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Adaptive Islet-Specific Regulatory CD4 T Cells Control Autoimmune Diabetes and Mediate the Disappearance of Pathogenic Th1 Cells In Vivo¹

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Adaptive regulatory T cells that develop from naive CD4 cells in response to exposure to Ag can act as immunotherapeutic agents to control immune responses. We show that effectors generated from murine islet-specific CD4 cells by TCR stimulation with IL-2 and TGF- β 1 have potent suppressive activity. They prevent spontaneous development of type 1 diabetes in NOD mice and inhibit development of pancreatic infiltrates and disease onset orchestrated by Th1 effectors. These regulatory T cells do not require innate CD25⁺ regulatory cells for generation or function, nor do they share some characteristics typically associated with them, including expression of CD25. However, the adaptive population does acquire the X-linked forkhead/winged helix transcription factor, FoxP3, which is associated with regulatory T cell function and maintains expression in vivo. One mechanism by which they may inhibit Th1 cells is via FasL-dependent cytotoxicity, which occurs in vitro. In vivo, they eliminate Th1 cells in lymphoid tissues, where Fas/FasL interactions potentially play a role because Th1 cells persist when this pathway is blocked. The results suggest that adaptive regulatory CD4 cells may control diabetes in part by impairing the survival of islet-specific Th1 cells, and thereby inhibiting the localization and response of autoaggressive T cells in the pancreatic islets. *The Journal of Immunology*, 2006, 176: 4730–4739.

Although the mechanisms by which they function remain poorly understood, CD4 T cells with the capacity to regulate immune responses (TR cells)⁴ have emerged as key components in the control of homeostasis and self-tolerance. TR cells are now recognized to be a diverse population comprised of distinct subsets that display a spectrum of often overlapping phenotypes, but which may have different roles in regulating specific aspects of immunity. They can, however, be subdivided into two major subgroups: naturally occurring or innate TR cells that arise in the thymus early in life, and adaptive TR cells that develop from naive CD4 cells in the periphery during the course of an immune response (1, 2). Although the innate population has received intense study, much less is known about adaptive TR cells that differentiate from naive cells in the periphery in response to the cytokine milieu that develops during their encounter with Ag to become effector CD4 cells.

Innate TR cells represent a developmentally distinct lineage that is characterized by a combination of surface markers that include CD25, CD62L, CD134 (OX40), CD152 (CTLA-4), and glucocorticoid-induced TNFR family-related protein, as well as the expres-

sion of the X-linked forkhead/winged helix transcription factor, FoxP3 (3). Innate TR cells can inhibit the responses of T cells by direct cell contact in vitro, but their activity in vivo depends upon the cytokines, IL-10 and TGF- β 1, whose precise roles in mediating their function remain elusive (4). In contrast, adaptive TR populations can acquire different phenotypes depending upon the conditions of their induction. They are thought to modulate immune responses exclusively via cytokine-mediated effects and can include Th1 and Th2 cells, as well as intermediate phenotypes (5), in addition to IL-10- and TGF- β 1-producing subsets (Tr1 and Th3, respectively; Refs. 6 and 7), which are typically favored by mucosal routes of immunization. When generated in vivo, the anatomic location of their priming can also be critical in determining their cytokine secretion phenotypes. Thus, intranasal administration of peptide Ag can promote development of IL-10-producing TR cells (8) whereas orally administered Ag can favor TGF- β 1 producing TR cells (9). Targeting of Ag to subsets of dendritic cells, and the immunogenicity of Ag are among the factors thought to contribute to the cytokine secretion phenotypes of adaptive TR cells (10).

Innate TR cells play a fundamental role in preventing autoimmunity through the recognition of self-Ag (4). They may also contribute to control of effector T cell expansion and inflammation in immune responses to bacterial and viral pathogens (11, 12), and to regulation of memory T cell homeostasis (13). Adaptive TR populations also have the potential to function in these capacities, and such cells may arise as acute effector responses wane leading to less immunogenic conditions. A key feature of adaptive CD4 populations is that they can be generated ex vivo from naive CD4 cells, and used to control naive CD4 cell responses to foreign Ag (14), as well as autoimmune responses (6). Importantly, adaptive CD4 cells can share functional and phenotypic characteristics of innate TR cells such as secretion of TGF- β 1 and/or IL-10, and perhaps the sustained expression of CD25 and FoxP3, suggesting

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⁴ Abbreviations used in this paper: TR cell, CD4 cell with regulatory function; Ltn, lymphotactin; LN, lymph node; Bodipy 558/568, 4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid; TFIIIB, transcription factor IIB.

that it will be possible to exploit such cells in the Ag-specific regulation of responses.

Type I diabetes is a progressive autoimmune disease, which becomes pathogenic when innate TR cells may no longer have the capacity to keep autoaggressive CD4 cells in check (15, 16), ultimately leading to the destruction of pancreatic islet β cells. Protection from disease can be achieved by expression of TGF- β 1 in islets (17), which may promote the recruitment, accumulation, and/or expansion of innate TR cells that inhibit the autoimmune response in the site (18). It is also possible that self-reactive islet-infiltrating CD4 cells acquire characteristics of TR cells in response to local cytokine effects such that they are not only no longer pathogenic, but protective. Although the approach of islet-localized cytokine expression is impractical in a clinical setting, administration of mitogenic and nonmitogenic anti-CD3 mAb can protect both mice and humans against diabetes onset via TGF- β -dependent mechanism(s) in the absence of innate TR cells (19), and remarkably can reverse pre-existing disease early after clinical onset. These findings suggest that adaptive TR cells generated in the periphery can be sufficient to control development of type I diabetes even after progression to acute disease. Although polyclonal stimulation can be used to generate such cells, it is possible that CD4 cells specific for the relevant autoantigens are the key populations to be affected, because restimulation of effector cells by Ag in the lymphoid compartment or in target organ is likely to be crucial to elicit their effector response.

In this study, we generated adaptive TR cells from islet-specific CD4 cells by TCR signaling together with IL-2 and TGF- β 1 in vitro. These cells displayed an effector phenotype, and responded to restimulation in vitro and after transfer in vivo by production of IL-10 and TGF- β 1 as well as the chemokines, lymphotactin (Ltn; Ref. 4; XCL1) and RANTES (CCL5), which are associated with activated CD4 cells that become recruited into the pancreas (20). This population not only prevented spontaneous development of diabetes, but also inhibited the rapid disease onset mediated by Th1 effectors that were also islet-specific, causing their disappearance from the draining lymph nodes (LN) and spleen. These cells did not require innate TR cells for development or function and could be generated from islet-specific CD4 cells derived from diabetic mice. We show that one mechanism by which these adaptive TR cells can inhibit the acute response of Th1 cells in vitro is via FasL-dependent cytotoxicity, a mechanism that may also contribute to their function in vivo. Our results indicate that ex vivo-generated, Ag-specific TR cells can be potent immunotherapeutic agents for the control autoimmune diabetes.

Materials and Methods

Mice

NOD/LtJ, NOD/LtJ-Scid^{-/-}, NOD/LtJ-Rag 1^{-/-}, and NOD.NON-Thy1.1LtJ mice were obtained from The Jackson Laboratory. BDC2.5 CD4 T cell (α 1, $\nu\beta$ 4) TCR transgenic mice (21) were from D. Mathis and C. Benoist (Joslin Diabetes Center, Boston, MA). PCX.NOD mice that express GFP under the actin promoter were from N. Sarvetnick (The Scripps Research Institute, La Jolla, CA). The mice were bred in the vivarium at Sidney Kimmel Cancer Center. BDC2.5 mice were maintained as heterozygous for the TCR transgene and bred to NOD, NOD, Rag^{-/-}, and PCX.NOD mice. Female mice were used in all experiments. All experiments in this study were approved by the Sidney Kimmel Cancer Center Institutional Animal Care and Use Committee.

Antibodies

Anti-CD3 (2C11), -CD28 (37.51), -IFN- γ (XMG1.2), -IL-4 (11B11), -IL-10 (JES-2A5), and -TGF- β 1 (1D11.16.8) were generated from the indicated hybridomas and purified as described (22). Reagents from BD Pharmingen were purified hamster IgG1, anti-CD178/FasL (MFL3), and anti-IL10R (1B1.3a), and PE-labeled mAb specific for: CD152/CTLA-4,

CD25, β 7 integrin, CD134/OX40, CD95/Fas, CD178/FasL, IFN- γ , IL-10, and anti-BrdU, and FITC and biotin-labeled anti- $\nu\beta$ 4. Allophycocyanin- and PE-labeled streptavidin and allophycocyanin anti-CD90/Thy1.2 were from eBioscience. PerCP-anti-CD4 was from Caltag Laboratories. Purified anti-Ltn (XCL1) was from R&D Systems. Biotin-anti-mouse IgG and PE-anti-rat κ from BD Pharmingen were used as second step reagents. mAb for ELISAs included capture and detection pairs for IL-2, -4, -10, and IFN- γ from BD Pharmingen, and for the chemokines, Ltn and RANTES from R&D Systems. Rabbit anti-FoxP3 sera was generated as described (23).

Generation of CD4 effectors

CD4 cells were enriched from the spleens and pooled LN of BDC 2.5 mice by negative selection using magnetic sorting with a mixture from BD Pharmingen (Imag). CD4 cells were seeded at 10⁶/ml in 6-well plates (Costar) in 5 ml of complete media (20) to which 10 ng/ml rIL-2 was added. For Th1 cells, plates were coated with 10 μ g/ml anti-CD3 and the cultures were supplemented with 5 ng/ml rIL-12 (Genetics Institute), 10 μ g/ml anti-IL-4, and 10 μ g/ml anti-CD28 (20). For TR cells, plates were coated with 50 μ g/ml anti-CD3, and cultures were supplemented 2 ng/ml human rTGF- β 1 (R&D Systems) and 10 μ g/ml anti-IFN- γ . After 60–72 h, the cells were expanded in media with rIL-2. Th1 cells were harvested at 4 days, and TR cells at 5 days.

Proliferation, cytokine secretion, and CTL activity

BDC CD4 cells were restimulated with plate-bound anti-CD3 (10 μ g/ml) in 200 μ l triplicate cultures in 96-well plates (Costar) as indicated in the text. For cytokine analysis, supernatants (100 μ l) were harvested at 48 h for naive cells and at 24 h for effectors, and tested by amplified ELISAs (24). Latent and active TGF- β 1 were assayed with ELISA kits from Promega. After collecting supernatants, [³H]thymidine (1 μ Ci/well) was added and uptake was assessed 18 h later to measure proliferation. To test CTL activity, Th1 cells were labeled with ⁵¹Cr (Na₂⁵¹CrO₄) (22) and 3 \times 10⁵ were added to triplicate cultures in 96-well plates with varying numbers of unlabeled TR cells for 6 or 14 h. ⁵¹Cr release into supernatants was measured using a gamma counter, and cpm from TR cell cultures were compared with those from unlysed and lysed Th1 cells without TR cells. Alternatively, Th1 cells were labeled by fluorescence with 4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (Bodipy 558/568) (Molecular Probes) for 20 min at 37°C (25) and 3 \times 10⁵ were mixed in various ratios with TR cells. Viable recovery was assessed by flow cytometry using 7-aminoactinomycin D to distinguish dead cells.

Gene expression analysis

RNA was isolated from Th1 and TR cells after 4 and 5 days of culture, respectively, using RNeasy Mini kits from Qiagen. Affymetrix mouse 430A 2.0 chips were used to profile gene expression levels. For each chip, 10 μ g of total RNA was labeled and scanned using Affymetrix's standard procedure (www.affymetrix.com/support/technical/manual/expression_manual.affx). Data were analyzed using GeneSifter software from 4 chips each for Th1 and Th2 cells generated from two separate experiments. A gene was considered to be differentially expressed between two different samples when expression was determined to be present in all samples under default parameters and there was a 2-fold or greater change in net fluorescence between the average of four samples ($p = 0.05$).

Adoptive transfer

BDC CD4 cells were injected i.v. into NOD, NOD Thy1.1, or NOD.Scid mice as indicated in the text for individual experiments. In Scid recipients, donor cells were distinguished by $\nu\beta$ 4. For transfers into NOD mice, the donor CD4 cells were from BDC PCX.NOD mice and marked by GFP. Thy 1.2 was used to track BDC CD4 cells in NOD Thy 1.1 mice. Blood glucose levels were determined using an AccuChek II monitor (Boehringer Mannheim Diagnostics) daily for 2 wk and weekly thereafter. Two consecutive readings >300 mg/dl were considered indicative of diabetes. For coinjection studies, naive BDC cells or Th1 cells were labeled with CFSE (Molecular Probes) (25) and TR cells with Bodipy 558/568 before injection of equal numbers (2 \times 10⁶) into recipients. To detect donor cell division after several days in vivo, recipients were given BrdU (Sigma-Aldrich) as described (26). Anti-FasL blocking or isotype control mAb were injected i.p. in a dose of 200 μ g/recipient at the time of cell transfer.

Intracellular staining

For BrdU analysis, PCX.NOD BDC cells were stained with CD4-PerCP and biotin- $\nu\beta$ 4/allophycocyanin-streptavidin, permeabilized with Cytoperm/Cytofix (BD Pharmingen), and then stained with PE-anti BrdU. For CTLA-4 staining, Th1 and TR cells were incubated with surface stains and

permeabilized as above. For intracellular cytokine staining, the cells were stimulated by overnight culture with 10 $\mu\text{g}/\text{ml}$ anti-CD3 at $10^6/\text{ml}$ in 24-well plates. Brefeldin A (10 $\mu\text{g}/\text{ml}$) was added after 2 h. After harvest, the cells were stained for surface markers, permeabilized, and stained with anti-cytokine Abs in concentrations that were optimized for the individual reagents.

Detection of FoxP3 protein

Analysis was performed on in vitro-generated Th1 or TR cells or in vivo-transferred TR cells that were FACS sorted on the basis of Thy 1.2 from the spleens and pancreatic LN 7 days after transfer into NOD Thy 1.1 recipients. The cells were washed with PBS, lysed in the presence of protease inhibitors (Roche Diagnostic Systems), and sonicated. Protein concentration was determined by bicinchoninic acid protein assay (Pierce). Lysates were separated on 4–12% gradient bis-Tris gels (Invitrogen Life Technologies), and transferred to nitrocellulose. FoxP3 was detected by immunoblotting with anti-FoxP3 and standard chemiluminescence. As a loading control, blots were probed for the transcription factor IIB (TFIIB) (Santa Cruz Biotechnology). A positive control lysate was from 293T cells transfected with mouse FoxP3 DNA.

Homing

Localization of BDC cells in tissues of NOD mice was evaluated after labeling with ^{51}Cr as described (22) and injection of $1\text{--}2 \times 10^6$ cells with a total of $5\text{--}6 \times 10^4$ cpm. Radioactivity present in blood, lymphoid, and nonlymphoid organs was determined at 16 h after cell transfer using a gamma counter.

Histology

Pancreata were fixed in 10% buffered Formalin and embedded in paraffin. Four-micrometer-thick sections were stained with H&E. Insulitis indices were performed by scoring islet infiltrates (20). Between 10 and 30 islets per pancreas were analyzed in five levels of sections that differed by 80 μm for a total of 120–125 individual islets for each treatment group.

Results

Characteristics of BDC CD4 effectors generated in the presence of IL-2 and TGF- β 1

Although CD4 cells from BDC2.5 TCR transgenic mice are exposed to islet Ag, they express a predominantly naive phenotype

with high levels of the homing receptors CD62L and β_7 integrin and low levels of CD44 (27) (data not shown). As reported, few cells express CD25 (27, 28). To generate effectors, BDC cells were stimulated with anti-CD3 together with rIL-2. Previous studies indicated that priming of naive CD4 cells in the presence of TGF- β and rIL-2 induces TGF- β -secreting effectors but the population includes Th1-like cells unless endogenous IFN- γ is neutralized (29). Thus, to elicit TGF- β -producing effectors with a potential to function as TR cells, anti-IFN- γ and rTGF- β 1 were added. We also used a higher dose of anti-CD3 (without anti-CD28) for generation of TR cells, which largely overcame the antiproliferative effects of TGF- β 1. Although Th1 cells developed after 4 days (20), TR cells exhibited a 24 h lag before both populations demonstrated an equivalent 3- to 5-fold expansion. Without IL-2, TGF- β 1 failed to support CD4 cell expansion (data not shown).

After culture, both populations were activated in appearance and expressed high levels of CD44 and CD25 (IL-2R α) (Fig. 1A). The cells were heterogeneous with respect to CD62L and regulation by IL-12 may account for the higher levels found on Th1 cells than on TR cells (30). β_7 integrin was maintained on TR cells but not Th1 cells, consistent with previously reported effects of TGF- β 1 on expression of this receptor (31). Both populations expressed OX40 and high levels of intracellular CTLA-4. Recent studies show that FoxP3 is induced in naive (CD25 $^-$) CD4 cells in response to TGF- β 1 (32) and is associated with development of a phenotype that is indistinguishable from that of innate TR cells (14). To determine whether this occurs during effector generation from BDC CD4 cells, we analyzed expression of the FoxP3 protein by Western blots. As shown in Fig. 1B, expression was detected in effectors from cultures containing IL-2 and TGF- β 1, but not IL-2 and IL-12.

We then assessed cytokine secretion by CD4 effectors after restimulation with anti-CD3 to determine the effects of TGF- β 1 on cytokine polarization. IL-2 was not detectable in the culture supernatants of either Th1 or TR cells (<20 pg/ml) as is typical for

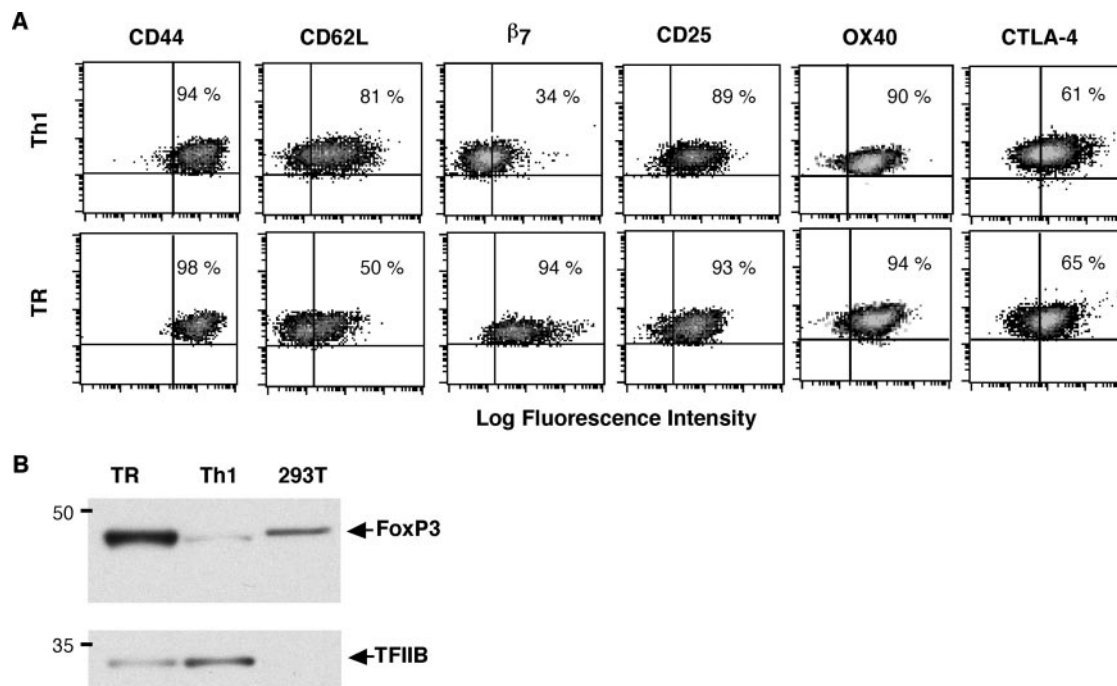


FIGURE 1. Phenotype of CD4 effector cells. *A*, Th1 and TR cells were induced from BDC 2.5 CD4 cells as described in *Materials and Methods*. At the time of harvest, the cells were analyzed for expression of the indicated markers. All were surface stains except CTLA-4, which was detected by intracellular staining. Plots are gated on CD4 $^+$ lymphocytes. Shown are $\nu\beta 4^+$ cells (y-axis) vs the various markers (x-axis). *B*, Western blots were used to analyze FoxP3 protein expression by 5×10^5 Th1 and TR cells effectors compared with transfected L293T cells. TFIIB was used as a loading control.

effectors which have active consumption. Th1, but not TR, cells secreted IFN- γ while TR, but not Th1, cells produced high levels IL-10 (Fig. 2A, left panel). Both active and latent TGF- β 1 were measurable (Fig. 2A, middle panel), but the active form was found only in supernatants of TR cells. The results demonstrate that TGF- β 1/IL-2 supported development of effectors that produce both IL-10 and TGF- β 1, cytokines which are associated with immunoregulatory functions. We also tested TR cells for secretion of chemokines and found that compared with Th1 cells, TR cells produced higher levels of Ltn and lower levels of RANTES (Fig. 2A, right panel). The results show that TR cells exhibit properties associated with activated CD4 effectors, including the capacity to produce cytokines and chemokines in response to TCR signaling.

To further evaluate functional activity, TR cells were tested for their ability to proliferate in response to anti-CD3. Their expansion was comparable to that of Th1 cells (Fig. 2B). The results show that both populations can divide in the absence of exogenously added cytokines, other T cells, or APC and that TGF- β 1 does not elicit a population that is anergic to TCR stimulation unless high doses of IL-2 are present. However, when added to cultures of Th1 cells, TR cells completely abolished secretion of IFN- γ by Th1 cells, while maintaining their production of IL-10 (Fig. 2C). The data show that TR cells function as a regulatory population that can down-modulate the response of Th1 cells.

Adaptive TR cells inhibit autoimmune diabetes

We showed previously that BDC Th1 cells induce diabetes when transferred into NOD.Scid recipients (20). We used this model to determine whether TGF- β /IL-2-induced TR cells affect the response of Th1 cells in vivo (Fig. 3A). As expected, Th1 cells caused rapid development of diabetes, with onset as early as 1 wk after cell transfer, and occurring in all recipients by day 10 (Fig. 3A). In contrast, 95% of the recipients of TR cells alone remained

disease-free throughout the experiment. When TR cells were coinjected with Th1 cells in a ratio of $2 \times 10^6:1 \times 10^6$, respectively, there was marked protection, with 80% of the recipients exhibiting normal blood glucose levels. Experiments conducted for >90 days confirmed that protection was long-lasting (data not shown). After titrating TR cells, we found that the 2:1 ratio was the optimum for disease prevention; with lower doses of TR cells, the numbers of protected recipients dropped in a dose-dependent manner (data not shown).

To assess in vivo changes that accompanied injection of TR cells, we examined the pancreata of NOD.Scid recipients for the presence of islet infiltrates (Fig. 3B). At the time of diabetes onset, the recipients of Th1 cells showed extensive mononuclear cell infiltrates and islets were no longer visible (left panel). In contrast, recipients of TR cells had very limited islet infiltration (middle panel). In mice given both Th1 and TR cells, pancreatic infiltrates were also very limited (right panel), suggesting that Th1 cells failed to accumulate in this site when TR cells were also present. To quantitate the levels of islet infiltration that occurred when TR cells were injected alone or together with Th1 cells, we analyzed the insulinitis indices for the two groups of recipients. As shown in Fig. 3C, at 30 days after transfer of TR cells, the majority of islets were either surrounded by, or invaded by infiltrating cells (perinsulinitis, and insulinitis, respectively). Although destruction of islets had been contained in recipients of both TR and Th1 cells, progression to a greater level of insulinitis had occurred. The data indicate that TR cells, like Th1 cells become established in the pancreas, but that the pathogenicity of Th1 effectors was kept in check by TR cells.

To determine whether TR cells have the potential to alter the spontaneous diabetes onset, TR cells were injected into NOD mice at 7 days of age during the preinsulinitis phase which extends to 3 wk of age. For these young recipients, TR cells were administered

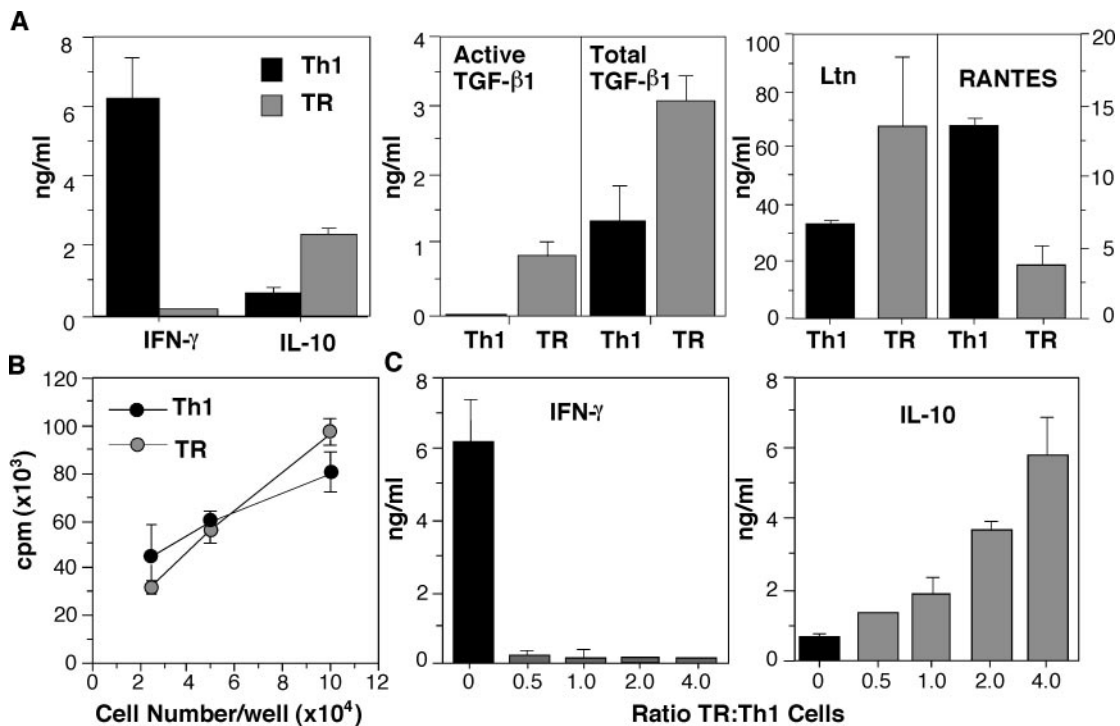


FIGURE 2. Responses of CD4 effector cells. A, 1×10^5 Th1 and TR cells that were induced as for Fig. 1 were restimulated in separate cultures with plate-bound anti-CD3. Supernatants were tested for the indicated cytokines and chemokines by ELISA. B, Th1 and TR cells were titrated as shown and cultured as for A. Proliferation was measured by [3 H]thymidine uptake. C, Th1 cells (1×10^5) were cultured alone or together with the indicated ratios of TR cells. Cytokine secretion was measured as for A.

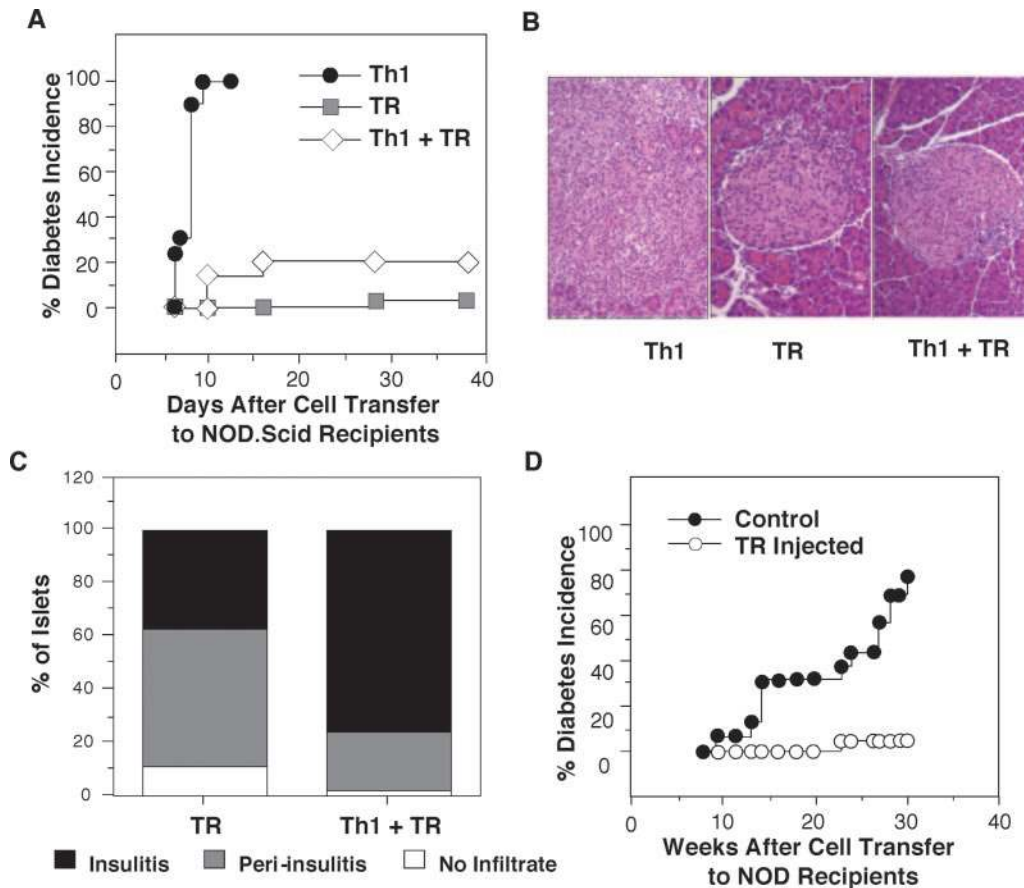


FIGURE 3. Adaptive TR cells inhibit autoimmune diabetes. *A*, Th1 cells (1×10^6) and TR cells (2×10^6) were injected i.v. alone or together into NOD.Scid female mice ($n = 20$ /group) who were monitored for onset of diabetes by blood glucose levels. *B*, Representative pancreatic histology for Th1 recipients (day 7), TR recipients, and Th1 + TR recipients (day 30) by H&E staining. *C*, Insulinitis indices for NOD.Scid recipients of TR cells or Th1 + TR cells were determined by scoring 125 individual islets for each treatment group for the presence of infiltrates. *D*, One-week-old NOD mice were injected i.p. with 1×10^5 TR cells and compared with age-matched NOD mice that were not injected ($n = 18$ females/group) for diabetes incidence.

i.p. in a dose 20-fold lower than that used for adult animals. As shown in Fig. 3C, TR cells protected female NOD mice from becoming diabetic. These data indicate that TGF- β 1/IL-2-induced TR cells controlled development of disease in addition to blocking the pathogenic response of islet-specific Th1 cells. When an equivalent number of Th1 cells were injected into young NOD mice, all recipients died by 1 wk after cell transfer (data not shown).

Adaptive TR cells arise from CD25⁻CD4 cells

Previous studies showed that BDC mice contain innate TR cells that express CD25 (28), and CD25⁺ TR cells have been reported to enhance TGF- β 1-dependent conversion of CD25⁻CD4 cells into CD25⁺ cells that exhibit regulatory functions (33). To investigate whether the innate TR cells contribute to development of TR cells induced with TGF- β 1/IL-2, we generated BDC Rag^{-/-} mice, which lack this population. As reported (34), these mice develop diabetes by 3–4 wk of age. In addition, as shown in Fig. 4A, BDC Rag^{-/-} CD4 cells isolated from 3-wk-old donors induce diabetes after transfer into NOD.Scid recipients, whereas an equal number of wild-type BDC CD4 cells have limited diabetogenicity unless they are previously activated (35). To determine whether TR cells could develop from BDC CD4 cells in the absence of innate TR cells, TGF- β 1/IL-2 effectors were induced from BDC Rag^{-/-} CD4 cells and cotransferred with Th1 cells into NOD.Scid recipients in a 2:1 ratio. The data in Fig. 4B demonstrate that despite their autoaggressive potential, BDC Rag^{-/-} CD4 cells had the capacity to develop into TR cells that control diabetes. The results

show that innate TR cells are not required for the generation or function of TR cells in response to TGF- β 1/IL-2.

In vivo localization, response, and phenotype of TGF- β 1/IL-2 TR cells

Because Th1 and TR cells express different levels of the homing receptors CD62L, and $\alpha_4\beta_7$ (Fig. 1A), we analyzed their capacity to localize in lymphoid as well as nonlymphoid tissues compared with naive BDC CD4 cells using ⁵¹Cr labeling of the donor populations to track their distribution in NOD recipients. As shown in Fig. 5A, the effectors were found in the blood and homed with similar efficiency to the spleen and pancreatic LN, but poorly to other peripheral LN compared with naive cells. As reported (36), the effectors showed a greater capacity than naive cells to enter the lungs and liver, but neither Th1 nor TR cells were detected in the pancreas. The data suggest the pancreatic LN and spleen are the sites in the lymphoid compartment where TR and Th1 cells have the potential to interact.

To determine the phenotype of TR cells *in vivo*, we assessed FoxP3 expression after adoptive transfer. For these experiments, NOD Thy 1.1 mice were used as recipients of TR cells generated from BDC CD4 cells to enable distinction of donor cells by Thy 1.2. After 7 days, TR cells were isolated by FACS sorting from the pancreatic LN and spleen, and tested for FoxP3. As shown in Fig. 5B, TR cells from both sites retained expression of FoxP3. We also examined surface marker expression in pancreatic LN at 1 wk after transfer of effectors generated from BDC PCX.NOD CD4 cells

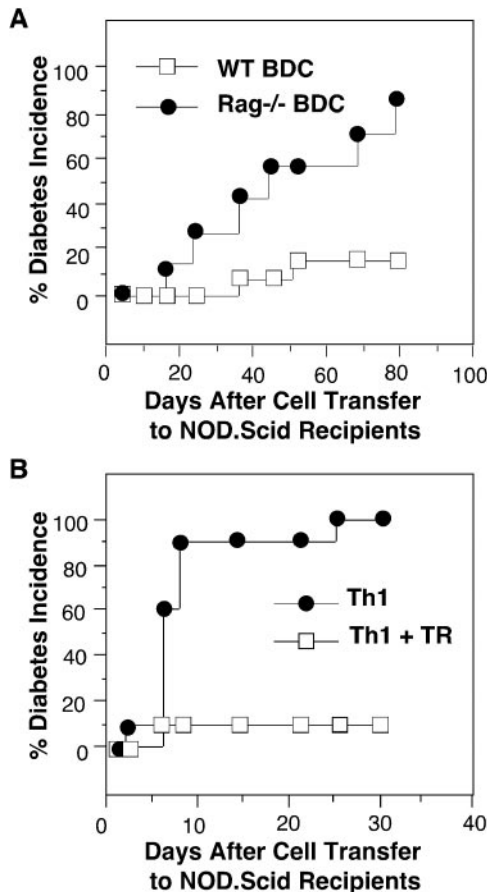


FIGURE 4. Adaptive TR cells develop in the absence of CD25⁺ cells. *A*, CD4 cells were isolated from wild-type BDC mice, or from BDC mice that were crossed to NOD Rag^{-/-} mice, and were injected into separate groups of NOD.Scid recipients (2×10^6 /mouse). Diabetes incidence is shown for recipients of wild-type ($n = 13$) or Rag^{-/-} ($n = 7$) BDC cells. *B*, TR cells were generated from BDC Rag^{-/-} CD4 cells and Th1 cells were induced from wild-type BDC CD4 cells as for Fig. 1. One $\times 10^6$ Th1 cells were injected into individual NOD.Scid recipients either alone ($n = 10$) or together with 2×10^6 TR cells ($n = 11$) and diabetes incidence assessed.

into separate groups of NOD recipients (Fig. 5C). Both subsets lost CD25 and remained heterogeneous for CD62L, indicating that unlike for innate TR cells, these markers do not distinguish adaptive TR cells in vivo.

We showed previously that Th1 cells expand in vivo after adoptive transfer and maintain their cytokine polarization (20). We now assessed in vivo expansion and cytokine secretion by TR cells. Like Th1 cells, TR cells showed higher levels of BrdU uptake in pancreatic LN compared with spleen, suggesting that the autoantigen recognized by BDC CD4 cells is present in sufficient levels to induce a response primarily in this site (Fig. 5D). To assess the stability of the cytokine phenotype of TR cells in vivo, we used intracellular staining. TR cells from the pancreatic LN but not the spleen retained the capacity to produce IL-10, TGF- β 1, and Ltn (Fig. 5E) suggesting that their response to Ag in this site plays a role in maintaining their effector phenotype. IL-2, IFN- γ , and TNF- α were not detected (data not shown), indicating that their cytokine secretion patterns are stable during the time of the experiment.

Selective effects of TR cells on Th1 cells

To study the effects of TR cells on naive or Th1 cells in vivo, they were cotransferred in a 1:1 ratio into NOD recipients after marking

the populations with the fluorescent dyes, Bodipy 558/568 or CFSE, respectively. After 4 days, naive BDC cells underwent several divisions in the pancreatic LN, and neither their numbers nor capacity to proliferate were affected by TR cells (Fig. 6A). In contrast, when Th1 cells were injected with TR cells, there was a marked disappearance of Th1 cells from the pancreatic LN (Fig. 6B), but not from nondraining LN (Fig. 6C, day 2 after cell transfer). Comparable results were obtained when the dyes used to label Th1 and TR cells were reversed. The response was not altered by treatment of recipients of Th1 and TR cells with mAb to TGF- β 1 or to IL10 and IL-10R compared with control Ab (data not shown), suggesting that the potential production of these cytokines by TR cells in vivo does not lead to impaired survival of Th1 cells.

Expression of FasL by Th1, but not Th2, cells

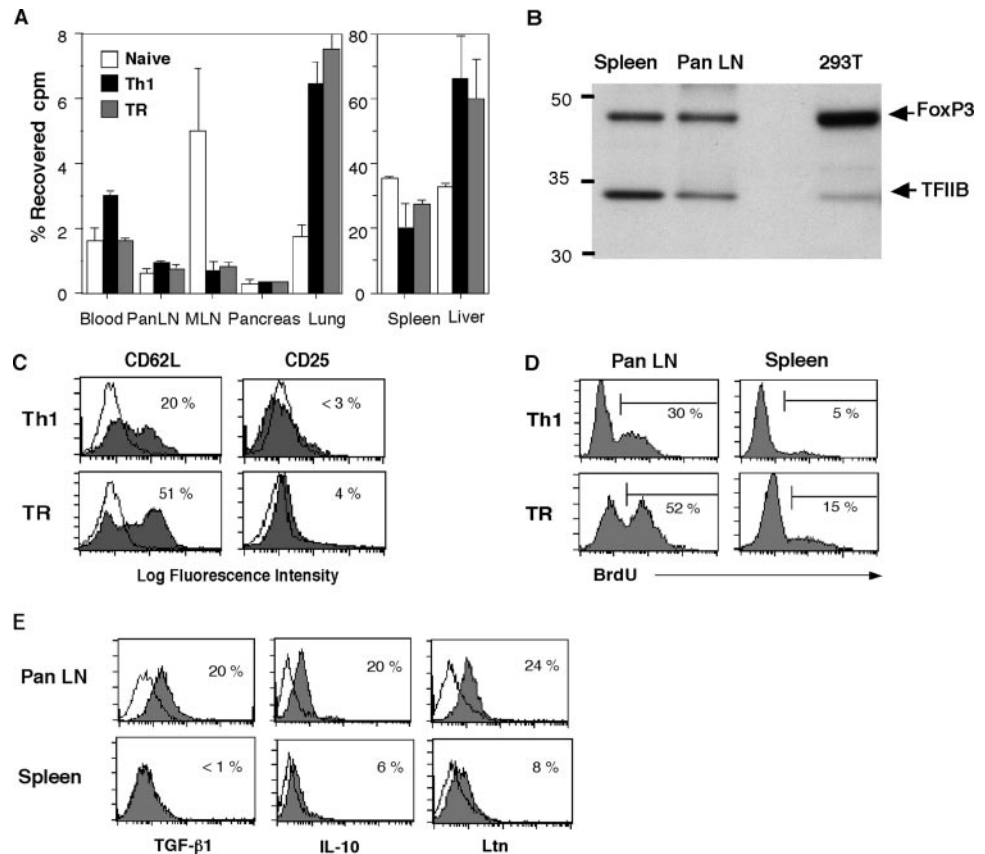
To identify possible molecular mechanism(s) that could participate in TR-mediated disappearance of Th1 cells in vivo, we performed gene profiling of Th1 and TR cells using RNA isolated from the activated effector populations from primary cultures. We detected 677 gene differences as measured by a >2-fold change in expression. Although perforin (37) and granzyme-mediated (38) cytotoxic activity has been associated with innate TR cells, we did not find these molecules to be expressed in greater levels than in Th1 cells (Fig. 7A). In contrast, Th1 cells expressed higher levels of the TNF superfamily member, FasL (4.2-fold), and of the Bcl-2 family proapoptotic protein member, Bim (3.2-fold), suggesting greater susceptibility to apoptosis. Although we did not detect differences between Th1 and TR cells in Bim expression by intracellular staining (data not shown), we confirmed that while both populations expressed similar levels of Fas, only Th1 cells expressed FasL (Fig. 7B). On the basis of this data, we determined whether TGF- β 1/IL-2-induced TR cells might be cytotoxic.

Cytotoxic activity of adaptive TR cells

Because our previous studies showed that effectors retain high viability for 48 h after withdrawal from stimulation (39), we used ⁵¹Cr labeling of Th1 cells to assess cytotoxicity by release of radioactivity after mixing with various numbers of TR cells. As shown in Fig. 8A, there was marked lysis after 14 h but not 6 h of coculture. To validate these results, we used fluorescent labeling of Th1 cells. Thus, Bodipy 558/568-labeled Th1 cells were mixed with unlabeled TR cells and recovery of viable Th1 cells was assayed after overnight culture. As shown in Fig. 8B, although both populations retained high viability and were recovered in similar numbers when cultured separately, when combined, there was a dose-dependent loss of Th1 cells. To determine whether the Fas/FasL pathway participated in this effect, we included a blocking mAb to this FasL in the cultures (Fig. 8B). The data demonstrate that cytotoxicity by TR cells was prevented by inhibition of Fas/FasL interactions. In contrast, in the same cultures, recovery of TR cells was unaffected by the presence of FasL-blocking mAb (Fig. 8C). The results show that TR cells can mediate direct killing of Th1 cells but do inflict fratricide on themselves. The data support the hypothesis that a cytotoxic mechanism might contribute to control of Th1 cells by TR cells in vivo.

To begin to test this prediction, anti-FasL-blocking or isotype control mAb were administered to NOD mice at the time of injection of CFSE-labeled Th1 cells and Bodipy-labeled TR cells. As shown in Fig. 8D, the disappearance of Th1 cells that occurs by day 2 in the presence of TR cells was reversed, suggesting that Fas/FasL interactions may contribute either directly or indirectly to the mechanism(s) underlying TR-mediated loss of Th1 cells in vivo. Although the in vitro assays of TR cytotoxicity and the in vivo induction of Th1 cell disappearance by TR cells suggest that

FIGURE 5. In vivo localization, phenotype, and responses of adaptive TR cells. *A*, Naive BDC CD4 cells, Th1 cells, or TR cells were ^{51}Cr -labeled and injected into separate NOD recipients ($n = 3/\text{group}$). After 16 h, the indicated tissues were harvested and the radioactivity counted to detect localization of the transferred cells. *B*, FoxP3 was analyzed as for Fig. 1 from 5×10^4 TR cells sorted from the spleens or pancreatic LN of NOD Thy 1.1 recipients at 7 days after transfer of 2×10^6 NOD BDC TR cells. *C*, Surface marker expression by Th1 and TR cells induced from BDC $2.5 \times \text{PCX.NOD}$ mice in pancreatic LN at 7–10 days after transfer into NOD mice. *D*, NOD mice injected with 2×10^6 Th1 cells or TR cells from BDC $\times \text{PCX.NOD}$ mice ($n = 4/\text{group}$) were given BrdU on day 3 after transfer. Uptake by donor cells in the spleen and pancreatic LN was assessed on day 7 for Th1 cells and day 10 for TR cells. *E*, TR cells from *D* were tested for synthesis of the indicated cytokines by intracellular staining.



TR cells induce apoptosis of Th1 cells, we were unable to directly demonstrate Th1 cells in the early stages of apoptotic death in TR-treated recipients by annexin V staining (data not shown). Not surprisingly, there were similar levels of the antiapoptotic protein Bcl-2 in Th1 cells recovered from recipients that did or did not receive TR cells (data not shown).

Discussion

In this study, we show that Ag-specific TR cells develop from autoreactive CD4 cells in response to TCR stimulation in the presence of IL-2 and TGF- β 1 and can be used as a cell-based therapy to inhibit the development of spontaneous and Th1 cell-mediated diabetes. After in vitro induction, these adaptive TR cells exhibit characteristics of an activated effector population which proliferates and produces the cytokines, IL-10 and TGF- β 1, and the chemokines, Ltn and RANTES. They home to, and respond to Ag in the draining pancreatic LN and they appear to target autoaggressive Th1 cells by eliminating them from this site and from the spleen. As a consequence, the autoaggressive response is controlled, and although islets become infiltrated, the massive inflammation and islet-structure that is orchestrated by Th1 cells is completely curtailed. These findings demonstrate that Ag-specific TR cells generated ex vivo can acquire properties that enable them to block an autoimmune response in the lymphoid tissues, and thereby may prevent accumulation of pathogenic cells in the target organ and development of disease. In autoimmune diabetes where autoantigen-specific CD4 cells are present in prediabetic individuals, this approach may be an effective alternative or adjunct therapy to in vitro expanded innate TR cells (28) for which it may prove more difficult to obtain Ag-specific populations.

The adaptive TR cells described in this study have several features that distinguish them. It has been reported that TGF- β 1 promotes conversion of naive CD25 $^{-}$ CD4 cells into a population that

is phenotypically and functionally indistinguishable from innate CD25 $^{+}$ TR cells (14, 32, 40). However, although CD4 cells responding to TCR stimulation in the presence of IL-2 and TGF- β 1 in our model up-regulate FoxP3, a characteristic marker of innate TR cells (41), they do not display an anergic phenotype in vitro. In contrast to a previous report (14), TR cells generated ex vivo with TGF- β 1 did not inhibit the proliferation of naive Ag-specific cells in vivo. Moreover, they failed to retain CD25 expression after in vivo transfer, underscoring a recent report that FoxP3 marks TR cells independently of CD25 (42). In addition, by gene array analysis, we find that adaptive TR cells, unlike innate TR cells, retain the capacity for synthesis of IL-2 (L. M. Bradley, unpublished observations). The development and function of islet-specific TR cells did not depend upon innate CD25 $^{+}$ cells which are present in low but detectable frequencies in BDC 2.5 mice (28). Indeed, TR cells could be generated in the absence of CD25 $^{+}$ cells from islet-specific populations that contained diabetogenic TR cells. This suggests that induction of adaptive TR cells ex vivo may remain possible after the autoaggressive response is underway, and that this strategy has the potential to be a feasible therapeutic approach after onset of clinical manifestations of disease when the activity of innate TR cells is no longer sufficient to maintain control.

Our results indicate that TGF- β 1 regulates the development of effector cells that secrete both TGF- β and IL-10. This phenotype is intermediate to that of Tr1 cells which predominantly produce IL-10 (6), and Th3 cells which primarily secrete TGF- β 1 (9). The results support the concept that adaptive TR cells can display a spectrum of cytokine phenotypes (5), and reveal the potential for using different cytokine milieus to promote the development of TR cells that produce specific cytokines that will be most effective in down-regulating autoimmune or allergic responses in particular sites. Because islet-specific TR cells that develop in response to TGF- β 1 retained their cytokine polarization patterns in vivo in

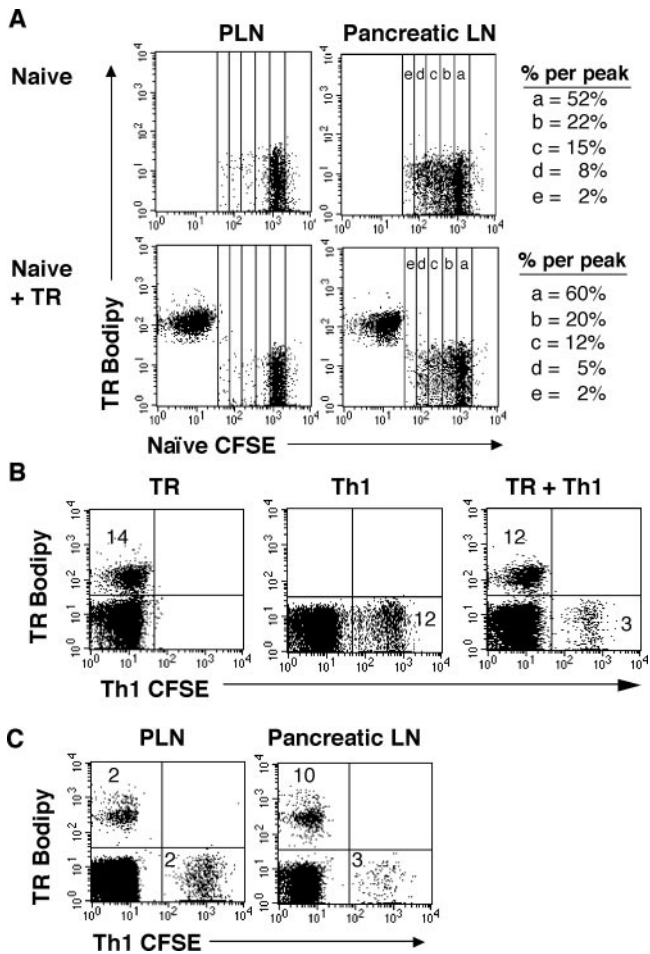


FIGURE 6. Selective effects of TR cells on Th1 cells. Naive BDC CD4 cells (A) or Th1 cells (B and C) were labeled with CFSE and 2×10^6 were injected alone or together with an equal number of Bodipy 558/568-labeled TR cells into NOD recipients. For A and B, the indicated tissues were assessed on day 4 after transfer for the presence of donor cells by flow cytometry. For C, analysis was performed on day 2.

draining LN and contribute to prevention of diabetes in NOD mice, both IL-10 and TGF- β 1 have the potential to participate in the long-term control of the autoreactive response.

Thus, TGF- β 1 produced by adaptive TR cells could block the development of autoaggressive Th1 cells from naive cells by inhibiting the induction of T-bet (29, 43), or cause deviation of islet-specific cells to a nonpathogenic phenotype, which may occur when TGF- β 1 is expressed in islets (17). TGF- β 1 can reduce cytokine secretion by activated CD4 cells (44) but it does not cause their apoptosis or limit their capacity to expand (45). Moreover, TGF- β 1 can induce Th1 cells to produce IL-10 (46). IL-10 can inhibit the differentiation and responses of Th1 by down-regulating IL-12 production by APC (47, 48). IL-10 can also directly inhibit cytokine production by Th1 cells, and enhance responses of activated T cells to TGF- β 1 through regulation of its receptors (45). Despite the potential for TGF- β 1/IL-10 cross-talk, neither cytokine exhibits cytotoxicity for T cells, and in blocking experiments in vivo, neither cytokine was required for the adaptive TR population to acutely control Th1 cells by impacting their survival (data not shown). Nevertheless, these cytokines, and TGF- β 1 in particular, could contribute to the maintenance of TR cells over more protracted periods.

We observed the loss of Th1 cells from pancreatic LN and the spleen, but not from pooled PLN when cotransfer with TR cells

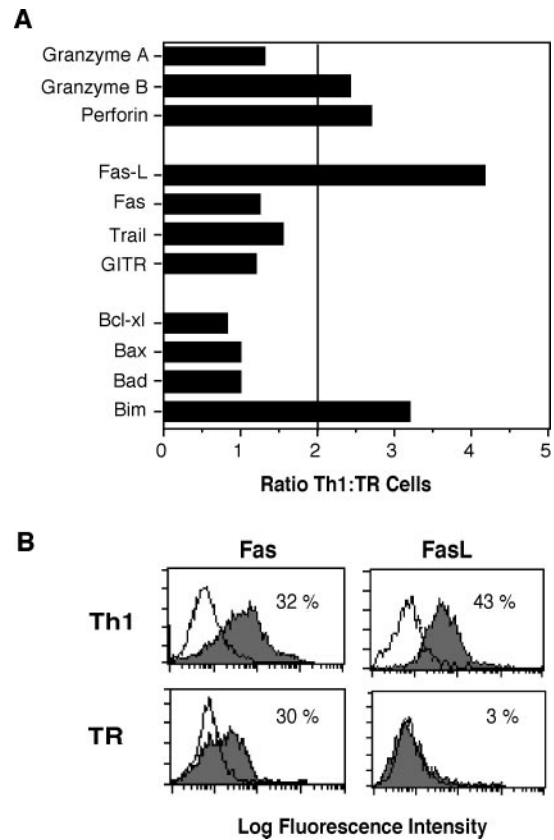


FIGURE 7. Expression of FasL by Th1 cells. A, RNA was isolated from BDC Th1 and TR cells that were induced as for Fig. 1, and used for gene expression analysis by Affymetrix microarrays. Expression of selected genes involved in apoptosis ($p = 0.05$). Gene differences ≥ 2 -fold were considered significant. B, Fas and FasL expression by Th1 and TR BDC \times PCX. NOD cells in the pancreatic LN at 7 days after their transfer into separate groups of NOD mice.

was performed, and have shown that TR cells can be directly cytotoxic for Th1 cells in the absence of either Ag or APC, supporting the concept that TR cells have the potential to regulate Th1 responses by this mechanism. The killing effect of TR cells in vitro was mediated by the Fas/FasL pathway, which also contributes to the in vivo disappearance of Th1 cells. Because TR cells do not kill themselves in vitro, other surface receptors, which could include and adhesion/costimulatory molecules, might also participate in this response by facilitating the interactions of Th1 and TR cells and thereby enable Fas/FasL engagement. However, it is exceptionally difficult to detect apoptotic cells in vivo due to their rapid clearance. In addition, in vivo, Ag may facilitate Th1 and TR interactions, possibly at the level of APC, and result in the engagement of other mechanism(s) that regulate survival of Th1 cells. Thus, although TR cells have the potential to directly impact the survival of Th1 cells through non-Ag-specific processes such as cytotoxicity, Ag may nonetheless confer specificity to the regulation by bringing TR and Th1 cells in close proximity at the level of APC. Although further study will be required to resolve the means by which TR cells inhibit development of diabetes, the results underscore that both adaptive and innate TR cells may use multiple processes to achieve immune regulation, and that by the use of ex vivo generation and/or expansion of TR cells, conditions that lead to specific functional readouts to target particular aspects of immune processes can be designed.

A potentially important aspect of adaptive TR cells is that they may have the capacity to give rise to memory cells that could

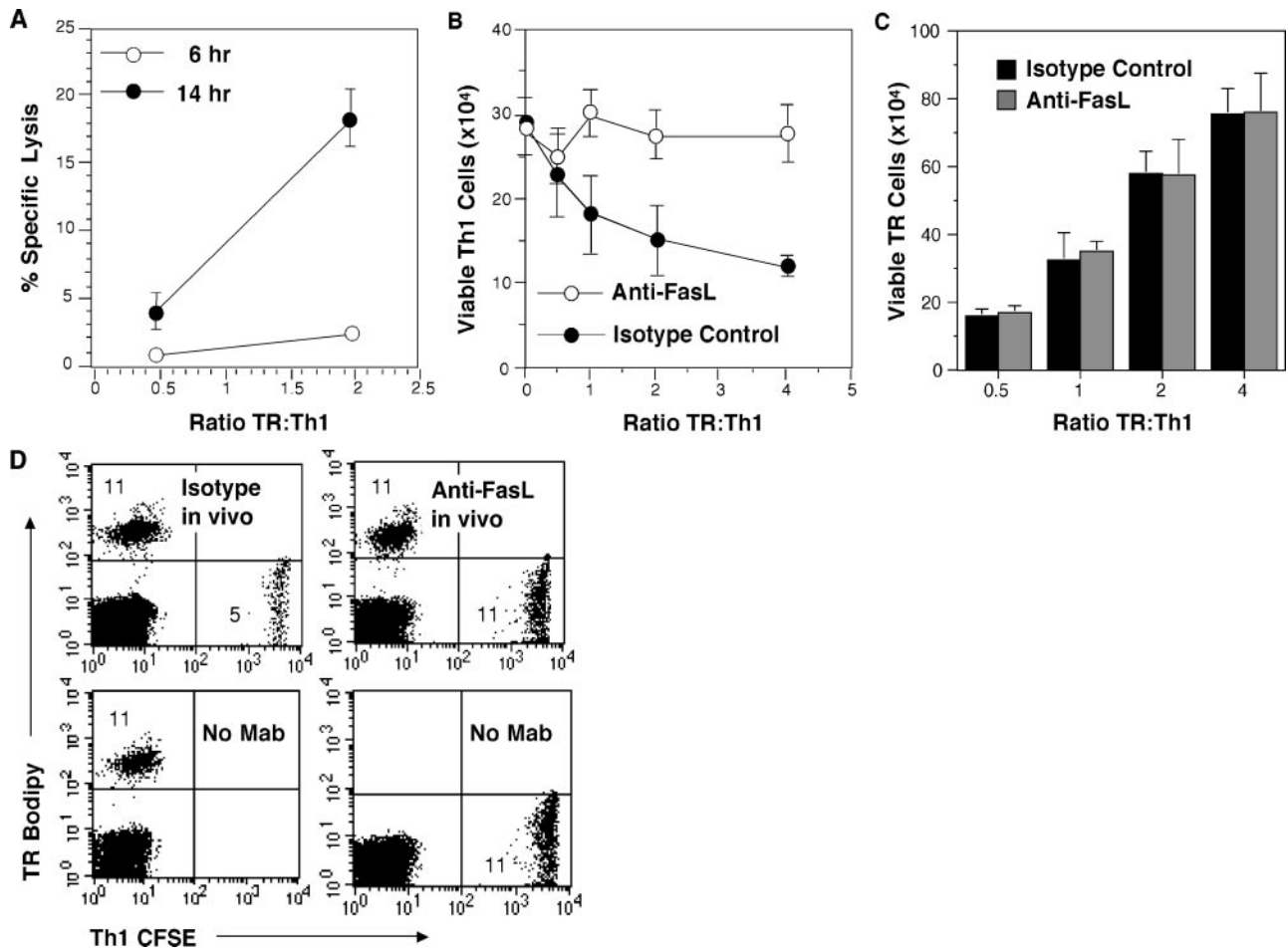


FIGURE 8. Cytotoxicity of TR cells for Th1 cells. *A*, Different numbers of TR cells were either incubated with 3×10^5 ^{51}Cr -labeled Th1 cells for 6 or 14 h. Release of radioactivity in amounts greater than that obtained without TR cells was used to calculate specific lysis at the indicated ratios as described in *Materials and Methods*. *B*, Various numbers of TR cells were incubated with 3×10^5 Bodipy 558/568-labeled Th1 cells. Identical sets of three replicate cultures were treated with either anti-FasL or a control Ab ($10 \mu\text{g/ml}$). At 14 h, viable Th1 cell recovery was analyzed by flow cytometry. *C*, In the same experiment, the recovery of viable TR cells in isotype control and anti-FasL-treated cultures is shown. *D*, Bodipy 558/568-labeled TR cells and CFSE-labeled Th1 cells were injected alone or together into NOD recipients as for Fig. 6. Mice that received both populations were injected with $200 \mu\text{g}$ of either hamster-IgG1 or anti-FasL. Shown are recoveries of Th1 and TR cells from the spleen on day 2 after cell transfer.

mediate long-term protection because FoxP3 was retained by TR cells. Their capacity to produce TGF- β 1 in sites where Ag is present may support their self-renewal through autocrine usage. Although our data suggest that adaptive TR cells can be sufficient to control diabetes, it is likely that adaptive and innate TR cells can work in concert. TGF- β 1 and IL-10 production by innate TR cells could augment the expansion and responses of adaptive TR cells. In view of a recent report that innate TR cells can use exogenous TGF- β 1 to mediate suppression (49), adaptive TR cells may also serve to augment their function. Because innate TR cells in NOD mice are inadequate to prevent diabetes onset, the use of adaptive TR cells that are specific for disease-associated Ag could serve to control the acute response, and thereby enable the innate TR population to regain long-term control.

Because adaptive TR cells share characteristics of effectors, these cells could arise in the context of immune responses to certain pathogens where the location of infection or the characteristics of the microorganism, itself, promote the development of cytokine-polarized effectors which down-regulate responses. The presence of such cells may account for the regulatory activities found to be associated with CD25 $^{-}$ cells immediately ex vivo (50), which have been identified previously in disease models (4). Harnessing the adaptive immune response using Ag-specific CD4 cells with

specific functional and phenotypic characteristics to regulate not only autoimmune diseases, but also allergy, and graft rejection, will add to the arsenal of strategies to combat these conditions.

Disclosures

The authors have no financial conflict of interest.

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