Autoimmune Diabetes Onset Results From Qualitative Rather Than Quantitative Age-Dependent Changes in Pathogenic T-Cells

Sylvaine You,[1,2] Mériam Belghith,[1,2] Stephen Cobbold,[3] Marie-Alexandra Alyanakian,[1,2] Christine Gouarin,[1,2] Samia Barriot,[1,2] Corinne Garcia,[2] Herman Waldmann,[3] Jean-François Bach,[1,2] and Lucienne Chatenoud[1,2]

Diabetogenic T-cells can be detected in pre-diabetic nonobese diabetic (NOD) mice after transfer in NOD-SCID recipients. Here we demonstrate that 6-week-old pre-diabetic NOD mice, >2 months before disease onset, already harbor pathogenic T-cells in equal numbers to overtly diabetic animals. The delay in diabetes appearance is explained by the presence of regulatory CD4+ CD25+ T-cells that control diabetogenic effectors and that are, in our hands, transforming growth factor (TGF)-β–dependent. Our present results suggest, however, that diabetes onset is only partly explained by a decline in this regulatory T-cell activity. Another major factor appears to be the progressive resistance of diabetogenic cells to TGF-β–dependent mediated inhibition. We propose that progression to overt disease correlates with the pathogenic T-cell's escape from TGF-β–dependent T-cell–mediated regulation. ***Diabetes*** **54:1415–1422, 2005**

Insulin-dependent diabetes (type 1 diabetes) is a genetically controlled autoimmune disease caused by selective destruction of insulin-secreting β-cells by pathogenic T-cells (1). In nonobese diabetic (NOD) mice, a spontaneous model of type 1 diabetes, pathogenic T-cells are easily assessed by adoptive transfer into syngeneic immunodeficient recipients (1–5). In NOD mice, rupture of self-tolerance is first evidenced at 3–4 weeks of age when the initial wave of mononuclear cells infiltrates pancreatic islets that is ~3–4 months before the first signs of overt disease, as assessed by glycosuria and hyperglycemia (1,6,7). During this quite long phase (i.e., pre-diabetes), insulitis progresses until ~12–14 weeks of age as a "benign" process that is not associated with massive β-cell destruction (1). Although major progress has been made in our knowledge on the candidate autoantigens (8–10) and the forces driving the emergence of diabetogenic T-cells (1,11–17), there are still uncertainties about the mechanisms underlying the progression to irreversible β-cell destruction. There is compelling evidence to show that regulatory T-cells mediating transferable tolerance actively control diabetogenic effectors (18–24). However, is this just a game of numbers? Does diabetes onset exclusively result from either the decrease in regulatory T-cells over time or from the progressive expansion of β-cell–specific T-cell effectors overriding the control afforded by regulatory T-cells?

Our current results point to an alternative possibility, which is that there are key age-dependent differences in effector T-cells that are qualitative rather than quantitative and render them progressively insensitive to T-cell–mediated regulation. This insensitivity involves progressive unresponsiveness of effector T-cells to the immunoregulatory cytokine transforming growth factor (TGF)-β.

RESEARCH DESIGN AND METHODS

NOD mice (K^d, $I\text{-}A^{g7}$, D^b) and NOD-SCID mice were bred in our animal facility under specific pathogen-free conditions. Glycosuria and glycemia were monitored using colorimetric strips (Glukotest and Hemoglukotest; Boehringer-Mannheim).

Fluorescence-activated cell sorting analysis. Lymphocytes were stained for surface markers and analyzed by flow cytometry. Antibodies to TGF-β (2G.7) were purified and fluoresceinated in our laboratory. Anti–glucocorticoid-induced tumor necrosis factor receptor (GITR) antibodies were kindly provided by Dr. Cobbold (Sir William Dunn School of Pathology, Oxford, U.K.). CD62L, CD25, CD4, and CD103 (α_E integrin subunit) antibodies were obtained from PharMingen-BD (San Diego, CA).

Cell preparations. Splenocytes were isolated from 4- or 6-week-old or diabetic NOD mice. Splenocytes were then purified on the basis of CD62L, CD25, or CD4 expression using magnetic bead cell sorting (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purity of the sorted cells was 90–97%, and recovery ranged from 50 to 70%.

Adoptive cell transfers. Recipients were adult 6-week-old NOD-SCID mice. Animals were injected intravenously with either a single cell population or, in the case of cotransfer experiments, a mixture of two distinct populations. The precise cell numbers used varied depending on the experiments and are detailed in the RESULTS section.

In vitro proliferation assays. Cells were cultured in complete RPMI 1640. $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells were seeded in 96-well microplates (2×10^4 cells/well) and stimulated with soluble anti-CD3 antibody (2.5 μg/ml 145-2C11; provided by J.A. Bluestone) in the presence of mitomycin-treated antigen-presenting cells (APCs). Neutralizing antibodies to TGF-β were added to the cocultures (10 or 50 μg/ml 2G.7). For other experiments, $CD4^+CD25^-$ and $CD4^+CD25^-CD62L^-$ T-cells were incubated with APCs, anti-CD3 antibody, and recombinant active TGF-β (0–4,000 pg/ml; R&D Systems, Abingdon, U.K.). For "criss-cross" cocultures, $CD4^+CD25^+$ T-cells from 6-week-old animals were coincubated with $CD4^+CD25^-$ T-cells isolated from diabetic

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From the [1]Institut National de la Santé et de la Recherche Médicale (INSERM) U580, Paris, France; the [2]Faculté de Medicine, René Descartes Paris 5, Paris, France; and the [3]Sir William Dunn School of Pathology, Oxford, U.K.

Address correspondence and reprint requests to Pr. Lucienne Chatenoud, INSERM U580, Hôpital Necker, 161 rue de Sèvres, 75015 Paris, France. E-mail: chatenoud@necker.fr.

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APC, antigen-presenting cell; GITR, glucocorticoid-induced tumor necrosis factor receptor; IFN-γ, γ-interferon; IL, interleukin; TGF, transforming growth factor.

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NOD mice, and, inversely, $CD4^+CD25^-$ T-cells from 6-week-old animals were coincubated with $CD4^+CD25^+$ T-cells isolated from diabetic NOD mice. After 72 h at 37°C, cells were pulsed with [^{3}H]thymidine (Amersham). Data were expressed as the percent inhibition, deduced as: percent inhibition = {1 − [cpm ($CD4^+CD25^-$ plus $CD4^+CD25^+$)/cpm $CD4^+CD25^-$]} × 100, where cpm is the counts per minute.

Similar assays were performed in 24-well plates using transwells (Costar). 5,6-carboxyfluorescein diacetate-succinimidyl ester–labeled $CD4^+CD25^-$ T-cells from 6-week-old NOD mice (2×10^5 cells/well) were stimulated with anti-CD3 antibody and APCs. $CD4^+CD25^+$ T-cells (2×10^5 cells/well) were added in the transwell with APCs. After 4 days, cells were stained with CD4 antibodies, and the $CD4^+CD25^-$ T-cell proliferation was analyzed by fluorescence-activated cell sorting.

Cytokine production. $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells from 6-week-old or diabetic mice were used and cultured as described in the proliferation assay. "Age-matched" or "crossed" cocultures were performed, and supernatants were recovered after 24, 48, and 72 h of culture. Interleukin (IL)-4, IL-10, and γ-interferon (IFN-γ) were measured by enzyme-linked immunosorbent assays, and TGF-β1 was measured using a DuoSet kit (R&D Systems).

Real-time quantitative RT-PCR. DNase I–treated total RNA from tissues was prepared using the SV Total RNA isolation system (Promega, Madison, WI). Reverse transcription was performed using a proStar kit with random hexamers (Stratagene, Cedar Creek, TX). Real-time quantification was performed using gene-specific fluorogenic probes and a Universal MasterMix kit (PE-Applied Biosystem). PCR and TaqMan analysis were performed using the ABI/PRISM 7700 sequence detector system (PE-Applied Biosystem, Warrington, U.K.). Multiplex PCRs were performed using appropriate gene-specific primers and VIC-labeled CD3γ and FAM-labeled *foxP3* probes. Standard curves of cDNAs from normal spleen were used to calibrate C_T to amounts of *foxP3* and to normalize cDNAs on each 96-well plate run. Normalized values for mRNA expression were calculated as (*foxP3* mean)/(CD3γ mean).

Statistical analysis. The occurrence of diabetes was plotted using the Kaplan-Meier method, i.e., a nonparametric cumulative survival plot. The statistical comparison between the curves was performed using the log-rank (Mantel-Cox) test. In addition, results were analyzed using Student's *t* test when needed.

RESULTS

Diabetogenic cells are present in large numbers long before onset of overt diabetes. Adoptive transfer of $CD4^+$ and $CD8^+$ spleen cells from diabetic NOD mice into immunodeficient syngeneic recipients, i.e., NOD-SCID mice, triggers diabetes (18,20). Using unseparated splenocytes, disease transfer was not observed after injection of cells from 4-week-old donors (data not shown). Diabetes transfer was, however, obtained on injection of cells from pre-diabetic donors aged ≥6 weeks, but later and at a lower rate when compared with that observed on transfer of total spleen cells from diabetic donors (Fig. 1*A*). This difference could be explained either by the progressive accumulation of diabetogenic T-cell effectors present in sufficient numbers only in overtly diabetic animals, or alternatively by the presence in pre-diabetic animals of subsets of regulatory T-cells mediating active tolerance whose number and/or functional capacity would decline with disease progression. To distinguish between these two hypotheses, transfer experiments were performed using splenocytes from NOD mice at different ages depleted of regulatory T-cells, i.e., expressing the α-chain of the IL-2 receptor (CD25) or L-selectin (CD62L) (3,20, 22–24). Results showed that diabetes incidence was similar in recipients injected with equal numbers of $CD25^-CD62L^-$ (e.g., enriched for effector cells) from 6-week-old NOD or diabetic mice (including same proportions of $CD4^+$ and $CD8^+$ T-cells); however, there was a slight delay in disease onset in recipients of effector cells from the younger mice (Fig. 1*B*). Such a pattern was comparable to that observed on transfer of total splenocytes from diabetic mice

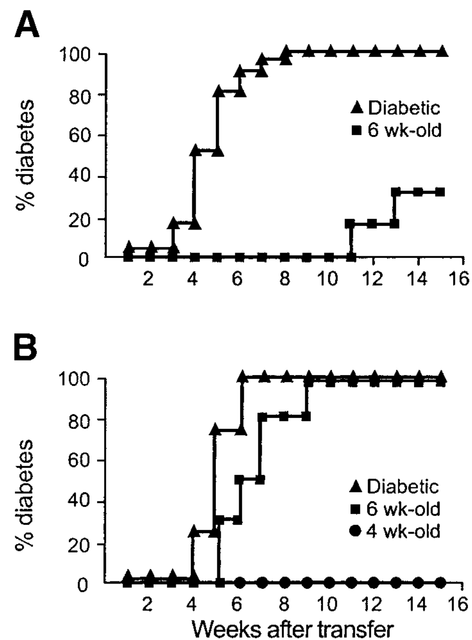

FIG. 1. Capacity of different NOD mice cell subsets to transfer diabetes. Cells were isolated from 4- or 6-week-old or overtly diabetic NOD mice. Diabetes was monitored in NOD-SCID recipients (n = 10–20 per group) injected with total spleen cells (5×10^6) (*A*) or $CD25^-CD62L^-$ spleen cells (4×10^5) (*B*). Splenocytes from 6-week-old NOD mice exhibit low diabetogenic capacities that are significantly increased after $CD25^+$ and $CD62L^+$ T-cell depletion ($P < 0.002$). wk, week.

(Fig. 1*A*). In contrast, $CD25^-CD62L^-$ cells from pre-diabetic 4-week-old NOD mice did not transfer diabetes.

These data show that fully competent diabetogenic $CD25^-CD62L^-$ cells appear at the periphery between 4 and 6 weeks of age. Moreover, diabetogenic cells from pre-diabetic 6-week-old mice and those from diabetic mice appear equally potent at transferring diabetes. Because spontaneous diabetes only appears 10 weeks later, these results stress the role of regulatory T-cells expressing CD25 and/or CD62L in controlling disease progression.

In vivo sensitivity of diabetogenic effectors to T-cell–mediated regulation. We next explored whether the deficient suppression observed in mice with overt disease was caused by either decreased functional capacity of regulatory T-cells or diminished effector cell sensitivity to regulatory cells, or both. To this end, we performed criss-cross in vivo experiments to analyze the sensitivity of diabetogenic cells from young or old NOD mice to regulatory T-cells recovered from young or old NOD donors.

In all of the experiments $CD25^-CD62L^-$ spleen cells, depleted in $CD25^+$ and $CD62L^+$ subsets, were used as effector diabetogenic cells. Figure 2 shows that $CD4^+CD25^+$ T-cells (e.g., enriched for regulatory T-cells) from young NOD mice were less efficient at controlling diabetes transfer induced by effector cells from diabetic mice than that elicited by injection of effector cells from young pre-diabetic donors. This was observed using equal numbers of effector cells ($CD25^-CD62^-$ cells) from two origins that, as mentioned above, were equally potent at transferring diabetes (Fig. 1*B*). Moreover, $CD4^+CD25^+$ T-cells from diabetic NOD mice significantly inhibited disease transfer elicited by effector cells from young NOD mice, albeit less efficiently than $CD4^+CD25^+$ T-cells from 6-week-old animals (Fig. 2). Thus, at the time of diabetes onset, the age-

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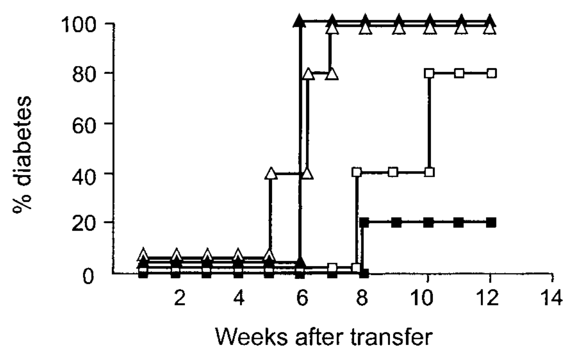

FIG. 2. Cotransfers using age-matched and unmatched diabetogenic and regulatory T-cells. CD25$^-$CD62L$^-$ cells were isolated from 6-week-old or diabetic NOD mice and cotransferred (4×10^5) into NOD-SCID recipients with CD4$^+$CD25$^+$ T-cells (5×10^5) isolated from either young or diabetic animals (n = 5 per group). Significant protection was observed only when the CD25$^-$CD62L$^-$ cells used were recovered from young mice ($P < 0.002$ and $P < 0.003$ with regulatory cells from 6-week-old and diabetic mice, respectively). ■, CD25$^-$CD62L$^-$ 6-week-old plus CD25$^+$ 6-week-old mice; □, CD25$^-$CD62L$^-$ 6-week-old plus CD25$^+$ diabetic mice; ▲, CD25$^-$CD62L$^-$ diabetic plus CD25$^+$ diabetic mice; △, CD25$^-$CD62L$^-$ diabetic plus CD25$^+$ 6-week-old mice.

dependent reduction in the functional capacity of regulatory cells is only partial and parallels a significantly reduced sensitivity of diabetogenic effectors to T-cell–mediated regulation.

Age-dependent decline in the in vitro sensitivity of CD4$^+$CD25$^-$ T-cells to CD4$^+$CD25$^+$ T-cells. To further investigate the cellular mechanisms mediating the resistance of effector cells to immunoregulation, we performed in vitro studies using the conventional non–antigen-specific coculture model. CD4$^+$CD25$^+$ T-cells have the capacity to suppress the proliferation of CD4$^+$CD25$^-$ T-cells in coculture (25–28). As detailed in Fig. 3*A*, a consistent age-dependent decrease in in vitro suppressive ability was observed in NOD mice. CD4$^+$CD25$^+$ T-cells from young 6-week-old pre-diabetic mice efficiently inhibited the proliferation of CD4$^+$CD25$^-$ T-cells from the same donors (Fig. 3*A*). In contrast to this, a significantly lower suppression was found using CD4$^+$CD25$^+$ cells from 8-week-old NOD mice cocultured with CD4$^+$CD25$^-$ T-cells from the same donors (Fig. 3*A*). Moreover, very low or no suppression (ranging from 0 to 10%) was found in 50% of cases when CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were collected from the same overtly diabetic donors (Fig. 3*A*). These low suppression indexes were not explained by an insufficient ratio of suppressor to effector cells because no significant effect was observed when increasing the CD25$^+$-to-CD25$^-$ ratio (Fig. 3*B*).

The in vitro functional capacity of CD4$^+$CD25$^+$ T-cells recovered from NOD H-2^k and B6 H-2^{g7} mice (used as controls) at 6, 16, and 35 weeks of age was not significantly different from that found in young pre-diabetic NOD mice and did not vary with aging, suggesting a genetic dependency of the decline of the suppressive ability in the NOD strain (data not shown). In normal non–autoimmune-prone mice, the CD4$^+$CD25$^+$-mediated suppressor activity was cell-to-cell contact dependent (25). Using 5,6-carboxyfluorescein diacetate-succinimidyl ester–labeled CD4$^+$CD25$^-$ T-cells, we observed that also in NOD mice the proportion of dividing cells significantly increased when CD4$^+$CD25$^+$ T-cells were physically separated from their targets using the transwell device (Fig. 3*C*).

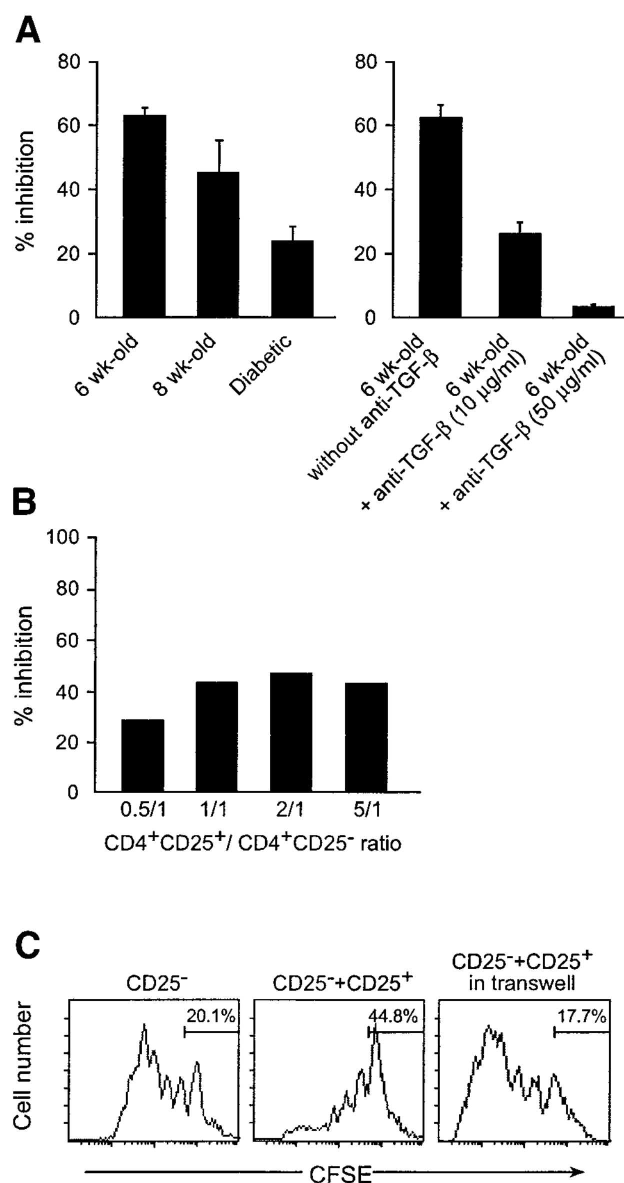

FIG. 3. In vitro functional capacity of CD4$^+$CD25$^+$ T-cells. *A*: We used 6-week-old (n = 22), 8-week-old (n = 5), and diabetic NOD (n = 17) mice. Cocultures were performed by incubating CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T-cells from the same mouse at a 1:1 ratio with APCs and anti-CD3 antibody. A significant difference was observed when comparing mean values between 6-week-old and diabetic NOD mice ($P < 0.0001$) and between 6- and 8-week-old NOD mice ($P < 0.042$). The addition of anti–TGF-β antibody, 2G.7, reversed the suppression in a dose-dependent manner (no antibody: 60.9 ± 2.6% inhibition; + 2G.7 10 µg/ml: 32.8 ± 3.6% inhibition, $P < 0.005$; + 2G.7 50 µg/ml: 4.5 ± 1.2% inhibition, $P < 0.001$). *B*: Suppression of CD4$^+$CD25$^-$ T-cell proliferation by increasing proportion of CD4$^+$CD25$^+$ T-cells. *C*: In vitro inhibition of CD4$^+$CD25$^-$ T-cells by CD4$^+$CD25$^+$ T-cells is cell contact–dependent. The proliferation of 5,6-carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labeled CD4$^+$CD25$^-$ T-cells from 6-week-old NOD mice was analyzed by immunofluorescence after being incubated 4 days with young NOD CD4$^+$CD25$^+$ T-cells separated or not separated by a transwell membrane. The results were representative of 5–8 experiments. wk, week.

We next analyzed whether the deficient in vitro suppression observed in diabetic mice was caused not only by decreased functional capacity of regulatory T-cells but also by a diminished sensitivity of proliferating cells to immunoregulation. Criss-cross in vitro experiments were performed, using a similar strategy to that described above

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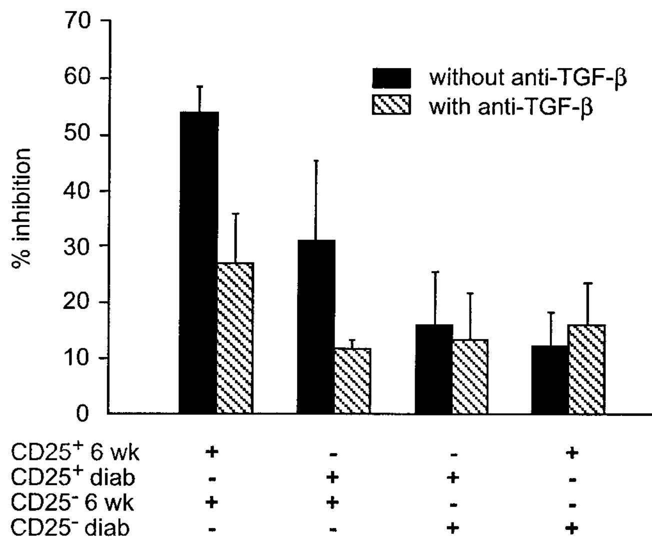
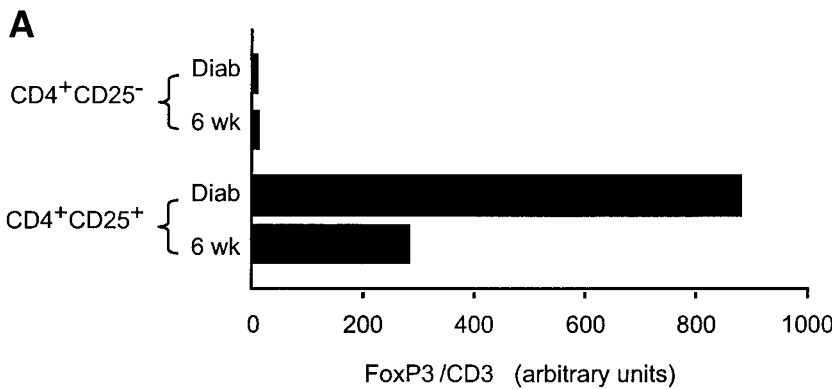


FIG. 4. Cocultures using age-matched and unmatched diabetogenic and regulatory T-cells. The suppressive capacity of CD4$^+$CD25$^+$ T-cells from 6-week-old NOD mice was measured after incubation with CD4$^+$ CD25$^-$ T-cells from either 6-week-old or diabetic mice. Similarly, CD4$^+$CD25$^+$ T-cells from diabetic mice were cultured with either diabetic or young NOD CD4$^+$CD25$^-$ mouse T-cells. The effect of anti–TGF-β antibody (10 μg/ml) was also analyzed. Data represent the mean of five experiments. diab, diabetic; wk, week.

for the in vivo cotransfers. Figure 4 shows that CD4$^+$ CD25$^+$ T-cells from diabetic NOD mice suppressed proliferation of CD4$^+$CD25$^-$ T-cells from young pre-diabetic mice, though less efficiently than CD4$^+$CD25$^+$ T-cells from young NOD mice. Conversely, CD4$^+$CD25$^+$ T-cells from young NOD mice were as ineffective as CD4$^+$CD25$^+$ T-cells from diabetic mice at suppressing the proliferation of CD4$^+$CD25$^-$ T-cells from overtly diabetic donors.

We analyzed the expression of several regulatory T-cell candidate markers on the various T-cell subsets that showed a protective or diabetogenic ability. *FoxP3* mRNA was detected in CD4$^+$CD25$^+$ T-cells but not in CD4$^+$ CD25$^-$ T-cells (Fig. 5*A*). Surprisingly, CD4$^+$CD25$^+$ T-cells from diabetic animals expressed higher levels of *foxP3* mRNA compared with those from 6-week-old NOD mice. The GITR was also exclusively expressed on CD4$^+$CD25$^+$ T-cells (Fig. 5*B*), with no significant difference between young and diabetic NOD mice. Finally, 18–20% of CD4$^+$ CD25$^+$ T-cells expressed CD103 (α_E subunit of the $\alpha_E\beta7$ integrin); similar levels and intensity were observed in young and diabetic NOD mice.

Age-dependent sensitivity of effector cells to TGF-β. TGF-β has been shown to be involved in CD4$^+$ T-cell–mediated regulation both in vivo and in vitro (25,29–34). We examined the ability of neutralizing anti–TGF-β antibodies to alter T-cell–mediated regulation in NOD mice. First, we observed that the in vitro suppression was abrogated in a dose-dependent manner on addition of an anti–TGF-β antibody (2G.7; 49.1 ± 4.5 and 92.2 ± 2.1% reversal of suppression [mean ± SE] for 10 and 50 μg/ml of antibody, respectively) (Fig. 3*A*). This is also in keeping with our data showing that in vivo, in the cotransfer model described above, administration of anti–TGF-β antibodies re-



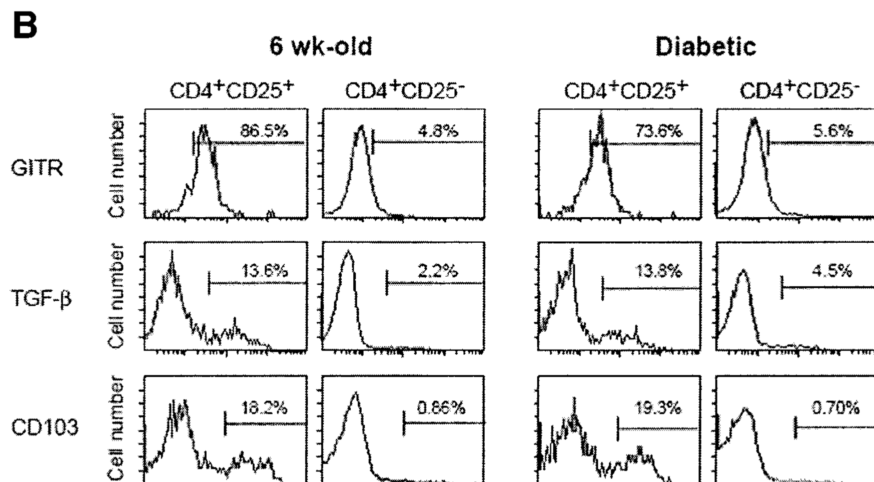


FIG. 5. *foxP3* mRNA, GITR, membrane TGF-β, and CD103 expression by regulatory T-cells from NOD mice. *A*: RNA samples from CD4$^+$CD25$^+$ and CD4$^+$ CD25$^-$ T-cells isolated from 6-week-old or diabetic NOD mice were analyzed for *foxP3* and normalized to CD3γ. Samples were tested in triplicate and plotted as the means ± SE. *B*: Fluorescence-activated cell analysis of lymph node CD4$^+$ CD25$^+$ and CD4$^+$CD25$^-$ T-cells for the expression of GITR, CD103, and membrane TGF-β. wk, week.

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TABLE 1
Cytokines produced by $CD4^+CD25^+$ or $CD4^+CD25^-$ T-cells from 6-week-old or diabetic NOD mice

	$CD25^+$ 6 weeks	$CD25^-$ 6 weeks	$CD25^+$ diabetic	$CD25^-$ diabetic
IL-4 (pg/ml)	13.1	11.9	12.1	21.2
IL-10 (pg/ml)	116.2	24.4	68.3	61.6
IFN-γ (pg/ml)	342.7*	593.6†	1,226.8*	2,788.3†
TGF-β (pg/ml)	133.1*	106.2	45.4‡	126.7

$CD4^+CD25^+$ or $CD4^+CD25^-$ T-cells from 6-week-old or diabetic mice were stimulated with CD3 antibody and APCs. Single-cell cultures were performed, and supernatants were recovered 24, 48, and 72 h later. *,†,‡$P < 0.05$ for comparisons between cell subsets.

versed protection from disease transfer afforded by $CD4^+CD25^+$ T-cells from young NOD mice (data not shown).

On in vitro stimulation with anti-CD3 antibody, $CD4^+CD25^+$ T-cells from young pre-diabetic NOD mice produced TGF-β at levels significantly higher that those produced by the equivalent subset from diabetic mice (Table 1). In addition, membrane TGF-β was spontaneously expressed, in the absence of any previous stimulation, on 13% of $CD4^+CD25^+$ T-cells independent of the age of the mouse (Fig. 5*B*). $CD4^+CD25^-$ T-cells expressed very low levels of membrane TGF-β (2–4%).

IL-10 was produced in higher amounts by $CD4^+CD25^+$ T-cells from young animals, whereas the opposite pattern was observed for IFN-γ (Table 1). We also observed that $CD4^+CD25^-$ T-cells from diabetic animals produced fourfold higher levels of IFN-γ compared with those recovered from 6-week-old mice. Similarly, in coculture, higher TGF-β concentrations were observed when $CD4^+CD25^+$ T-cells from young animals were present, whereas high IFN-γ production was observed when $CD4^+CD25^-$ T-cells from diabetic animals were present (Table 2).

Given the central role of TGF-β, it was important to study more directly its influence on effector cells. This was performed in vitro by adding increasing amounts of recombinant TGF-β on anti-CD3 antibody–stimulated $CD4^+CD25^-$ and $CD4^+CD25^-CD62L^-$ T-cells from pre-diabetic and diabetic NOD mice. Importantly, for all T-cell subsets studied, strictly identical cell numbers were used. Proliferating $CD4^+CD25^-$ and $CD4^+CD25^-CD62L^-$ T-cells from 6-week-old NOD mice were significantly more sensitive to TGF-β–mediated inhibition than those from diabetic mice (Fig. 6). Ten to twenty times more recombinant

TABLE 2
Cytokines produced during age-matched or unmatched cocultures using $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells

	$CD25^+$ 6 weeks + $CD25^-$ 6 weeks	$CD25^+$ diabetic + $CD25^-$ 6 weeks	$CD25^+$ diabetic + $CD25^-$ diabetic	$CD25^+$ 6 weeks + $CD25^-$ diabetic
IL-4 (pg/ml)	18.1	20.5	29.8	26.7
IL-10 (pg/ml)	251.1	146.2	190.9	337.4
IFN-γ (pg/ml)	434.2	558.8	2,184.7	1,815.9
TGF-β (pg/ml)	121.3	60.6	69.6	120.7

$CD4^+CD25^+$ or $CD4^+CD25^-$ T-cells from 6-week-old or diabetic mice were stimulated with CD3 antibody and APCs. "Age-matched" or "crossed" cocultures were performed, and supernatants were recovered 24, 48, and 72 h later.

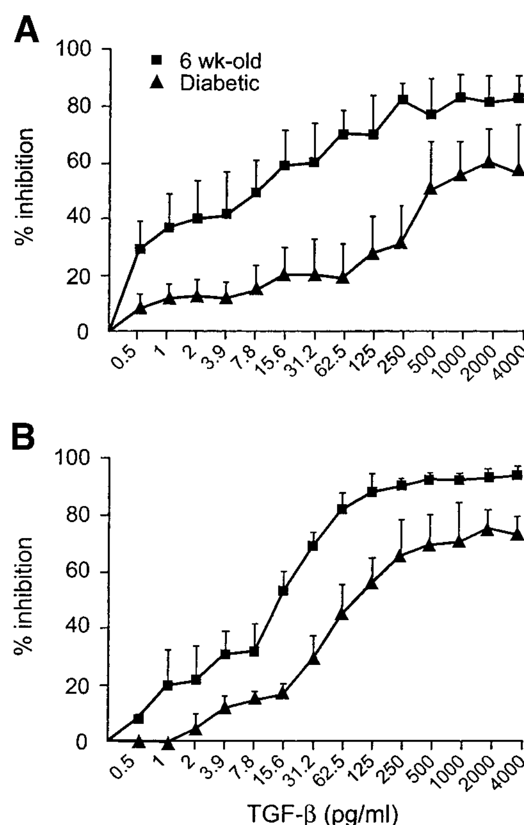

FIG. 6. Suppression of $CD4^+CD25^-$ T-cell proliferation by recombinant human TGF-β. $CD4^+CD25^-$ T-cells (*A*) or $CD4^+CD25^-CD62L^-$ T-cells (*B*) from 6-week-old or diabetic NOD mice were stimulated with CD3 antibody and APCs and incubated with increasing amounts of recombinant human TGF-β. The results were representative of four experiments. wk, week.

TGF-β was necessary to suppress proliferation of pathogenic cells recovered from diabetic animals compared with those recovered from young animals. Expression of TGF-β receptors I and II was investigated by Western blot, and both were detected on $CD4^+CD25^-CD62L^-$ T-cells recovered from young NOD mice as well as diabetic animals (data not shown). In addition, the expression of TGF-β receptor signaling molecules such as phosphorylated Smad2, also assessed by Western blot, did not appear to significantly differ in extracts from effector T-cells recovered from young NOD mice and diabetic mice (data not shown).

DISCUSSION

Direct evidence for loss of β-cell–specific self-tolerance as assessed by mononuclear cell infiltration of pancreatic islets is present very early in NOD mice (3 weeks of age), a long time before onset of overt disease (1,6,7). So far, it has been generally assumed that this is mainly caused by a "game of numbers" in which islet-specific pathogenic T-cells progressively accumulate, overriding T-cell–mediated regulation that controls pathogenic aggressors (7,18–24). This is also compatible with the observation that NOD mice have a general failure in tolerance caused by defective negative selection in the thymus (35,36). However, this emergence of autoreactive T-cells in abnormally high numbers is not sufficient per se. In addition, there is compelling evidence to suggest that β-cell autoantigen(s)–

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driven T-cell activation is a mandatory step for the emergence of the aggressive "diabetogenic" lymphocyte response (1,8–10,12).

Our current data are the first to demonstrate that, besides quantitative changes that might occur in autoantigen-specific T-cell populations as disease progresses, autoimmune diabetes also results from qualitative differences in diabetogenic T-cells. As assessed in adoptive transfer, pathogenic effectors are detectable in young pre-diabetic 6-week-old NOD mice. These diabetogenic T-cells can only be identified if purified on the basis of their lack of both CD25 and CD62L (3,4) to eliminate regulatory T-cells that effectively slow down diabetes transfer when total spleen cell populations are used.

Our data also confirm the reported important role of regulatory $CD4^+CD25^+$ and $CD62L^+$ T-cells in controlling pathogenic effectors (18,20–23,37). Thus, $B7^{-/-}$ and $CD28^{-/-}$ NOD mice, which lack $CD4^+CD25^+$ T-cells, show accelerated disease onset (23). Infusion of wild-type syngeneic $CD25^+$ T-cells back into $CD28^{-/-}$ NOD recipients prevents disease (23). Regulatory T-cells present in NOD mice share some similarities with those described in other models, notably the polyautoimmune syndrome induced by day 3 thymectomy in BALB/c mice (38,39) and diabetes observed after thymectomy and sublethal irradiation of PVG rats (30,40). They express high levels of the transcription factor *foxP3* and other membrane molecules considered "candidate" markers for regulatory T-cells such as GITR (41,42), membrane TGF-β (25,31), and CD103 (43). Moreover, in vitro they effectively suppress the anti-CD3 antibody–induced proliferation of autologous $CD4^+CD25^-$ T-cells.

Given this high number of diabetogenic cells in young pre-diabetic mice, we wanted to understand why it is only 2–3 months later that overt irreversible disease develops. The first hypothesis was that regulatory T-cells progressively decrease in numbers and/or functional capacity until they become incapable of controlling pathogenic effectors. Results from in vivo cotransfer experiments mixing diabetogenic and regulatory T-cells from the same donors (either young pre-diabetic or overtly diabetic) show that good protection from disease transfer is obtained with regulatory T-cells from young, but not aged donors, which would favor this explanation. However, the situation is more complex. In criss-cross cotransfers it became apparent that regulatory T-cells from diabetic NOD mice still protected from disease transfer caused by $CD25^-CD62L^-$ pathogenic cells from young donors. In addition, $CD25^-CD62L^-$ diabetogenic T-cells from diabetic donors were insensitive to the protective effect of $CD4^+CD25^+$ T-cells from young NOD mice that appeared highly protective when cotransferred with $CD25^-CD62L^-$ cells from young donors.

In vitro, regulatory T-cells from young NOD mice efficiently inhibited anti-CD3 antibody–induced proliferation of $CD4^+CD25^-$ T-cells from the same donors but were ineffective at suppressing $CD4^+CD25^-$ T-cells from diabetic mice. Moreover, regulatory T-cells from diabetic NOD mice did not efficiently suppress proliferation of $CD4^+CD25^-$ T-cells from diabetic mice, whereas they were operational (albeit less efficiently than regulatory T-cells from young donors) on $CD4^+CD25^-$ T-cells from young mice. Thus, in such a non–antigen-specific system, results fully paralleled the data from in vivo criss-cross transfers discussed above.

Therefore, although regulatory T-cells appeared less effective in diabetic than in young NOD mice, they were still functional at the time of overt diabetes. This is well in keeping with the observation that the proportions of regulatory T-cells (i.e., $CD25^+$, $CD62L^+$, and $GITR^+$) did not decrease with aging. Concerning *foxP3* expression, it was in fact higher in diabetic versus young NOD mice. Besides these qualitative interpretations of the results, quantitative arguments cannot be excluded. Although $CD25^+$ T-cells are certainly enriched for cells exhibiting a regulatory phenotype, this does not imply that all $CD25^+$ T-cells express regulatory functions. In diabetic mice $CD4^+CD25^+$ T-cells may in fact include activated nonregulatory T-cells.

In our hands, the in vitro suppression assay was cell-to-cell contact dependent, and TGF-β, both soluble and membrane-bound, appeared to play a central role, as shown by the dose-dependent effective reversal of suppression observed on addition of a neutralizing TGF-β antibody. The relevance of TGF-β, initially described in the mouse (25) and subsequently in humans (44), has been challenged by the observations showing that $CD4^+CD25^+$ T-cells from TGF-β–deficient mice could mediate effective suppression (28). It is fair, however, to quote the various reports arguing for the role of TGF-β in T-cell–mediated regulation, although it is still difficult to conclude on its action as a mediator of the regulation and/or as a factor promoting the differentiation of regulatory cells. Among those reports are the oral tolerance models (33,34) and the ones showing protection from autoimmune diabetes mediated by transgenic expression of TGF-β1 in pancreatic islets (45), by TGF-β–producing $CD4^+$ T-cell clones (46,47), or by islet-infiltrating $CD4^+CD25^+$ T-cells (31). Neutralizing TGF-β antibodies have also been shown to reverse the ability of distinct subsets of $CD4^+$ regulatory T-cells to protect from autoimmune or immune-mediated diseases (29,30).

We further investigated whether the age-dependent decrease in sensitivity of diabetogenic effectors to immunoregulation involved TGF-β. Indeed, $CD4^+CD25^-$ and $CD4^+CD25^-CD62L^-$ T-cells from young NOD mice were more sensitive than those from diabetic animals to the effect of recombinant TGF-β (i.e., they required less TGF-β to be negatively regulated). Mechanisms underlying this defect must be elucidated. Preliminary Western blot analysis revealed the presence of TGF-β receptors I and II on the diabetogenic cells at a similar level, irrespective of the age of the donors. However, it has to be determined whether this upregulated resistance to TGF-β in overtly diabetic mice relies on variations in the number of TGF-β receptors and/or in their signaling capacity, including variations in the level of expression of molecules such as Smad7. Correlating with this enhanced resistance to regulation, $CD4^+CD25^-$ T-cells from older mice exhibited an accentuated Th1 phenotype. Thus, higher IFN-γ–to–IL-10 and IFN-γ–to–IL-4 ratios were observed in diabetic versus young mice.

To conclude, our results indicate that the buildup of a large pool of diabetogenic T-cells in NOD mice occurs very early and is not sufficient to promote disease onset, thus

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stressing the role of regulatory T-cells in diabetes control (3,19,22–24,31). In addition to this, we provide new information that disease onset is associated with loss of sensitivity of effector cells to TGF-β–dependent T-cell–mediated regulation. This concept is reminiscent of different in vitro and in vivo previous data (37,48,49). In particular, in vivo data in lupus-prone mice (NZB/NZW) showed that increased antierythrocyte autoantibody production was insensitive to regulatory T-cells, which were detectable in these animals (48). More recently, in autoimmune-prone Cbl-b$^{-/-}$ mice that express CD28-independent T-cell hyper-reactivity (50), effector CD4^{+}CD25^{-} T-cells were shown to be resistant to both TGF-β and CD4^{+}CD25^{+} regulatory T-cells, which were present and functional in these mice (49).

The question remains of the mechanisms underlying this resistance. One possibility is that at a certain point during the disease process, pathogenic T-cells express an autoantigen-dependent increase in their activation or avidity. This interpretation does not explain, however, the results obtained in our in vitro cocultures that were based on a non–antigen-specific system. Another possibility is an age-dependent change in the sensitivity of effector T-cells to regulatory cytokines, namely TGF-β in autoimmune-prone mice. Independent of interpretation, these data open interesting new therapeutic perspectives aimed at the selective elimination of the subset of TGF-β–resistant effector cells or at their pharmacological modulation, using agents affecting TGF-β–specific receptors and/or their signal transduction pathways.

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