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$CD4^+CD25^+$ T Regulatory Cells Dependent on ICOS Promote Regulation of Effector Cells in the Prediabetic Lesion

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Abstract

$CD4^+CD25^+$ T regulatory cells (Tregs) prevent autoimmune disease, yet little is known about precisely where they exert their influence naturally in a spontaneous autoimmune disorder. Here, we report that Tregs and T effector cells (Teffs) coexist within the pancreatic lesion before type 1 diabetes onset. We find that BDC2.5 T cell receptor transgenic animals contain a small subset of FoxP3 positive $CD4^+CD25^+CD69^-$ cells in the pancreas, actively turning over, expressing the clonotypic receptor, and containing functional regulatory activity. Gene expression profiling confirms that the $CD4^+CD25^+CD69^-$ cells in pancreatic tissue express transcripts diagnostic of regulatory cells, but with significantly higher levels of interleukin 10 and inducible costimulator (ICOS) than their lymph node counterparts. Blockade of ICOS rapidly converts early insulitis to diabetes, which disrupts the balance of Teffs and Tregs and promotes a very broad shift in the expression of the T regulatory–specific profile. Thus, $CD4^+CD25^+69^-$ Tregs operate directly in the autoimmune lesion and are dependent on ICOS to keep it in a nondestructive state.

Key words: autoimmunity • diabetes • costimulation • tolerance • microarray

Introduction

Type 1 diabetes is an autoimmune disease that develops when tolerance mechanisms fail to control immune responses to pancreatic islet β cell proteins (1). Studies of autoimmune diabetes are facilitated by mouse models, such as NOD mice that develop diabetes spontaneously and TCR transgenic mice that express autoimmune TCR specificities present within the natural NOD repertoire (1–8). Among these, BDC2.5 TCR transgenic (BDC2.5) mice express a TCR derived from a diabetogenic $CD4^+$ T cell clone, restricted by the MHC class II molecule A^{g7} (4, 9). Studies of diabetes pathogenesis in both BDC2.5 and NOD mice demonstrate that diabetes unfolds in stages (10). In BDC2.5 NOD mice, cells invade at 15–18 d of age and set up a massive infiltrate inside the islet (insulitis; references 4, 11). Yet, progression to diabetes occurs in only 10–20% of animals at ~20 wk of age. In most animals, the lesion settles into a state of "respectful" insulitis, with the inflammatory infiltrate clearly demarcated from functional β cells. CTLA-4 blockade in BDC2.5/NOD or crossing to the $B6^{H2\text{-}g7}$ background results in a far more aggressive lesion with β cell destruction and rapid diabetes onset (11–13). What cellular mechanisms explain nondestructive insulitis in BDC2.5/NOD? Either T cells entering the islets in young mice are phenotypically incompetent to cause disease, or they are actively prevented from doing so by a CTLA-4–dependent immunoregulatory mechanism.

Immune regulation by $CD4^+CD25^+$ T regulatory cells (Tregs) limits autoimmunity in several mouse models (14–16). The FoxP3 transcription factor appears to play a unique role as a master regulator of their phenotypic and functional properties (17–19). In vivo, $CD4^+CD25^+$ Tregs depend on mediators such as IL-10, TGF-β, and/or CTLA-4, but their exact mechanism of action has not been fully elucidated (20–25). Several studies of Tregs in transgenic models have demonstrated that the cells expand in vitro and in vivo to antigen presented by mature dendritic cells (26, 27), or in draining LNs of sites expressing cognate antigen

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Abbreviations used in this paper: FPR, false positive rate; ICOS, inducible costimulator; ILN, inguinal LN; MLN, mesenteric LN; PLN, pancreatic LN; Teff, T effector cell; Treg, T regulatory cell.

(28–30). Transfer studies showed that $CD4^+CD25^+$ Tregs home to the lamina propria and proliferate there during regulation of induced colitis, although proliferation in this system could not be separated from the lymphopenic state of the host (30). Still at issue is the localization, proliferative capacity, and function of Tregs in a spontaneous autoimmune disorder. Do the Tregs prevent the initial activation of effector cells in the lymphoid organs, or do they act secondarily on effector cells in the target tissues?

Multiple lines of evidence suggest that $CD4^+CD25^+$ Tregs are important in preventing responses in diabetes settings. Depletion of $CD25^+$ cells in a transfer setting or genetic absence of CD28 (leading to low numbers of Tregs in NOD mice) provoked exacerbation of disease (31, 32). Administration of IL-10, vitamin D, or TNF-α to NOD mice promoted the generation of Tregs in conjunction with amelioration of diabetes (33–35). Similarly, $CD4^+CD25^+$ Tregs may be reduced in the peripheral blood of people with recent onset diabetes (36). Restoration of this cell type may explain exciting recent advances in human diabetes treatment. The generation of TGF-β–producing Tregs by administration of anti-CD3 appears responsible for the efficacy of this drug in early clinical trials (37, 38). Thus, understanding the mechanism and point of action of this important cell type may yield advances in human type 1 diabetes therapy.

Previous studies of regulatory cells in BDC2.5 mice described clonotype^{lo} suppressor cells that came up in older animals (39) or $DX5^+$ populations effective in preinsulitic mice (13). Here, we find that a defined $CD4^+CD25^+$ T cell population with active regulatory properties can account for respectful insulitis in the prediabetes state in the BDC2.5 mouse model of type 1 diabetes. Tregs coexist in balance with aggressive effector cells directly in the autoimmune infiltrate, and express high levels of the immunoregulatory cytokine IL-10 in the pancreas compared with the draining LN. Loss of this balance through blockade of the costimulatory receptor inducible costimulator (ICOS) leads to the rapid onset of diabetes.

Materials and Methods

Flow Cytometry. Single cell suspensions were prepared from whole pancreas or indicated LNs using glass slide disruption. Debris was allowed to settle and removed (repeated two to three times for pancreas). Staining with live cell dye was critical, especially with pancreas. We used either 7-AAD (BD Biosciences) or Hoescht 33342 (CN Corp.). Cells were stained using standard methodology with a combination of the following: B220-PE-Texas red (RA3-6B2; Caltag), CD4-PE or PE-Cy7 (GK1.5), ICOS-PE (7E.17G9), CD69-FITC (H1.2F3), CD25-allophycocyanin (7D4 or PC61), or isotype controls rat IgG1 (R3-34) and rat IgG2b (A95-1; BD Biosciences). Cells were analyzed on a MoFlo® flow cytometer using Summit® software (DakoCytomation). Sorted cells were collected into media with 5% FBS and assessed for purity (93–98% in transfer studies). For RNA preparation, cells were sorted twice to >98% purity.

Mice and Transfer Experiments. BDC2.5/NOD mice were maintained in the Joslin Diabetes Center barrier facility (IACUC protocol 99-19, 99-20). Pancreata or total pooled LNs were isolated from 3–4-wk-old BDC2.5 mice. Live $B220^-CD4^+$ cells were sorted according to CD25/CD69 phenotypes and injected i.p. into 4–12-wk-old NOD.scid recipients (The Jackson Laboratory). Diabetogenic BDC2.5 splenocytes were obtained from 6–10-wk-old mice. Mice were monitored for diabetes as described previously (4).

Real-Time PCR Experiments. Total RNA was isolated from sorted cells by standard TRIzol method from 10^4 cells of each group, and cDNA was prepared using Superscript II® reverse transcriptase (Invitrogen). cDNA from 1,250 cell equivalents was used for real-time PCR for FoxP3 or IL-10 mRNA, using HPRT as a standard. $2^{-\Delta\Delta CT}$ calculations were used to present amounts of expression relative to the whole LN control.

Gene Expression Profiling. Tregs ($CD4^+CD25^+CD69^-$) and T effector cells (Teffs; combined $CD4^+CD25^-CD69^-$ and $CD4^+CD25^{lo}CD69^+$) were sorted from pancreas preparations to >98% purity as aforementioned. For ICOS blockade comparisons, mice were treated with two doses of anti-ICOS mAb or control rat IgG as indicated. Cells were lysed in TRIzol, and total RNA was prepared according to the manufacturer's instructions (Invitrogen). A method developed in-house was used to consistently amplify RNA from small cell populations (Goldrath, A., personal communication). Amplification is primarily linear; additionally, any distortion of data is eliminated when samples amplified the same number of times are directly compared. This procedure permitted analysis of transcripts from as few as 6,000 cells. We performed three to five independent experiments for each cell type. RNA was amplified for two rounds using the MessageAmp® aRNA kit according to manufacturer's instructions (Ambion). The last biotinylation step was performed using the Enzo Life Sciences BioArray HighYield RNA Transcript Labeling Kit® (T7). 5–15 μg of twice-amplified biotinylated aRNA was submitted to the Joslin Genomics Core for hybridization to murine genome U74Av2 array GeneChips® as per the manufacturer's instructions (Affymetrix, Inc.). The raw data were processed with the RMA algorithm for probe-level normalization (40), (S^+ Array Analyzer; Insightful; modified by R. Park [Joslin Diabetes Center, Boston, MA], D. Mathis, and C. Benoist), and composite expression values were calculated for each of the genes on the chip (averaging after outlier elimination; Distill software). Gene-wise p-values were calculated with Welch's modified Student's *t* test and converted to LOD scores as $-\log_{10}$.

Antibody Treatments. ICOS-specific 7E.17G9.G1 (41) and CTLA-4–specific UC10.4F10.11 (American Type Culture Collection) mAbs were purified from hybridoma culture supernatant by standard methods, either in-house or by Bio-Express or Harlan, and tested for endotoxin levels (<1.4 EU/mg protein). Each lot was titered, and doses were set based on Ab equivalents by flow cytometric activity (unpublished data). 7E.17G9.G1 mAb was injected i.p. in two doses of 50–100 μg at 9 and 12 d, or mice were injected with an equal amount of rat IgG2b isotype control (A95-1; BD Biosciences), rat IgG (Sigma-Aldrich), or PBS; or 400 μg of UC10.4F10.11 anti–CTLA-4 mAb as indicated. For anti-ICOS treatment of older mice, two doses of 200–400 μg 7E.17G9.G1 mAb were injected i.p. in PBS at 21 and 24 d, or starting at 26 or 35 d as indicated. In Fig. 6 A (right), an equal volume of post-in-house mAb elution supernatant was used as a control for endotoxin contamination (contains small amounts of residual 17G9).

Online Supplemental Material. Specific genes identified by GeneChip® array data from pancreatic Tregs and Teffs are presented in Table SI. Datasets are available in the public Gene Ex-

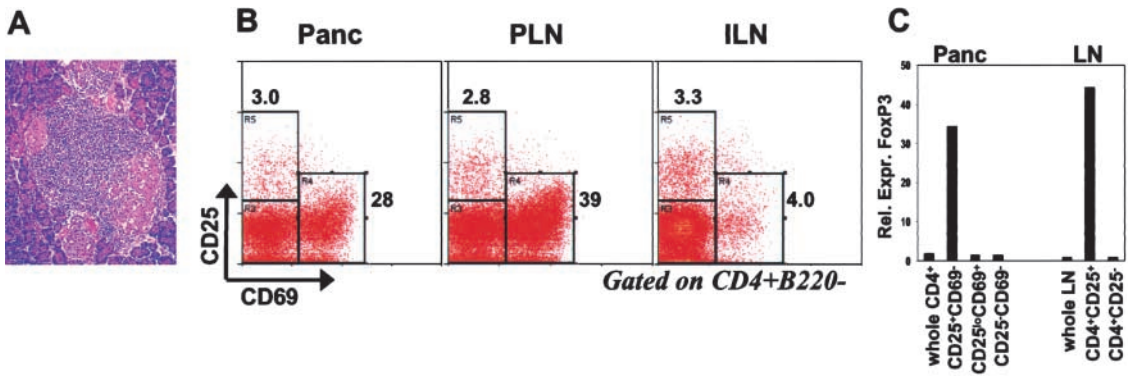

Figure 1. Infiltrate in the pancreatic lesion contains subsets of $CD4^+$ cells with activated or regulatory phenotypes. (A) Islets of BDC2.5 mice at 4 wk of age. (B) Cells from pancreatic infiltrate of 3–4-wk-old BDC2.5 mice were isolated as aforementioned and analyzed for CD4, B220, live cell dye, CD25, and CD69 markers. Plots are gated on $CD4^+B220^-$ live lymphocytes, and expression of activation markers CD25 and CD69 is shown. Data represent 10 separate experiments. (C) Pancreatic cells gated for $CD4^+$ expression, live lymphocytes, and $B220^-$ cells were sorted (whole $CD4^+$) or sorted separately into the distinct CD25/CD69 subsets observed in B. Whole BDC2.5 LNs, or LN sorted into $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets were also prepared. cDNA was prepared from each indicated group and assessed for expression of FoxP3 cDNA by real-time PCR analysis. Data are presented as relative expression of FoxP3 compared with the value in whole LNs (set to 1). Averaged results of three experiments are shown.

pression Omnibus database (accession no. GSE1419). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20040179/DC1.

Results

The Pancreatic Lesion Includes $CD4^+$ T Cell Subpopulations with Both Effector and Regulatory Phenotypes. Initially, we asked whether cells in the prediabetes pancreatic lesion of BDC2.5 mice (Fig. 1 A) are simply incompetent to cause disease, or whether there is an active regulatory process occurring in the islets. Preparation of islets followed by isolation of lymphocytes proved detrimental to identifying all T cells because heavily infiltrated islets fell apart during isolation and were not included in the final yield. Therefore, infiltrating T cells were prepared by mechanical disruption of whole pancreata of young 3–4-wk-old BDC2.5/NOD mice after careful removal of any LNs. The cells obtained (routinely 3–5 $\times$ 10^6 per BDC2.5/NOD pancreas) were a true component of the infiltrate in that lymphocytes could not be isolated from pancreata of strains with no islet infiltration (unpublished data). We divided the $CD4^+$ population into three subsets on the basis of the early activation marker CD69 and the activation/regulatory marker CD25: $CD4^+CD25^+CD69^-$, $CD4^+CD25^{lo}CD69^+$, and $CD4^+CD25^-CD69^-$ (Fig. 1 B). Although $CD4^+$ cells in the irrelevant inguinal LN (ILN) were mainly naive, those from the pancreatic LN (PLN) or from the pancreatic lesion were highly activated, with 15–40% of $CD4^+$ cells displaying a $CD25^{lo}CD69^+$ profile consistent with recent activation. 3–11% of pancreatic infiltrating cells had a $CD4^+CD25^+CD69^-$ phenotype, consistent with Tregs.

This phenotype suggested that the $CD4^+CD25^+CD69^-$ cells might contain the regulatory fraction, whereas those $CD69^+$ cells that were also $CD25^+$ might reflect expression of the CD25 marker as a sign of recent activation. To examine this issue, we sorted each cell type independently from the pancreatic lesion, and assessed the expression of the regulatory cell transcription factor FoxP3. Real-time PCR analyses of the three populations indicated that FoxP3 expression was highly enriched (10–35-fold in different experiments) in the $CD4^+CD25^+CD69^-$ population relative to the $CD4^+CD25^{lo}CD69^+$ or $CD4^+CD25^-CD69^-$ populations (Fig. 1 C), as in $CD4^+CD25^+$ cells isolated from LNs. Even though the $CD25^{lo}CD69^+$ cells express some CD25, this did not reflect increased FoxP3 expression. CD69 appears to be a useful marker to separate recently activated cells (which also express CD25) away from $CD25^+$ cells expressing the regulatory transcriptional controller FoxP3. A majority of the $CD25^+CD69^-$ cells (90%) expressed the clonotype receptor, as

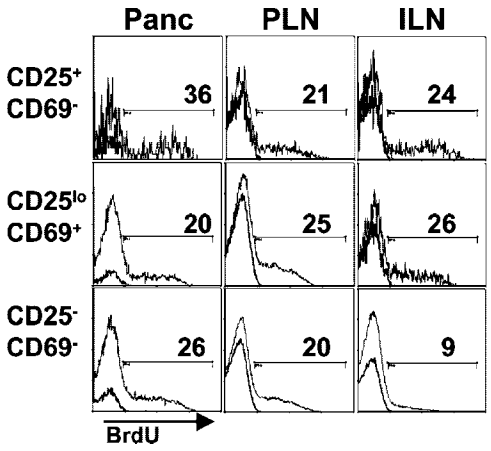

Figure 2. $CD4^+CD25^+CD69^-$, $CD25^{lo}CD69^+$, and $CD25^-CD69^-$ subsets are proliferating in the pancreas and LNs of unmanipulated BDC2.5 mice. 3–4-wk-old BDC2.5 mice were fed BrdU in their drinking water for 5 d. Pancreas (Panc), pancreatic LNs (PLN), or inguinal LN (ILN) were isolated and analyzed by five-color flow cytometry for CD4, B220, CD25, CD69, and BrdU expression. Cells were gated for lymphocytes, $CD4^+B220^-$ cells, and indicated CD25/CD69 subsets (as in Fig. 1 B). Isotype control (thick line) versus BrdU (thin line) staining is shown. Data are representative of three experiments.

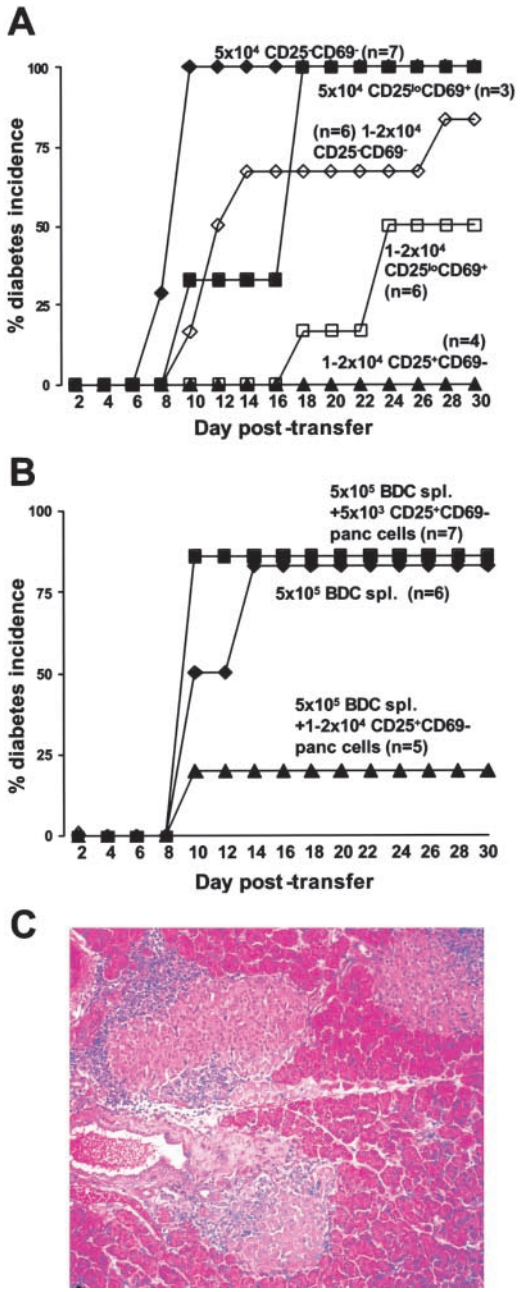

Figure 3. $CD4^+CD25^+CD69^-$ cells isolated directly from the pancreatic lesion protect from diabetes, whereas $CD4^+CD25^{lo}CD69^+$ and $CD25^-CD69^-$ cells are pathogenic. (A) Cells from the pancreatic lesion of 3–4-wk-old BDC2.5 mice were sorted into $CD4^+CD25^+CD69^-$ (▲$CD25^+CD69^-$), $CD4^+CD25^{lo}CD69^+$ (■$CD25^{lo}CD69^+$), and $CD4^+CD25^-CD69^-$(◆$CD25^-CD69^-$) subsets by flow cytometry. Sorted cells were transferred i.p. separately into NOD.scid recipients at two doses as follows: 50,000 cells (closed symbols) or 10–20,000 cells (open symbols). Mice were monitored every other day after 7 d for the onset of hyperglycemia by urine, and diabetes was confirmed by blood glucose levels >250 mg/dl. Data represent pooled results of three separate experiments with similar results. (B) $CD4^+CD25^+CD69^-$ cells were sorted as in A. Either 10–20,000 (▲), 5,000 (■), or no (◆) $CD4^+CD25^+CD69^-$ cells were transferred i.p. into NOD.scid recipient animals in combination with 5×10^5 diabetogenic BDC2.5 splenocytes. Mice were monitored as described in A. Data represent pooled results of three separate experiments with similar results. (C) Histological sections were prepared from pancreata of transferred NOD.scid animals. Hematoxylin and eosin–stained sections from recipients that did not develop diabetes (protected by Treg) by the end of 30 d after receiving the 10–20,000 cell dose of $CD4^+CD25^+CD69^-$ cells and 5×10^5 diabetogenic BDC2.5 splenocytes are shown.

did the other phenotypes (unpublished data). These data suggest that a balance of putative Tregs and Teffs expressing the BDC2.5 receptor resides together directly in the target organ.

Cells with Both Effector and Regulatory Phenotypes Are Actively Proliferating in the Pancreatic Lesion An interesting issue was whether the T cells were actively proliferating in the pancreatic lesion because previous analyses of Treg proliferation have shown expansion in the draining node or in the intestine of lymphopenic hosts (30), but not the target tissue of autoimmunity in mice with a full lymphoid compartment. Flow cytometric analysis of mice fed with BrdU for 5 d showed that all $CD4^+$ cell phenotypes in the pancreatic lesion proliferated (Fig. 2). If anything, $CD25^+CD69^-$ cells were the most active. This was also the case in the draining PLN, but not in the irrelevant ILN. Thus, the insulitic lesion is not inert, and cells with both regulatory and effector phenotypes are turning over rapidly in the presence of a full lymphoid compartment.

Functional Pathogenic and Regulatory Cells Coexist in the Pancreatic Lesion. These data are suggestive of an active balance of regulatory and pathogenic $CD4^+$ T cells in the pancreatic lesion. To evaluate function, we sorted $CD25^+CD69^-$, $CD25^{lo}CD69^+$, and $CD25^-CD69^-$ cells from BDC2.5/NOD pancreata were transferred into NOD.scid recipients. Mice that received $CD25^-CD69^-$ cells or $CD25^{lo}CD69^+$ cells developed diabetes shortly after transfer (Fig. 3 A). As few as 10–20,000 cells of these phenotypes were enough to provoke diabetes in 50–80% of recipients within 1 mo after transfer. All mice that received $CD25^-CD69^-$ cells or $CD25^{lo}CD69^+$ cells, at the doses tested, developed insulitis (unpublished data). In contrast, $CD25^+CD69^-$ cells did not cause diabetes or develop insulitis after transfer. We asked whether the $CD25^+CD69^-$ cells residing in the pancreas did, in fact, have protective capacity by transferring 10–20,000 $CD25^+CD69^-$ cells isolated from the lesion with 5×10^5 diabetogenic BDC2.5/NOD splenocytes into NOD.scid recipients. This small dose of cells, 10–100-fold lower than the dose used in other models of regulation using polyclonal populations (20, 42), provided significant protection from diabetes in this cotransfer context (Fig. 3 B). BDC2.5 LN Tregs ($CD4^+CD25^+CD69^-$) were also effective at this dose (unpublished data). In vivo regulation was dose dependent because protection from diabetes was lost if only 5,000 Tregs were cotransferred (Fig. 3 B). In protected mice that had received 20,000 $CD25^+CD69^-$ Tregs and 5×10^5 diabetogenic BDC2.5/NOD splenocytes, we observed formidable, but respectful, insulitis in the islet after 30 d (Fig. 3 C), similar to that seen in islets of unmanipulated BDC2.5/NOD mice (compare with Fig. 1 A). Interestingly, Tregs taken directly from the pancreatic lesion were unable to set

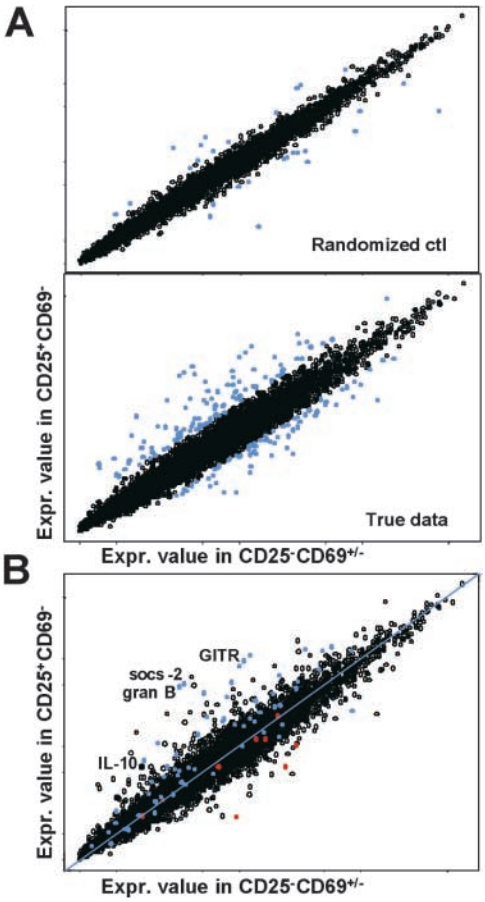

Figure 4. $CD4^+CD25^+CD69^-$ cells have a gene expression profile of Tregs, whereas $CD25^{lo}CD69^+$ and $CD25^-CD69^-$ cells are distinct. (A) Cells were sorted to high purity directly from the pancreas lesion of 3–4-wk-old BDC2.5 mice for the $CD4^+CD25^+CD69^-$ population, or the $CD25^{lo}CD69^+$ and $CD25^-CD69^-$ combined populations. RNA was prepared and hybridized to Affymetrix U74Av2 array GeneChips® as described in Materials and Methods. RMA analysis was used to compare relative expression of data from the $CD4^+CD25^+CD69^-$ population to the $CD25^{lo}CD69^+$ and $CD25^-CD69^-$ combined populations. The cut-off for significant differences was determined using a random dataset to identify a 10% FPR for the real data, in this case a 2.1-fold increase (or decrease) in gene expression (highlighted in blue). For each cell type, three to five separate experiments were performed. (B) The gene list of 77 from McHugh et al. (reference 45) is highlighted on our relative gene expression graph in blue (up in LN $CD25^+$) and red (up in LN $CD25^-$).

up a presence there in the absence of other T cells in the NOD.SCID host, suggesting that they may require signals from Teffs to access the pancreas or persist there. These data demonstrate that Teff and Treg subsets coexist in the pancreatic lesion, and that T effectors capable of disease transfer are being actively suppressed by $CD25^+CD69^-$ Tregs during the nondestructive period of insulitis.

The Gene Expression Profile of Pancreatic Tissue Tregs Subsets Is Distinct. To better understand the function and phenotype of effector and regulatory cells residing in the target organ during autoimmunity, we compared the gene expression profiles of $CD25^+CD69^-$ Tregs to the combined Teff populations of $CD25^{lo}CD69^+$ and $CD25^-CD69^-$ cells. We sorted cells from the lesion, isolated RNA, prepared double amplified biotinylated aRNA, and hybridized each sample to an Affymetrix murine genome U74Av2 array GeneChip® for at least three replicates of each population. A false positive rate (FPR) was estimated from a randomized dataset generated by shuffling expression values between samples (Fig. 4 A, randomized control). The $CD25^+CD69^-$ Tregs and Teff cell populations from the prediabetic lesion could be distinguished by the expression of a limited set of genes; with a 10% FPR cutoff, 96 genes were overexpressed in $CD25^+CD69^-$ cells, and 73 were underrepresented, significantly more than in the randomized dataset (Fig. 4 A). Several of the genes overexpressed in $CD25^+CD69^-$ cells such as GITR, CD103, Nrp-1, IL-10, and CTLA-4 are diagnostic of Tregs with suppressive activity (Table SI, available at http://www.jem.org/cgi/content/full/jem.20040179/DC1; references 14–16, 43–46). Overall, this group was dominated by cell surface or cytokine genes. Conversely, the genes underrepresented in Tregs seemed dominated by transcription or signaling factors (OBF-1, Tcf7, Eomesodermin, and Smad1). We compared our data to 77 genes shown previously to be overrepresented in LN $CD4^+CD25^+$ cells from normal mice or from in vitro–activated cultures (45); as illustrated in Fig. 4 B, many of these were also overrepresented in pancreatic $CD25^+CD69^-$ Tregs (21 out of 77 overlap with our list at 10% FPR; Table SI). However, many of the genes on our list have not been identified previously as particularly expressed by Tregs. They might represent a unique subset of genes either induced in a subset of Tregs to make them capable of accessing the tissues, or in Tregs once within the lesion.

Tregs in Pancreatic Tissue Express Higher Levels of the Regulatory Cytokine IL-10, GITR, and ICOS than Those Found in LNs. To compare more directly the differences between Tregs found in tissue and their LN counterparts, we examined the expression of several regulatory molecules by flow cytometry or real-time PCR. IL-10 is an important effector molecule for Tregs in several in vivo systems (for review see references 14–16) and has been shown to be critical for regulation in the BDC2.5 model (47). We observed that IL-10 was highly up-regulated in Treg in the pancreatic lesion compared with effector cells (Table SI). We compared mRNA levels of IL-10 in Tregs isolated from the pancreatic infiltrate or LN. Unlike FoxP3 levels, which were similar between LNs and pancreatic Tregs (Fig. 1), IL-10 levels were 15–36-fold higher in pancreatic Tregs compared with those isolated from the PLN or irrelevant mesenteric LN (MLN; Fig. 5 A). The GITR molecule was also consistently present at somewhat higher levels on pancreatic versus LN Tregs (Fig. 5 B), as well as increasing on activated cells in the pancreas. ICOS, a CD28 family member, is known to be an important costimulatory receptor for the production of IL-10 (48–51). Although ICOS is usually thought to act as a positive costimulator, genetic deficiency or Ab blockade of ICOS leads to exacerbation of disease in mouse models of multiple sclerosis (48, 51). ICOS was expressed on a small subpopulation (5–10%) of $CD25^+CD69^-$ Tregs in the LN, although interestingly ICOS was always lower or absent on PLN Tregs (Fig. 5

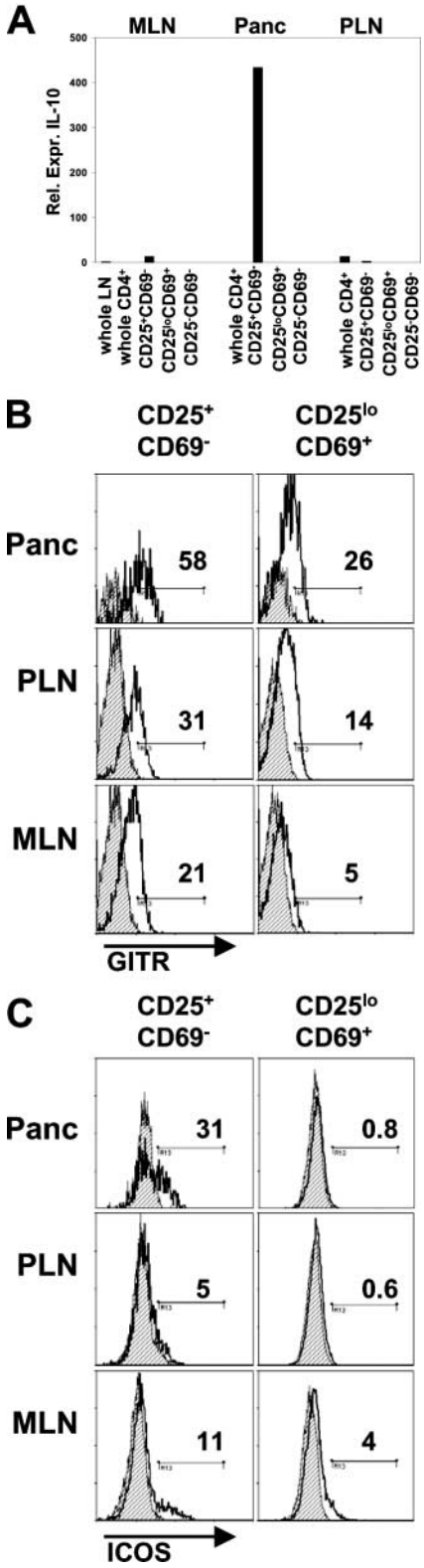

Figure 5. IL-10, GITR, and ICOS are specifically up-regulated in the pancreatic lesion compared with the draining lymph node. (A) Cells were sorted to high purity from the pancreas lesion, pancreatic LNs (PLN), or mesenteric LNs (MLN) of 3–4-wk-old BDC2.5 mice for the $CD4^+CD25^+CD69^-$, $CD25^{lo}CD69^+$, or $CD25^-CD69^-$ populations. mRNA was prepared from each indicated group and assessed for IL-10 expression by real-time PCR analysis. Data are presented as relative expression of IL-10 compared with the value in whole LNs (set to 1). The average of three experiments is shown. (B) GITR expression (unshaded histograms) was examined on $CD4^+B220^-$ live lymphocytes gated on the CD25/CD69 profiles indicated (Fig. 1 B) from the pancreas, PLN, or MLN and compared with isotype control (shaded histograms). The data shown are representative of three experiments. (C) ICOS expression (unshaded histograms) was examined on subsets as in B. The data shown are representative of five experiments.

C). Strikingly, 30–80% of $CD25^+CD69^-$ Tregs in the pancreas expressed ICOS. Recently activated cells ($CD25^{lo}CD69^+$) showed no comparable ICOS expression. In some experiments, ICOS was present on $CD25^{lo}CD69^+$ cells in the pancreas (2–16%), but was of lower intensity. $CD25^-CD69^-$ cells did not express ICOS (unpublished data). These data suggest that Tregs in the autoimmune lesion acquire functional and phenotypic properties distinct from their LN counterparts, in particular marked by high expression of ICOS. Higher levels of the regulatory cytokine IL-10, GITR, and ICOS in Tregs specifically from the pancreatic lesion suggest that Tregs function directly in the target organ to mediate regulation.

ICOS Blockade Disrupts the Balance of Tregs and T Effectors in the Lesion and Promotes Loss of Expression of Important Regulatory Molecules by Tregs. Abrogation of costimulatory signals in both the NOD and BDC2.5 models has been shown to lead to rapid onset of diabetes (32); in particular, blockade of CTLA-4, known to be important for Treg function, led to immediate onset of diabetes in young BDC2.5/NOD mice if administered when they first exhibited insulitis (11, 21, 22). To test if ICOS on Tregs in the pancreas was important to maintain the balance of regulation, we treated young BDC2.5/NOD mice with anti-ICOS mAb. These animals developed diabetes rapidly at the onset of insulitis, all becoming diabetic by 25 d of age (Fig. 6 A, left). In contrast with the CTLA-4 blockade, which is only effective when given before islet infiltration, ICOS blockade could promote diabetes for a period after insulitis onset as well (Fig. 6 A, right), although the effect also waned in older animals.

We analyzed the frequency of $CD4^+$ CD25/CD69 T cell subsets within the pancreas after ICOS blockade. ICOS blockade led to an increased frequency of $CD25^{lo}CD69^+$ Teff cells and a decreased frequency of $CD25^+CD69^-$ Tregs, resulting in a significant change in the ratio of the two subpopulations (Fig. 6 B). This change only occurred in the pancreatic infiltrate, but not in the PLN or irrelevant LN, reflective of ICOS expression specifically in the pancreatic infiltrate. Anti-ICOS treatment did not lead to deletion of Tregs because the absolute numbers of Tregs in the lesion did not change; rather, an increase in Teff was responsible for the skewing of the Teff/Treg ratio (unpublished data). The blockade of ICOS perturbed the Teff/Treg ratio, thereby disrupting the balance of regulation in the lesion.

Microarrays were used to analyze gene expression changes induced by ICOS blockade in the two populations. Teff or Treg $CD4^+$ subsets were sorted as aforemen-

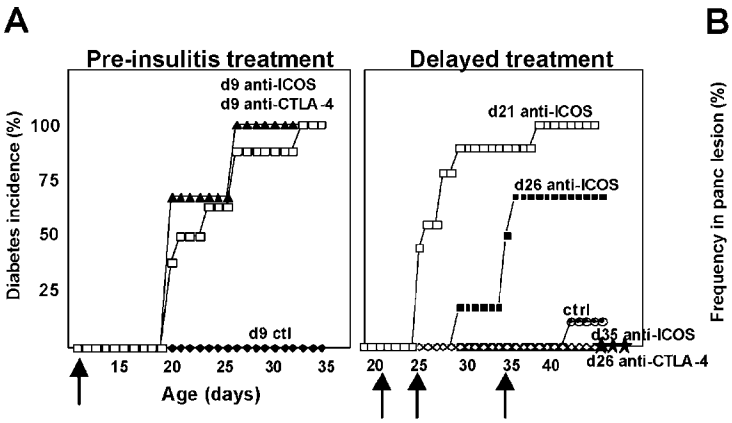

Figure 6. ICOS or CTLA-4 blockade in young BDC2.5 animals leads to rapid diabetes onset and correlates with a disruption of the T regulatory/T effector balance. (A) BDC2.5 mice were treated at 9 and 12 d of age with anti–CTLA-4 or ICOS mAb, indicated by arrow (left). Mice were treated with two doses of anti-ICOS mAb starting at 21, 26, or 35 d of age (arrows); with anti–CTLA-4 mAb starting at day 26; or with controls as described in Materials and Methods (right). Diabetes was monitored as aforementioned. (B) BDC2.5 mice were treated at 21 and 24 d with anti-ICOS mAb or control and killed at 26 d of age before diabetes onset. Pancreatic lesion, PLN, and ILN cells were prepared and stained with anti-CD4, B220, CD25, CD69, and live cell dye. Percentages are shown of pancreatic $CD4^+CD25^{lo}CD69^+$ cells ($CD25^{lo}CD69^+$) or $CD4^+CD25^+CD69^-$ cells ($CD25^+CD69^-$), gated on live lymphocytes and $B220^-$ cells ($n = 5$; $P < 0.008$, $CD25^{lo}CD69^+$ cells; $P < 0.008$, $CD25^+CD69^-$ cells by pairwise Student's *t* test). PLN and ILN are not shown because there were no significant differences.

tioned from 4-wk-old BDC2.5/NOD mice 5 d after treatment with anti-ICOS or control rat IgG (Fig. 7 A), and RNA probes were prepared for microarray analysis as aforementioned. Changes induced by ICOS blockade were more limited in the effector population than in Tregs; the comparative plot was much tighter for Teff than for Tregs (Fig. 7, B and C). In Teff, only 33 genes were induced by a factor of ≥1.5-fold, and only 1 was repressed. Interestingly,

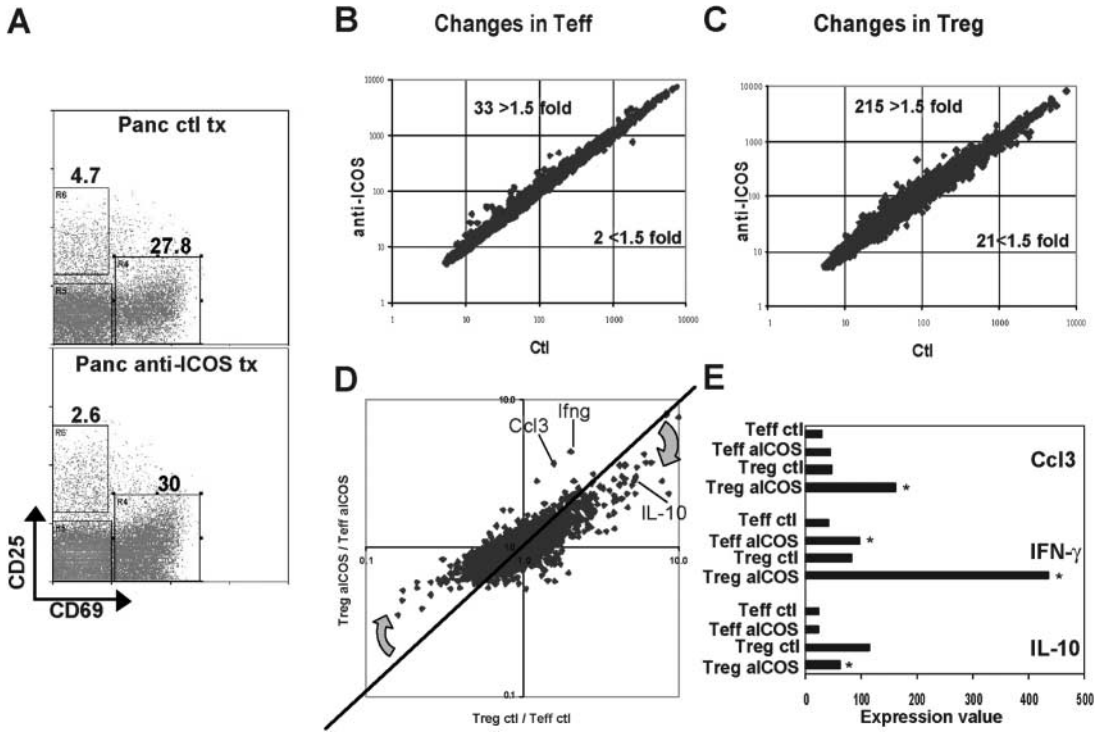

Figure 7. ICOS blockade dampens the immunoregulatory gene profile and correlates with expression of proinflammatory cytokines. (A) Cells from pancreatic infiltrate of 3–4-wk-old BDC2.5 mice were isolated after treatment with anti-ICOS mAb or rat IgG control as indicated, and analyzed for CD25/CD69 expression profiles on $CD4^+B220^-$ live lymphocytes. Data represent five separate experiments. Cells were sorted to high purity directly from the pancreatic lesion of 3–4-wk-old BDC2.5 mice 5 d after indicated treatments for the $CD4^+CD25^+CD69^-$ Treg population, or the $CD25^{lo}CD69^+$ and $CD25^-CD69^-$ combined Teff populations. RNA was prepared and hybridized to Affymetrix U74Av2 array GeneChips®, and data were analyzed as described in Materials and Methods and in Fig. 4. (B) Expression value comparison plot for Teff populations with anti-ICOS versus control treatment. (C) Similar plot for Treg populations. (D) A ratio plot comparing the ratio of Treg anti-ICOS–treated/Teff anti-ICOS–treated profiles to the same ratio in the Treg and Teff control-treated profiles. Diagonal line indicates where points would fall if nothing changed between the treatments. A selection of genes that are highly over- or underexpressed after anti-ICOS treatment are highlighted. (E) Changes in expression values for cytokines within the lesion in Treg and Teff cells from each treatment group are shown. Asterisks indicate fold changes >1.9.

a number of these induced genes have also been found activated in a recent analysis of pancreatic destruction induced by cyclophosphamide treatment of BDC2.5/NOD mice, including IFN-γ and several IFN-γ–regulated genes (Matos, M., personal communication).

In contrast, the changes affecting Tregs after ICOS blockade are far more profound, 215 and 21 genes were affected at the 1.5-fold cutoff. The plot of Fig. 7 D is highly informative. First, it shows that essentially all genes that are under- or overexpressed in control-treated Tregs compared with control-treated Teff remain so after anti-ICOS administration. This provides an independent validation of the "Treg signature" described in Fig. 4. Most importantly, it also becomes clear that the differential expression of the entire profile between Treg and Teff is lessened after ICOS blockade, as evidenced by the off-diagonal shift. This "muting" of the Treg signature affects genes that are both over- or underrepresented in Tregs (IL-10, GITR, and SOCS-2 or Tcf7, Eomesodermin, and Myb, respectively). It is as if ICOS blockade induced a broad shift of gene expression away from the Treg profile, toward one that is more similar to other $CD4^+$ cells. We considered the caveat that the $CD25^+CD69^-$ cell population might be more contaminated with effector cells after ICOS blockade, but ruled out this possibility by simulating computationally what the Treg gene expression values would look like given 5, 10, or 50% contamination by the Teff population. Greater than 35% contamination would be required for the shift seen in Fig. 7 D, an unlikely scenario given the decreased frequency yet static absolute numbers of $CD25^+CD69^-$ cells after ICOS blockade (Fig. 6 B and not depicted), and given the similar FACS® profiles with anti-ICOS or control treatment (Fig. 7 A). Another possible explanation is that a population of $CD25^+ICOS^+FoxP3^+$ cells is lost via antibody-induced cell death, and replaced by a $CD25^+CD69^-$ effector cell population, thus keeping the numbers of cells with this phenotype constant. However, $ICOS^+$ cells could still be detected at similar frequencies after anti-ICOS treatment using labeled anti–rat IgG (unpublished data), suggesting that there was no large composition change within the $CD25^+$ population.

The two standout genes that were exceptions to the down-modulation of the Treg profile were IFN-γ and Ccl-3 (MIP1α). Paradoxically, these proinflammatory genes were overrepresented in Tregs without treatment and further induced by anti-ICOS in both Treg and Teff populations. Cytokines represented in the lesion switch from an immunoregulatory to a proinflammatory profile (Fig. 7 E). Thus, ICOS blockade induces changes in the relative numbers and in the broad gene expression profiles of the Treg population, suggesting that ICOS engagement is necessary to maintain the activity of the Treg populations in the lesion, and thus prevent β cell destruction.

Discussion

Several mouse models of autoimmune diabetes share the feature of exhibiting a long quiescent period between the initiation of insulitis and the onset of diabetes (10). Similarly, the presence of islet-directed antibodies and T cells in the blood of prediabetic individuals years before diagnosis of diabetes suggests that the human disease might unfold in these same stages (52, 53). In animal models, where it can be assessed, the prediabetic state coincides with a local autoimmune infiltrate that refrains from terminal β cell destruction. This work aimed to unravel the localization as well as cellular and molecular mechanisms that control nondestructive insulitis, critical because this is likely to be the stage at which many therapeutic interventions will be attempted. We demonstrated the presence of both pathogenic Teffs and active $CD4^+CD25^+$ Tregs within the lesion of young prediabetic mice. Both were highly effective, very few cells being required to effect full islet destruction or, conversely, to exert full protection. The ability to recreate a nondestructive lesion much like the one in unmanipulated young BDC2.5 or NOD mice by cotransferring diabetogenic splenocytes and Tregs provides evidence that locally acting Tregs are an important element in the establishment of nondestructive insulitis.

How do the tissue Tregs described here relate to previously described regulatory populations in the BDC2.5 system? Kanagawa et al. (39) found that BDC2.5 clonotype^{lo} cells had regulatory properties, but these cells were not $CD25^+$ and developed as the mice aged, thus likely represent a different regulatory population. Gonzalez et al. (13) demonstrated that cells capable of protecting 10-d-old BDC2.5/RAG^o mice from fulminant diabetes were not enriched in the $CD25^+$ fraction, but resided, at least in part, in a population with markers, such as DX5, typical of NK T cells. This population also promoted a state of "respectful" insulitis, and had an effect directly in the islet lesion. The explanation for the divergence of these findings from the current ones may reflect differences in timing between the two experimental systems as follows: the protective effect of the $DX5^+$ fraction required administration before the onset of insulitis. One might speculate that these cells are needed upstream of $CD4^+CD25^+$ Tregs, perhaps to promote their establishment.

We observed that both Teffs and Tregs were actively cycling in pancreatic infiltrate. Similarly, Tregs could be observed proliferating in LNs draining their specific antigen site (28, 29), or in the lamina propria during regulation of colitis in a lymphopenic environment (30). Together, these observations clearly indicate that, in vivo, Tregs are not anergic. Previous studies have suggested that antigen-specific Tregs are not required for immunoregulation in TCR transgenic models of multiple sclerosis, but are more effective than nonspecific cells (54, 55). Here, we found that $CD25^+CD69^-$ Tregs essentially all expressed the clonotypic TCR, but although CD3 levels were equivalent to clonotype levels, we could not rule out the presence of dual TCR-expressing cells. The numbers of BDC2.5 Tregs required for effective protection were 10–100-fold lower than those needed in polyclonal cotransfer models of inflammatory bowel disease or autoimmune gastritis (20, 42).

These data imply that TCR specificity of Tregs for antigens in the target organ may be important for their effectiveness, probably because it allows triggering of the cells at the appropriate location.

The molecular mechanisms behind immunoregulation in the lesion are partially revealed by the gene expression profiles of the cells residing there. Although there were similarities to expression profiles reported for LN Tregs (45), some of the genes overexpressed in $CD25^+$ Tregs are highly evocative of local suppressive action. The chemokine receptors CCR-2, CCR-5, and CXCR3 may control the entry of cells into the site, especially given reported expression of the cognate chemokines in the islets (56). CCR-2 has been found recently to correlate with regulatory activity in a model of autoimmune arthritis (57). Expression of mRNA for the cytokine IL-10 was highly up-regulated in the pancreas compared with the LNs, which was consistent with its antiinflammatory activity and its implication in Treg function in several studies (for review see references 14–16). In BDC2.5 mice, IL-10 was observed to be protective and important for dominant regulation of diabetes in older animals (47). The striking enhancement of IL-10 transcripts in the pancreas suggests that regulation specifically occurs within the target organ. Localization and increased production of immunoregulatory molecules in the target tissue is likely critical for preventing spontaneous autoimmunity from evolving into overt autoimmune disease.

This important balance of regulation is disrupted by the loss of ICOS signals in the pancreatic lesion. Tregs are diluted out of the lesion and turn down the complete Treg signature. Dependence on ICOS for expression of regulatory molecules such as IL-10 by other Treg types has been shown in a model of airway hypersensitivity (58). Our data demonstrate that ICOS is expressed on $CD4^+CD25^+$ Treg particularly in the target organ during autoimmunity. There, it is essential for maintaining the Treg gene program and, hence, preventing a switch to a proinflammatory environment during the early phase of insulitis. Although we cannot completely exclude that rather than dampening the Treg gene profile, ICOS blockade somehow leads to an increase in a $CD25^+CD69^-$ effector population that contaminates the detection of $CD25^+CD69^-$ Treg genes, cytometric analysis shows no evidence that the $CD25^+CD69^-$ populations are significantly altered by ICOS blockade, nor that ICOS blockade causes the loss of $CD25^+CD69^-$ $ICOS^+$ cells. It had been postulated that a Th1/Th2 switch of effector cells was responsible for the exacerbation of autoimmunity after ICOS blockade in EAE models (48, 51). The present data demonstrate that a loss of immunoregulatory capacity by $CD4^+CD25^+$ Tregs is actually the mechanism by which ICOS blockade functions early to exacerbate autoimmunity.

Understanding the molecular and cellular basis of immunoregulation in the lesion could lead to the development of therapies that favor Tregs and promote long-term tolerance, even after an immune response has been well-established in the islet lesion. Costimulatory receptor blockade of ICOS upsets the balance of regulation directly in the lesion when it is first being established, and not in the draining node. It is interesting to consider what other signals might provoke diabetes after establishment of nondestructive insulitis in mice or humans. Perhaps viral infections, other environmental triggers, genetic factors, or age-dependent waning of Treg efficacy observed in NOD mice (31) disrupt the fragile balance of immunoregulation in the lesion and provoke diabetes.

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