In Vivo Control of Diabetogenic T-Cells by Regulatory CD4⁺CD25⁺ T-Cells Expressing Foxp3

Dorthe Lundsgaard, Thomas Lindebo Holm, Lars Hornum, and Helle Markholst

To understand the ability of regulatory T-cells to control diabetes development in clinically relevant situations, we established a new model of accelerated diabetes in young DP-BB rats by transferring purified T-cells from DR-BB rats made acutely diabetic. Transfer of 3, 5, 10, or 23 million pure in vitro-activated T-cells accelerated diabetes onset in >90% of the recipients, with the degree of acceleration being dosage dependent. Cotransfer of unfractionated leukocytes from healthy donors prevented diabetes. Full protection was achieved when protective cells were transferred 3–4 days before diabetogenic cells, whereas transfer 2 days before conferred only partial protection. Protection resided in the CD4⁺ fraction, as purified CD4⁺ T-cells prevented the accelerated diabetes. When CD25⁺ cells were depleted from these cells before they were transferred, their ability to prevent diabetes was impaired. In contrast, two million CD4⁺CD25⁺ cells (expressing Foxp3) prevented the accelerated diabetes when transferred both before and simultaneously with the diabetogenic T-cells. In addition, 2 million CD4⁺CD25⁺ T-cells prevented spontaneous diabetes, even when given to rats age 42 days, whereas 20 million CD4⁺CD25⁻ cells (with low Foxp3 expression) were far less effective. We thus demonstrated that CD4⁺CD25⁺ cells exhibit powerful regulatory potential in rat diabetes. Diabetes 54:1040–1047, 2005

Autoimmune destruction of pancreatic β-cells arises as a result of a loss of tolerance. Regulatory T-cells, particularly subsets of CD4⁺ T-cells, are essential for maintaining self-tolerance. Several forms of regulatory CD4⁺ cells are known: in vitro-generated Tr1 and Th3 and naturally occurring CD4⁺CD25⁺ cells. Regulatory CD4⁺CD25⁺ T-cells can control the expansion of other CD4⁺ subsets (CD25⁻) and pathogenic subsets (CD4⁺CD45RBhigh) in vitro (1–4). They also control naive CD8⁺ cells (5) and memory CD8⁺ cells (6,7). However, reports on their ability to control pathogenic cells in vivo are fewer and more recent. In vitro–generated regulatory CD4⁺ T-cell clones prevent colitis (8) and gastritis (9). Natural CD4⁺CD25⁺ cells prevent Th1- and Th2-induced colitis as well as Leishmania major infection (10), control active experimental autoimmune encephalomyelitis (11), and ameliorate established colitis (12).

In the NOD mouse model of human type 1 diabetes, regulatory T-cells were first defined as a subpopulation of cells expressing CD4⁺CD45RBlow or CD4⁺CD62L⁺ (13–15). Later, these regulatory T-cells were shown to co-express CD62L and CD25 (16).

In the other spontaneous model of type 1 diabetes, the DP-BB, regulatory cells have not been characterized with regard to their expression of CD25. Previously, spontaneous disease was prevented by a single transfer of spleen cells (17) and the prevention was enhanced by enrichment for CD4⁺ cells (18). However, transfer of leukocytes from normal rats has so far been successful only if the transfer takes place before the initiation of insulitis (i.e., at about age 1 month) (17,19); transfers conducted at 2 months accelerate diabetes (19). Protection is dependent on the engraftment of the ART2⁺ (formerly RT6) cells (20); in the control strain, the DR-BB, depletion of these induces diabetes (21). We recently showed that accelerated diabetes induced by the transfer of in vitro phorbol myristate acetate (PMA) plus ionomycin–activated unfractionated splenocytes from acutely diabetic donors is delayed by transfer of DR-BB–derived leukocytes (22).

In this study, we proposed to prevent diabetes using a new rat model where diabetes onset was accelerated by transfer of pure, in vitro PMA plus ionomycin–activated T-cells; that is, without activated antigen-presenting cells as were used in our previous study (22). Using pre-activated diabetogenic T-cells in the current study allowed us to explore a situation that is relevant to understanding how such already activated T-cells can be controlled in patients with ongoing β-cell destruction. Establishing the conditions required for preventing diabetes by cotransferring various leukocytes from healthy DR-BB rats allowed us to narrow a powerful regulatory potential to CD4⁺CD25⁺ T-cells. This new model in rats is well suited to testing preventive strategies for preclinical proof-of-concept, as such studies in mice alone have not been entirely predictive (23). Moreover, it allowed us to track diabetes-inducing and -protective subsets in vivo.
TABLE 1
Accelerated diabetes after transfer of purified, diabetogenic T-cells

<table>
<thead>
<tr>
<th></th>
<th>No transfer</th>
<th>3 $\times$ 10^6 cells</th>
<th>5 $\times$ 10^6 cells</th>
<th>10 $\times$ 10^6 cells</th>
<th>23 $\times$ 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes incidence</td>
<td>455 of 491 (93%)</td>
<td>18 of 18 (100)</td>
<td>30 of 30 (100)</td>
<td>32 of 34 (94)</td>
<td>3 of 3 (100)</td>
</tr>
<tr>
<td>Median age (days) at diabetes onset</td>
<td>76 (72–81)</td>
<td>58 (56–61)</td>
<td>52 (50–54)</td>
<td>50 (46–53)</td>
<td>47</td>
</tr>
</tbody>
</table>

Data are n (%) or median (interquartile range) and were pooled from 14 experiments. *P < 0.0001 vs. 3, 5, 10, and 23 $\times$ 10^6 cells; †P < 0.0004 vs. 5 $\times 10^6$ cells, P < 0.0023 vs. 10 $\times 10^6$ cells, and P < 0.0001 vs. 23 $\times 10^6$ cells; ‡P < 0.0001 vs. 23 $\times 10^6$ cells, but NS vs. 10 $\times 10^6$ cells; §NS vs. 23 $\times 10^6$ cells.

The purity of CD4 Oslo, Norway, according to the manufacturer’s description, except that only data not shown). Cell subset distribution was as previously described (22, as indicated below. CD90, which discriminates recent thymic emigrants from activated, pure T-cells. The percentage of CD4 T-cells positive for CD25 was 69 ± 4% being CD8^+ T-cells, 69 ± 4% being CD8^+ T-cells and 44 ± 8% of the CD8^+ T-cells. A minor fraction of T-cells (6 ± 2%) expressed the CD4 T-cell receptor or anti-CD25 antibody and then streptavidin-APC conjugated and processed for flow cytometry, as previously described. Foxp3 expression in CD4^+CD25^+ and CD4^+CD25^− T-cells. RNA was extracted from an aliquot of the purified CD4^+CD25^+ and CD4^+CD25^− T-cells (before in vivo testing) with an RNA Microrep Kit (Stratagene). Quantitative differences in the levels of Foxp3 were determined using semiquantitative RT-PCR on cDNA made with Superscript (Life Technologies). ß-28]-labeled dCTP was added to the PCRs, which were then run on standard sequencing gels (Gel-Mix 6; Life Technologies). Primer-specific bands were quantitated using a Typhoon 8600 Variable Mode Imager (Amersham-Pharmacia Biotech, Little Chalfont, U.K.). Primers specific for Foxp3 were 5'-GCTTTGTGCTGT GCGGAGAC-3' and 5'-GTTTCTGAAATGAGGCAAT-3' (from GenBank accession no. XM_228771), and primers specific for the housekeeping gene G6pd were 5'-GACCTGACAGCTCCAATCCAC-3' and 5'-CACGACCTCAGTACAAAGGG-3' (from GenBank accession no. NM_017000) were used as internal control in all reactions.

Statistical analysis. Frequency data were analyzed by a logrank test and differences in T-cell ratios were evaluated by Student’s t test. Equal or nonequal variance was evaluated by the F test.

RESULTS
Characterization and transfer of purified diabetogenic T-cells to prediabetic DP-BB rats. Before the transfer, the frequency of T-cells among reactivated, live cells from diabetic DP-BB donors was 96 ± 3%, with >85% CD26^L^+ (69 ± 4% being CD4^+ and 31 ± 4% being CD8^−) (n = 10). The percentage of CD4^+ T-cells positive for CD25 was 55 ± 5%; 77 ± 7% expressed the CD4 blast marker CD134 and <2% expressed CD122. Among the CD8^+ T-cells, 69 ± 9% were positive for CD25. The marker CD90, which discriminates recent thymic emigrants from more experienced T-cells, was expressed on 10 ± 7% of the CD4^+ T-cells and 44 ± 16% of the CD8^+ T-cells. A minor fraction of T-cells (6 ± 2%) expressed ART2 on the day of transfer.

We transferred these in vitro–activated, pure T-cells intravenously into DP-BB rats age 25–35 days and screened the rats for diabetes. Doses of 3, 5, 10, and 23 $\times 10^6$ T-cells all resulted in an accelerated onset of diabetes compared with no transferred cells (Table 1). Diabetes
Control of diabetes-inducing cells by unpurified cells from healthy donors. We then tested the ability of unfractionated leukocytes from healthy DR-BB rats to prevent accelerated diabetes induced by transfer of $10^4$ purified T-cells (Fig. 1). We transferred cells containing $64 \times 10^6$ T-cells as a means to prevent spontaneous diabetes (22). Subgroup analysis of the protective cells before transfer was similar to that previously described (22). Transfer of protective cells 3 days before rats received diabetogenic cells resulted in 0 of 4 rats becoming diabetic, while transfer 4 days before resulted in 1 of 10 rats becoming diabetic by age 62 days; transfer of only diabetogenic cells resulted in 0 of 4 rats becoming diabetic, whereas transfer 3 days before rats received protective cells resulted in 32 of 34 rats developing diabetes by age 42–59 days (Fig. 1). Transfer of protective cells 2 days before diabetogenic cell transfer produced a partial but significant prevention of accelerated diabetes, as 4 of 8 rats developed diabetes by age 43–51 days. In all experiments, a group of DP-BB rats receiving only protective cells was included; of these, 97% ($n = 29$) were protected from developing spontaneous diabetes (data not shown).

Protection resides in the CD4$^+$ subset of T-cells. To test whether the observed protection resides in the CD4$^+$ compartment of T-cells, we purified CD4$^+$ T-cells from mLNs by negative methods. The CD4$^+$ subset displayed a similar distribution of ART2$^{\text{high}}$ and CD25$^+$ cells before and after purification (65 and 8.5%, respectively). We transferred different numbers of these cells 4 days before transferring diabetogenic cells (Table 2). The $43 \times 10^6$ CD4$^+$ T-cells corresponded to the number present in the previous experiment with unfractionated cells. Accelerated diabetes induced by $5 \times 10^6$ diabetogenic T-cells was effectively prevented by $43 \times 10^6$ CD4$^+$ T-cells, whereas accelerated diabetes induced by $3 \times 10^6$ diabetogenic T-cells was prevented by both $21.5 \times 10^6$ and $10 \times 10^6$ CD4$^+$ T-cells. Two rats developed diabetes late in life; thus, the rats were protected against diabetes for the first period after transfer, but this protection was not lasting. The rest of the cotransfer recipients all remained free of diabetes up to at least age 120 days (Table 2).

Homing of diabetogenic T-cells and CD4$^+$ protective T-cells. To evaluate the migration pattern of the diabetogenic and protective T-cells, we transferred protective CFDA-SE$^+$-labeled CD4$^+$ T-cells and then 4 days later transferred diabetogenic CTO-labeled T-cells. We found that 2 or 4 days after the last transfer diabetogenic cells had homed to panLNs, mLNs, and cLNs as well as the spleen, but with a significant preference for the panLNs (Fig. 2A). Protective cells displayed distinct CFDA-SE dilution, indicating cell divisions in all lymph nodes (Fig. 2B) and were found at equal frequencies (11–21% of total live T-cells), with no difference between panLNs and other lymph nodes irrespective of transfer of protective cells only or of both cell types (data not shown). The lymph node cells were harvested 7 days after transfer of protective cells alone and stained with an anti-CD25 antibody (Fig. 2C). Both dividing and dividing fractions of the CD4$^+$ T-cells contained cells that coexpressed CD25.

Protection of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ subsets in the accelerated model. We next attempted to elucidate whether protection resided within the CD25$^+$ or CD25$^-$ subset of the $21.5 \times 10^6$ CD4$^+$ T-cells. We transferred protective T-cells 4 days before transferring $3 \times 10^6$ diabetogenic T-cells (Fig. 3A). Diabetes onset was signifi-

### TABLE 2
Prevention of accelerated diabetes by prior transfer of pure CD4$^+$ T cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Diabetogenic T-cells $\times 10^6$</th>
<th>Protective CD4$^+$ T-cells $\times 10^6$</th>
<th>Rats</th>
<th>Rats rendered diabetic before age 120 days</th>
<th>Degree of protection against diabetes (%)</th>
<th>Age at diabetes onset (days)</th>
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<tbody>
<tr>
<td>1A</td>
<td>5</td>
<td>None</td>
<td>12</td>
<td>12</td>
<td>100</td>
<td>52 (49–53)</td>
</tr>
<tr>
<td>1B</td>
<td>None</td>
<td>43</td>
<td>10</td>
<td>0</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>1C</td>
<td>5</td>
<td>43</td>
<td>11</td>
<td>1</td>
<td>91</td>
<td>82</td>
</tr>
<tr>
<td>1D</td>
<td>5</td>
<td>21.5</td>
<td>2</td>
<td>0</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>2A</td>
<td>3</td>
<td>None</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>58 (58–59)</td>
</tr>
<tr>
<td>2B</td>
<td>3</td>
<td>21.5</td>
<td>3</td>
<td>0</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>2C</td>
<td>3</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>83</td>
<td>109</td>
</tr>
</tbody>
</table>

Data are median (interquartile range) unless otherwise indicated. DP-BB rats (age 32–35 days) received CD4$^+$ T-cells in the indicated amounts. After 4 days, diabetes-inducing T-cells were transferred in the indicated amounts. Data are pooled from four experiments. $^*P < 0.0001$ vs. groups 1B and 1C, $P < 0.0175$ vs. group 1D; $^1P < 0.0045$ vs. group 2B, $P < 0.0003$ vs. group 2C.
significantly delayed in rats receiving $2 \times 10^6$ CD4$^+$CD25$^+$ (1 of 6 developed diabetes at age 93 days), compared with controls receiving diabetogenic T-cells only (8 of 8 developed diabetes at age 52–66 days). We also observed that five of eight rats receiving $20 \times 10^6$ CD4$^+$CD25$^+$ T-cells became diabetic at age 59–76 days, which was significantly different from the finding in controls (Fig. 3A).

Next, we performed the protective transfers simultaneously with diabetogenic transfer of $3 \times 10^6$ T-cells (Fig. 3B). Diabetes onset was significantly delayed in rats receiving both diabetogenic and $2 \times 10^6$ CD4$^+$CD25$^+$ T-cells (2 of 12 became diabetic at age 78 and 85 days, respectively) compared with rats receiving diabetogenic cells only (8 of 9 developed diabetes at age 57–71 days). However, this was not the case in rats receiving both diabetogenic and $20 \times 10^6$ CD4$^+$CD25$^-$ cells (5 of 11 developed diabetes at age 63–97 days).

**Protection of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ subsets in the spontaneous diabetes model.** Because $2 \times 10^6$ CD4$^+$CD25$^+$ T-cells appeared to harbor a powerful regulatory potential, we tested whether transfer of these cells to otherwise untreated DP-BB rats could prevent spontaneous diabetes (Fig. 4). When the cells were transferred to 14 rats age 27 ± 2 days (Fig. 4A), only 3 rats became diabetic (at age 91–103 days) as opposed to 93% in rats not receiving the transferred cells (age 77 ± 10 days). Moreover, only 2 of 18 rats became diabetic (age 71 and 85 days, respectively) among littermates that received transfer of the reciprocal subset of $20 \times 10^6$ CD4$^+$CD25$^-$ T-cells.

When $2 \times 10^6$ CD4$^+$CD25$^+$ T-cells were transferred to 10 rats age 42 days, none became diabetic (Fig. 4B). By contrast, 6 of 10 littermates became diabetic at age 83–91 days after the reciprocal subset of $20 \times 10^6$ CD4$^+$CD25$^-$ T-cells were transferred, which was significantly different from the former group as well as from the controls.

**Foxp3 expression in CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ subsets.** Because the transcription factor, Foxp3 is specifically expressed in CD4$^+$CD25$^+$ regulatory T-cells in mice and humans (24–27), we evaluated Foxp3 mRNA levels in our cell subsets by RT-PCR. We found that the CD4$^+$CD25$^+$ regulatory T-cell subset expressed 40-fold more message for Foxp3 than the CD4$^+$CD25$^-$ subset (Fig. 5). Moreover, Foxp3 levels were almost undetectable in cells used for diabetogenic transfers (data not shown).

**DISCUSSION**

We previously observed that unfractionated cells from healthy donors could not fully prevent diabetes induced by unpurified PMA plus ionomycin—activated splenocytes, but only delayed onset (22). In this study, we found that such protective cells prevent diabetes induced by puri-
fied PMA plus ionomycin–activated T-cells. Thus, there appears to be a large number of cells within diabetes-inducing unpurified splenocytes that cannot be controlled. What subset this is needs to be formally tested, but antigen-presenting cells that are activated by PMA plus ionomycin are obvious candidates. In this study, however, we focused on defining subsets within the unfractionated cells that protect against diabetes in the purified T-cell model and against spontaneous diabetes because of the clinical relevance of these situations.

Indeed, transfer models have been widely used for studying diabetes development in BB rats (22,28–32). In the present study, we extended these models and showed that a wide range (3 to 23 × 10^6) of reactivated, pure diabetogenic T-cells is able to accelerate diabetes in young DP-BB rats. The finding that the high number (23 × 10^6) is associated with more rapid diabetes development contrasts with the inflammatory bowel disease transfer model studied by Barthlott et al. (33), where only 1 × 10^6 or fewer cells transferred colitis. In that model it appears that massive homeostatic expansion of colitis-inducing cells is required within the recipient for disease to develop. In our model this seems not to be the case.

We found that the transfer of protective leukocytes needs to precede the transfer of diabetogenic T-cells to be 100% effective. There were ~43 × 10^6 CD4⁺ T-cells within the unfractionated pool, which contained considerable and normal fractions of both ART2^high⁺ and CD25⁺ cells and, in an interesting finding, as many as 17 × 10^6 CD45RC^high⁺ cells (data not shown), which are known to transfer wasting disease and autoimmunity, including diabetes (34–36).

We further found that pure CD4⁺ T-cells control accelerated diabetes. This finding is new, as previously it has been reported only that the transfer of cells enriched for diabetogenic cells of 1:3 seems to approach the lower limit for complete prevention. When we fractionated CD4⁺ T-cells according to their level of CD25 expression, we found that pure CD4⁺CD25⁻ T-cells protected the DP-BB recipient fully when they were transferred before diabetogenic cells.

From our data with 10 × 10^6 protective, pure CD4⁺ cells containing <1 × 10^6 CD4⁺CD25⁺ T-cells and 3 × 10^6 diabetogenic T-cells, we can deduce that a ratio of protective to diabetogenic cells of 1:3 seems to approach the lower limit for complete prevention. When we fractionated CD4⁺ T-cells according to their level of CD25 expression, we observed that CD4⁺CD25⁻ cells at a ratio of 2:3 protective to diabetogenic cells prevent diabetes. This protection was evident whether cells were transferred before or together with the diabetogenic subset. This finding correlates with a true regulatory phenotype (37) compared with other models of autoimmunity in which regulatory T-cells are able to prevent adoptively transferred disease at similar ratios (11,33,38).
The most important finding was that CD4⁺CD25⁺ T-cells were able to prevent spontaneous diabetes development during the interval where the activation of autoreactive cells is believed to occur (19); that is, as late as age 42 days. Previous studies have shown that reconstitution by transfer of purified and normal T-cells into DP-BB recipients age 2 months precipitates the onset of diabetes rather than confers protection (19). In contrast, we show that transfer of the reciprocal 20 × 10⁶ CD4⁺CD25⁻ subset is far less protective, although the diabetes incidence is lowered. Other studies (39,40) in rat models of induced diabetes also point to a role for the CD4⁺CD25⁺ regulatory T-cells. Thus, CD4⁺CD25⁺ T-cells protect in the virus-induced diabetic DR-BB model (39). In the adult thymectomy and split-dose gamma irradiation PVG rat model, CD4⁺ single positive thymocytes expressing CD25 control diabetes, whereas peripheral CD4⁺ T-cells both positive and negative for CD25 were found to have regulatory functions (40). The fact that we also observed some protective potential in the CD4⁺CD25⁻ fraction supports the hypothesis that regulatory CD4⁺CD25⁻ cells also exist in rats, as in mice (38).

In the NOD mouse, simultaneous cotransfer of CD4⁺CD25⁺ T-cells and autoreactive cells isolated from inflamed pancreatic islets only delay diabetes development (16). This apparent discrepancy may be explained by the source of regulatory T-cells in the NOD mouse, as these, and those in all reported experiments, are harvested from prediabetic mice that later in life develop diabetes.

The fact that CD25 is also expressed on conventionally activated T-cells hampers its use as a marker for regulatory cells. In contrast, the transcription factor Foxp3 ap-
pears to be a specific marker, as it has been found only in cells with regulatory function and is expressed in naturally occurring CD4+CD25+ cells in both mice (24–26) and humans (27). Recently, Foxp3-expressing CD4+CD25+ T-cells were shown to prevent diabetes in a mouse model (41), and retroviral transduction of Foxp3 in nonregulatory T-cells rendered these cells capable of inhibiting colitis (24,25). We found Foxp3 to be upregulated in rat CD4+CD25+ T-cells compared with CD4+CD25− T-cells. Ours is the first study to report that Foxp3 is also a marker of naturally occurring regulatory CD4+CD25+ T-cells in the rat.

The transfer of CD4+ T-cells into the lymphopenic recipients was associated with immediate and massive expansion of the protective cells (Fig. 2), as evidenced by the transfer of as few as 2 × 10^6 CD4+CD25+ T-cells to 42 day-old DP-BB rats completely prevented diabetes. Because we tracked the autoreactive cells with CTO, we could not detect the number of cell divisions. However, in the inflammatory bowel disease model, it was shown that CD4+CD25+CD45RBlow regulatory T-cells regulate cell division in the CD4+CD45RBhigh compartment early after cotransfer, and that they themselves display a limited potential for expansion (38). It was thus demonstrated that CD4+CD25+ T-cells can regulate other CD4+ T-cells in lymphopenic recipients. In contrast, it has not been previously shown that regulatory cells control autoreactive effector CD8+ T-cells in vivo, albeit CD4+CD25+ T-cells can prevent intra-islet differentiation of CD8+ T-cells into effector cytotoxic T-lymphocytes in the Tet− tumor necrosis factor-α/CD80 B6 model for type 1 diabetes (44). In DP-BB rats, both CD4 and CD8 T-cells act synergistically in inducing β-cell destruction (28); our observation that this can be prevented by CD4+CD25+ T-cells is new.

We further found that diabetogenic T-cells preferentially homed to the panLNs. The ability to enter the lymph nodes correlated well with the high levels of CD62L on the cells before transfer. In NOD mice, the panLNs have been shown to be important for diabetes development (45); however, the involvement of CD62L for homing in transfer models is not clear (46,47).

In human type 1 diabetes, loss of β-cell mass is believed to have considerably slower kinetics than in the BB rat model. Thus, the finding that regulatory cells controlled highly activated diabetogenic cells in our models suggests that they could possibly do so in human type 1 diabetes also. Tolerogenic strategies (achieved by T-cell–depleting antibodies) have been able to halt or prevent human type 1 diabetes, apparently by reducing the number of autoreactive T-cells and thus favoring the number of regulatory T-cells (48,49). Because the feeding of insulin β-chain has been shown to slow disease progression in NOD mice via induction of bystander immune suppression (50), another possibility would be to increase the number of regulatory cells in humans.

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