Rapid Publication

Antigen-Specific FoxP3-Transduced T-Cells Can Control Established Type 1 Diabetes

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$CD4^+CD25^+$ T-cells can be used to interfere with spontaneous autoimmune diseases such as type 1 diabetes. However, their low frequency and often unknown specificity represent major obstacles to their therapeutic use. Here we have explored the fact that ectopic expression of the transcription factor Foxp3 can confer a suppressor phenotype to naïve $CD4^+$ T-cells. We found that retroviral transduction of polyclonal CD4 T-cells with FoxP3 was not effective in interfering with established type 1 diabetes. Thus, more subtle and more organ-specific regulation might be required to prevent type 1 diabetes, as well as to avoid systemic immunosuppression. However, a single injection of 10^5 FoxP3-transduced T-cells with specificity for islet antigen stabilized and reversed disease in mice with recent-onset diabetes. By comparing FoxP3-transduced T-cells with various antigen specificities, it became clear that the in vivo effect correlated with specific homing to and activation in pancreatic lymph nodes and not with in vitro suppressor activity or cytokine production. Our results complement recent results on in vitro–amplified antigen-specific T-cells in ameliorating type 1 diabetes and suggest that FoxP3 transduction of expanded T-cells might achieve the same goal. *Diabetes* 54:306–310, 2005

Recessive tolerance toward a single autoantigen was so far unable to completely prevent spontaneous autoimmune diseases such as type 1 diabetes (1,2). Therefore, intervention by means of dominant tolerance seems more favorable, as autoreactive T-cells with various specificities can be modulated at the same time (3). $CD4^+CD25^+$ T-cells can be used to prevent type 1 diabetes (4,5). However, their low frequency and diverse specificity represent major obstacles to their therapeutic use. Ectopic expression of the transcription factor FoxP3 can confer a suppressor phenotype to naïve $CD4^+$ T-cells (6,7). However, retroviral transduction of FoxP3 to polyclonal $CD4^+$ cells was not effective in interfering with the disease in a nonlymphopenic model of recent-onset diabetes. Instead, a single injection of as few as 10^5 β-cell–specific FoxP3-transduced T-cells stabilized and reversed disease in mice with recent-onset diabetes. By comparing FoxP3-transduced T-cells with various antigen specificities, we show that the in vivo effect correlated with specific homing and activation in pancreatic lymph nodes and not with in vitro measures of suppressor activity or cytokine production. Our results complement recent results on naturally occurring, in vitro–amplified antigen-specific T-cells in type 1 diabetes (8,9). However, because naïve or activated cells are easier to isolate and amplify, our studies might be a novel approach to cellular immunotherapy of autoimmunity.

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Received for publication 11 October 2004 and accepted in revised form 24 November 2004.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

Posted on the World Wide Web at http://diabetes.diabetesjournals.org on 10 December 2004.

CFSE, 5,6-carboxyfluorescein diacetate-succinimidyl ester; eGFP, enhanced green fluorescent protein; IL, interleukin.

RESEARCH DESIGN AND METHODS

NOD/Ltj mice were purchased from The Jackson laboratories (Bar Harbor, ME) or bred in our facility. Diabetes incidence in females was 88%. Diabetes development was monitored by tail bleeding analyzed with the Accu-Chek Advantage device (Roche Diagnostics). Two subsequent measurements >200 mg/dl at least 2 days apart were considered to indicate type 1 diabetes. All animal experiments were performed according to National Institutes of Health guidelines, and experimental protocols were approved by the animal care and use committee of the MHH.

Generation of FoxP3 retrovirus. FoxP3 was cloned from mRNA of NOD splenocytes by RT-PCR using the following primers: FoxP3fw 5′-ACGTCTC GAGAACCCAATGCCCAACCCTAGGC-3′ and FoxP3rv 5′-CAGTCTCGAGTC AAGGGCAGGGATTGGAGCAC-3′. RT-PCR was performed with pfu-Turbo polymerase (Stratagene, La Jolla, CA). Sequence analysis was performed after subcloning into the retroviral vector to confirm identity. The 1.3-kb cDNA fragment was cloned into a modified Moloney murine leukemia virus (MMLV)-based retroviral vector (CMMP) (10) containing an enhanced green fluorescent protein (eGFP) under control of an internal ribosomal entry site (Clontech, Palo Alto, CA) via *Xho*I restriction sites of the vector. Retroviral supernatants were generated by transient transfection of the human embryonic kidney epithelial cell line 293T with these retroviral constructs and appropriate packaging plasmids. Viruses were pseudotyped with VSV-G to increase transduction efficiency in the NOD strain. High-titer retroviruses (>10^9/ml) were generated by ultracentrifugation of the supernatants (16.5 K for 2 h at 4°C). Concentrated supernatants were stored at −80°C. RT-PCR for FoxP3 expression was performed with the above primers on RNA isolated from retrovirally transduced or freshly isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ BDC T-cells using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany) including a DNase digestion step. β-Actin cDNA was amplified as an internal control using intron-spanning primers 5′-TGGAATCCTGTGGCATCC ATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

Retroviral transduction of $CD4^+$ T-cells. $CD4^+$ T-cells were enriched from splenocytes using SpinSep murine $CD4^+$ T-cell enrichment cocktail (StemCell Technologies); $CD25^+$ cells were removed using biotinylated anti-CD25 and SA-MACS beads (Miltenyi Biotech). In a 24-well plate, 7.5×10^5 $CD4^+CD25^-$ T-cells were incubated with 2×10^6 T-cell–depleted irradiated splenocytes (3,000 rad) in the presence of 5 μg/ml anti-CD3 (2C11) (Becton Dickinson) and 50 units/ml interleukin (IL)-2. Spin infection (1,000*g* at 4°C for 2 h) with high-titer VSV-G pseudotyped retrovirus at a multiplicity of infection of 5–10 was performed on days 2 and 3 in the presence of polybrene at 8 μg/ml. On day 4, eGFP $CD4^+$–positive cells were sorted.

Characterization of transduced T-cells

Inhibition assay. 2×10^4 sorted $CD4^+CD25^-$ T-cells (spleen) from NOD mice (6–8 weeks of age) were cocultured with various numbers of sorted $CD4^+CD25^+$ T-cells (spleen) from NOD mice or eGFP or FoxP3-transduced $CD4^+$ T-cells in the presence 2×10^5 T-cell–depleted irradiated (3,000 rad) splenocytes and anti-CD3 (clone 2C11 at 5 μg/ml). Culture was pulsed with [^{3}H]-thymidine for 24h after 72 h of incubation. Incorporated radioactivity was measured using scintillation fluid in a β-counter.

IL-10 enzyme-linked immunosorbent assay. After a 48-h culture, supernatants were harvested and assayed for IL-10 concentration by enzyme-linked immunosorbent assay with the Opti-EIA mouse Opti-EIA IL-10 kit (BD PharMingen). $CD4^+$ T-cells were labeled with 5,6-carboxyfluorescein diacetate-succinimidyl ester (CFSE) (Molecular Probes) by incubation for 10 min at 37°C in 10 μmol/l CFSE in PBS/0.1% BSA at a density of 1×10^7 cells per ml and injected into the lateral tail vein in a volume of 200 μl PBS (4×10^6 cells).

Antibodies and fluorescence-activated cell sorter analysis. Biotin-conjugated monoclonal antibodies to CD4 (H129.19), phycoerythrin-conjugated monoclonal antibodies to CD4 (GK1.5), CD25 (PC61) and allophycocyanin-conjugated monoclonal antibodies to CD4 (RM4–5), and CD25 (PC61) were purchased from Becton Dickinson. Fc receptor–blocking monoclonal antibody 2.4G2 was used as culture supernatant. Surface stainings were performed according to standard procedures at a density of $2–4 \times 10^6$ cells per 50 μl, and volumes were scaled up accordingly. Flow-cytometric analysis was performed on a FACSCalibur (Becton Dickinson) by using CELLQUEST (Becton Dickinson) and FlowJo (Treestar) software. Sorting of $CD25^-$ and $CD25^+$ populations as well as sorting of eGFP-positive cells was performed on a MoFlow cell sorter (DakoCytomation, Fort Collins, CO).

Histology. Histology and insulin staining was performed as described (11).

Statistical analysis. Results of proliferation assays and cytokine enzyme-linked immunosorbent assay were analyzed by student's *t* test. Differences in diabetes incidence were analyzed by χ quadrate test. The cumulative diabetes onset was compared by Kaplan-Meier analysis. All differences reported in the results were significant ($P < 0.05$).

RESULTS

FoxP3 transduction of T-cells causes in vitro suppressor function irrespective of antigen specificity. We developed a modified transduction protocol with multiple rounds of spin infection with high-titer retroviruses pseudotyped with VSV-G protein to overcome the low transduction efficiency seen in NOD T-cells, as compared with T-cells of other strains. Transduction efficiencies of 5–15% were achieved with this protocol. Therefore, FoxP3-transduced cells were subsequently enriched by cell sorting for eGFP-positive cells. We used a retroviral construct with FoxP3 expression driven by the retroviral long-terminal repeat, followed by an internal ribosomal entry site directing translation to an eGFP. Retroviruses with eGFP were used as controls. For the following experiments, we used either polyclonal $CD4^+$ T-cells from NOD mice ($CD4^+$), $CD4^+$ T-cells from T-cell receptor transgenic mice (TCR-tg) recognizing the p286 peptide of GAD65 I-A^{g7} restricted (12) (GAD), or $CD4^+$ T-cells from BDC2.5 TCR-tg mice recognizing an unknown islet antigen I-A^{g7} restricted (13) (BDC). Freshly isolated $CD4^+CD25^-$ from BDC-transgenic mice showed no FoxP3 expression by RT-PCR in contrast to $CD4^+CD25^+$ T-cells (Fig. 1*A*). FoxP3 expression was strongest in sorted FoxP3-transduced BDC T-cells, while eGFP-transduced BDC T-cells showed only very weak FoxP3 expression (Fig. 1*A*). All three T-cell populations showed an enhanced proliferation to anti-CD3 after transduction with the eGFP control virus, possibly due to the previous in vitro activation for retroviral transduction. After transduction with FoxP3, all three populations were anergic to anti-CD3 in vitro (Fig. 1*B*). FoxP3-transduced T-cells suppressed the proliferation of naïve $CD4^+CD25^-$ responder cells in coculture assays. The effect of suppression was independent of the antigenic specificity of the FoxP3-transduced T-cell population and was still effective at a 1:9 ratio of suppressor to responder cells (Fig. 1*C*). This highly efficient suppressor activity might be due to the strong activation of these cells during retroviral transduction. This effect was also observed with amplified Tregs after activation (8,9). IL-10 production after stimulation with anti-CD3 was only seen in FoxP3-transduced T-cells and not in T-cells without transduction or eGFP-transduced control cells (Fig. 1*D*). The level of IL-10 production after FoxP3 transduction was independent of the antigen specificity of the T-cells.

Site-specific homing and activation of FoxP3-transduced T-cells determines the in vivo effect. $CD4^+$ T-cells with above-mentioned specificities were labeled with CFSE and adoptively transferred into 10-week-old NOD females. Their specific homing and activation was studied after 72 h. BDC T-cells showed specific homing to (Fig. 2*A*) and activation in draining pancreatic lymph nodes (Fig. 2*B*). On the contrary, polyclonal $CD4^+$ and GAD-specific T-cells did not home specifically and were not activated in draining pancreatic lymph nodes, as measured by CFSE dilution. The same homing and activation pattern was seen after transfer into 16-week nondiabetic NOD females (with higher levels of insulitis and ~50% incidence of type 1 diabetes) (11), suggesting that the advanced autoimmune disease did not influence these parameters (data not shown).

Diabetes can be prevented in young NOD mice by numerous means. However, the later you interfere in the course of the disease, the more difficult it gets to prevent disease. In fact, there are only few ways to stabilize disease in NOD females that are already diabetic, i.e., either application of the CD1-restricted antigen α-galctosyl-ceramide (14,15), T-cell depletion with monoclonal antibodies (16), or application of anti-CD3 (17). It is most interesting that the latter approach is the only one with proven benefit in the ongoing human disease (18). Therefore, interventions at this late stage in the NOD model might be predictive of possible effectiveness in the human disease. We therefore used the model of recent-onset diabetes as the hardest test to prove therapeutic efficiency in advanced autoimmunity.

When we tried to recapitulate the results of FoxP3-transduced regulators obtained in lymphopenic models of autoimmune gastritis and colitis (6,7), we saw no effect of polyclonal FoxP3-transduced cells (Fig. 3), even if up to 1×10^6 cells were given to mice with recent-onset diabetes (data not shown). These results are remarkable because 2×10^5 polyclonal–nonactivated $CD4^+CD25^+$ cells given repeatedly could prevent the development of the disease (5). Likewise GAD-specific FoxP3-transduced T-cells did not interfere with the disease, and mice developed progressive type 1 diabetes. In contrast, a single injection of

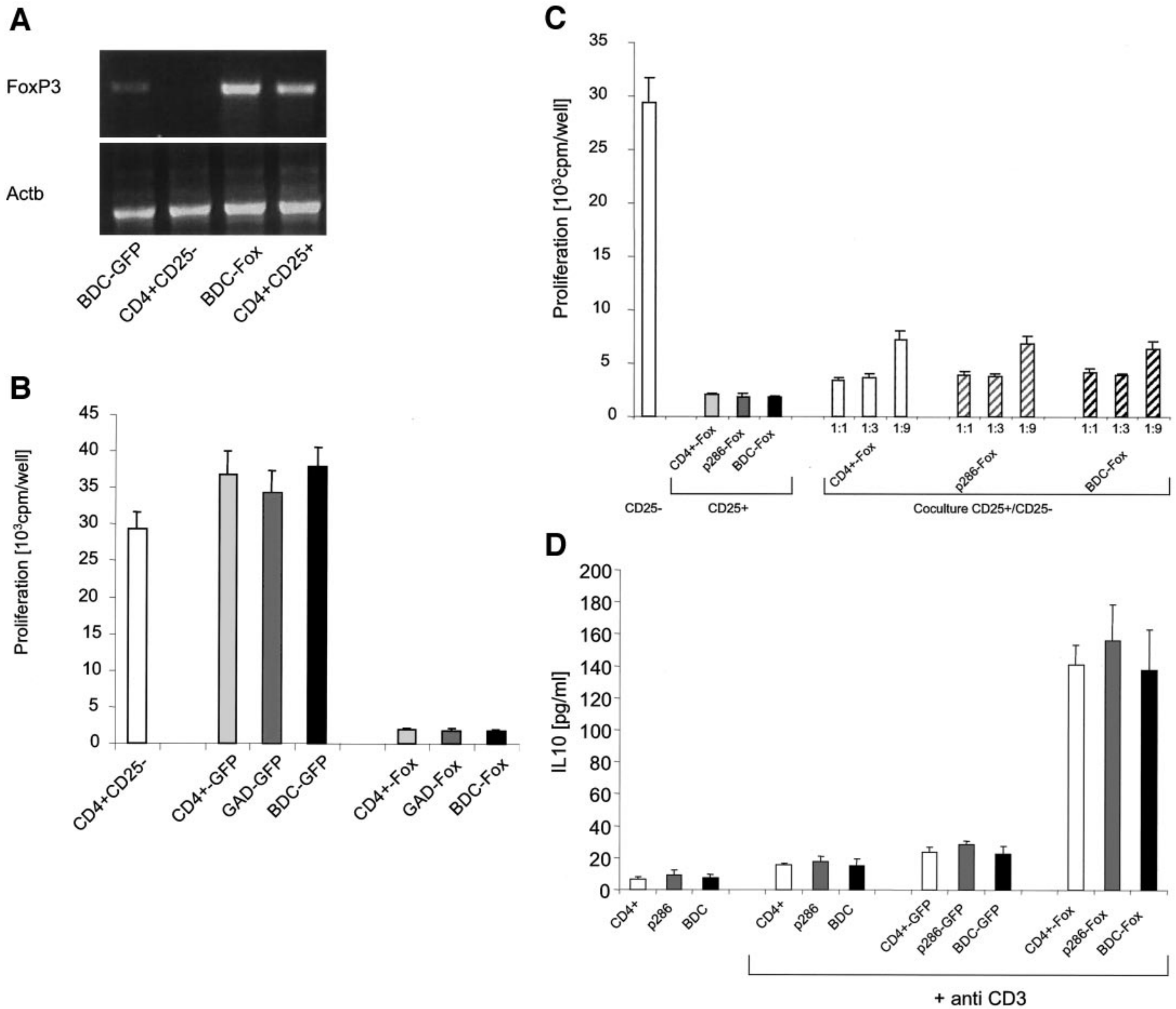

FIG. 1. Functional characterization of $CD4^+$ T-cells with various antigen specificities after transduction with FoxP3. *A*: Expression of FoxP3 mRNA by RT-PCR. β-Actin cDNA was amplified as an internal control. *B*: In vitro proliferation assay in response to anti-CD3 stimulation of polyclonal-naïve $CD4^+CD25^-$ cells (white bar) and polyclonal ($CD4^+$) (light gray bar) p286 of GAD65 (GAD)-specific (dark gray bar) and $CD4^+$ cells from BDC2.5 mice (black bar) after retroviral transduction with eGFP control virus or FoxP3. *C*: Coculture of 2×10^4 $CD4^+CD25^-$ cells with various numbers of FoxP3-transduced polyclonal (CD4), GAD p286–specific (GAD), and BDC2.5 cells. *D*: IL-10 production in response to anti-CD3 stimulation of polyclonal (CD4), GAD p286–specific (GAD), and BDC2.5 cells without retroviral transduction and after transduction with eGFP control virus and FoxP3.

1×10^5 BDC FoxP3-transduced Tregs stabilized the hyperglycemia for almost 6 weeks, after which blood glucose levels returned to levels <200 mg/dl ($P < 0.0001$ vs. CD4-Fox and p286-Fox) (Fig. 3). The single injection stabilized the disease for >100 days. The effect was due to FoxP3 transduction ($P < 0.0001$ vs. BDC-GFP), as mice receiving eGFP-transduced BDC2.5 cells showed a faster deterioration of their blood glucose levels. This also demonstrates that autoreactive effector cells in advanced autoimmune disease can be efficiently controlled, although there have been reports that these might be less susceptible to regulation than autoreactive T-cells in earlier stages of the disease (19). Histology performed after 100 days in mice receiving BDC FoxP3-transduced T-cells showed peri-insulitis with detectable β-cells (online appendix Fig. 1, available at http://diabetes.diabetesjournals.org). It is unclear whether glucose control is mediated by newly generated β-cells or by an improved function of the nondestroyed β-cells, but this was not the scope our experiments. Reisolation of FoxP3 transduced after 100 days was not possible, which may be due to the small number of initially transferred cells.

DISCUSSION

While FoxP3-transduced $CD4^+$ T-cells with various specificities had similar regulatory properties in in vitro assays, this did not predict their in vivo effectiveness. In fact, the in vivo effect was linked to specific homing and activation of T-cells in pancreatic lymph nodes. This demonstrates

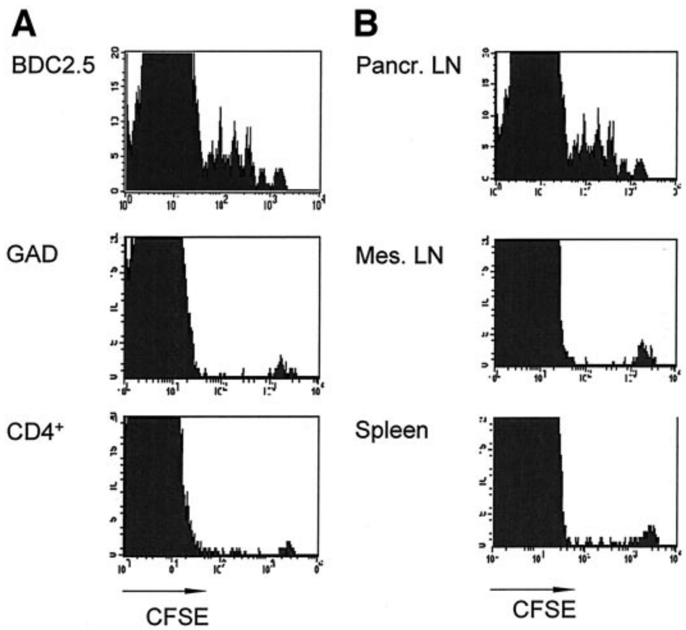

FIG. 2. Homing and proliferation of antigen-specific T-cell (*A*) CFSE staining of polyclonal (CD4), GAD p286–specific (GAD), and BDC2.5 cells in pancreatic lymph nodes 48 h after adoptive transfer. *B*: Homing and proliferation of BDC2.5 in various lymphatic compartments 48 h after transfer of CFSE-labeled $CD4^+$ T-cells.

once more (20) that the in vitro and in vivo properties of $CD4^+CD25^+$ Tregs might be quite different.

It has already been suggested that antigen-specific T-cells are important for the regulation of effector T-cells in nonlymphopenic models (8,9,21–23). It is remarkable that an originally diabetogenic T-cell (13), after extensive in vitro activation, can be turned into an efficient regulator of the disease by FoxP3 transduction.

One might be afraid to use similar experimental settings to treat human autoimmune diseases, as contaminations with nontransduced T-cells may worsen the autoimmune disease. To this end, even our sorted eGFP-positive cells may contain some untransduced cells. Second, due to low transduction efficiencies in the NOD strain, we did not sort $eGFP^{high}$ cells but rather all eGFP-positive cells, although it was shown that the $eGFP^{high}$ cells express higher levels of surface markers associated with $CD4^+CD25^+$ Tregs, such as CTLA4 and GITR (6). Finally, we transferred 10^6 FoxP3–transduced unsorted BDC2.5 T-cells (transduction efficiency of 15%) to a recently diabetic NOD female and could stabilize the disease (data not shown). Therefore, even small contaminations with activated effector cells might be controlled in vivo by the antigen-specific regulators. It was somehow surprising that Tregs specific for the p286 epitope of GAD65 were largely ineffective. Although p286-specific T-cells can be activated in vivo after immunization with the peptide (12), we did not see any specific homing or proliferation in pancreatic lymph nodes. This could either be due to the low affinity of the T-cell receptor, as these T-cells are negatively selected by thymic expression of GAD65 (12,24), or to the fact that GAD65 is just expressed at very low levels in β-cells of NOD mice (25). In line with this, it was shown that most of the T-cell receptor transgenic T-cells in these animals express an additional endogenous T-cell receptor α-chain to survive negative selection and that adoptive transfer of p286-specific T-cells rather delayed diabetes development in NOD mice (12).

Our results are in line with recently published studies using natural occurring antigen-specific $CD4^+CD25^+$ (9) or $CD4^+CD25^+CD62L^{high}$ (8) cells from T-cell receptor transgenic animals after in vitro expansion. As few as 5×10^4 in vitro–amplified Tregs prevented type 1 diabetes in a adoptive transfer into NOD-scid mice (9), while 10^7 in vitro–amplified BDC2.5 Tregs were necessary to stabilize disease in females with recent-onset diabetes (8). However, the latter two studies amplified naturally occurring Tregs, possibly induced in the thymus, while we investigated naïve or activated T-cells after transduction with FoxP3. It must be determined by future molecular studies whether these populations represent a similar phenotype. At least their development is completely different.

Although the results with in vitro–amplified Tregs are very exciting, amplification was only 10- to 100-fold. This means that a large number of naturally occurring antigen-specific Tregs must be initially obtained. But $CD4^+CD25^+$ T-cells and especially the $CD62L^{high}$ subfraction only rep-

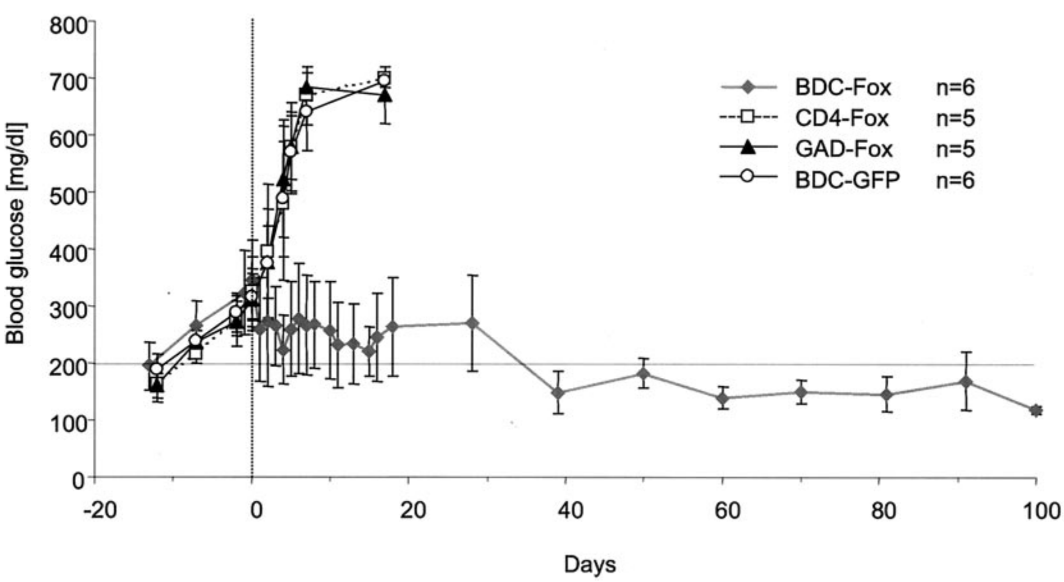

FIG. 3. Application of FoxP3-transduced $CD4^+$ T-cells in NOD mice with recent-onset diabetes. Blood glucose concentration in mice receiving 10^5 polyclonal (□), GAD65 p286–specific (▲), and BDC2.5 (◆) T-cells transduced with FoxP3 or BDC2.5 T-cells transduced with eGFP (○). Values are averages per group.

resent very small populations. It might thus be difficult to obtain relevant numbers of natural Tregs to a given antigen from blood of patients with a polyclonal T-cell repertoire. Besides this, we know that the precursor frequencies of autoantigen-specific T-cells are very low in spontaneous autoimmune diseases such as NOD (2,11) mouse or human (26) type 1 diabetes. One could therefore assume that the number of Tregs to a given antigen should be even smaller than the number of effector cells to the same antigen in individuals developing the disease.

On the other hand, we are able to detect antigen-specific autoreactive effector T-cells in mouse (2) and humans, and in vitro amplification of these cells is much more efficient than amplification of natural Tregs (8). Furthermore, ectopic FoxP3 expression can also confer a suppressor phenotype to $CD4^{-}CD8^{+}$ T-cells (27), thereby possibly enlarging the repertoire of Tregs to major histocompatibility complex I–restricted epitopes. Future studies must repeat the experiments with T-cells cloned from the polyclonal T-cell repertoire instead of using T-cell receptor transgenic T-cells. Although we demonstrated stability of a single injection of antigen-specific Tregs over 100 days, regulatory therapies might be less stable in humans than in our animal model. Therefore, repeated applications of Tregs might be necessary.

Our results show that few FoxP3-transduced antigen-specific naïve or activated Tregs can efficiently interfere with ongoing autoimmunity in a nonlymphopenic model. Therapeutic interventions that are effective in already-diabetic NOD females have so far been limited (14,15,17) but have already proven to be effective in human type 1 diabetes (18). We therefore believe that FoxP3-transduced antigen-specific T-cells might have a therapeutic potential in patients at risk to develop type 1 diabetes, patients with recent-onset type 1 diabetes, or patients after islet cell transplantation.

ACKNOWLEDGMENTS

This study was supported in part by grant 281541 of the Juvenile Diabetes Research Foundation Center for Islet Cell Transplantation at Harvard (to H.V.B. and E.J.), fellowship grant JA977/1-1 by the Deutsche Forschungsgemeinschaft (to E.J.), start-up grants of the HILF I program of the Hannover Medical School (to E.J.), and a project grant of the Deutsche Diabetes Gesellschaft (to E.J.).

The authors thank Y. Lee and R. Mulligan for providing the CMMP vector and X. Li and C. Jordanidis for excellent technical support.

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