Transfer of Regulatory Properties from Tolerogenic to Proinflammatory Dendritic Cells via Induced Autoreactive Regulatory T Cells

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Transfer of Regulatory Properties from Tolerogenic to Proinflammatory Dendritic Cells via Induced Autoreactive Regulatory T Cells

Fleur S. Kleijwegt,* Sandra Laban,* Gaby Duinkerken,* Antoinette M. Joosten,* Bobby P.C. Koeleman,† Tatjana Nikolic,* and Bart O. Roep*

Infectious tolerance is a term generally assigned to the process through which regulatory T cells (Tregs) transfer immunoregulatory properties to other T cells. In this study, we demonstrated that a similar process applies to human dendritic cells (DCs), albeit through a different mechanism. We induced and cloned proinsulin-specific Tregs using tolerogenic DCs and investigated mechanisms by which induced Ag-specific regulatory T cells (iaTregs) endorse the suppressive effects. iaTregs expressed FOXP3, programmed death-1, and membrane-bound TGF-β and upregulated IL-10 and CTLA-4 after stimulation with the cognate Ag. The iaTregs suppressed effector T cells only when both encountered the cognate Ags on the same APCs (linked suppression). This occurred independently of IL-10, TGF-β, programmed death-1, or CTLA-4. Instead, iaTregs used a granzyme B-mediated mechanism to kill B cells and monocytes, whereas proinflammatory DCs that resisted being killed were induced to upregulate the inhibitory receptors B7 (family) homolog 3 and ICOS ligand. These re-educated mature monocyte-derived dendritic cells (mDCs) suppressed effector T cells and induced IL-10–producing cells from the naive T cell pool. Our data indicated that human tolerogenic DCs confer infectious tolerance by inducing Ag-specific Tregs, which, in turn, re-educate proinflammatory mature DCs into DCs with regulatory properties. The Journal of Immunology, 2011, 187: 000–000.

R egulatory T cells (Tregs) are important for maintaining peripheral tolerance in immune homeostasis (1, 2). A reduction in Treg numbers or functionality leads to the loss of self-tolerance and an increase in autoimmunity (3, 4). Type 1 diabetes is an autoimmune disease caused by the destruction of insulin-producing β cells by autoreactive T cells lacking effective immune regulation (5). Multiple studies investigating natural Tregs (nTregs) in type 1 diabetes showed normal frequencies but decreased function (6–8). Furthermore, the resistance of effector T cells to regulation in type 1 diabetes adds to an imbalance between nTregs and effector T cells (9). Current efforts to treat type 1 diabetes evaluate the administration of polyclonal nTregs expanded in vitro, which raises safety concerns related to the purity and stability of the Treg phenotype after injection, intransient differences, and a possibility of uncontrolled in vivo expansion (10). Treatment with Ag-specific Tregs seems to be more effective at preventing diabetes than does treatment with nonspecific Tregs (11). Ag-specific Tregs can be induced and stimulated with tolerogenic dendritic cells (tDCs) (10), rendering DC-based immunomodulatory therapy an attractive alternative for induction of tissue-specific immune regulation in type 1 diabetes.

Dendritic cells (DCs) are the natural directors of the immune response that are instructed by environmental triggers to polarize naive T cells into effectors or Tregs (12). Previously, we demonstrated that modulation of human DCs with vitamin D3 in vitro generates tDCs, which induce Ag-specific Tregs (13, 14). Programmed death ligand (PDL)-1 and membrane-bound TNF play important roles in the mechanisms by which DCs induce Ag-specific Tregs (13, 15). In this article, we report cloning of induced Ag-specific Tregs (iaTregs), as well as detailed analyses of their phenotype and mechanism of action. iaTregs bear a strong resemblance to naturally occurring Ag-specific Tregs (6). Yet, rather than killing mature monocyte-derived DCs (mDCs) presenting the cognate peptide, iaTregs changed the phenotype and functionality of mDCs, re-educating them to upregulate inhibitory receptors, lose the ability to stimulate autoreactive effector T cells, and induce IL-10–producing cells from the naive T cell pool. Our data indicated that tDCs use infectious tolerance to change proinflammatory mDCs through the induction of Ag-specific Tregs.

Materials and Methods

**DC culture and Treg induction**

Human tDCs were generated, as previously described (13). In short, CD14+ monocytes were isolated from buffy coats of healthy donors and cultured with GM-CSF (800 U/ml), IL-4 (500 U/ml) (both from Invitrogen, Breda, The Netherlands) and vitamin D3 (10−8/ml) (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) for 6 d to obtain immature DCs. Mature tDCs were obtained by incubating 0.5 × 106 DCs with 0.2 × 106 CD40L-
expressing L cells for 48 h. For induction of iaTregs, mature tDCs were loaded with proinsulin peptide (C19-A3 specific for DRB1*0401; 10 μM) for 4 h at 37°C.

An induced Ag-specific iaTreg line was generated by culturing naïve CD4+ T cells (Dynal, Invitrogen) isolated from PBMCs with autologous mature tDCs loaded with proinsulin peptide, and the Treg line was obtained following the same Treg protocol, as described previously (15).

**Cloning of induced Ag-specific Tregs**

The iaTreg line was cloned by limiting dilution cloning (on average one cell per three wells in 96-well plates). Clones were expanded by consecutive expansion rounds with proinsulin peptide–loaded HLA-DRB1*0401+ PBMCs and feeder mix (PBMCs pooled from six donors) and nonspecific expansion with 2% PHA and feeder mix. Clones were expanded in X-Vivo medium (Biowhittaker, Verviers, Belgium) with 10% human serum. On day 3, 40 ng/ml IL-15 (Peprotech, London, UK.) was added. After several expansion rounds, clones were harvested, and proinsulin peptide–specific suppression was confirmed.

**mAbs and flow cytometry**

mAbs used in this study were purchased from eBiosciences (San Diego, CA) FITC–conjugated anti–Ig-like transcript (ILT)-4 (clone 27D6), IgM (clone eBMG2b), perforin (clone dG9), CTLA-4 (clone 14D3), programmed death (PD)-1 (clone J116), IgG2b (clone eB2a), IgG2a (clone eB2a), IgG1 (clone P3); PE–conjugated anti–granzyme B (clone GB11), PD-1 (clone MIIH4), CD95 (Fas) (clone DX2), PDL-1 (clone MIIH4), glucocorticoid-induced TNFR family related gene (GITR; clone eBioAITR), and Foxp3 (clone eB98); allophycocyanin–conjugated anti–rat IgG2a (clone eB2a), FoxP3 (clone PCH101); biotinylated anti–CD209 (eB-h209), ICOS ligand (ICOSL; clone MIIH 12), B2 (family) homolog (B7-H3; MIIH35) followed by streptavidin–allophycocyanin. Ab against CD103–allophycocyanin (clone LF 61) was purchased from Invitrogen. FITC–labeled anti–CXCR3 (clone 1c6), CD25 (clone 2A3, and IgG1 (clone X40); PE–labeled anti–CD134 (clone ACT-35), CD28 (clone CD282), CD152 (clone BNE3), CD80 (clone L37.4), CD86 (clone IT2.2), HLA-DR (clone G46-6), IFN-γ (clone 4S.B3), and IgG1 (clone X40); and allophycocyanin–labeled anti–IL-10 (clone JES5-19F1) and IgG2a (clone R3959) were all obtained from BD Pharmingen (San Diego, CA). ILT-3/–PE–CY7 (clone ZM3.8) and IgG PE–CY7 (clone SFC12T4D11) were from Beckman Coulter (Marseille, France). Purified PE–anti–TFG-β (clone TB21) were from IQ Products (Groningen, The Netherlands). Anti–IL-10R was from R&D Systems (Abingdon, UK.).

For flow cytometry analysis, aliquots of 2 × 10^5 cells were incubated with a mixture of mAbs, incubated on ice for 30 min, and washed with PBS supplemented with 0.5% BSA. For intracellular cytokine analysis, cells were incubated with GolgiStop (BD, San Diego, CA) and Cytofix/Cytoperm fiximation kit, according to the manufacturer’s protocol. Flow cytometric staining was analyzed on a FACScalibur (Becton Dickinson). Analyses were performed using FlowJo 7.6 (Tree Star, Ashland, OR).

**Suppression assays with Th1 clone**

The Th1 clone (PM1#11) was derived from a prediabetic donor (16). This HLA-DR3–restricted clone is specific for a glutamic acid decarboxylase (GAD)65 epitope (339–352). For suppression assays, we made use of the different HLA restrictions for the clones: GAD clone is DR3 restricted, and Treg clones are DR4 restricted. Mature monocyte–derived DCs (mDCs) were generated following the monocyte–derived DC protocol and matured with 100 ng/ml LPS (Sigma Aldrich Chemie) for 24 h. To measure linked suppression, mDCs were loaded with proinsulin peptide (5 μg/ml) and/or GAD peptide (1 μg/ml) for 2–4 h. These peptide concentrations were determined by titration to avoid over- or understimulation of each T cell clone. Loading of mDCs with similar concentrations of peptide also gives suppression of Th1 clone (data not shown). To measure bystander suppression, DR3* /noDR4* mDCs were loaded with GAD peptide, and DR4* /noDR3* mDCs were loaded with proinsulin peptide for 2–4 h.

For maximal proliferation, Th1 clone was cultured with Treg clone in the presence of GAD–pulsated DR3* DCs and unpulsed DR4* DCs in a 10:10:1:1 ratio. For suppression, Th1 clone was cultured with Treg clone, GAD–pulsated DR3* DCs, and proinsulin–loaded DR4* DCs in a 10:1:1 ratio. Tregs were added in different amounts to the Th1 clone (1:2, 1:1, or 2:1). In the blocking experiments, suppression assays of DR3*/DR4* DCs with Th1 clone and Treg clone in a 1:1:20 ratio were used. Neutralizing Abs for anti–TFG-β (TB21; IQ Products), anti–IL-10R (clone 90220; R&D, UK), anti–CTLA-4 (14D3; eBiosciences), and anti–PD-1 (J116; eBiosciences) were used in concentrations of 10 μg/ml.

**Analysis of mDC modulation by iaTregs**

mDCs were preincubated with Tregs, with or without their cognate proinsulin epitope, in a 1:10 or 1:20 ratio (DC/iaTreg) for 24 h, and iaTregs were removed using anti–human TCR αβ (clone WT3; BD Biosciences, San Diego, CA) and Dynal Beads negative–isolation protocol (Dynal; Invitrogen). Separation yielded 95–98% pure mDCs. Isolated mDCs were subsequently cocultured with CFSE–pulsed effector Th1 clone labeled with 1 μM CFSE for 3 d, and proliferation was measured by CFSE dilution. To analyze surface receptor changes on altered mDCs, Tregs were not removed, and mDCs were gated as CD209+ or CD11c+ cells.

**Cytotoxicity assay**

HLA–matched PBMCs were split into two groups; one half was labeled with 0.1 μM CFSE (CFSElow) and incubated with proinsulin (5 μg/ml) peptide for 4 h at 37°C. The other half was labeled with 1.0 μM CFSE (CFSEhigh), without peptide as a source of target APCs. After labeling, PBMCs were washed twice, and CFSElow and CFSEhigh were mixed in a 1:1 ratio. Next, 1 × 10^5 mixed PBMCs were incubated with 1 × 10^5 iaTregs for the cytotoxicity assay. Experiments were performed in triplicate. After 24 h, cells were harvested and labeled with anti–CD19–PE or anti–CD19–PE and the percentage of CD19+ or CD19+ cells within each group of CFSE was measured by FACS. Killing was calculated as the ratio of CD19+ or CD19+ CFSE+/CD19+ or CD19+ CFSE−. The ratio obtained from cultures without Tregs was set to 0% killing. Perforin–based cytotoxic activity was inhibited by 50 nM concanamycin A (CMA; Sigma Aldrich Chemie), and granzyme B was inhibited by EGTa (4 mM; Sigma Aldrich Chemie) in the cultures. CMA and EGTa were added to the cocultures at the start of the experiment and analyzed after 24 h of culture.

**Induction of IL-10–secreting cells by iaTreg–altered mDCs**

mDCs, with or without proinsulin, were cocultured with Tregs for 24 h at a ratio of 1:10. Thereafter, naïve CD4+ T cells were added to the wells at a ratio of 10:1:10 (T cell/DC/Treg). These naïve CD4+ T cells were cultured with or without DR3+ /DR4+ mDCs, with or without DR4+ mDCs but HLA identical to Tregs, avoiding a reaction of naïve T cells to Tregs. After 4 d, cells were harvested and stained for intercellular production of IL-10 or IFN-γ. Baseline was set at the isotype control value. For blocking of ICOSL, functional purified anti–ICOSL (clone MIIIH2, eBiosciences; blocking Ab) or purified control mouse IgG1 (eBiosciences) was added, together with naïve CD4+ T cells, to the coculture of the Tregs and the mDCs, at a concentration of 10 μg/ml.

**Statistical analysis**

To determine differences between the groups, data were compared by a two–tailed Student t test (paired or unpaired, as indicated in the figure legend) using the SPSS 18 software package. Results were obtained from at least three independent experiments and presented as the average ± SEM. The divisions (d) of responder cells were calculated by a proliferation index as shown in the formula below (n = number of divisions; events are the number of cells per CFSE peak):

\[
P(d) = \frac{\sum \text{Events}(d)}{\sum \text{Events}(d)/2^n}
\]

A proliferation index of 1.0 (no division) forms the 100% inhibition value. Proliferation index of Th1 cells without iaTregs is set as the 0% inhibition.

**Results**

Cloning and phenotype of proinsulin–specific Tregs induced by tDCs

Vitamin D3–modulated tDCs induce Ag–specific Tregs (iaTregs) (13). To assess the mechanism of action of iaTregs, we generated an iaTreg line specific for a common diabetogenic epitope of proinsulin (C19-A3) (17) by two rounds of Ag–specific stimulation using tDCs pulsed with the epitope. Proinsulin–specific iaTregs were cloned and expanded by alternating rounds of specific stimulation with proinsulin peptide–loaded HLA–DR4 PBMCs and nonspecific stimulation with PHA. Next, we analyzed phenotypical and functional characteristics of suppressive clone iaTreg clone #78.
The nonactivated iaTregs expressed low FOXP3 and intermediate CD25 and were CD103 and CD28 positive and CD127 and CCR7 negative (Fig. 1A). Other markers associated with Tregs that were expressed included PD-1, GITR, CD95 (Fas), OX40, and membrane-bound TGF-β. There was partial expression of CD69, and iaTregs had high HLA-DR and CXCR3 expression. Upon Ag-specific activation, iaTregs expressed CTLA-4 on the surface, as well as intracellular granzyme B and IL-10 (Fig. 1B, 1C). iaTregs did not express ICOS, ICOSL, perforin, CCR7, or CD73 at rest or after activation (Fig. 1B).

### iaTregs suppress diabetogenic Th1 clone through linked suppression

To test whether iaTregs suppress effector T cells, a potent diabetogenic Th1 clone (PM1#11) specific for the islet auto-Ag GAD65 (peptide 339–352) was used (16). Using different HLA restriction

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**FIGURE 1.** Phenotype of Treg-clone FS1-78. A, Surface expression of general and Treg-specific markers by proinsulin-specific Tregs at rest. Filled graphs represent expression of the depicted markers, and empty graphs represent isotype control. B, Phenotype of Tregs at rest (light gray graphs) and after activation (dark gray graphs). Dotted line represents isotope control. Tregs were cultured with proinsulin-loaded mDCs for 24 h at a 10:1 ratio, and surface CTLA-4, ICOS, and ICOSL and intracellular granzyme B and perforin were measured by FACS Calibur and analyzed using FlowJo software. C, Cytokine profile of Tregs after activation with mDCs loaded with proinsulin peptide. Intracellular IFN-γ and IL-10 expression were measured by FACS Calibur and analyzed using FlowJo software. Quadrants of IL-10 and IFN-γ were set based on isotype control.
of the Th1 and iaTreg clones (Th1 peptide is restricted by HLA-DR3, and Treg peptide is restricted by HLA-DR4), we investigated whether iaTregs suppress through linked or bystander suppression. Linked suppression applies when Tregs suppress effector T cells only when both effector and suppressor T cells recognize their epitopes on the same APC (suppression mediated through the APC; Fig. 2A, left panel, Fig. 2B), whereas bystander suppression is acting when Tregs suppress effector T cells, even when these recognize their Ags on distinct APCs (Fig. 2A, right panel). iaTregs suppressed the Th1 clone in a dose-dependent fashion through linked suppression. Furthermore, iaTregs suppressed Th1 cells only when mDCs were pulsed with their cognate peptide (Supplemental Fig. 1). When iaTregs and Th1 cell Ags were presented on distinct mDCs, proliferation of the effector clone was not affected.

Because iaTregs express multiple inhibitory receptors and produce immunomodulatory cytokines, we analyzed which of these were engaged in the suppressive capacity. IL-10R and TGF-β were blocked in the iaTreg/Th1 coculture using anti–IL-10R and anti–TGF-β Abs. Blocking these receptors did not abrogate suppression of Th1 cell proliferation (Fig. 3A). Similar experiments in which PD-1 and CTLA-4 were blocked by anti–PD-1 and anti–CTLA-4 Abs, respectively, were performed, and these receptors did not affect the suppression of Th1 cell proliferation (Fig. 3B).

**FIGURE 2.** iaTregs function via linked suppression. A, Schematic representation of linked (left panel) and bystander suppression (right panel). For linked suppression, mDCs (HLA-DR3+/DR4+) were loaded with Treg peptide (proinsulin C19-A3) and Th1 peptide (GAD65 339-352) and cultured with CFSE-labeled Th1 clone and unlabeled Treg clone in different ratios. iaTregs suppressed Th1 cell proliferation in a dose-dependent manner. For the bystander suppression, mDCs (DR3, not DR4) were loaded with Th1 peptide, and mDCs (DR4, not DR3) were loaded with Treg peptide and cultured with CFSE-labeled Th1 clone and unlabeled Treg clone in different ratios. Graphs show dilution of CFSE representing Th1 cell proliferation. iaTregs suppressed Th1 cell proliferation only through linked suppression and not through bystander suppression. Experiment is representative of three independent experiments. B, Proliferation index of the Th1 clone without iaTregs (maximum division) was set as the 0% inhibition. Proliferation index is calculated by the formula given in Materials and Methods. Cumulative data for linked suppression of three independent experiments (mean ± SEM). 2x Treg: **p = 0.0008; 1x Treg: *p = 0.0034; 0.5x Treg: ***p = 0.0001 (paired t test).

**FIGURE 3.** Tregs do not suppress Th1 cells via IL-10, TGF-β, PD-1, or CTLA-4. A, Suppression is measured by inhibition of proliferation of a Th1 clone labeled by CFSE. mDCs (HLA-DR3/DR4) loaded with Treg peptide and Th1 peptide were cultured with clonal Th1 and Tregs at a 1:10:20 ratio, respectively. The black-filled graph (Th1) shows maximal proliferation of the Th1 clone cultured with mDCs. Addition of iaTregs (gray-filled graph) suppresses proliferation of Th1 cells. When IL-10 and TGF-β are blocked by anti–TGF-β and anti–IL-10R Abs, suppression of proliferation is not abrogated. All experiments were performed in triplicate. Graphs show representative of three independent experiments. B, Similar experiments as in A were performed in which PD-1 and CTLA-4 are blocked by anti–PD-1 and anti–CTLA-4 Abs. Neither receptor blockades abrogated the suppressive function of the Tregs. All experiments were performed in triplicate, and results displayed are representative of three independent experiments.
TGF-β Abs alone or in combination (Fig. 3). Blockade of IL-10 and TGF-β did not change the suppressive activity of iaTregs. Likewise, inhibition of CTLA-4 and PD-1 did not affect the suppressive capacity of the iaTregs (Fig. 3), indicating that IL-10, TGF-β, CTLA-4, and PD-1 appear redundant for the suppression of Th1 cells.

**Mechanism of linked suppression depends on the type of the APC presenting the Ag to iaTregs**

Naturally occurring Tregs are able to lyse APCs presenting their cognate Ag (6). Using the same assay, we assessed whether iaTregs kill B cells and monocytes loaded with proinsulin peptide. After a 24-h coculture, on average 28.4% of the B cells and 28.7% of the monocytes were killed by iaTregs (Fig. 4A). In contrast, monocyte-derived mDCs were resistant to lysis by iaTregs. Inhibition of perforin by CMA reduced killing of B cells (p = 0.308) and did not change the capacity of iaTregs to lyse monocytes (p = 0.352). The destruction of B cells and monocytes was mainly mediated by granzyme B, because the inhibitor of granzyme B (EGTA) entirely prevented the killing of APCs (p < 0.01).

Because mDCs were resistant to killing by iaTregs, we analyzed whether iaTregs modified the ability of mDCs to stimulate Th1 cells. mDCs were pulsed with Th1 peptide alone or Th1 peptide and Treg peptide, cultured with iaTregs for 24 h, and subsequently separated from iaTregs (Fig. 4B, left panels). The separation procedure does not influence the mDC phenotype or T cell-stimulation capacity (data not shown). Next, CFSE-labeled Th1 cells were added to the mDCs, and proliferation of Th1 cells was analyzed. mDCs that had been preincubated with activated iaTregs were incapable of stimulating proliferation of the Th1 cells (Fig. 4B). Importantly, iaTregs diminished the capacity of mDCs to stimulate Th1 cells only when mDCs were pulsed with the iaTreg epitope during preincubation, implying that Ag-specific recognition of iaTregs leads to changes in the function of mature, proinflammatory mDCs, conferring inhibitory properties on mDCs that act even if iaTregs are removed.

**iaTregs upregulate inhibitory receptors ICOSL and B7-H3 on mDCs**

To study the mechanism underlying the diminished stimulatory capacity of iaTreg-altered mDCs (@DCs), we compared the expression of costimulatory, Ag-presenting and inhibitory molecules on the surface of mDCs after coculture with iaTregs for 24 h with or without cognate peptide. mDCs presenting proinsulin peptide upregulated ICOSL significantly and induced expression of B7-H3 after coculture with iaTregs (Fig. 5). However, the expression of costimulatory receptors CD80 and CD86 or HLA-DR did not differ from that of mDCs incubated with iaTregs in the absence of the Treg epitope. Upregulation of PDL-1 in the mDCs loaded with proinsulin was not reproducible when different mDC donors were analyzed. The expression of inhibitory receptors ILT-3 and ILT-4 and PDL-2 was similar between mDCs with or without proinsulin peptide.

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**FIGURE 4.** iaTregs kill monocytes and B cells and change stimulatory capacity of mDCs. A. Killing assay of B cells, monocytes, and mDCs. For B cells and monocytes, CFSE<sup>hi</sup> and CFSE<sup>lo</sup>-labeled target APCs were mixed and cultured with iaTregs at a 1:1 ratio. Killing is depicted as the difference in the percentages between the two CFSE peaks within the live B cell or monocyte gate (CD19<sup>+</sup> or CD14<sup>+</sup>). APCs without iaTregs is set to 0% killing. Treg peptide-loaded or -unloaded mDCs were cultured with iaTregs. For mDCs (CD209<sup>+</sup>), percentages of live cells are compared between the Treg-peptide loaded mDCs and the unloaded mDCs. Black bars represent killing of B cells, monocytes, and mDCs when incubated with iaTregs. Light gray bars and dark gray bars show killing by iaTregs in the presence of a perforin-inhibitor or granzyme B inhibitor, respectively. Data represent mean ± SEM from two independent experiments analyzed in triplicate. *p = 0.0014, **p = 0.0006, ***p = 0.0007, ****p = 0.0038 and iaTregs versus iaTregs with perforin inhibitor for B cells (p = 0.308) or monocytes (p = 0.352); paired t test. B. Reduced proliferation of Th1 cells when stimulated with iaTreg-modulated mDCs. First, mDCs loaded with Th1+Treg peptides or Th1 peptide alone were cultured with iaTregs for 24 h (1:10 ratio). Next, iaTregs were removed, and mDCs were cultured with CFSE-labeled Th1 cells. Representative proliferation of Th1 cells (measured by CFSE dilution) cultured with mDCs loaded with Th1 peptide (gray graph) and mDCs cultured with both Treg peptide and Th1 peptide (black graph). Independent experiments were performed in triplicates (n = 2).
@DCs induce naive T cells to produce IL-10, changing the IL-10/IFN-γ ratio

Additionally, we investigated the functional properties of @DCs. mDCs pulsed with or without proinsulin were cultured with iaTregs. After 24 h, naive CFSE-labeled CD4+ T cell were added to the coculture. Naive T cells were HLA mismatched with mDCs but haplo-identical to iaTregs to avoid interaction of naive T cells with iaTregs. After 4 d, intercellular IL-10 and IFN-γ production by CFSE+ cells was analyzed using flow cytometry. The @DCs induced an increased amount of IL-10-producing cells (Fig. 6A). Furthermore, the ratio of IL-10/IFN-γ-producing cells induced by @DCs was inverted compared with the ratio induced by unchanged mDCs (Fig. 6B). Because the expression of ICOSL on mDCs was shown to convey tolerogenic capacities by inducing IL-10-producing cells (18), we investigated whether induction of IL-10-producing cells is due to upregulation of ICOSL. mDCs were pulsed with or without proinsulin and cocultured with iaTregs for 24 h, after which naive CD4+ T cells were added to the culture with anti-ICOSL Ab or isotype control. Blockage of ICOSL prevented induction of the high IL-10/IFN-γ-producing cell ratio in the presence of @DCs (Fig. 6C).

Although our experiments point to a cell contact-dependent mechanism transforming naive T cells into IL-10–producing cells by @DCs, this would not rule out a direct effect of iaTregs remaining in the cocultures of @DC and naive T cells or additional regulatory effects of soluble factors, such as IL-35. To investigate any direct effects of iaTregs or soluble factors produced by these cells on the induction of IL-10–producing T cells by @DCs, we performed smaller-scale experiments in which iaTregs were removed from mDCs or @DCs after 24 h of coculture, and mDCs or @DCs were subsequently incubated with naive CFSE-labeled CD4+ T cells (Supplemental Fig. 2). For comparison, we included a group exposed to the same T cell-removal procedure and culture medium exchange, but without removing the anti-TCR Ab (sham depletion of iaTregs), to check the effect of the separation procedure with magnetic beads on the cultures alone, as well as cytokines produced by iaTregs. Again, cultures with @DCs inverted the fraction of IL-10–producing cells over IFN-γ–producing cells compared with mDCs. These results implied that neither suppressive factors secreted by iaTregs nor the presence of iaTregs themselves are necessary for @DCs to induce IL-10–producing cells from the naive T cell pool.

**Discussion**

We report that vitamin D3-modulated tDCs induce potent Ag-specific Tregs (iaTregs) that suppress diabetogenic Th1 cells via linked suppression. We further demonstrated that iaTregs re-educate proinflammatory mDCs in an Ag-specific manner by upreg-
interaction with the cognate peptide on the surface of mDCs. This ration (28). Instead of killing mDCs, iaTregs altered mDCs after regulation of antiapoptotic proteins, such as Bcl-xL, upon maturation (27). Indeed, Ag-specific Tregs endorse linked suppression, suggesting redundancy of IL-10 and TGF-β. The activity of iaTregs also was not mediated by suppressive molecules PD-1 or CTLA-4. Ag-specific Tregs were shown to exert their function via cytolysis of the APCs or the effector cells, metabolic disruption, or by modulating the immunogenic function of DCs (27). Intermediate expression of CD25 and low expression of FOXP3 distinguishes iaTregs from nTregs, yet both cell types share OX40, GITR, CD95 (Fas), and PD-1 expression (20, 21). iaTregs expressed CD103, which is common to most types of Tregs (21, 22). Intermediate expression of CD25 and low expression of FOXP3 distinguishes iaTregs from nTregs, yet both cell types share OX40, GITR, CD95 (Fas), and PD-1 expression (20, 23). Expression of activation markers HLA-DR and CD69 and a lack of CD127 are similar to both nTregs and Tr1 cells (19, 20). Like nTregs and Tr1 cells, iaTregs express the inhibitory receptor CTLA-4 on the surface. iaTregs are most comparable to the recently described natural occurring ISIS-Tregs secreting IL-10 in response to their epitope, with similar phenotype and function (high IL-10 secretion, granzyme B secretion, and Ag specificity) (6), because unlike Tr1, IL-10 was not apparently involved in their suppressive action. It is unknown whether ISIS-Tregs share the capacity to modulate proinflammatory DCs with iaTregs.

In terms of the mechanism by which iaTregs suppress Th1 cells, blocking IL-10 and TGF-β signaling in the coculture did not affect suppression, suggesting redundancy of IL-10 and TGF-β. The activity of iaTregs also was not mediated by suppressive molecules PD-1 or CTLA-4. Ag-specific Tregs were shown to exert their function via cytolysis of the APCs or the effector cells, metabolic disruption, or by modulating the immunogenic function of DCs (27). Indeed, Ag-specific Tregs endorse linked suppression by killing the APCs (6). Although iaTregs could destroy B cells and monocytes, proinflammatory mDCs were not lysed. To our knowledge, this is the first evidence that mDCs are resistant to killing by Tregs. This resistance may result from upregulation of antiapoptotic proteins, such as Bcl-xL, upon maturation (28). Instead of killing mDCs, iaTregs altered mDCs after interaction with the cognate peptide on the surface of mDCs. This specific alteration of Ag-bearing mDCs persisted upon removal of iaTregs.

We revealed that auto-Ag–specific Tregs induce upregulation of B7-H3 and ICOSL on mDCs. Both molecules are involved in immune regulation: B7-H3 preferentially dampens Th1-mediated responses (29), whereas ICOSL promotes IL-10 secretion by T cells (30) and induces Tr1 cells that produce IL-10 and inhibit experimental airway inflammation in mice (18, 31). In our human model, the induction of IL-10–producing T cells by @DCs was also mediated through ICOSL. Modulation of mDCs into @DCs by iaTregs did not reduce the expression of HLA-DR or co-stimulatory molecules CD80 and CD86, nor did iaTregs increase inhibitory molecules PDL-1 and ILT-3, and ILT-4 on mDCs, in contrast to DCs modulated by regulatory alloreceptor CD4 and CD8 T cells (32, 33). This implied that different types of Tregs educate DCs in a distinct fashion, leading to different mechanisms used by tDCs to exert immunoregulatory function. The phenotype of @DCs also differs from the tDCs used to generate iaTregs: tDCs have low CD80 and CD86, high ILT-3 and PDL-1, low ICOSL, and no

![Image](http://www.jimmunol.org/)

**FIGURE 6.** @DCs increase induction of IL-10–producing CD4+ T cells and change the IL-10/IFN-γ ratio, which is mediated by ICOSL. mDCs, with or without proinsulin peptide, were cultured with iaTregs for 24 h. Thereafter, naive CD4+ T cells were isolated, labeled with CFSE, and added to the iaTreg and mDC coculture. After 4 d, cells were stained for intracellular IL-10 and IFN-γ. A, Percentage of IL-10–producing (left panel) and IFN-γ–producing (right panel) CFSE+ T cells stimulated with mDCs (black symbols) or @DCs (light gray symbols) are shown. The symbols represent T cells from different donors. B, Ratio of IL-10/IFN-γ–producing CFSE+ T cells per donor. Experiments were performed in duplicate or triplicate. Mean values per donor are shown (n = 4), p = 0.045, two-tailed t test. C, Following a 24-h incubation of mDCs with iaTregs, CFSE+–labeled naive CD4+ T cells were added to the culture together with anti-ICOSL Ab or isotype control. Figure depicts the ratio of IL-10/IFN-γ–producing CFSE+ T cells after 4 d of coculture in the presence of isotype control (black bars) or anti-ICOSL Abs (striped bars). Two independent experiments were performed in triplicate.

![Image](http://www.jimmunol.org/)

**FIGURE 7.** Transfer of regulatory properties from tolerogenic to proinflammatory DCs via autoreactive Tregs. Monocytes can be modulated by 1,25(OH)2vitamin D3 to differentiate into tDCs, which, in turn, induce iaTregs. The latter process is mediated by PDL-1 and membrane-bound TNF (mTNF). iaTregs, in turn, functionally modify proinflammatory mDCs to become anti-inflammatory DCs (@DCs) by upregulating inhibitory receptors ICOSL and B7-H3. @DCs lose the capacity to stimulate Th1 responses and instead induce IL-10–producing T cells from the naive T cell pool.
B7-H3 expression (13). The difference may be caused by the modulation method: tDCs result from modulation of monocytes with vitamin D3 during differentiation, whereas iaTregs alter fully differentiated mature DCs.

Infectious tolerance is a process in which a tolerance-inducing state is transferred from one cell population to another (34) and is supported by different mechanisms, including IL-35, TGF-β production, or essential amino acid-depleting enzymes (35–37). In this study, we demonstrated a transfer of tolerogenic properties from tDCs to mature proinflammatory DC via iaTregs, which operate in an Ag-dependent manner (modeled in Fig. 7). In the microenvironment of pancreas-draining lymph nodes, DCs may present different peptides of the same tissue simultaneously. Modulation of these DCs into @DCs by iaTregs recognizing proinsulin epitope would inactivate all other autoreactive T cells and induce IL-10–producing cells of different tissue-derived specificities. When applied as intervention therapy in type 1 diabetes, this implies that Tregs generated by tDCs and specific for a single, given auto-Ag could alter DCs that subsequently reduce autoimmunity responses to a diverse range of β cell Ags. Because both tDCs and iaTregs express CXCXR3, these cells have the potential to migrate to distressed β cells in the pancreas (38) and pancreatic tissue, including lymph nodes (39). We propose that iaTregs are potentially powerful regulators of the autoimmune response that can overcome the issue of impaired nTregs and the presence of strong effector T cells as existing in type 1 diabetes patients. The potential of tDCs to selectively induce Ag-specific infectious tolerance by re-educating proinflammatory DCs harboring the same Ag, as well as other auto-Ags, renders these an attractive candidate for immunotherapeutic and tissue-specific intervention cell therapy in patients with autoimmune diseases.

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References


