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Polyclonal Adaptive Regulatory CD4 Cells That Can Reverse Type I Diabetes Become Oligoclonal Long-Term Protective Memory Cells[1]

Elana Godebu,*† Daphne Summers-Torres,† Melissa M. Lin,† Bas J. G. Baaten,† and Linda M. Bradley[2†]

Type 1 diabetes is a CD4 cell-dependent disease that results from destruction of insulin-producing β cells in pancreatic islets. An ideal therapy would reverse diabetes shortly after onset when islet function in not yet fully ablated, and also prevent re-emergence of disease through the generation of memory cells that control the autoimmune response. In this study, we show that adaptive/induced polyclonal regulatory (TR) cells, which contain islet-reactive cells, fulfill these criteria in the NOD mouse model. CD4 cells induced to express FoxP3, IL-10, and TGF-β1 in response to TCR signaling and TGF-β1 can reverse diabetes with clinical restoration of prediabetic serum levels of IL-10. Unlike naturally occurring TR cells, these adaptive TR cells persist indefinitely (>1 year) as FoxP3$^+$, CD25$^-$ memory cells that self-renew. Establishment of memory is accompanied by narrowing of the T cell repertoire to usage of a single TCR β-chain, Vβ11, implying selection by Ag. With islet-specific adaptive TR cells, we show that memory is functionally stable and transferable. Therefore, adaptive TR cells, which can be readily generated from normal CD4 populations and become focused by Ag with induction of memory, may provide a treatment and a vaccine for the long-term cure of diabetes making them attractive as immunotherapeutic agents. ***The Journal of Immunology,*** **2008, 181: 1798–1805.**

It is well established that CD4 T cells with regulatory function (TR)[3] can develop from naive CD4 cells that lack expression of the forkhead winged transcription factor, FoxP3 (1), which accounts for in vivo specification of cells of the innate TR lineage (2). These adaptive/induced TR cells can be elicited ex vivo by TCR stimulation in the presence of IL-10 or TGF-β1 (3–5). They can also be generated in vivo by oral tolerance regimens (6) and by systemic administration of Ag in the absence of adjuvants (7). In models of induced autoimmunity, disease prevention by adaptive TR cells can be complete and irreversible under conditions where there are no overt perturbations of the immune system (8). These findings provide a strong rationale for analysis of adaptive TR cells as potential immunotherapeutic agents for control of autoimmune diseases. One stumbling block has been that Ag-specific TR cells are likely to be the most effective, and in diseases such as type I diabetes (T1D), the autoantigens have yet to be identified. Moreover, it is not yet known whether adaptive TR cells become functional as memory cells that contribute to the maintenance of tolerance after overt symptoms of disease subside, or whether they primarily function in inhibiting disease and enable a temporary resetting of pre-existing immune mechanisms to restore homeostasis.

The NOD mouse model of T1D has provided several insights into potential uses of TR CD4 cells as cell-based therapies (9). The development of disease is heralded by appearance of cellular infiltrates in the islets (insulitis) that become progressively more pathogenic, ultimately leading to destruction of insulin-producing β cells (10). Protection against spontaneous disease can be achieved during the preinsulitis and established insulitis phases of T1D by adoptive transfer of islet-specific innate TR cells that express FoxP3 and by adaptive TR cells that are either FoxP3$^+$ or FoxP3$^-$ (11–14). Reversal of diabetes by TR cells that are specific for islet-Ag has also been achieved (13, 15). The majority of treated animals remain protected for extended periods despite continued expression of the relevant autoantigens. Because the onset of T1D may in part be due to the inability of innate TR cells to contain the progressive autoreactive T cell response (16), the infusion of exogenous TR cells could not only contribute to acute control but also the long-term maintenance of tolerance if they can function as protective memory cells. From a clinical perspective, an ability to generate such protective adaptive TR cells ex vivo from normal CD4$^+$ cells would be an important advance that would facilitate their use in treatment of T1D.

In this study, we generated FoxP3$^+$ polyclonal adaptive TR cells from CD25$^-$CD4 cells from NOD mice using TCR stimulation together with TGF-β1. In adoptive transfer studies, we show that these TR cells can reverse T1D shortly after onset and become established as oligoclonal memory cells that persist indefinitely (>1 year) as functionally stable FoxP3$^+$, CD25$^-$ memory cells that transfer protection against T1D. The results indicate that adaptive TR cells have significant potential to provide an avenue for both treatment of, and vaccination against T1D.

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[3] Abbreviations used in this paper: TR, regulatory T cell; T1D, type 1 diabetes; LN, lymph node; PLN, peripheral LN; ICS, intracellular staining.

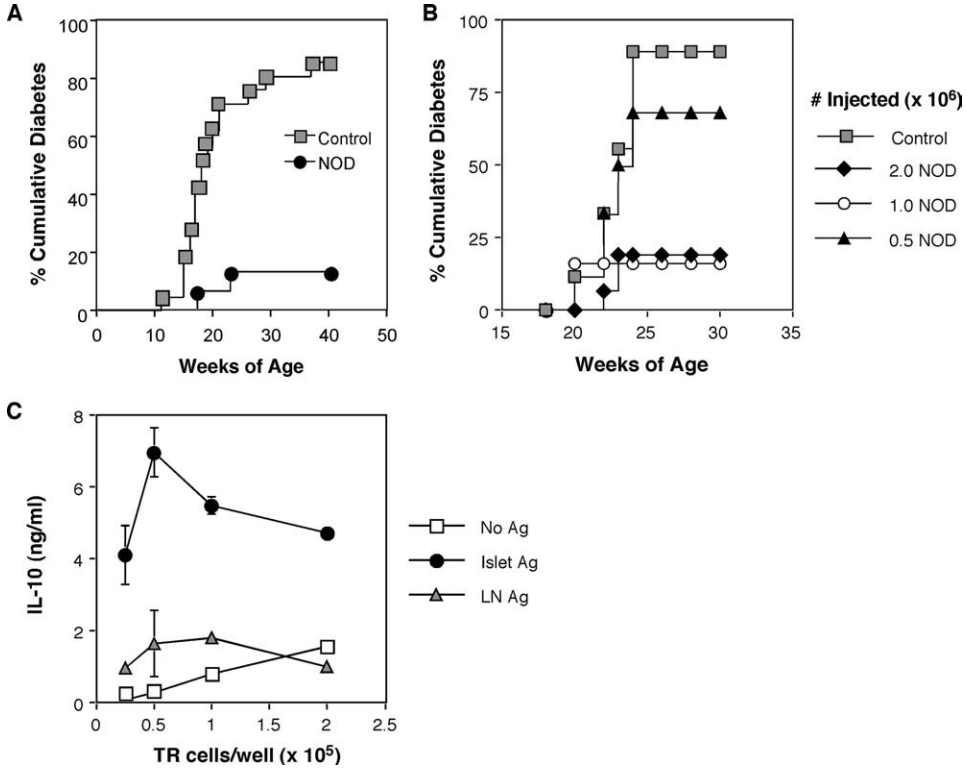

FIGURE 1. Polyclonal adaptive TR cells prevent T1D in adult NOD mice. Ex vivo-generated polyclonal (NOD Thy 1.1) TR cells were injected (*A*) into nondiabetic 8-wk-old NOD mice ($n = 15$, 2×10^6/recipient). Diabetes onset was evaluated in recipients of polyclonal TR cells ($n = 15$) and compared with age-matched NOD controls ($n = 21$); and (*B*) injected into NOD mice in doses of $0.5–2 \times 10^6$ cells ($n = 6$/group) and compared with age-matched controls ($n = 12$) for development of diabetes. *C*, Polyclonal TR cells were cultured in various doses with 5×10^4 splenic APC that were precultured for 1 day with 3 μg/ml islet proteins, LN proteins, or no Ag. After 48 h, the culture supernatants were tested for IL-10 secretion by cytokine bead array.

Materials and Methods

Mice

NOD/LtJ, NOD.NONThy1.1LtJ, and NOD.C-(Ptprc-D1Mit262)/WehiJ (NOD.CD45.2) mice were obtained from The Jackson Laboratory. BDC2.5 TCR-transgenic mice (17) were obtained from D. Mathis (Joslin Diabetes Center, Boston, MA). These mice were bred in the vivarium at the Sidney Kimmel Cancer Center. All experiments were approved by the institutional animal care and use committee. Only female mice were used.

Abs and staining reagents

Anti-CD3 (2C11), -CD28 (37.51), and -IFN-γ (XMG1.2) were produced by hybridomas and purified by Biolegend. Reagents from BD Pharmingen were anti-Vβ4 (KT4-FITC), -CD25 (PC61, biotin) -CD4 (GK1.5, Pacific Blue), -CD90.1 (Thy1.1, OX-7, PerCP), -BrdU (3D4, FITC), and isotype controls. Anti-FoxP3 (FJK-16S, PE) was obtained from eBioscience, anti-IL-10 (JES5-16E3, biotin) was obtained from Biolegend, and TGF-β1, 2, 3 (1D11, PE) was obtained from R&D Systems. Streptavidin-labeled with allophycocyanin or PE-Cy7 were obtained from eBioscience.

Antigens

Islets were freed from pancreata by collagenase P digestion, isolated by centrifugation through histopaque and pelleted. Single-cell suspensions from superficial lymph nodes (LN; inguinal, axillary, brachial) were prepared and pelleted. The proteins were solubilized with Nonidet P-40 and concentrated by membrane filtration for use to stimulate cytokine production in vitro. The H-2 I-Ag^7-restricted peptide, 1040-63 (18), was synthesized by Sigma-Genosys, and used to recall memory in vivo.

Generation of adaptive TR cells

CD4 cells were enriched from the spleens and peripheral LNs (PLN) of 8-wk-old NOD Thy1.1 or BDC 2.5, Thy 1.1 female mice by negative selection using magnetic sorting with an Ab mixture obtained from Imag (BD Pharmingen) according to the manufacturer's protocol. The cells were then depleted of CD25$^+$ cells by using positive selection with biotin-labeled anti-CD25 and streptavidin-conjugated magnetic beads. CD4 cells were cultured for 3 days with plate-bound anti-CD3 (50 μg/ml) in medium containing anti-CD28 (5 μg/ml), anti-IFN-γ (10 ng/ml), TGF-β1 (2 ng/ml), and IL-2 (5 ng/ml). The cells were then expanded for 2 days in medium containing IL-2 as previously described (14).

Adoptive transfer and immunizations

For most experiments, adaptive TR cells were injected i.v. into 8- to 10-wk-old NOD mice in a dose of 2×10^6 cells (14). However, different doses of TR cells from NOD CD45.2 CD4 cells were compared with those from BDC 2.5 Thy1.1 CD4 cells for the ability to prevent spontaneous T1D onset when injected into 15-wk-old NOD mice (Thy 1.2, CD45.1), as described in the text. To induce in vivo responses of BDC 2.5 TR cells, recipients were challenged i.v. with H-2 I-Ag^7-restricted peptide, 1040-63 (18), in a dose of 100 μg with 50 μg of LPS (*Escherichia coli*; List Biologicals).

Reisolation of memory TR cells and assay for protection against induced diabetes

Donor TR cells were isolated by positive selection with magnetic sorting using anti-Thy1.1 and 10^6 cells were injected i.v. into NOD recipients that were given 4×10^6 diabetogenic T cells from recently diagnosed NOD mice (19). For analysis of TR cells immediately ex vivo, CD4 cells were magnetically enriched by negative selection as indicated above.

Intracellular staining

To detect donor cell division in vivo, recipients were given BrdU (Sigma-Aldrich) as described (20). The donor cells were identified by surface Thy 1.1 and CD4, and intracellular BrdU. Intracellular staining (ICS) was used to detect FoxP3 following the protocol obtained from Biolegend. The capacity of splenic TR cells to produce the cytokines IL-10 and TGF-β1 at 2 mo after adoptive transfer was tested by ICS. At 2 h after restimulation of 2×10^6 cells cultured in 24-well plates with PMA (5 ng/ml) and ionomycin (500 ng/ml), brefeldin A (10 μg/ml) was added to block cytokine secretion (21). After 3 additional hours, the cells were stained with anti-CD4 and -Thy1.1 and, after permeabilization and fixation with reagents from BD Pharmingen, were stained with PE-anti-TGF-β1, 2, 3 and allophycocyanin anti-IL-10.

Cytokine secretion

Cytokine bead arrays (Luminex) with reagents and the protocol from Bio-Rad were used to detect cytokines that were present in 20 μl of mice sera before and after reversal of diabetes by TR cells and to assess the in vitro production of cytokines in 50 μl of culture supernatants of NOD TR cells that were restimulated with splenic APC pulsed with 3 μg/ml protein extracted from isolated islets (see above) or from LN. For this latter assay, TR

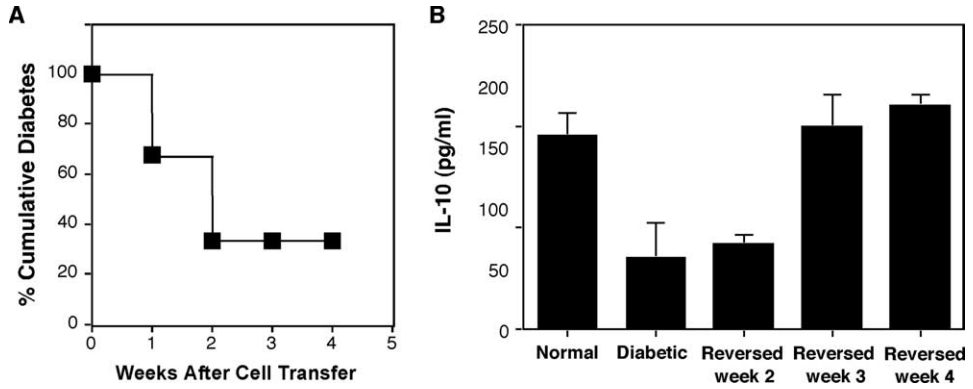

FIGURE 2. TR cells reverse recent onset diabetes. *A*, Ex vivo generated polyclonal TR cells were injected into NOD mice (2×10^6/recipient) at 1 day following an initial blood glucose reading ≥300 mg/dl ($n = 12$). Blood glucose levels were monitored weekly thereafter. *B*, Sera were taken from nondiabetic mice ($n = 6$), recent onset diabetic mice ($n = 5$), as well as recent onset diabetic mice that received ex vivo-generated polyclonal TR cells (2×10^6/recipient) and became normoglycemic ($n = 4$). Levels of IL-10 were tested by cytokine bead arrays.

cells that were generated in culture were plated in various doses in triplicate in 96-well plates with 5×10^4 splenic APC (22).

TCR Vβ analysis

RNA was isolated from TR cells generated in vitro using NOD Thy 1.1^+ CD4 cells and from the same population of TR cells at 4 mo after transfer into 8-wk-old NOD recipients using Qiagen kits. The TR donor population from the spleens and LN of the recipients was isolated by sorting Thy 1.1^+ cells using an Aria flow cytometer (BD Biosciences). PCR was used by BioMed Immunotech to analyze the RNA for the Vβ repertoire.

Diabetes detection

Blood glucose levels were measured using an AccuChek II monitor (Boehringer Mannheim Diagnostics) weekly after initiation of the experiments. Two consecutive readings >300 mg/dl were considered indicative of diabetes (14).

Results

Polyclonal TR cells generated ex vivo protect against T1D

We showed previously that $CD25^-CD4$ cells from islet-specific BDC 2.5 CD4 cells develop into $FoxP3^+$ TR cells after TCR stimulation in the presence of TGF-β1 (14). These induced TR cells controlled acute onset of T1D elicited by Th1 effectors in SCID recipients and protected NOD mice from spontaneous disease when administered during the preinsulitis phase. We now investigated whether adaptive TR cells could be generated from CD4 cells from NOD mice and whether protection could be extended to adult animals during the established insulitis phase. $CD4^+CD25^-$ cells were isolated from NOD Thy1.1 mice, cultured under TR conditions, and then transferred into 8-wk-old NOD female mice in a dose of 2×10^6/recipient. The results in Fig. 1*A* show that like islet-specific monoclonal TR cells (14), polyclonal TR cells protected NOD mice from the spontaneous development of T1D. To assess the efficacy of polyclonal TR cells, various numbers were compared for protection against T1D after transfer into diabetes-free 16-wk-old recipients. As shown in Fig. 1*B*, doses of TR cells ranging from 1 to 2×10^6 showed comparable protective activity in the spontaneous disease model. In contrast, at the lowest dose, 0.5×10^6, TR cells had a reduced ability to prevent disease, unlike monoclonal TR cells which remained effective (data not shown). The data suggested that frequency of TR cells specific for islets Ag cells might contribute to the efficacy of disease inhibition.

To test whether of TR cells derived from NOD CD4 cells were reactive to islet Ag, whole islets were isolated from the pancreata of NOD mice and the islet proteins were solubilized. This preparation was used to restimulate TR cells in vitro together with splenic APC. For comparison, we also tested responsiveness to proteins extracted from cells derived from PLN. As shown in Fig. 1*C*, islet Ag elicited IL-10 production by the TR cells, but as expected there was no apparent response to proteins from PLN. The data suggest that TR cells specific for relevant autoantigen within the polyclonal population may be specifically restimulated upon transfer in vivo, thereby eliciting an effector response that controls disease.

Because humans with T1D do not present clinically until after insulitis is established and symptoms of dysregulated glucose metabolism are present, we next asked whether adaptive TR cells could reverse diabetes when transferred into recently diabetic mice, 1 wk after a blood glucose reading ≥300 mg/dl. The results in Fig. 2*A* show a reversal rate of 67% at 4 wk after transfer of TR cells in a dose of 2×10^6/recipient ($n = 12$), which is comparable to the 73% diabetes reversal that was achieved with the same number of BDC 2.5 TR cells ($n = 11$) (data not shown). Furthermore, while the serum concentration of IL-10 dropped when diabetes developed, the levels of this cytokine returned to normal in mice that that regained normoglycemia in response to TR cells (Fig. 2*B*), but not in those that did not (data not shown). The data show that adaptive TR cells generated from $FoxP3^-$, $CD25^-CD4$ cells

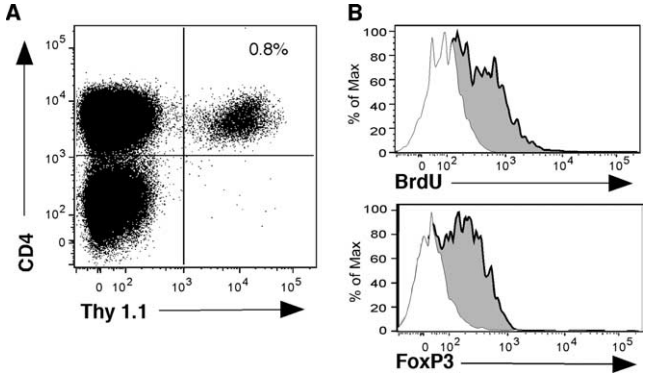

FIGURE 3. Maintenance of polyclonal adaptive TR cells. *A*, At >1 year after transfer of 2×10^6 polyclonal TR cells into 8-wk NOD mice ($n = 3$), donor cells were identified in lymphocytes pooled from lymphoid tissues by Thy1.1 and CD4 staining and flow cytometry after enrichment of CD4 cells. *B*, BrdU uptake (*top panel*) and FoxP3 expression (*bottom panel*) were determined by gating on Thy 1.1^+ donor cells. Unfilled histograms represent the isotype controls.

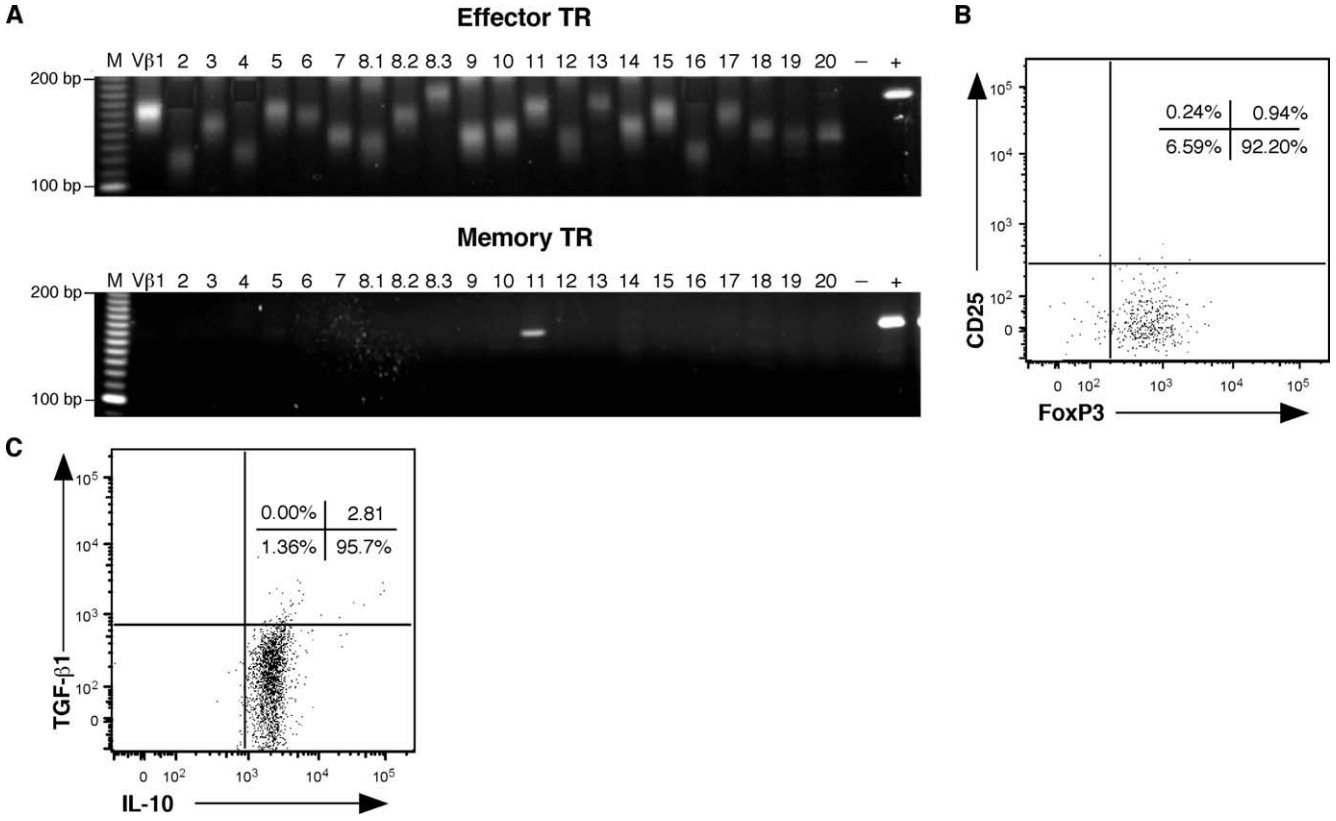

FIGURE 4. Vβ repertoire narrowing after adoptive transfer of adaptive TR cells. *A*, RNA was isolated from ex vivo-generated polyclonal effector (NOD Thy 1.1) TR cells (*top panel*) and from FACS-sorted Thy 1.1$^+$ memory TR cells that were reisolated at 4 mo after injection into nondiabetic 8-wk-old NOD mice ($n = 8$, 2×10^6/recipient) (*bottom panel*). TCR Vβ usage was analyzed by PCR. *B*, CD4 cells from 16-wk-old NOD recipients of NOD CD45.2 TR cells were isolated at 4 mo after cell transfer. Shown are CD25 and FoxP3 expression by Vβ11$^+$, CD45.2$^+$ donor cells. *C*, TGF-β1 and IL-10 production by the same donor cells as in *B* after stimulation with PMA and ionomycin for 5 h.

can regulate the spontaneous development of T1D when administered to adult animals, that such cells can be generated from prediabetic individuals, and that they have the capacity to re-establish normoglycemia. The results also demonstrate that loss of IL-10 is an important diagnostic indicator of progression to diabetes and that restoration of normal levels of this cytokine is associated clinically with the reversal of hyperglycemia and the protective function of TR cells.

Adaptive TR cells are maintained indefinitely by homeostatic turnover

To assess the long-term maintenance of TR cells, we examined NOD recipients for the presence of donor cells (Fig. 3*A*) >1 year after transfer. Donor TR cells were readily detected and the majority retained FoxP3 (Fig. 3*B*). Using BrdU to study their turnover, we also found that the donor cells continued to undergo

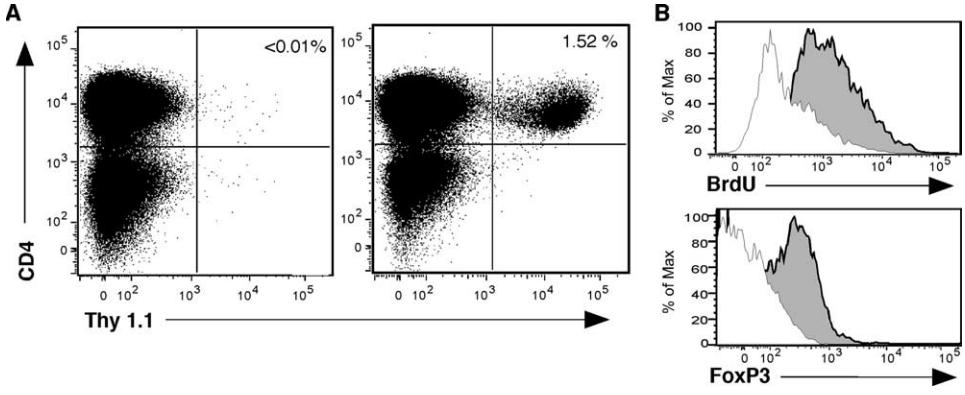

FIGURE 5. Expansion of Ag-specific adaptive TR cells to mimotope peptide challenge. *A*, Ex vivo-generated BDC 2.5 TR cells were injected into 8-wk-old NOD mice (2×10^6/recipient) ($n = 6$). At >1 year after transfer, donor cells were quantitated in CD4 cells enriched from pooled lymphoid tissues by flow cytometry using Thy1.1 and CD4 staining (*right panel*). Mice carrying BDC 2.5 TR cells for >1 year were challenged with the 1040-63 mimotope peptide with LPS at 6 days before analysis (*left panel*). *B*, To assess division, BrdU was administered from the time of immunization, and reisolated donor cells were analyzed for BrdU uptake (*top panel*) and FoxP3 expression (*bottom panel*). Unfilled histograms represent the isotype controls.

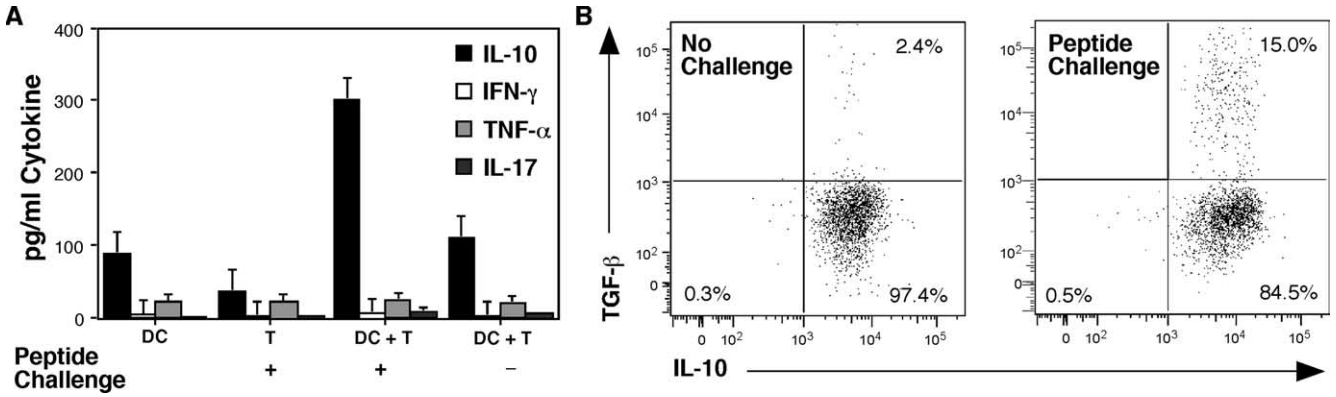

FIGURE 6. Cytokine secretion by in vivo-reactivated adaptive TR cells memory cells. *A*, BDC 2.5 TR cells were injected into 8-wk-old NOD mice (2×10^6/recipient). After 2 mo, the mice were challenged with the 1040-63 peptide and LPS ($n = 3$) or LPS alone ($n = 2$). Five days later, memory TR cells were reisolated from the lymphoid tissues by magnetic sorting. The cells (10^6) were stimulated with splenic DC (10^5) and 1040-63 peptide. Supernatants were tested at 48 h for cytokines by cytokine bead arrays. *B*, BDC 2.5 memory TR cells from recipients that were challenged with 1040-63 and LPS or were unimmunized were tested for expression of IL-10 and TGF-β1 by intracellular staining after restimulation for 5 h with PMA/ionomycin. Shown are plots gated on $CD4^+$, Thy 1.1^+ cells.

homeostatic division in the lymphoid compartment. These findings suggested a continued response to self-Ag in vivo and the development of persisting memory cells.

Polyclonal adaptive TR cells become established as oligoclonal memory cells

From these data, we predicted that selection of TR cells by self-Ag might occur over time in vivo. To test this prediction, we compared the usage of Vβ chains of the TCR by adaptive TR cells immediately after generation in vitro from NOD Thy 1.1 CD4 cells and again at 4 mo after transfer in vivo. As shown in Fig. 4*A*, adaptive TR cells generated from NOD CD4 cells were polyclonal as evidenced by the representation of multiple TCR Vβ chains. With time in vivo, however, the TR population became focused to usage of a single chain, Vβ11. This finding implies Ag recognition in vivo selects for the persistence and survival of an oligoclonal population of TR cells. Indeed, the $V\beta11^+$ cells were exclusively $FoxP3^+$, $CD25^-$ (Fig. 5*B*), and retained the capacity to produce IL-10 in response to stimulation immediately ex vivo (Fig. 5*C*), confirming that they expressed a phenotype identical with adaptive TR cells generated from BDC 2.5 CD4 cells (14). The data thus provide a rationale for comparisons with islet-specific adaptive BDC 2.5 TR cells where we could further study the function of memory cells using a mimotope peptide, 1040-63 (18).

Adaptive TR cells persist as functional memory cells

Because a hallmark of memory cells is the ability to greatly re-expand as effector cells after challenge with Ag, we immunized recipients of BDC 2.5 TR cells with peptide 1040-63, a treatment that does not cause diabetes (data not shown). As depicted in Fig. 5*A*, the memory TR population present at >1 year after adoptive transfer was nearly undetectable in magnetically enriched CD4 cells, but underwent ≥150-fold expansion by day 5 after immunization. Moreover, the majority of the TR cells underwent

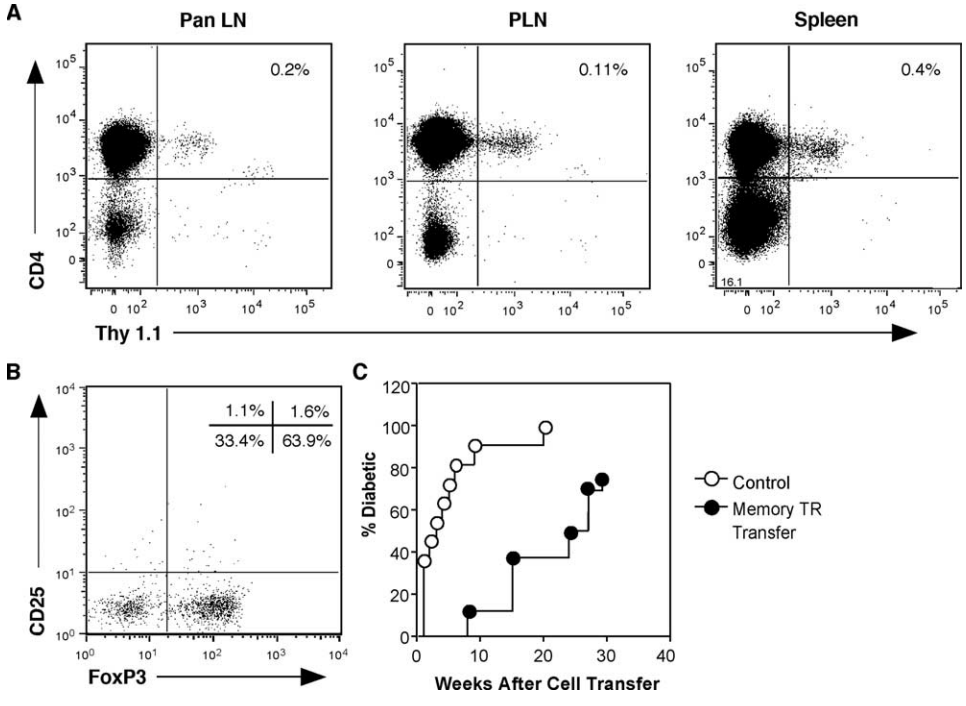

FIGURE 7. Persistence of protection against diabetes by adaptive TR memory cells. *A*, Islet-specific BDC 2.5 TR cells were injected into nondiabetic 8-wk NOD mice (2×10^6). After 2 mo, CD4 cells were enriched from lymphoid tissues and donor cells were quantitated by flow cytometry using Thy1.1 and CD4 staining. *B*, FoxP3 and CD25 expression was determined by gating on Thy 1.1^+ donor cells. *C*, Memory TR cells were reisolated 6–8 wk after transfer from recipients of BDC 2.5 TR cells and injected into new recipients at a dose of $0.2–1.0 \times 10^6$ cells ($n = 7$). The recipients were also given T cells from diabetic mice in a dose of 4×10^6/recipient. The mice were compared with age-matched controls that received diabetogenic T cells only ($n = 9$).

division as measured by uptake of BrdU from the time of immunization. The data demonstrate that adaptive TR cells retain characteristics of memory cells with the capacity for recall responses indefinitely. Importantly, the donor cells remained FoxP3$^+$ (Fig. 5*B*).

Cytokine responses of adaptive memory effector TR cells

To test the ability of memory effectors to produce cytokines, BDC TR cells were injected into adult NOD recipients that were challenged after 2 mo with the 1040-63 peptide and LPS, or were given only LPS. The donor cells were reisolated from the lymphoid tissues of these mice by magnetic sorting and stimulated by culture with splenic DC and the 1040-63 peptide. These in vivo-reactivated memory effector cells primarily secreted IL-10 (Fig. 6*A*). To verify that memory TR cells were themselves producing IL-10, the donor cells were tested by ICS following stimulation with PMA/ionomycin. As shown in Fig. 6*B*, the TR cells produced IL-10 immediately ex vivo both before and after peptide challenge, whereas recall with peptide in vivo was necessary for the production of TGF-β1. The maintenance of their ability to produce IL-10 and TGF-β1 demonstrates that the adaptive TR memory cells are phenotypically quite stable over time and retain responsiveness to specific Ag.

Adaptive TR cells retain the capacity to mediate protection against T1D

Although adaptive TR cells persisted indefinitely, their function might be altered over time with exposure to autoantigen. To address this question, BDC 2.5 TR cells were reisolated 6–8 wk after transfer to adult NOD recipients when they remained detectable (Fig. 7*A*). Like memory adaptive TR cells generated from NOD CD4 cells, these islet-specific TR cells expressed FoxP3, but lacked CD25 (Fig. 7*B*). These cells were injected into NOD recipients that were also given T cells from diabetic mice (4×10^6/recipient) to accelerate diabetes induction. As shown in Fig. 7*C*, the memory population transferred protection against diabetes in this acute model as measured by the delay in onset. These findings show that Ag-specific adaptive TR cells persist as functional protective memory cells. The results suggest that as memory cells, adaptive TR cells continue to contribute to control of the autoimmune response in T1D.

Discussion

In this study, we analyzed polyclonal adaptive/induced regulatory TR cells as immunotherapeutic agents for treatment of, and long-term protection against, T1D. We show that in the NOD mouse model of this human disease, polyclonal CD4 cells that acquire regulatory function and FoxP3 expression ex vivo contain cells reactive with islet Ag and not only reverse recent onset diabetes, but also persist indefinitely and have the capacity to actively protect against reoccurrence of disease. Remarkably, the establishment of memory is accompanied by a narrowing of the T cell repertoire to an oligoclonal population with usage of a single TCR β-chain, implying selection by Ag recognition in vivo. As a further indication of the clinical relevance of this model, we find that reversal of diabetes correlates with restoration of prediabetic levels of the immunodampening cytokine, IL-10, providing readout of protection. Our previous work showed that adaptive TR cells could be induced from BDC 2.5 cells from diabetic NOD Rag$^{-/-}$ mice which display diabetes at 3–4 wk of age due to the absence of innate TR cells (14). This result showed that precursors with the capacity to give rise to adaptive TR are present in individuals with T1D. Together, our findings show that polyclonal CD4 cells can be used not only to treat T1D by restoring normoglycemia, but also to vaccinate against re-emergence of diabetes.

Although several reports now show that adaptive TR cells generated ex vivo or in vivo control of autoimmune diseases (7, 23–26), whether such protection involves the generation of memory cells that contribute to the maintenance of tolerance has not been previously explored. Here, we show that as memory cells, adaptive TR cells become exclusively Vβ11$^+$, maintain FoxP3 expression without CD25, are indefinitely stable with respect the ability to control T1D, and produce the signature cytokines IL-10 and TGF-β1 (14). These cells behave as bona vide memory cells that are maintained by homeostatic turnover. Upon challenge with Ag, these cells have the capacity for extensive expansion and for development of effector function, as measured by cytokine production. Although they do not maintain CD25 after transfer in vivo, there are previous examples of TR cells that express FoxP3 but not CD25 (27), which suggests that unlike innate TR cells (28, 29), these adaptive TR are regulated independently of IL-2. Because memory CD4 cells depend on IL-7 for survival (30), we predict that this γ_c cytokine will regulate CD25$^-$ adaptive TR memory cells and that this property will account for their long-term maintenance.

It is becoming increasingly evident that adaptive TR cells are generated in vivo, particularly by interactions with DCs in mucosal lymphoid sites where regulation occurs via retinoic acid (24–26). In vitro, the development of adaptive TR cells via DCs is dependent upon TGF-β1 (12). Although many of the in vivo-generated populations are thought to be phenotypically indistinguishable from thymus-derived innate CD25$^+$ TR cells (31), accumulating evidence suggests that naturally occurring FoxP3$^+$ TR cells represent mixtures of innate and adaptive populations that can include both CD25$^+$ and CD25$^-$ cells. In addition, ex vivo-generated IL-10-producing FoxP3$^-$ cells such as Tr1 cells can have potent regulatory activity in T1D (11) and IL-10 production is associated with both Foxp3$^+$ and FoxP3$^-$ TR cells that develop in vivo (32). Indeed, IL-10 is a key cytokine made by memory adaptive TR cells that persist in vivo in our model, and its loss from the serum provides a clinical measure of progression to diabetes whereas its restoration to prediabetic levels is a readout of TR cell-mediated reversal of disease.

Although there are many studies of innate polyclonal TR cells (FoxP3$^+$, CD25$^+$) in autoimmune models, including T1D (33), these cells are not highly effective at reversing the full-blown autoimmune response such as at the time of diabetes onset (13). However, two studies showed that in vitro-expanded innate CD25$^+$ TR cells from BDC 2.5 CD4 cells control the development of T1D in NOD mice in response to diabetogenic T cells (34) and restore normoglycemia in diabetic NOD mice (13). These findings suggest that innate TR cells enriched for the relevant autoantigen are more effective as a cell-based therapy than are polyclonal cells. A recent report showed that islet-specific innate TR cells became undetectable with time and concluded that the cells did not persist but rather re-establish mechanisms of tolerance (15). However, this study did not explore whether innate TR cells could be re-expanded by Ag as shown here for adaptive TR cells. Our results suggest that adaptive TR cells contributed to long-term protection. This conclusion is strengthened by our observation that after several weeks of maintenance in vivo, adaptive TR cells transferred protection to new recipients. Moreover, our results show that polyclonal adaptive TR cells are maintained indefinitely. Overall, the data support the concept that innate and adaptive TR cells differ with respect to homeostatic regulation, as implied by the differences in CD25 expression. The ability to use adaptive instead of innate TR cells as a therapy obviates the requirement to select for,

and to greatly expand a minor cell population, and allows for the generation of protective memory.

Of note, a recent study using a nonautoimmune model examined ex vivo-induced adaptive TR cells generated from FoxP3$^-$CD4 cells of FoxP3 reporter mice and showed that FoxP3 is lost by polyclonal CD4 cells after adoptive transfer in vivo (35). Thus, our results suggest that exposure to Ag may be key to maintaining FoxP3 and regulatory function. Indeed, we demonstrate that polyclonal TR cells that contain islet-reactive cells and persist in higher numbers as memory cells than do those elicited from monoclonal BDC 2.5 CD4 cells. This suggests that the polyclonal population could be selected for clones with higher affinity TCRs that become more numerous with time. The TCR repertoire narrowing from polyclonal to oligoclonal (Vβ11$^+$) in vivo suggests that repeated low-level exposure to Ag under weakly immunogenic conditions may help to sustain not only self-renewal of memory TR cells but also their functional fitness (1).

The mechanisms engaged by memory adaptive TR cells to maintain control of the autoaggressive response in T1D could be several. They may include direct inhibition of autoreactive CD4 cell priming or effector function via TGF-β and IL-10 production. In addition, as memory cells with elevated frequencies and potentially enhanced responsiveness, TR cells might out-compete naive CD4 cells with pathogenic potential for access to DCs, and through such interactions may directly or indirectly alter APC function (13). Other molecules such as galectins (36) and IL-35 (37) that have been associated with innate CD25$^+$ TR cells could also function in the suppressive activity of adaptive TR cells. Furthermore, it is possible that adaptive TR cells could contribute to the functional restoration of innate TR cells through cytokine effects on their function or frequency, or both.

Our finding that adaptive TR cells can be generated from polyclonal CD4$^+$CD25$^-$ CD4 cells and used as a cell-based therapy to control T1D development in prediabetic NOD mice as well as to restore normoglycemia in mice with recent onset diabetes underscores that these cells may have significant potential for use as therapeutic agents because their generation is straightforward and the protection they afford is long-lived. Because the majority of adaptive TR cell-treated mice in which diabetes was reversed remained normoglycemic for several weeks, therapeutic administration of adaptive TR cells could extend the time during which additional treatment modalities could be pursued to achieve long-term control of T1D. It is also important that polyclonal adaptive TR cells give rise to persisting memory cells because studies of anti-CD3 administration as a treatment for T1D indicate that regulatory function is associated with both CD25$^+$ and CD25$^-$ CD4 cells when diabetes is reversed (38). In addition, systemic anti-CD3 treatment together with mucosal administration of a peptide epitope from the candidate autoantigen, proinsulin, led to the generation of Ag-specific FoxP3$^+$CD25$^+$ TR cells with the capacity to cause remission from diabetes (39). These findings suggest that innate and adaptive TR cells can work in concert to control the autoaggressive response in T1D. Thus, we predict that initial expansion of peripheral CD4 cells with islet Ag or candidate autoantigen in the presence of TGF-β1 may be an effective general strategy to generate adaptive TR cells that ultimately provide Ag-specific memory that controls pathogenic responses in T1D. As memory cells, adaptive TR cells may also limit the pathogenic consequences of responses to infections that trigger release of islet Ag, such as those caused by enteroviruses (40) which are linked to diabetes onset in humans (41). Thus, the capacity to exploit memory in adaptive TR may have far-reaching consequences for immune-based therapies of human autoimmune diseases.

Disclosures

The authors have no financial conflict of interest.

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