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Randal K. Gregg, J. Jeremiah Bell, Hyun-Hee Lee, Renu Jain, Scott J. Schoenleber, Rohit Divekar and Habib Zaghouani

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IL-10 Diminishes CTLA-4 Expression on Islet-Resident T Cells and Sustains Their Activation Rather Than Tolerance1

Randal K. Gregg,2 J. Jeremiah Bell, Hyun-Hee Lee, Renu Jain, Scott J. Schoenleber, Rohit Divekar, and Habib Zaghouani3

IL-10, a powerful anti-Th1 cytokine, has shown paradoxical effects against diabetes. The mechanism underlying such variable function remains largely undefined. An approach for controlled mobilization of endogenous IL-10 was applied to the NOD mouse and indicated that IL-10 encounter with diabetogenic T cells within the islets sustains activation, while encounter occurring peripheral to the islets induces tolerance. Insulin-β-chain (INSβ) 9-23 peptide was expressed on an Ig, and the aggregated (agg) form of the resulting Ig-INSβ triggered IL-10 production by APCs, and expanded IL-10-producing T regulatory cells. Consequently, agg Ig-INSβ delayed diabetes effectively in young NOD mice whose pathogenic T cells remain peripheral to the islets. However, agg Ig-INSβ was unable to suppress the disease in 10-wk-old insulitis-positive animals whose diabetogenic T cells have populated the islets. This is not due to irreversibility of the disease because soluble Ig-INSβ did delay diabetes in these older mice. Evidence is provided indicating that upon migration to the islet, T cells were activated and up-regulated CTLA-4 expression. IL-10, however, reverses such up-regulation, abolishing CTLA-4-inhibitory functions and sustaining activation of the islet T lymphocytes. Therefore, IL-10 supports T cell tolerance in the periphery, but its interplay with CTLA-4 sustains activation within the islets. As a result, IL-10 displays opposite functions against diabetes in young vs older insulitis-positive mice. The Journal of Immunology, 2005, 174: 662–670.

Type 1 or insulin-dependent diabetes mellitus (IDDM)4 is regarded as an immune-mediated disease in which the β cells of the pancreatic islets of Langerhans are destroyed as a consequence of inflammatory reactions triggered by activation of T cells specific for β cell-associated Ags (1, 2). The NOD mouse develops spontaneous diabetes that shares many of the features associated with human IDDM, providing a well-characterized animal model for this complex autoimmune disease (3). In the NOD mouse model, like in human IDDM, self-reactive Th1 cells play a major role in the initial stages of the disease (4). IL-10, a powerful anti-Th1 cytokine, has in recent years shown variable effects against type 1 diabetes (5–9). The mode of delivery of the cytokine (5–7) as well as the age of the animals (8, 9) are believed to be contributing factors to the erratic behavior of IL-10. The question then is how IL-10 in the blood affects diabetes differently from IL-10 expressed in the islet. Also, how does IL-10 suppress diabetes in young animals whose diabetogenic T cells remain peripheral to the islets, but display no effectiveness in older animals whose diabetogenic T cells are spread both in the periphery and the islets? One potential response to these questions is that peripheral and islet-resident diabetogenic T cells display differential susceptibility to regulation by IL-10. The studies presented in this work devised a unique strategy for mobilizing and targeting endogenous IL-10 to diabetogenic T cells and attempted to explore this postulate.

We have previously shown that expression of myelin peptides on Ig facilitates internalization through FcyR and increases peptide presentation to T cells (10). In addition, aggregation of the Ig-mycelin chimera, which cross-links FcyR, induced IL-10 production by APCs and sustained effective suppression of experimental allergic encephalomyelitis (11–13). Recently, IL-10 has been shown to serve as a growth factor for T regulatory (Treg) cells (14, 15). In fact, in vitro (16) as well as in vivo (17) regimes using IL-10 successfully induced Treg cells that produce IL-10 and support tolerance against pathogenic T cells.

In this study, the I-Aβ7-restricted insulin-β-chain (INSβ) 9-23 peptide (18, 19) was genetically engineered into the V region of an Ig molecule, and the resulting Ig-INSβ was aggregated (agg) and tested for induction of IL-10-producing Treg cells and suppression of diabetes. Both young NOD mice that have not progressed to insulitis and older animals positive for insulin autoantibody (IAA), which is indicative of insulitis, were included in the studies. The results indicate that agg Ig-INSβ induced IL-10 production by APCs and sustained the development of IL-10-producing Treg cells in vivo. Moreover, when given to 4-wk-old NOD mice, agg Ig-INSβ suppressed diabetogenic T cells and protected the mice against diabetes. This effect is most likely due to down-regulation by IL-10 from APCs and/or Treg cells because: 1) soluble (sol) Ig-INSβ, not inducing IL-10, was less effective against the disease; 2) agg Ig-INSβ was unable to protect young IL-10-deficient mice from diabetes; and 3) depletion of Treg cells at young age also

Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia, MO 65212
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2 Current address: University of Virginia, Beirne B. Carter Center for Immunology Research, MR4 Building, Charlottesville, VA 22908-1386.

3 Address correspondence and reprint requests to Dr. Habib Zaghouani, University of Missouri School of Medicine, Department of Molecular Microbiology and Immunology, M616 Medical Sciences Building, Columbia, MO 65212. E-mail address: zaghouanib@health.missouri.edu

4 Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; agg, aggregated; DC, dendritic cell; Foxp3, forkhead/winged helix transcription factor gene; GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; IAA, insulin autoantibody; INSβ, insulin β-chain; sol, soluble; Treg, T regulatory.

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hinders agg Ig-INSβ-mediated delay of the disease. Surprisingly, however, agg Ig-INSβ was unable to delay diabetes in IAA-positive mice despite the fact that the disease remained reversible as the sol form of Ig-INSβ was able to reverse it. Evidence is provided indicating that T cells up-regulate CTLA-4 upon migration to the islets and agg Ig-INSβ reverses such expression both in vitro and in vivo through APC and/or Treg cell-derived IL-10. The end result is sustained activation of the diabetogenic T cells. Given the fact that IAA-positive IL-10−/− mice were able to reverse their diabetes upon treatment with agg Ig-INSβ, it is suggested that down-regulation of CTLA-4 by IL-10 nullifies its inhibitory functions and sustains T cell activation and lack of protection against diabetes.

Materials and Methods

**Mice**

NOD (H-2k) and NOD.scid mice were purchased from The Jackson Laboratory, and IL-10-deficient (IL-10−/−) NOD mice were previously described (20). The experimental procedures performed on these animals were conducted according to the guidelines of the institutional animal care and use committee.

**Assessment of diabetes**

Mice are bled from the tail vein weekly, and the blood samples are used to assess glucose content using test strips and an Accu-Chek Advantage monitoring system (Roche Diagnostics). A mouse is considered diabetic when the blood glucose is above 300 mg/dl for 2 consecutive wk.

**Antigens**

**Peptides.** All peptides used in this study were purchased from Metabion and purified by HPLC to >90% purity. INSβ peptide encompasses the diabetogenic INSβ 9-23 amino acid sequence (SHLVEAALVCGGER). Glutamic acid decarboxylase 2 (GAD2) peptide corresponds to aa residues 206-220 of GAD65 (21). Hen egg lysozyme (HEL) peptide encompasses a nondiabetogenic epitope corresponding to aa residues 11-25 (AMKRKGLDNLRYGYS) of HEL (22). INSβ, GAD2, and HEL peptides are presented to T cells in association with I-Ag7 MHC class II molecules.

**Ig chimeras.** Ig-INSβ, Ig-GAD2, and Ig-HEL express INSβ, GAD2, and HEL peptide, respectively. Insertion of INSβ, GAD2, and HEL nucleotide sequences into the CDR3 of the H chain V region of the 91A3 IgG2b, λ Ig was conducted as described (10). Large-scale cultures of transfectedoma cells were conducted in DMEM medium containing 10% iron-enriched calf serum (BioWhittaker). Purification of the chimeras was conducted on separation columns of rat anti-mouse γ/δ mAb coupled to CNBr-activated 4B Sepharose (Amersham Biosciences). Aggregation of the Ig chimeras was conducted by precipitation with 50% saturated (NH4)2SO4, as described (11).

**T cell lines**

T cell lines specific for INSβ, GAD2, and HEL peptides were generated by immunizing NOD mice s.c. with 100 μg of peptide in CFA and in vitro peptide stimulation, followed by restim, as described (11). These lines are of Th1-type T cells and produce IFN-γ, but not IL-4 or IL-10 upon stimulation with the corresponding peptide or sol Ig chimera (data not shown).

**Isolation of T cells**

CD4 and CD8 T lymphocytes were isolated from splenic or islet cells by positive selection on Miltenyi (Miltenyi Biotec) microbeads, according to the manufacturer’s instructions. Isolation of islet CD4 and CD8 T cells was performed, as described (23). Isolation of splenic CD4+ CD25+ T cells was conducted by negative selection of CD4 T cells, followed by positive selection by anti-CD25 Ab coupled to microbeads, according to Miltenyi’s instructions.

**Isolation of APCs**

Splenic dendritic cells (DC) were purified, according to a standard collagenase/differential adherence method (13). Briefly, the spleen was disrupted in a collagenase solution, and isolated DC floated on a dense BSA gradient. Subsequently, the cells were allowed to adhere to petri dishes for 90 min at 37°C, washed, and incubated overnight. The DC were then harvested and further purified on anti-CD11c-coupled microbeads, according to Miltenyi’s instructions. Partial purification of splenic APCs was conducted by floating the cells on a dense BSA gradient as for the DC, and the cells were washed in plain culture medium and used in presentation assays. These APCs are designated BSA-APCs.

**Flow cytometry analyses**

For staining of CD4+ CD25 T cells, purified splenic CD4 T cells (1 × 10⁶ cell/ml) were incubated with anti-CD4-FITC and anti-CD25-APC or isotype control rat IgG1 APC for 30 min at 4°C and washed with buffer. The cells were then fixed with 2% formaldehyde for 20 min at 25°C and then analyzed. Events (30–50 × 10⁴) were collected on a FACSVantage flow cytometer (BD Biosciences) and analyzed using CellQuest software 3.3 (BD Biosciences). Staining for CTLA-4 was conducted, as follows: purified islet and splenic CD4 T cells (1 × 10⁶ cells/ml) were incubated with anti-CTLA-4-PE or isotype control hamster IgG1 for 2 h at 37°C, followed by anti-CD4-FITC for 30 min at 4°C. The cells were then washed, fixed with 2% formaldehyde, and analyzed on a FACSVantage flow cytometer, as above.

**Proliferation assays**

For T cell line proliferation assay, irradiated (3000 rad) NOD female splenocytes (5 × 10⁵ cells/50 μl/well) were incubated with graded amounts of either Ig-INSβ or Ig-HEL (100 μg/well), and 1 h later INSβ-specific T cells (5 × 10⁵ cells/well/50 μl) were added. After 72 h, 1 μCi of [3H]thymidine was added per well, and the culture was continued for an additional 14.5 h. The cells were then harvested on a Trilux 1450 Microbeta Wallac Harvester, and incorporated [3H]thymidine was counted using the Microbeta 270.004 software (EG & G Wallac).

For evaluation of T cell responses in vivo, purified splenic CD4 T cells (2.5 × 10⁵ cell/well) isolated from 16-wk-old untreated or agg Ig-INSβ-treated mice were stimulated with irradiated (3000 rad) BSA-APCs (5 × 10⁵ cells/well) and 30 μg/ml peptide, and proliferation was measured, as above.

For proliferation of Treg cells, purified CD4+ CD25+ and CD4+ CD25− T cells (2 × 10⁵ cells/well) were incubated for 72 h with 18 μM INSβ or HEL and irradiated (3000 rad) BSA-APCs (4 × 10⁵ cells/well), and proliferation was assessed.

**T cell cytokine assays**

All cytokine analyses were done by ELISA using anti-cytokine Abs from BD Pharmingen, as described (13).

Analysis of the effect of the APC’s IL-10 on IFN-γ production was done as follows: the T cell line (0.2 × 10⁶ cells/well) was incubated with purified NOD splenic DC (5 × 10⁵ cells/well) and Ig chimeras for 24 h, and IFN-γ as well as IL-10 were measured by ELISA. In some experiments, blockade of IL-10 was performed by supplementing the culture with 40 μg/ml anti-IL-10 Ab JES5-2A5. The isotype control used 40 μg/ml rat IgG2a.

For evaluation of cytokine T cell responses in vivo, purified splenic CD4 T cells (2.5 × 10⁵ cell/well) isolated from 16-wk-old untreated or agg Ig-INSβ-treated mice were stimulated with irradiated (3000 rad) BSA-APCs (5 × 10⁵ cells/well), and 30 μg/ml peptide and IFN-γ as well as IL-10 were measured by ELISA after 48-h incubation.

For assessment of IL-10 production by Treg cells, purified CD4+ CD25+ and CD4+ CD25− T cells (2 × 10⁵ cells/well) were incubated for 48 h with 10 μg/ml plate-bound anti-CD3 Ab (2C11), and the cytokine was measured by ELISA.

For evaluation of IFN-γ production by islet-resident T cells, bulk islet cells (5 × 10⁵ cells/well) were stimulated with 18 μM INSβ peptide or 1 μM Ig chimeras for 48 h, and IFN-γ was measured by ELISA. In the case of purified islet lymphocytes, the CD4 or CD8 T cells (2 × 10⁵ cells/well) were incubated with irradiated BSA-APCs (5 × 10⁵ cells/well) and 1 μM Ig chimeras. IFN-γ was measured 48 h later by ELISA.

**RT-PCR for Foxp3 expression**

Total RNA was extracted from cells using TRIzol reagent and used to determine relative mRNA levels of forkhead/winged helix transcription factor gene (Foxp3). Reverse transcription and DNA amplification were performed using 300 ng of total RNA, 100 ng of Foxp3 and β-actin primers (24), and the Quant iTect SYBR Green RT-PCR kit from Qiagen, as described (25).

**Adoptive transfer**

CD4+ CD25+ and CD4+ CD25− T cells were purified from the spleen of 6-wk-old agg Ig-INSβ-treated mice, and 5 × 10⁵ cells were cotransferred i.v. with 10 × 10⁶ diabetic splenocytes into NOD.scid mice (4–8 wk of age). The animals were monitored for blood glucose levels weekly.
Depletion of Treg cells

For depletion of CD25+ T cells in vivo, NOD mice were given 1 mg/mouse anti-CD25 mAb (PC61) alone or concurrently with agg Ig-INSβ injection. Rat IgG (1 mg/mouse) was used as a control.

Detection of IAA

The following was conducted by ELISA: microtiter plates were coated with 50 μl of sodium bicarbonate solution (pH 9.6) containing 10 μg/ml porcine insulin (Sigma-Aldrich) for 16 h at 4°C. The plates were then washed three times with PBS-0.05% Tween 20 and saturated with 2.5% casein (in 0.3 M NaCl, pH 7) for 2 h. Serum samples (1/200 dilutions) were then added for 16 h at 4°C, followed by biotin-conjugated rat anti-mouse α mAb (100 μl at 1 μg/ml). The plates were then incubated with avidin peroxidase (2.5 mg/ml) for 30 min at 25°C, and the assay was revealed by addition of ABTS substrate. The samples were read at 405 nm on a Spectramax 190. A sample is considered IAA positive when the OD405 is >0.2. This cutoff line of 0.2 was chosen because serum samples from 10 SJL mice, which are nonprone to diabetes development and presumably do not produce insulin-specific autoantibodies, never exceeded 0.05 OD405 (4-fold less than cutoff).

Statistical analysis

The χ² test was used for data analysis among experimental and control groups. Cytokine levels were compared using Student’s t test for unpaired samples.

Results

Agg Ig-INSβ triggers IL-10 production by APCs and supports the development of Treg cells

Recent studies have revealed that delivery of myelin peptides on IgG enhances presentation to T cells (12). Moreover, aggregation of IgG-myelin chimeras induced IL-10 production by APCs and sustained effective down-regulation of myelin-reactive T cells (11, 13). Because IL-10 can serve as a growth factor for Treg cells (14), delivery of self peptides on IL-10-inducing agg IgGs could support the development of Treg cells and sustain additive tolerogenic functions that should be effective against complex autoimmune diseases such as type 1 diabetes. To test this premise, the I-A<sup>e</sup>-restricted diabetogenic INSβ peptide was expressed on an Ig and the resulting Ig-INSβ chimera was tested for presentation to INSβ-specific T cells, triggering of IL-10 production by APCs, and induction of Treg cells. Fig. 1A shows that Ig-INSβ, but not the control Ig-HEL, induced significant proliferation of INSβ-specific T cells. These results indicate that Ig-INSβ was taken up by the APCs and processed, and an INSβ peptide was generated and presented to T cells. Also, agg, but not sol INSβ induced IL-10 production by DC (Fig. 1B). As IL-10 can serve as a growth factor for Treg cells (14, 15), treatment with agg INSβ+ may support the development of Treg cells in vivo. Fig. 1, C and D, shows that agg INSβ increased CD4<sup>+</sup>CD25<sup>+</sup> T cells from 4.4% in untreated to 7.1% in agg INSβ-treated nondiabetic NOD mice. Moreover, these CD4<sup>+</sup>CD25<sup>+</sup> T cells had increased Foxp3 mRNA expression relative to their CD4<sup>+</sup>CD25<sup>-</sup> counterparts (Fig. 1E), but displayed reduced proliferation upon stimulation with INSβ peptide (Fig. 1F). CD4<sup>+</sup>CD25<sup>-</sup> T cells from untreated mice also had 4-fold higher Foxp3 expression (data not shown). Interestingly, stimulation with anti-CD3 Ab induced increased IL-10 production by the expanded relative to the natural CD4<sup>+</sup>CD25<sup>+</sup> T cells or the CD4<sup>+</sup>CD25<sup>-</sup> counterparts (Fig. 1G). The lack of increased IL-10 production by the natural CD4<sup>+</sup>CD25<sup>-</sup> T cells may be related to lower frequency of IL-10-producing cells among this heterogeneous population, while treatment with agg INSβ specifically expands IL-10-producing T cells. Finally, upon transfer to NOD.scid mice, the CD4<sup>+</sup>CD25<sup>-</sup>, but not CD4<sup>+</sup>CD25<sup>+</sup> T cells conferred protection against passive diabetes mediated by diabeticogenic splenocytes (Fig. 1H). Thus, these CD4<sup>+</sup>CD25<sup>+</sup> T cells represent Tregs rather than activated CD4<sup>+</sup> T cells because they have higher Foxp3 expression relative to their CD4<sup>+</sup>CD25<sup>-</sup> counterparts as did CD4<sup>+</sup>CD25<sup>+</sup> T cells from untreated mice, were not proliferative upon stimulation with INSβ peptide, and suppressed diabetes upon transfer into NOD.scid mice along with pathogenic splenocytes. Overall, these results indicate that agg INSβ supports the development of IL-10-producing Treg cells endowed with suppressive functions.

Agg Ig-INSβ suppresses T cell responses

IL-10 produced by the DC upon presentation of agg Ig-INSβ displays down-regulatory functions on the activation of specific T cells engaged to the DC through INSβ peptide. Indeed, when an
INSβ-specific Th1 cell line was incubated with DC and agg Ig-INSβ, the secretion of IFN-γ by the T cell line decreased as production of IL-10 by the DC increased (Fig. 2A). Such down-regulation of IFN-γ did not occur with sol Ig-INSβ, which did not induce IL-10 secretion by the DC (Fig. 2B). Neutralization of IL-10 during stimulation with agg Ig-INSβ restored IFN-γ production by the T cells (Fig. 2C).

In vivo, when NOD mice were given agg Ig-INSβ at a young age and then tested for T cell responses at a later time point, there was effective suppression of proliferation and IFN-γ production (Fig. 2, D and E). Untreated mice, whether diabetic or not, developed significant proliferation and IFN-γ production upon stimulation with INSβ, but not HEL peptide. Interestingly, agg Ig-INSβ-treated, but not untreated mice developed IL-10 responses upon stimulation with INSβ, but not HEL peptide (Fig. 2F). Overall, these findings indicate that agg Ig-INSβ induces tolerance of diabetogenic T cells most likely through IL-10 from APCs and/or Treg cells.

**Agg Ig-INSβ delays diabetes in young NOD mice through IL-10-producing Treg cells**

Agg Ig-INSβ was then tested for protection of young NOD mice against diabetes. Accordingly, animals were given agg Ig-INSβ at the preinsulitis stage (weeks 4, 5, and 6 of age), and the mice were monitored for blood glucose weekly up to week 26. As shown in Fig. 3A, agg Ig-INSβ delayed diabetes in all mice, except one up to week 20. Such delay remained significant by week 26, at which point only 30% of the mice had high blood glucose levels, while 80% of the untreated mice became diabetic. It is worth noting that agg Ig-HEL displayed a significant delay of diabetes up to week 16. Because Ig-HEL is made of the same Ig backbone (IgG2b isotype) as Ig-INSβ and upon aggregation cross-links FcγR on the presenting cells and induces IL-10 production by APCs, such a delay is most likely due to IL-10 bystander suppression. In fact, we have previously observed similar bystander suppression unrelated to Ag specificity with Ig-myelin chimeras (11–13). In contrast, sol Ig-INSβ, which does not induce IL-10 by APCs, was not as effective as agg Ig-INSβ in delaying the onset of diabetes (Fig. 3B). Although no animals were hyperglycemic by week 16 of age and some delay persisted until week 20, most of the mice became hyperglycemic by week 26 of age.

**FIGURE 2.** Agg Ig-INSβ down-regulates INSβ-specific T cells both in vitro and in vivo. Down-regulation of INSβ-specific Th1 cell line in vitro by agg (A) or sol Ig-INSβ (B) was assessed by measurement of IFN-γ using ELISA. IL-10 production by the presenting DC was also measured in the same culture well by ELISA. C, The effect of DC’s IL-10 on IFN-γ secretion by the Th1 cell line was assessed by stimulation with graded amounts of agg Ig-INSβ in the presence of 40 μg of anti-IL-10 Ab or rat IgG control. Each bar represents the mean ± SD of triplicates. D–F, Mice were untreated (Nil) or given 300 μg of agg Ig-INSβ (Agg Ig-INSβ) at weeks 4, 5, and 6 of age, and their responses were analyzed on week 16. The analysis used purified splenic CD4 T cells that were stimulated with INSβ or HEL peptide presented on BSA-APCs. Splenic cells from untreated diabetic (Nil/dia) mice were included for comparison purposes. The proliferative responses (D) were measured by [3H]thymidine incorporation, while IFN-γ (E) and IL-10 (F) production were assessed by ELISA.

**FIGURE 3.** Expansion of CD4+CD25+ IL-10-producing T cells is required for effective suppression of diabetes in young NOD mice. Female NOD mice (10 per group) were given an i.p. injection of a saline solution containing 300 μg of either agg (A) or sol Ig-INSβ or Ig-HEL (B) at weeks 4, 5, and 6 of age, and then monitored for blood glucose levels weekly up to 26 wk of age. A group of mice that did not receive any injection (Nil) was included for control purposes. C, Groups (10 mice per group) of female wild-type (WT) and IL-10−/− NOD mice were given agg Ig-INSβ according to the same treatment regimen and monitored for blood glucose levels. A group of IL-10−/− mice that did not receive any treatment with agg Ig-INSβ was included for control purposes. D, Groups (10 mice per group) of mice were given agg Ig-INSβ according to the same treatment regimen, except that each injection was accompanied by 1 mg of anti-CD25 Ab or rat IgG control. A group of mice given anti-CD25 Ab without agg Ig-INSβ was included to serve as control. a, p < 0.05 compared with untreated mice; b, p < 0.05 compared with IL-10−/−/agg Ig-INSβ-treated mice; c, p < 0.05 compared with untreated mice.
diabetic by week 26. Sol Ig-HEL did not display any significant delay of diabetes onset, indicating that the effect observed with Ig-INSβ is Ag specific. The role of IL-10 against diabetes at this young age became evident when agg Ig-INSβ was unable to delay the onset of diabetes in NOD mice deficient for IL-10 (Fig. 3C).

Indeed, the incidence of diabetes was similar in agg Ig-INSβ-treated and untreated IL-10−/− NOD mice, but significantly higher than in the treated wild-type mice. Interestingly, when depleting anti-CD25 Ab accompanied the treatment, delay of disease did not occur (Fig. 3D). Indeed, the incidence of diabetes increased from 20 to 50% at weeks 20 and 30 to 70% at week 26 in animals treated with agg Ig-INSβ + rat IgG vs agg Ig-INSβ + anti-CD25 Ab. These results indicate that agg Ig-INSβ, which sustains IL-10 production from both APCs and Treg cells, down-regulates diabeticogenic T cells and effectively protects young mice against diabetes.

**Endogenous IL-10 opposes protection against diabetes upon treatment of IAA-positive mice with agg Ig-INSβ**

Recently, it has been shown that IAA can be used as a marker for insulitis (26) and prediction of type 1 diabetes in young NOD mice (27). Similarly, among 58 female NOD mice that seroconverted to IAA production at the age of 8–11 wk, 84% had become diabetic by 30 wk of age, suggesting that our assay for detection of autoantibody is reliable and supports the notion that IAA can predict both diabetes (27) and most certainly insulitis (26). This offers a reference point to evaluate agg Ig-INSβ for reversal of diabetes at an early stage of the disease. Accordingly, NOD mice were given agg Ig-INSβ upon IAA seroconversion, as indicated, and monitored for blood glucose levels up to week 26 of age. Surprisingly, no significant delay of disease was observed, and the incidence of diabetes was similar in the mice treated with agg Ig-INSβ and Ig-HEL (Fig. 4A). The sol Ig-INSβ though showed some delay on week 20 relative to untreated or sol Ig-HEL-treated mice (Fig. 4B).

Moreover, when a continuous treatment regimen was applied, a significant delay of the disease was observed with the sol, but not the agg form of Ig-INSβ (Fig. 4, C and D). Indeed, only 20% of sol Ig-INSβ-treated mice developed diabetes by week 20, and such a delay remained significant as only an additional 10% of mice became diabetic by 26 wk of age (Fig. 4D). The delay is Ag specific, as Ig-HEL had no significant delay or protection against diabetes at any time point and Ig-HEL-treated animals had a similar pattern of disease as the untreated mice. The disease pattern observed in agg Ig-INSβ-treated groups was also comparable to those seen with untreated or Ig-HEL-treated mice (Fig. 4C). Histological analysis at week 26 indicated that the mice treated continuously with sol Ig-INSβ and remaining free of diabetes had islet infiltration, but to a lesser extent than mice given sol Ig-HEL (Fig. 4E). The lack of efficacy of agg Ig-INSβ against diabetes was not due to irreversibility of the disease, but most likely to endogenous IL-10 induced by agg Ig-INSβ. This statement is supported by the observation that IAA-positive IL-10−/− mice reverse their diabetes upon treatment with agg Ig-INSβ, while the untreated mice do not (Fig. 4F). Indeed, the incidence of diabetes in these mice was 30% at week 26 of age, while the untreated animals had 70% incidence like wild-type NOD mice treated with agg Ig-INSβ. Overall, these results indicate that agg Ig-INSβ is not effective against diabetes upon IAA seroconversion most likely due to an undefined regulatory function of IL-10.

**Agg Ig-INSβ stimulates rather than tolerizes islet-resident T cells**

IL-10 has been ineffective against diabetes when expressed locally in β cells (5). Similarly, mobilization of IL-10 by agg Ig-INSβ is also ineffective against the disease after IAA seroconversion, a stage in which diabeticogenic T cells would have migrated to the islets. One possible interpretation of these observations is that islet T cells are resistant to the modulatory function of IL-10. To test this premise, splenic (peripheral) and islet cells from diabetes-free 12-wk-old naive NOD mice were stimulated with agg Ig-INSβ and their IFN-γ responses were measured. Fig. 5 shows that agg Ig-INSβ reduced IFN-γ responses by the splenic cells, while the sol form of Ig-INSβ as well as free INSβ peptide did not (Fig. 5A). Addition of IL-10, however, reduced the response of the cells against free INSβ and sol Ig-INSβ to levels similar to those observed with agg Ig-INSβ. In contrast, agg Ig-INSβ stimulated significant IFN-γ responses by islet cells, while the sol form and free peptide did not (Fig. 5B). Interestingly, exogenous IL-10 boosts free INSβ and sol Ig-INSβ to support significant IFN-γ responses by the otherwise unresponsive islet cells. Neutralization of IL-10 with an anti-IL-10 Ab during stimulation with agg Ig-INSβ inhibits the IFN-γ responses by islet cells, while isotype-matched control Ab did not (Fig. 5C). Because islet infiltration includes CD8

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**FIGURE 4.** Treatment of diabetes in IAA-seropositive mice is much more effective when the regimen is devoid of IL-10. Mice (10 per group) that tested positive for IAA between the age of 8 and 11 wk were given an i.p. injection of 300 µg of agg (A) or sol (B) Ig-INSβ ( ), or Ig-HEL ( ) on the week of seroconversion, as well as 7 and 14 days later. Other groups of mice were given a weekly injection of agg (C) or sol (D) Ig-INSβ ( ) or Ig-HEL ( ) up to week 12. Subsequently, these mice received another 300 µg of Ig chimera every 2 wk until the age of 24 wk. This regimen is referred to as continuous treatment regimen. All mice were monitored for blood glucose from weeks 12 to 26 of age. An untreated group of mice ( ) was included in all experiments for comparison purposes. E. Shows an H&E staining of islet sections and the percentage of noninfiltrated islets in the IAA-positive mice treated continuously with sol Ig-INSβ or Ig-HEL. The histological analyses illustrated in E were performed on non diabetic mice at week 26 of age. F. Groups of IAA-positive IL-10−/− female NOD mice were subject to a continuous treatment regimen with agg Ig-INSβ (IL-10−/−/Agg Ig-INSβ), and their incidence of diabetes is compared with untreated IL-10−/− (IL-10−/−/Untreated) as well as wild-type NOD female mice treated with agg Ig-INSβ (WT/Agg Ig-INSβ), p < 0.05 compared with untreated mice; b, p < 0.05 compared with WT/agg Ig-INSβ-treated mice.
FIGURE 5. Islet INSβ-specific T cells develop IFN-γ responses upon stimulation with Ag in the presence of IL-10, while splenic T cells undergo down-regulation. Whole splenic (A) and islet (B) cells from 12-wk-old NOD female mice were stimulated with 18 μM INSβ, 1 μg/ml agg, or sol Ig-INSβ in the absence or presence of 1 ng of rIL-10, as indicated, and their IFN-γ responses were measured. C. The stimulation of islet cells was conducted in the presence of 40 μg of anti-IL-10 Ab or isotype control rat IgG. D. Purified islet CD4 and CD8 T cells were incubated with BSA-APCs and 1 μM agg or sol Ig-INSβ with or without 40 μg/ml anti-IL-10 Ab. In all experiments, the incubation lasted 48 h, and cytokine measurement was done by ELISA using 100 μl of culture supernatant. Each bar represents the mean ± SD of triplicates after deduction of background levels obtained from cultures without Ag stimulation. These background levels were 3- to 8-fold lower than sol Ig-INSβ for the spleen (A) or agg Ig-INSβ for the islets cells (B–D). *, p < 0.05 compared with sol Ig-INSβ; **, p < 0.01 compared with sol Ig-INSβ.

Among other T cells (28), the INSβ peptide contains a CD8 epitope (29, 30), and IL-10 has been shown to stimulate CD8 T cells (31), the IFN-γ responses obtained with islet cells could be due to cross-presentation of agg Ig-INSβ to CD8 T cells. Therefore, bulk islet cells were fractionated into CD4 and CD8 T cells, and stimulation with agg Ig-INSβ was reassayed. The results in Fig. 5D indicate that the postulate is incorrect, and CD4, but not the CD8 T cells were able to produce IFN-γ upon stimulation with agg Ig-INSβ. Furthermore, neutralization of IL-10 with an anti-IL-10 Ab inhibits IFN-γ responses by the CD4 T cells. These data indicate that islet and peripheral INSβ-specific CD4 T cells display differential susceptibility to IL-10.

Agg Ig-INSβ down-regulates CTLA-4 expression on islet T cells through endogenous IL-10

Upon migration to the islets, T cells would be exposed to Ag and undergo activation. Hypothetically, these cells would up-regulate CTLA-4 to deliver negative signals and control such activation (32, 33). IL-10 may down-regulate CTLA-4 to interfere with its inhibitory function and sustain activation of islet-resident T cells. Indeed, Fig. 6 shows that in the spleen of unmanipulated 12-wk-old mice, only 2.5% of CD4 T cells express surface CTLA-4 (Fig. 6A), while in the islets CTLA-4 expression was seen on 11% of the resident CD4 T cells (Fig. 6B). Interestingly, stimulation of the islet CD4 T cells with agg Ig-INSβ in the presence of anti-CTLA-4 Ab inhibited stimulation of IFN-γ production, while isotype control Ab did not (Fig. 6C). Moreover, anti-CTLA-4 Ab did not confer stimulatory function to sol Ig-INSβ, indicating that signaling through, rather than blockade of, CTLA-4 is the operative mechanism in this setting.

To test whether IL-10 interferes with expression of CTLA-4, islet CD4 T cells were purified by positive selection on anti-CD4 Ab-coated Miltenyi microbeads and stained with anti-CD4-FITC and PE-conjugated anti-CTLA-4 Ab or isotype control hamster IgG. The cells were gated on CD4 and analyzed for binding of anti-CTLA-4 or isotype control hamster IgG. The marker, M1, represents the cells positive for CTLA-4. C. Purified islet CD4 T cells were incubated with BSA-APCs and 1 μM agg or sol Ig-INSβ with or without 100 μg/ml anti-CTLA-4 Ab, then IFN-γ was measured by ELISA. The 4F10 Ab used here triggers rather than blocks the CTLA-4-inhibitory pathway. Each bar represents the mean ± SD of triplicates. D. The islet CD4 T cells were incubated with BSA-APCs and a 1 μM mixture of either agg or sol Ig-INSβ + Ig-GAD2 (1/1) in the presence or absence of 1 ng of rIL-10. The cells were then stained with anti-CD4-FITC and anti-CTLA-4-PE and analyzed as in A and B. For investigation of in vivo down-regulation of CTLA-4 by agg Ig-INSβ, IAA-positive NOD female mice were untreated (E), given a three-injection regimen (as in Fig. 4A) of agg Ig-INSβ alone (F), agg Ig-INSβ accompanied by anti-IL-10 Ab (500 μg/ injection), (G) or sol Ig-INSβ (H). Seven days later, the splenic CD4 T cells were purified and stained with anti-CD4 and anti-CTLA-4, as above.

FIGURE 6. IL-10 reverses up-regulation of CTLA-4 expression upon treatment with agg Ig-INSβ. A and B. Splenic and islet CD4 T cells were purified by positive selection on anti-CD4 Ab-coated Miltenyi microbeads and stained with anti-CD4-FITC and PE-conjugated anti-CTLA-4 Ab or isotype control hamster IgG. The cells were gated on CD4 and analyzed for binding of anti-CTLA-4 or isotype control hamster IgG. The marker, M1, represents the cells positive for CTLA-4. The islet CD4 T cells were incubated with BSA-APCs and 1 μM agg or sol Ig-INSβ with or without 100 μg/ml anti-CTLA-4 Ab, then IFN-γ was measured by ELISA. The 4F10 Ab used here triggers rather than blocks the CTLA-4-inhibitory pathway. Each bar represents the mean ± SD of triplicates. D. The islet CD4 T cells were incubated with BSA-APCs and a 1 μM mixture of either agg or sol Ig-INSβ + Ig-GAD2 (1/1) in the presence or absence of 1 ng of rIL-10. The cells were then stained with anti-CD4-FITC and anti-CTLA-4-PE and analyzed as in A and B. For investigation of in vivo down-regulation of CTLA-4 by agg Ig-INSβ, IAA-positive NOD female mice were untreated (E), given a three-injection regimen (as in Fig. 4A) of agg Ig-INSβ alone (F), agg Ig-INSβ accompanied by anti-IL-10 Ab (500 μg/ injection), (G) or sol Ig-INSβ (H). Seven days later, the splenic CD4 T cells were purified and stained with anti-CD4 and anti-CTLA-4, as above.
Ig-INSβ-treated animals (Fig. 6, E and F). In fact, when tested for IFN-γ production, these cells showed higher levels of cytokine than untreated animals (248 pg/ml ± 46 vs 128 pg/ml ± 27). Moreover, coadministration of anti-IL-10 Ab with agg Ig-INSβ restored CTLA-4 expression, and the number of islet cells with significant surface CTLA-4 was similar to that observed in mice recipient of sol Ig-INSβ, which does not induce IL-10 production by APCs (Fig. 6, G and H). These results indicate that IL-10 produced by the APCs and/or Treg cells down-regulates CTLA-4 expression on islet-resident T cells.

**Discussion**

IL-10, an anti-Th1 cytokine and growth factor for Treg cells, prompted high expectations for modulation of autoreactive T cells and suppression of autoimmunity (14, 15, 17, 34, 35). Success has been achieved in a number of autoimmunity models, but IL-10 has shown variable results in type 1 diabetes (5–9). In this study, an approach for controlled mobilization of IL-10 was developed and used both in young insulinitis-free and older IAA-positive mice to determine how the cytokine regulates diabetogenic CD4 T cells within and peripheral to the islets. It is shown that Ig-INSβ, an Ig expressing the diabetogenic INSβ peptide, can, upon aggregation, cross-link FcγRs and trigger the production of IL-10 by APCs (Fig. 1). In vitro, agg Ig-INSβ suppressed IFN-γ responses of INSβ-specific T cells, and such modulation was dependent upon IL-10 (Fig. 2). In vivo, young mice exposed to agg Ig-INSβ developed IL-10-producing Treg cells (Fig. 1), reduced their proliferative and IFN-γ responses (Fig. 2), and delayed their diabetes (Fig. 3). This protection against the disease was also IL-10-dependent as NOD mice deficient for the IL-10 gene were unable to delay their disease upon treatment with agg Ig-INSβ (Fig. 3). Moreover, depletion of IL-10-producing Treg cells abrogated agg Ig-INSβ-mediated protection against diabetes (Fig. 3). These observations suggest that endogenous IL-10, whether from APCs or Ag-expanded Treg cells, contributes significantly to the down-regulation of peripheral T cells in these young mice and sustains protection against the disease. IL-10 exercises anti-Th1 function through down-regulation of the expression of costimulatory molecules (31, 36). Our own investigation indicates that agg Ig-INSβ does not up-regulate B7 or CD40 on APCs (data not shown), which agrees with our previous reports showing that agg Ig-myelin chimeras made of the same Ig backbone as Ig-INSβ modulate T cells through lack of costimulation (11, 12). Thus, the mechanism we propose for protection against diabetes in the young mice suggests that IL-10 from the APCs and/or Treg cells most likely interferes with costimulation (see Fig. 7, left panel). This does not, however, exclude the possibility that Treg cells may be exercising additional suppressive function (37, 38) or that IL-10 may be directly affecting the diabetogenic T cells (39).

In contrast, this IL-10-driven protection against diabetes was not effective in older animals positive for IAA. Indeed, when agg Ig-INSβ was administered upon IAA seroconversion, protection was not achieved, despite the fact that the disease remains reversible and the sol form of Ig-INSβ delayed diabetes effectively (Fig. 4). Given the fact that in young animals most of the diabetogenic T cells remain peripheral to the islets, while in older mice a significant number of these cells would have become islet resident, we suspected that peripheral and islet-resident T cells display differential susceptibility to regulation by IL-10. This hypothesis proved correct, and splenic INSβ-specific T cells down-regulated IFN-γ production upon stimulation with agg Ig-INSβ, while islet T cells responded to such stimulation and produced significant amounts of IFN-γ (Fig. 5). However, sol Ig-INSβ, which does not induce IL-10 production by APCs, displayed opposite effects and stimulated IFN-γ responses by the splenic, but not islet T cells. IL-10 has previously been shown to stimulate CD8 T cells (31). Given the fact that INSβ encompasses a CTL epitope (29, 30), we thought that agg Ig-INSβ is cross-presented on MHC class I through the exogenous pathway and stimulates CD8 T cells that would be frequent in the islets during insulitis (40). Our prediction, however, proved incorrect, and upon separation of islet CD4 and CD8 T cells and stimulation with agg Ig-INSβ only the CD4 T cells responded and produced IFN-γ (Fig. 5D). Overall, these observations indicate that IL-10 is stimulatory for islet-resident diabetogenic CD4 T cells, but down-regulatory for the same cells when the encounter occurs peripheral to the islets.

Upon migration to the islets, T cells are presumably exposed to Ag and most likely undergo activation. CTLA-4 expression arises when the encounter occurs peripheral to the islets. Through down-regulation of the expression of costimulatory molecules (31, 36), CTLA-4-expressing T cells are then likely to become islet resident, providing a means to control excessive responses (32, 33). Thus, it is possible that upon IAA seroconversion, the islet-resident T cells up-regulate CTLA-4 expression. Upon treatment with agg Ig-INSβ, it may be that IL-10 interferes with CTLA-4-inhibitory function and stimulates T cell responses rather than tolerance. This postulate proved correct, and islet, but

![FIGURE 7. Proposed model for IL-10 regulation of peripheral and islet-resident diabetogenic T cells.](http://www.jimmunol.org/)

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not splenic T cells from the same animal displayed up-regulated expression of surface CTLA-4 (Fig. 6, A and B). Interestingly, addition of anti-CTLA-4 Ab during incubation with agg Ig-INSβ restored the inhibitory function of CTLA-4 and the T cells were not able to produce IFN-γ (Fig. 6D). Moreover, stimulation of islet T cells with agg Ig chimeras reduced the surface expression of CTLA-4, while stimulation with sol chimeras did not, unless supplemented with exogenous IL-10 (Fig. 6D). In vivo, treatment of IAA-positive mice with agg Ig-INSβ down-regulated CTLA-4 expression on islet T cells (Fig. 6, E and F). However, neutralization of IL-10 during administration of agg Ig-INSβ restored CTLA-4 expression (Fig. 6G). Thus, IL-10 sustains stimulation of previously activated islet-resident T cells by down-regulation of CTLA-4 expression and interference with its inhibitory function. In fact, administration of anti-CTLA-4 upon IAA seroconversion completely abrogated the onset of diabetes (data not shown). Therefore, interruption of CTLA-4-inhibitory function by IL-10 promotes activation rather than tolerance. The median panel of Fig. 7 proposes that IL-10 down-regulates both costimulatory molecules and CTLA-4, resulting in loss of inhibitory control of diabetogenic T cells. This will ultimately sustain stimulation, as previously activated lymphocytes do not require costimulation (41, 42). The fact that sol Ig-INSβ, not inducing IL-10, was able to delay disease at this stage bodes well with the findings. The right panel of Fig. 7 proposes that sol Ig-INSβ does not sustain activation of the cells because the APC at this inflammatory site express costimulatory molecules that should engage CTLA-4, which is not down-regulated by the sol Ig-INSβ (Fig. 6H). The end result then is inhibition of T cell activation and delay of diabetes.

Overall, agg Ig-INSβ tolerizes T cells in the periphery and limits input into the islets, thus effectively suppressing the disease when given at a young age before insulin. Upon IAA seroconversion, agg Ig-INSβ will exercise down-regulation of peripheral T cells, limiting the seeding of islets by naive T cells, but will compensate for the shortage by stimulating and sustaining vigorous activation of islet-resident cells that have migrated before the treatment or have escaped peripheral tolerance. Sol Ig-INSβ is less effective in tolerizing peripheral T cells due to the lack of IL-10. However, upon IAA seroconversion, sol Ig-INSβ will compensate for the moderate tolerance in the periphery by not sustaining activation of islet-resident T cells. This mechanism will require continual treatment and show reduced infiltration. This model agrees with the report showing that anti-CTLA-4 Ab delays passive diabetes induced by transfer of activated pathogenic T cells (43). The findings are also in good standing with observations indicating that local expression of IL-10 exacerbates the disease (5, 7) and delivery of IL-10 at an older age is not effective against diabetes (9). Thus, the model reconciles the variable functions associated with IL-10 (6).

The notion that encounter of the T cells with IL-10 before migration to the islets has a different outcome from encounters that happen within the islets is also supported by studies demonstrating that delivery of IL-10 at a young age (before insulitis) delays diabetes, while it is ineffective against disease in older animals with progressive insulitis (9).

Another point we emphasize is that this interplay between IL-10 and CTLA-4 may contribute to the development of spontaneous diabetes. Treg cells develop in the normal T cell repertoire and are presumed to sustain peripheral tolerance (37, 38). An initial exposure of β cell-associated self Ags would activate diabetogenic T cells, but could also expand Treg cells to control pathogenicity (44). However, if those Treg cells produce IL-10, an interplay with CTLA-4 would be put into motion and their function would be rather counterproductive, resulting in sustained T cell activation and exacerbation of diabetes. This possibility, however, remains to be investigated. Recently, we found that decline of membrane-bound TGFβ can also nullify the suppressive function of Tregs, leading to development of diabetes (45).

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