may be termed bystander suppression.[11] This is important, as one need not necessarily know the nature of a particular autoantigen responsible for an autoimmune response in order to develop a specific therapy. Rather, this approach requires only the feeding of a related antigen from the same tissue that can induce regulatory cells that migrate to the target tissue and suppress inflammation. With regard to oral insulin and NOD mice, amelioration of diabetes and insulitis is thus not necessarily related to interrupting autoreactivity to insulin. Indeed, various antigens may serve as targets of the autoimmune response in diabetes, including glutamate decarboxylase, insulin, insulin receptor, glucagon, carboxypeptidase H, sialoglycolipid, and heat shock proteins.[18,19] Our findings suggest that oral administration of any islet cell antigen capable of triggering cells that localize to the pancreas could suppress diabetes in this model,[20] and thus provide the basis for the oral administration of islet antigens in IDDM irrespective of the specific islet cell autoantigens involved in the disease.

References

1. Weiner HL, Friedman A, Miller A, Khoury SJ, Al-Sabbagh A, Santos L, Sayegh M, Nussenblatt RB, Trentham DE, Hafler DA: Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu Rev Immunol 1994, 12:809–837
2. Zhang ZJ, Davidson L, Eisenbarth G, Weiner HL: Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc Natl Acad Sci USA 1991, 88:10252–10256
3. Bergerot I, Fabien N, Maguer V, Thivolet C: Oral administration of human insulin to NOD mice generates $CD4^+$ T cells that suppress adoptive transfer of diabetes. J Autoimmunity 1994, 7:655–663
4. Mottram PL, Han WR, Purcell LJ, McKenzie IFC, Hancock WW: Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and IFN-γ in long-surviving mouse heart allografts after brief CD4 monoclonal antibody therapy. Transplantation 1995, 59:559–565
5. Sempe P, Richard MF, Bach JF, Boitard C: Evidence of $CD4^+$ regulatory T-cells in the non-obese diabetic male mouse. Diabetologia 1994, 37:337–343
6. Bradley BJ, Haskins K, Larrosa FG, Lafferty KJ. CD8 T-cells are not required for islet destruction induced by a $CD4^+$ islet-specific T-cell clone. Diabetes 1992, 41; 1603–1608
7. Rapoport MJ, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska J, Delovitch TL. Interleukin 4 reverses T-cell proliferative unresponsiveness, and prevents the onset of diabetes in non-obese diabetic mice. J Exp Med 1993, 178:87–99
8. Pennline KJ, Roque-Gaffney E, Monahan M: Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. Clin Immunol Immunopathol 1994, 71:169–175
9. Shehadeh NN, LaRosa F, Lafferty KJ: Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. J Autoimmunity 1993, 6:291–300
10. Yang X-D, Tisch R, Singer SM, Cao ZA, Liblau RS, Schreiber RD, McDevitt HO: Effect of tumor necrosis factor α on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. J Exp Med 1994, 180:995–1004
11. Miller A, Al-Sabbagh A, Santos LMB, Das MP, Weiner HL: Epitopes of myelin basic protein that trigger TGF-β release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression. J Immunol 1993, 151:7307–7315
12. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 1994, 265:1237–1240
13. Daynes R, Araneo B, Dowell T, Huang K, Dudley D: Regulation of murine lymphokine production *in vivo*. III. The lymphoid tissue microenvironment exerts regulatory influences over T helper cell function. J Exp Med 1990, 171:979–997
14. Coffman R, Lebman D, Shrader B: Transforming growth factor β (TGFβ) specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J Exp Med 1989, 169:170–177
15. Rabinovitch A: Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Diabetes 1994, 43:613–621
16. Khoury SJ, Hancock WW, Weiner HL: Oral tolerance to myelin basic protein and natural recovery from exper-

imental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor-β, interleukin-4, and prostaglandin-E expression in the brain. J Exp Med 1992, 176:1355–1364

17. Snijdewint FGM, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. Prostaglandin-E2 differentially modulates cytokine secretion profiles of human T-helper lymphocytes. J Immunol 1993, 150:5321–5329
18. Weiner HL: Oral tolerance. Proc Natl Acad Sci USA 1994, 91:10762–10765
19. Atkinson MA, Maclaren NK: Islet cell autoantigens in insulin-dependent diabetes. J Clin Invest 1993, 92: 1608–1616
20. Zhang ZJ, Shoelson S, Miller A, Weiner HL: Insulitis is suppressed in NOD mice by oral administration of insulin peptides and glucagon. FASEB J 1992, 6:1693 (Abstr.)