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Therapeutic Regulatory T Cells Subvert Effector T Cell Function in Inflamed Islets To Halt Autoimmune Diabetes

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Therapeutic regulatory T cells (Tregs) can reverse pre-established autoimmune pathology. In this study, using a mouse model of autoimmune diabetes, we aimed to determine the means by which therapeutic Tregs control islet inflammation. Islet Ag-specific Tregs infiltrated inflamed islets soon after infusion into prediabetic mice, which was quickly followed by a selective reduction of mRNA associated with effector T cells in the islets. This change was partially due to decreased CD8+ T cell accumulation in the tissue. CD8+ T cells that remained in the islets after Treg treatment were able to engage dendritic cells in a manner similar to that found in untreated mice, consistent with the retention of an activated phenotype by islet dendritic cells shortly after Treg treatment. Nonetheless, Treg treatment abrogated IFN-γ production by intraislet CD8+ and CD4+ T cells at the protein level with minimal effect on IFN-γ mRNA. Sustained expression of IFN-γ protein by effector T cells was dependent on common γ-chain cytokine activation of the mTOR pathway, which was suppressed in islet CD8+ T cells in vivo after Treg treatment. These multifaceted mechanisms underlie the efficacy of therapeutic Treg subversion of effector T cell functions at the site of inflammation to restore normal tissue homeostasis. The Journal of Immunology, 2015, 194: 3147–3155.

R egulatory T cells (Tregs) are essential for maintaining immune homeostasis and preventing autoimmune diseases. Treg control of immune responses can be divided into three distinct phases: homeostatic control, damage control, and infectious tolerance (1). Treg prevention of dendritic cell (DC) activation in lymphoid organs is important in the maintenance of immune homeostasis and prevention of self-reactive T cell priming (2, 3). In an ongoing immune response when T cell priming is established, such as in the setting of chronic autoimmune diseases, Tregs must act in the target tissues to mitigate further damage by preactivated cells. In this context, Tregs have been found to suppress established CD4+ T cell–mediated inflammation in the intestine (4, 5). These studies have shown that Tregs can suppress further T cell proliferation and activation, as well as effector T cell survival, migration into the target tissue, or their function. Tregs have also been shown to suppress CD8+ T cell degranulation and killing of target cells in vivo (6). Once inflammatory tissue destruction is under control, Tregs can impart regulatory properties onto other cells in a process called infectious tolerance for long-term immune quiescence (7, 8).

Type 1 diabetes is a highly localized, tissue-specific autoimmune disease, and research in the NOD mouse has demonstrated that Treg function and impairments are highly localized to the inflamed islets (9, 10). Moreover, infusion of islet Ag–specific Tregs from TCR transgenic NOD.BDC2.5 mice can prevent and reverse diabetes (11, 12). In a recent report, autologous Treg therapy stalled the progressive decline of c-peptide in children with new-onset type 1 diabetes (13). Understanding how therapeutic Tregs control disease progression may help to optimize Treg cell therapy and shed light on the pathogenic mechanisms that drive disease progression. Although the effects of Treg therapy in the draining pancreatic lymph node (PLN) have been previously reported (14), in this work, we sought to elucidate the primary impacts of therapeutic Tregs in the suppression of an ongoing immune response in the target tissue itself, the pancreatic islets. In doing so, we have identified distinct mechanisms by which Tregs control effector T cells in inflamed islets.

Materials and Methods

Mice

NOD.CD28−/−, NOD.CD11c-YFP.CD28−/−, NOD.Foxp3ΔDTR+ (15), NOD.BDC2.5.Thyl.1 TCR transgenic, NOD.µGFP.BDC2.5.Thyl.1 TCR transgenic, and NOD.8.3.Thyl.1 TCR transgenic mice were housed and bred at the University of California, San Francisco (UCSF) Animal Barrier Facility. The UCSF Institutional Animal Care and Use Committee approved all experiments.

Quantitative real-time PCR

Islets were isolated as previously described (16). Whole islets or sorted cells were lysed in TRizol (Invitrogen). RNA was extracted using RNeasy Micro columns (Qiagen). Reverse transcription was done using SuperScript III (Invitrogen). Quantitative real-time PCR (qRT-PCR) SYBR Green Mastermix and primers were from Qiagen, and reactions were run on a CFX 96 (Bio-Rad). An RT2 Profiler Custom PCR Array (Qiagen) was used for whole islet experiments.

Immunofluorescence microscopy

Pancreas cryosections were fixed in 4% PFA and stained with anti-phospho-S6 ribosomal protein (2F9; Cell Signaling Technology), anti-CD8, anti-CD4, and DAPI (Invitrogen). Images were acquired on a Leica SP5 confocal microscope using a 63× water immersion objective. Acquisition and postacquisition analyses and visualization were performed...
using Leica Application Suite Advanced Fluorescence Lite software and Imaris software (Bitplane AG). T cells were enumerated using Imaris or manually by a blinded party unaware of the treatment conditions. Enumerating the number of phosphorylated ribosomal S6 protein–positive (pS6*) T cells was done manually by a person blinded to the experimental conditions.

**Two-photon microscopy**

Islets were stained with Hoechst for 15 min at room temperature and embedded in RPMI 1640 containing 0.5% low melting point agarose (Invitrogen). Embedded islets were imaged on a custom-built two-photon microscope as previously described (14). For time-lapse image acquisition, z-stacks with up to 40 μm spacing were acquired every 30 or 60 s for 20–60 min. Data were visualized and analyzed using Imaris software.

**Flow cytometry**

Islet and lymph node single-cell suspensions were prepared as previously described (16). The following Abs were used to stain the cells: anti–CD4-PE (RM4-5), anti–CD8-Pacific orange (5H10), anti–CD45-phycoerythrin-cyanin-7 (30-F11), anti–Thy1.1-PerCP (OX-7), anti–Thy1.2-AL700 (30H12), anti–B220-Pacific blue (RA3-6B2), anti–CD11c-PE-Cy7 (N418), anti–Ki67–FITC (SolA15), anti–Bcl2-PE (3F11), anti–I-Ag7-AL700 (20-2.16), anti–CD40-PE (3/23), anti–CD80-biotin (16-10A1), anti–PD-1–FITC (J43), and anti–CD86-allophycocyanin (GL-1). For intracellular cytokine staining, PLN cells were restimulated in vitro with 10 ng/ml PMA and 0.5 μM ionomycin in the presence of 10 μg/ml brefeldin A (BFA; Sigma-Aldrich) for 2–3 h. For direct ex vivo intracellular cytokine staining in islet T cells, in vivo BFA treatment was applied from a previously published method (17). Mice were i.v. injected with 250 μg BFA 4 h before sacrifice. Islet isolation was done with the addition of 10 μg/ml BFA throughout. Islets were cultured at 37°C for an additional 2 h in RPMI 1640 with BFA and dissociated in the presence of BFA. For both PLN and islet cells, cells were fixed with 4% PFA for 5 min at room temperature after surface staining. Fixed cells were permeabilized in 0.1% saponin and stained with anti–IFN-γ (XMG1.2; eBioscience). Analyses were performed on a LSRII or Fortessa flow cytometer (BD Biosciences) with FACS Diva analysis software (BD Biosciences).

**Cell transfers**

Tregs from NOD.BDC2.5.Thy1.1 or NOD.uGFP.BDC2.5.Thy1.1 TCR transgenic mice were FACS-sorted to high purity (>99%) and expanded as previously described (11). A total of 10^6 expanded BDC2.5 Tregs were transferred to prediabetic mice via i.p. injection or, in the case of two-photon experiments, via i.v. injection. CD8* T cells were enriched from spleens of NOD.8.3.Thy1.1 TCR transgenic mice against CD4, CD19, and CD11b followed by depletion with Dynabeads Biotin Binder (Invitrogen), CD4*CD25* BDC2.5 T cells were negatively selected from spleens of NOD.BDC2.5.Thy1.1 TCR transgenic mice using Abs against CD8, CD25, CD19, and CD11b. For two-photon experiments, enriched CD8* T cells were labeled with CMTMR or CFSE (Invitrogen), and 8–10 × 10^6 cells were injected i.v. For in vivo proliferation and cytokine experiments, head-enriched cells were further negatively sorted by FACS to high purity, labeled with CFSE, and 10^6 of each cell type was injected i.v.

**Ex vivo islet cell culture and phosphoflow**

Dissociated islet cells were incubated overnight at 37°C in RPMI 1640 + 10% FCS in a 96-well U-bottom plate with 2–3 × 10^5 cells/well. Anti–CD25 (clone 3C7) was added at 10 μg/ml. Rapamycin was added at 100 ng/ml. Tofacitinib (Selleckchem) was added at 10 μM. IFN-γ concentrations in supernatants were measured using ELISA. Cells were stained with a fixable viability dye (eBioscience), fixed in Lyse/Fix buffer (BD Biosciences), permeabilized in Perm buffer III (BD Biosciences), and stained according to manufacturer’s instructions.

**In vivo Treg depletion**

Fifteen- to 17-wk-old female NOD.Foxp3^{DTR} mice and transgene-negative control littermates were injected i.p. with 40 ng/kg body weight diphtheria toxin (DT, Sigma-Aldrich) on 2 consecutive days and analyzed 1 d after the second injection (15).

**Statistical analysis**

Statistical analyses were performed with the aid of Prism software (GraphPad).

**Results**

Tregs traffic to inflamed islets where they downregulate an effector T cell signature

We used NOD mice deficient in the costimulatory molecule CD28 (NOD.CD28^−/−), which develop diabetes more rapidly than their wild-type counterparts with 100% penetrance by 12 wk of age, primarily because of their deficiency in Tregs (18). BDC2.5 Treg treatment of prediabetic NOD.CD28^−/− mice at 5–7 wk of age confers 100% long-term protection against diabetes development (11). The stark contrast in disease outcomes between Treg-treated and untreated NOD.CD28^−/− mice offers an ideal in vivo setting in which to study Treg function in the control of a multifaceted, polyclonal T cell–mediated autoimmune disease. BDC2.5 Tregs rapidly trafficked to the islets within 18 h of i.v. infusion into 5- to 6-wk-old NOD.CD28^−/− mice, in agreement with a previous report that inflamed islets can directly recruit T cells from the circulation without the cells needing to pass through the draining PLN (19). Tregs preferentially trafficked to infiltrated islets over non-infiltrated ones, because greater numbers of Tregs were observed in islets with more severe insulitis, regardless of the time point analyzed (Fig. 1A). Upon arrival, BDC2.5 Tregs were seen to make dynamic interactions with a continuous network of islet DCs (Supplemental Video 1). BDC2.5 Tregs persisted long term in the islets, where they proliferated and maintained high levels of Foxp3 (Supplemental Fig. 1). However, they did not completely clear insulitis (Fig. 1B). Despite this, none of the treated NOD.CD28^−/− mice progressed to overt diabetes (11 and data not shown).

To examine how therapeutic Tregs gain control over the immune infiltrate, we developed a qRT-PCR array that contained genes relevant to the immunopathology of type 1 diabetes, including indicators of β cell function, markers of immune cell populations, and molecules associated with immune effector functions (Supplemental Table I). We analyzed whole-islet mRNA from NOD.CD28^−/− mice at the time of BDC2.5 Treg treatment and at 3 and 7 d after treatment, along with islet mRNA from age-matched untreated litters. Untreated mice showed a progressive decline of insulin expression, demonstrating rapidly advancing β cell destruction in these mice (Fig. 1C). In contrast, mRNA for both insulin 1 and insulin 2 genes was preserved and showed a trend of increase in Treg-treated mice when compared with baseline.

The most notable change in immunological genes at 3 d after Treg treatment was a downregulation of granzyme A and granzyme B mRNA (Fig. 1D), which became more pronounced at 7 d after Treg transfer, along with a reduction in IFN-γ and the chemokines Cxcl9 and Xcl1 (Fig. 1E). Genes upregulated at 7 d after Treg treatment included the immunosuppressive cytokine IL-10, as well as IL-6, which has been shown to have direct cytotoxic effects on β cells and to improve β cell function (20, 21). NK cells express granzymes and IFN-γ, and have been implicated to be a primary Treg target in BDC2.5 TCR transgenic mice (15). Consistent with this previous report, we found that the NK cell marker KlrD1 was also reduced after Treg treatment (Fig. 1E). However, NK cells, which exhibit functional and numeric defects in NOD mice (22, 23), made up, on average, <2% of the total immune cell infiltrate. In contrast, CD8* T cells represented an average of ~10% of the immune infiltrate (data not shown). CD8α was reduced 5.3-fold 7 d after Treg treatment (Fig. 1E), whereas markers for other major cell populations remained relatively unchanged (Supplemental Table I). The reduction of CD8α mRNA could not be attributed to a decrease in CD8* DCs because DCs in inflamed islets were predominantly CD11b^−CD8α^− (16). Altogether, these changes implicated cytotoxic CD8* T cells as the cells immediately impacted by Treg therapy in inflamed islets.
Tregs reduce CD8\(^+\) T cell accumulation in the islets

We next enumerated intraislet CD8\(^+\) T cells using immunofluorescence microscopy in pancreas sections from NOD.CD28\(^{−/−}\) mice at 7 d post-Treg treatment and in age-matched controls. Significantly fewer CD8\(^+\) T cells per islet section were observed in Treg-treated mice than in controls (Fig. 2A). This decrease in CD8\(^+\) T cells was not due to a decrease in CD8\(^+\) T cell proliferation, as measured by Ki67 expression (Fig. 2B), or decreased expression of the prosurvival protein Bcl2, measured as the median fluorescence intensity of Bcl2 protein in CD8\(^+\) T cells (Fig. 2C), or by the percentage of Bcl2\(^+\) cells (data not shown).

In addition, we examined expression of the exhaustion marker PD-1 and found that Treg treatment moderately decreased PD-1 expression on CD8\(^+\) T cells (Fig. 2D). Together, these data indicated that the reduced numbers of islet CD8\(^+\) T cells was likely caused by factors other than reduced in situ expansion and survival of CD8\(^+\) T cells.

CD8\(^+\) T cells use the chemokine receptor Cxcr3 to home to sites of inflammation in multiple disease settings (24). Because peripheral CD8\(^+\) T cells in NOD mice express Cxcr3 (Supplemental Fig. 2), and one of its ligands, Cxc9, was reduced in the islets after Treg treatment (Fig. 1D), we assessed whether Treg treatment inhibited CD8\(^+\) T cell trafficking to the islets. Using two-photon microscopy, we examined the overnight islet accumulation of adoptively transferred 8.3 CD8\(^+\) T cells, which express a transgenic TCR that is specific for the B cell Ag IGRP (25).

Significantly fewer 8.3 T cells were seen within the islets of NOD.CD28\(^{−/−}\) mice that had been treated with Tregs 7 d prior than in the islets of age-matched control mice (Fig. 2E). Together, these data suggest that the reduction in islet CD8\(^+\) T cells observed histologically and by quantitative PCR profiling was due, in part, to reduced recruitment and/or decreased retention shortly after their arrival in the tissue.

Treg treatment does not inhibit islet T cell–DC interactions

Although Treg treatment significantly reduced CD8\(^+\) T cell accumulation in the islets, large numbers of CD8\(^+\) T cells persisted, along with the rest of the islet immune infiltrate (Fig. 2A). DCs are essential for sustaining inflammation in the islets (26, 27), and we have previously shown that islet infiltration by T cells leads to massive recruitment and activation of DCs, which amplifies the autoimmune response (16). In addition, other studies have implicated DCs as targets of Treg suppression (3, 28). Therefore, we tested the hypothesis that Tregs control CD8\(^+\) T cells indirectly by suppression of DCs.
Intraislet DCs largely maintained an activated profile 7 d after Treg treatment, with high levels of expression of MHC class II and CD80 that were comparable with age-matched untreated NOD.CD28<sup>−/−</sup> littermates (Fig. 3A). Expression of CD86 and CD40 on islet DCs was moderately and significantly decreased in Treg-treated mice (Fig. 3A); however, despite these decreases, expression levels of these molecules on Treg-treated islet DCs remained as high as or higher than the levels observed on DCs in the draining PLN.

We and others have previously shown that Tregs inhibit DC interactions with effector T cells in the lymph nodes, thereby preventing their activation (14, 29). To determine whether Treg treatment altered the ability of DCs to activate CD8<sup>+</sup> effector T cells in the inflamed islets, we transferred dye-labeled 8.3 CD8<sup>+</sup> T cells into NOD.CD28<sup>−/−</sup> mice 7 d after BDC2.5 Treg infusion and performed time-lapse imaging of isolated islets the next day using two-photon microscopy. Significantly fewer 8.3 T cells entered the draining PLN, Treg treatment did not diminish the frequency of IFN-<bold>γ</bold> T cells in the PLN produced less IFN-<bold>γ</bold> upon ex vivo restimulation, indicating an inhibition of activation of newly arrived effectors in the PLN (Supplemental Fig. 3C). However, among endogenous T cells in the PLN, Treg treatment did not diminish the frequency of IFN-γ–competent cells, but rather increased it (Supplemental Fig. 3D). We suspect this to be due to a buildup of previously differentiated effector T cells in the PLN after Treg treatment.

In contrast with the PLN, transferred T cells in the islets proliferated extensively, regardless of BDC2.5 Treg treatment (Fig. 4A, 4B), consistent with the lack of change in islet T cell Ki67 expression (Fig. 2B) or in islet T cell–DC dynamics (Fig. 3B) after Treg treatment. BDC2.5 T conventional cells showed no differences in proliferation in the presence of BDC2.5 Tregs, whereas a moderate attenuation of 8.3 T cell proliferation was observed. Overall, this inhibition in the tissue was slight when compared with that observed in the draining PLN. These results suggest that although the primary impact of Tregs in the lymph node is the suppression of T cell clonal expansion and priming, the Tregs at the site of inflammation largely do not inhibit T cell activation.

Distinct from T cells in the PLN that did not produce detectable IFN-γ protein without in vitro restimulation (data not shown), islet...
T cells readily expressed IFN-\(\gamma\) ex vivo after short-term in vivo BFA treatment and without in vitro restimulation. Despite their robust proliferation in the tissue, we observed a near-total inhibition of IFN-\(\gamma\) production by both transferred 8.3 and BDC2.5 T cells in Treg-treated mice, whereas transferred T cells in islets of untreated littermate controls made considerable amounts of the protein (Fig. 4C). Importantly, and in contrast with the PLN, IFN-\(\gamma\) expression in endogenous islet T cells was also significantly suppressed in Treg-treated mice (Fig. 4D). Surprisingly, quantitative PCR analysis of CD8+ and CD4+ T cells FACS-purified from Treg-treated islets showed that these cells expressed similar amounts of IFN-\(\gamma\) mRNA when compared with cells isolated from untreated control mice (Fig. 4E). This suggests that the downregulation of IFN-\(\gamma\) observed at the level of total islet RNA is due to decreased total numbers of islet effector T cells, in agreement with Fig. 2. Furthermore, isolated T cells that persist in Treg-treated mice appear to be similar to anergic cells that express cytokine mRNA, but not protein (30). These results show that Tregs in the target tissue can block effector T cell function at the final stage of effector cytokine protein production.

**Tregs suppress mTOR signaling in islet CD8\(^+\) T cells that is critical for IFN-\(\gamma\) production**

Previous studies have shown that T cell anergy can result from inhibition of mTOR signaling (31), and activation of the mTOR pathway is critical in the generation and maintenance of effector cell differentiation (32–36). To determine the requirement for mTOR signaling in sustaining islet T cell effector function, we treated ex vivo islet cell cultures with rapamycin, a pharmacological inhibitor of mTOR. pS6, an indicator of active mTOR signaling (37), could be readily detected in islet CD8+ and CD4+ T cells after overnight culture (Fig. 5A). Addition of rapamycin to the culture strongly inhibited pS6, along with a significant reduction of IFN-\(\gamma\) (Fig. 5B), demonstrating that mTOR signaling was required for maintaining IFN-\(\gamma\) production by effector T cells from inflamed islets.

mTOR activation occurs downstream of PI3K/AKT signaling, which can be triggered by cytokines (37). Because Tregs have been shown to suppress effector cells through deprivation of IL-2 (38), we examined the effects of blocking IL-2 signaling on mTOR activation and IFN-\(\gamma\) production. Blocking the high-affinity IL-2R CD25 moderately reduced pS6 (Fig. 5C), which was associated with a moderate reduction of IFN-\(\gamma\) (Fig. 5D). This suggests that maintenance of IFN-\(\gamma\) production is partially dependent on continued IL-2 signaling. IL-7, another common \(\gamma\)-chain cytokine, has been shown to be important for promoting effector and memory T cells in inflamed islets (39). Therefore, we further determined whether blocking all common \(\gamma\)-chain cytokine signaling would have a stronger impact on IFN-\(\gamma\) secretion. We cultured islet cells in the presence of a Jak3 inhibitor, tofacitinib,
which led to downregulation of pS6 signaling (Fig. 5E) and blocked IFN-γ production (Fig. 5F). To determine whether Treg treatment suppressed IFN-γ production by blocking mTOR signaling in vivo, we examined pS6 protein in intraislet CD4+ and CD8+ T cells using in situ immunofluorescence. Treg treatment led to a significant reduction in pS6 + CD8+, but not pS6 + CD4+, T cells in the inflamed islets (Fig. 5G). Taken together, these results demonstrate that sustained IFN-γ secretion by intraislet effector cells requires constant common γ-chain cytokine stimulation and activation of mTOR signaling. Treg therapy blocked mTOR signaling in islet CD8+ T cells, likely through cytokine deprivation.

Lastly, to determine whether endogenous Tregs present in the NOD mice functioned in a similar manner to the BDC2.5 Tregs, we examined effector T cell number, IFN-γ production, and pS6 expression in NOD mice upon acute depletion of endogenous Tregs. After 2 d of DT treatment in NOD.Foxp3DTR+ mice, endogenous Tregs were extensively diminished in peripheral lymphoid organs and in the islets as previously reported (15 and data not shown). We found a trend toward an increase of islet CD8+ T cell numbers in Treg-depleted mice, whereas CD4+ T cells decreased in number, likely reflecting the depletion of CD4+ Tregs (Fig. 6A). Endogenous islet T cells, both CD4+ and CD8+, increased their IFN-γ production after Treg depletion (Fig. 6B).
Furthermore, a significant increase in the number of islet CD8+ T cells expressing pS6 was observed. This increase in pS6 was again specific to CD8+ T cells, because no similar increase was observed in CD4+ T cells (Fig. 6C). These results complement the findings from CD28-deficient NOD mice treated with BDC2.5 Tregs to suggest that Tregs suppress islet destruction primarily by controlling CD8+ T cell accumulation and effector T cell functions in the inflamed islets.

**Discussion**

In this study, we investigated the impact of therapeutic Tregs on ongoing chronic autoimmune responses in vivo. We found that Tregs preferentially traffic to sites of more severe inflammation. The immediate impact of Tregs in inflamed islets is primarily on CD8+ T cells by reducing their recruitment and rapidly suppressing IFN-γ protein production without altering IFN-γ mRNA on a per-cell basis.

This study highlights the differences in Treg control of an autoimmune response in the target tissue versus the draining lymph node. Although Tregs have previously been shown to suppress T cell proliferation and differentiation in the lymph node, this work demonstrates that therapeutic Tregs can suppress previously differentiated effector T cells at the site of inflammation to halt further destruction of the tissue. Given the acuteness of the changes in effector molecule expression after Treg treatment, we believe this rapid reversal of effector function happens primarily in the inflamed islets.
FIGURE 6. Treg depletion in NOD mice enhances islet T cell effector function, CD8+ T cell numbers, and mTOR activation. NOD.Foxp3<sup>3DTR</sup>- mice and transgene-negative littermate controls were sacrificed for analysis after 2 d of DT treatment. (A) Numbers of CD8+ T cells (left panel) and CD4+ T cells (right panel) per islet section were quantified by immunofluorescence microscopy. (B) Islet T cell IFN-γ production was determined by ex vivo intracellular cytokine staining as in Fig. 4. Data are from six mice per group combined from two independent experiments. Bar graph depicts mean ± SEM. (C) Numbers of pS6+ CD8+ T cells (left panel) and pS6+ CD4+ T cells (right panel) per islet section were quantified by immunofluorescence microscopy. Data for (A) and (C) are combined from two independent experiments with a total of three to five mice for each condition. Each point represents an individual islet; bold lines indicate the mean. The p values were determined by Mann–Whitney U test.

early after Treg treatment. The suppression in the PLN may complement the effects of Tregs in the tissue by reducing the supply of primed T cells so that long-term immune quiescence is maintained.

In prediabetic mice and patients newly diagnosed with type 1 diabetes, islets are infiltrated by activated inflammatory cells, particularly cytotoxic CD8+ T cells that accumulate with disease progression (40–42). Prompt subversion of T cell effector function may underlie the efficacy of Treg therapy in preserving the function of the target tissue. In NOD.CD28<sup>-/-</sup> mice, the critical role of CD8+ T cells in disease pathogenesis is demonstrated by the protection against disease onset observed when CD8+ T cells are depleted in mice with established islet insulitis by Ab treatment (data not shown). Our laboratory has also found inhibition of CD8+ T cells to be the primary effect of therapeutic Tregs in a mouse model of islet transplantation (43). Recent work by others has shown the importance of Treg suppression of NK cells (44–46). In particular, Treg depletion in NOD.BDC2.5.Foxp3<sup>3DTR</sup>- mice leads to rapid diabetes because of activation of NK cells in the pancreas (15, 46). Because the BDC2.5 mice express a CD4<sup>+</sup> TCR transgene, few CD8<sup>+</sup> T cells are present in these mice. This difference may account for the difference in the primary cytotoxic effectors (NK cells versus CD8<sup>+</sup> T cells) in our two studies. Nonetheless, both studies emphasize the importance of Treg suppression of cytotoxic effectors in the control of an autoimmune response.

Clues to the mechanism behind our observed discrepancy between persistent IFN-γ mRNA expression but lack of IFN-γ protein in islet T cells of Treg-treated mice may come from recent work from Pearce and colleagues (47) demonstrating a direct requirement for aerobic glycolysis for translation of IFN-γ mRNA, but not for cell proliferation or survival of CD4<sup>+</sup> T cells. This work elegantly showed that GAPDH, an enzyme involved in aerobic glycolysis, binds to and suppresses translation of IFN-γ mRNA when its enzymatic activity is not used. Although mTOR is a known regulator of aerobic glycolysis, this process was independent of mTOR signaling in the CD4<sup>+</sup> T cells studied by Chang et al. (47). Studies in CD8<sup>+</sup> T cells have shown a requirement for mTOR activity and aerobic glycolysis to maintain effector cell differentiation (34–36), consistent with our finding that mTOR signaling is required to sustain the effector function of intraislet CD8<sup>+</sup> T cells. This may help to explain why the inhibition of mTOR activation in our system was specific for CD8<sup>+</sup> T cells only, despite the fact that Tregs inhibited effectors function of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and points to differential signaling requirements between these two cell types. Although only a small subset of CD8<sup>+</sup> cells were seen to express pS6 at any given time, we consider this to be due to the nonsynchronous nature of cell signaling in this in vivo system as opposed to population heterogeneity. Our findings suggest that Tregs suppress CD8<sup>+</sup> effector functions via modulation of their metabolism, and further investigation is needed to define the precise molecular target of Treg action.

Overall, we have demonstrated that Tregs function efficiently at the site of inflammation at multiple levels to halt tissue destruction by subverting fully differentiated effector T cells. In addition to their homeostatic function to suppress T cell priming in the draining lymph node (14), Tregs exert damage control in the inflamed tissue by inhibiting the accumulation of effector cells and by repressing effector function in fully differentiated T cells. This multifunctional capacity of Tregs is likely critical for their remarkable efficacy. Because therapeutic Tregs are being evaluated for treatment of autoimmune diseases and transplant rejection in humans, findings from this study will help to guide mechanistic studies associated with therapeutic Treg clinical trials and to identify adjunct therapies most likely to synergize with Tregs.

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Disclosures

The authors have no financial conflicts of interest.
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