The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 01 2004

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J Immunol (2004) 173 (7): 4407–4416.
https://doi.org/10.4049/jimmunol.173.7.4407

Perturbed Homeostasis of Peripheral T Cells Elicits Decreased Susceptibility to Anti-CD3-Induced Apoptosis in Prediabetic Nonobese Diabetic Mice[1]

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Activation-induced cell death (AICD) plays a key role in the homeostasis of the immune system. Autoreactive T cells are eliminated through AICD both from the thymus and periphery. In this study, we show that NOD peripheral T cells, especially $CD8^+$ T cells, display a decreased susceptibility to anti-CD3-induced AICD in vivo compared with T cells from diabetes-resistant B6, nonobese diabetes-resistant, and NOD.B6*Idd4* mice. The susceptibility of NOD $CD8^+$ T cells to AICD varies in an age- and dose-dependent manner upon stimulation in vivo with either a mitogenic or nonmitogenic anti-CD3. NOD T cells preactivated by anti-CD3 in vivo are less susceptible than B6 T cells to TCR-induced AICD. Treatment of NOD mice with a mitogenic anti-CD3 depletes $CD4^+CD25^-CD62L^+$ but not $CD4^+CD25^+CD62L^+$ T cells, thereby resulting in an increase of the latter subset in the spleen. Treatment with a nonmitogenic anti-CD3 mAb delays the onset of T1D in 8.3 TCR transgenic NOD mice. These results demonstrate that the capacity of anti-CD3 to protect NOD mice from T1D correlates with its ability to perturb T cell homeostasis by inducing $CD8^+$ T cell AICD and increasing the number of $CD4^+CD25^+CD62L^+$ T cells in the periphery. ***The Journal of Immunology,*** **2004, 173: 4407–4416.**

Recent evidence implicates a crucial role for $CD8^+$ effector T cells in islet β cell destruction during the onset of type 1 diabetes (T1D)[5] in NOD mice (1, 2). Spleen $CD4^+$ T cells from prediabetic NOD mice transfer insulitis but not T1D to NOD.*Scid* mice (3). β_2-microglobulin-deficient ($\beta_2m^{-/-}$) and anti-CD8 mAb-treated NOD mice deficient in $CD8^+$ T cells do not develop either insulitis or T1D (4–6). Restoration of expression of MHC class I on cells from $\beta_2m^{-/-}$ NOD mice restores their development of insulitis (7). Spleen cells from prediabetic NOD mice do not transfer insulitis into $\beta_2m^{-/-}$ NOD.*Scid* mice efficiently (8). Diabetogenic $CD8^+$ T cells cloned from islet infiltrates of young and diabetic NOD mice recognize the MHC class I-restricted insulin B9-23 peptide (9). The development of T1D is accelerated by the presence of islet β cell-specific cytotoxic $CD8^+$ T cells in NOD8.3 TCR$\alpha\beta$ transgenic (Tg) mice (10). Thus, it is important to determine the parameters that give rise to islet β cell-autoreactive $CD8^+$ T cells in NOD mice.

Apoptosis may represent one such parameter, as it regulates the homeostasis of the immune system (11) and can result in the deletion of autoreactive T cells in the thymus and periphery (12, 13). NOD mice are defective in both central and peripheral tolerance, as NOD thymocytes show decreased susceptibility to Fas-dependent and Fas-independent apoptosis (14), and mitogen-activated NOD peripheral T cells become less sensitive to apoptosis after IL-2 withdrawal (15). NOD peripheral T cells are also less sensitive to glucocorticoid- (16), cyclophosphamide- (17) and gamma-irradiation-induced apoptosis (18), and display a decreased susceptibility to activation-induced cell death (AICD) in vitro (19, 20).

An anti-CD3ϵ mAb (anti-CD3) is an effective immunosuppressant (21) and can reverse renal allograft rejection in the clinic (21, 22). In NOD mice, anti-CD3 treatment induces long-term remission of overt T1D by depletion of autoreactive T cells (23–25). A randomized multicenter trial has demonstrated that a nonmitogenic anti-CD3 can intervene with the deterioration in insulin production and improve metabolic control in T1D patients (26).

Previously, we reported that decreased susceptibility of $CD8^+$ T cells to anti-CD3-stimulated AICD in vitro may mediate the breakdown of self-tolerance in female NOD mice (20). In this study, we extend these analyses and investigate the susceptibility of female NOD T cells to anti-CD3-induced AICD in vivo by addressing two central questions. First, why does anti-CD3 treatment of female NOD mice effectively protect them from T1D only if administered immediately after the onset of T1D (24)? Second, is this age-dependent protection induced by anti-CD3 related to its capacity to

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Received for publication January 14, 2004. Accepted for publication July 21, 2004.

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[1] This work was supported by a grant from the Canadian Diabetes Association in honor of the late Olive I. Moore, and a grant from the Ontario Research and Development Challenge Fund. W.Y. and S.H. are recipients of a postdoctoral fellowship from the Canadian Diabetes Association. T.L.D. is the Sheldon H. Weinstein Scientist in Diabetes at the Robarts Research Institute and University of Western Ontario. P.S. is supported by operating funds from the Canadian Institute of Health Research and is a Scientist of the Alberta Heritage Foundation for Medical Research.

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[5] Abbreviations used in this paper: T1D, type 1 diabetes; β_2m, β_2-microglobulin; AICD, activation-induced cell death; NOR, nonobese diabetes resistant; Tg, transgenic; PI, propidium iodide; BGL, blood glucose level; FasL, Fas ligand.

0022-1767/04/$02.00

elicit the AICD and deletion of NOD peripheral T cells? The susceptibility to AICD of NOD T cells activated by a mitogenic or nonmitogenic anti-CD3 was compared, as these mAbs either do or do not activate a proinflammatory cytokine response, respectively (27–29). We found that the mitogenic anti-CD3 rapidly deleted C57BL/6 (B6) but not NOD spleen T cells, and that this deletion is dependent on TCR-induced activation of the T cells. $CD8^+$ T cells from NOD mice are less sensitive to this deletion than those from age- and sex-matched diabetes-free, nonobese diabetes-resistant (NOR), and NOD.B6*Idd4* congenic mice. The susceptibility of NOD $CD8^+$ T cells to AICD varies in an age-dependent manner upon stimulation in vivo with either a mitogenic or nonmitogenic anti-CD3. Consistent with our previous study (20), NOD T cells preactivated by anti-CD3 in vivo are less susceptible to TCR-induced AICD. Treatment of NOD mice with a mitogenic anti-CD3 depletes $CD4^+CD25^-CD62L^+$ T cells and increases the number of $CD4^+CD25^+CD62L^+$ T cells in the spleen. Nonmitogenic anti-CD3 treatment delays the onset of $CD8^+$ T cell-mediated T1D in 8.3 TCR Tg NOD mice. Thus, anti-CD3 protects NOD mice from T1D in part by inducing $CD8^+$ T cell AICD and depletion and increasing the number of $CD4^+CD25^+CD62L^+$ peripheral T cells.

Materials and Methods

Mice

NOD, NOR, NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C* and 8.3 TCR$\alpha\beta$ Tg NOD mice were bred in a specific pathogen-free barrier facility at the Robarts Research Institute (London, Ontario, Canada). B6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free mouse colony, and only female mice were used in these studies. The female diabetic NOD mice used were 15–25 wk of age.

T cell activation in vivo with anti-CD3 mAb

NOD and B6 mice of different ages were injected i.p. with 20 μg of either control hamster IgG or the mitogenic 2C11 anti-CD3ϵ mAb (Cedarlane Laboratories, Hornby, Ontario, Canada). After 2 h, spleen cells were fluorescently stained with FITC- or PE-conjugated anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD25 (PC61), anti-CD69 (H1.2F3) or anti-CD62L (MEL-14) mAbs, FITC-conjugated annexin V (BD Pharmingen, Mississauga, Ontario, Canada) or propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO). Alternatively, NOD, NOR, NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* mice at 20 wk of age were injected i.p. with 50 μg of either control IgG or the 2C11 anti-CD3. At different times thereafter, spleen cells were fluorescently stained as above. NOD mice at 4, 12, and 18 wk of age or new-onset ($\leq$7 day after onset) diabetic NOD mice (15- to 25-wk-old) were similarly treated with control IgG or anti-CD3. In another experiment, NOD mice were injected i.p. with 100 μg of either control IgG or a nonmitogenic anti-CD3-IgG3-Fc mAb (29), and 16 h later, spleen cells were fluorescently stained as above.

T cell activation in vivo and restimulation in vitro with anti-CD3 mAb

NOD and B6 mice were injected i.p. with 100 μg of either control hamster IgG or anti-CD3. At 16 h postinjection, spleen cells were harvested, suspended in complete RPMI 1640 (supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 10 mM HEPES, 0.06 μg/ml L-glutamine, 0.05 M sodium pyruvate, 0.05 mM, 0.005 mM 2-ME, and 10% FCS) and cultured in vitro (10^6 cells/ml) in anti-CD3ϵ mAb precoated (1 μg/ml) 12-well plates. At various times, spleen cells were harvested and stained by FITC- or PE-conjugated anti-CD4, anti-CD8, anti-CD25, anti-CD69, anti-Fas (Jo2) or anti-Fas ligand (FasL) (MFL3) mAbs (BD Pharmingen), PI, and FITC-conjugated annexin V.

Apoptosis assay

Apoptotic $CD4^+$ and $CD8^+$ T cells were detected by double staining cells (1×10^6) with FITC-conjugated annexin V and PI according to the manufacturer's protocol. Cells were analyzed by flow cytometry. Live gated cells in the annexin V^+PI^- quadrant were identified as early apoptotic cells, and ungated cells in the annexin V^+PI^+ compartment were identified as late apoptotic/dead cells, as described (20).

Anti-CD3 treatment of 8.3 TCR$\alpha\beta$ Tg NOD mice

8.3 TCR$\alpha\beta$ Tg NOD mice (7 wk old) were injected i.p. twice with 100 μg of either control IgG or nonmitogenic anti-CD3-IgG3-Fc mAb every 3 day. The onset of T1D was monitored by screening twice weekly for glycosuria. Glycosuric mice were tested for hyperglycemia by measurement of their blood glucose levels (BGL) twice weekly using a Glucometer (Bayer, Toronto, Ontario, Canada). Mice that displayed a BGL $>$ 11.1 mmol/l on two consecutive readings were considered to be diabetic.

Flow cytometry

Spleen cells were stained (30 min, 4°C) with the following FITC- or PE-conjugated mAbs (1 μg/10^6 cells): anti-CD4, anti-CD8, anti-CD69, anti-Fas (CD95), anti-CD25, anti-FasL, or anti-CD62L. To analyze FasL expression, the cells were stained with biotin-conjugated mAbs and then streptavidin-PE (0.015 μg/10^6 cells) (BD Pharmingen), washed twice, and analyzed by flow cytometry on a FACScan using CellQuest Software (BD Biosciences, San Jose, CA) (20).

Cytokine assay

Concentrations of IL-10, TNF-α, IFN-γ, and IL-4 in cell supernatants were quantitated by ELISA (30).

Histology and immunohistochemistry

Pancreata for histology were fixed in 10% buffered neutral formalin, and for immunohistochemistry were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and snap frozen in liquid nitrogen. Histology sections (5-μm thick) were stained with H&E. For immunohistochemistry, 5-μm-thick sections were adhered to positively charged slides, fixed in cold acetone (30–60 s), rehydrated in PBS, and stained at room temperature for 1 h with rat anti-mouse anti-CD8 (Ly-2) mAb (BD Biosciences) (1/25 dilution) after blocking with 10% horse serum in PBS. The slides were then washed two times in PBS and incubated with biotin-conjugated goat anti-rat polyclonal Ab (BD Biociences) (1/200 dilution) for 30 min at room temperature. After a 5-min wash with PBS, the sections were incubated with an avidin-biotin complex (Vectastatin ABC kit; Vector Laboratories, Burlingame, CA) for 30 min at room temperature before developing in diaminobenzidine tetrahydrochloride substrate (Sigma-Aldrich) and counterstaining with hematoxylin. Sections not incubated with primary Ab served as a negative control.

Statistical analysis

The statistical significance of the data was determined by the one-way ANOVA test. When the ANOVA was significant, the Fisher's least significant difference multiple-comparison test was applied. Differences were considered significant when $p < 0.05$.

Results

Decreased susceptibility of NOD T cells to anti-CD3-induced activation, apoptosis, and depletion occurs in an age-dependent manner

We set out to determine whether the ability of anti-CD3 to protect or not protect from T1D is related to its capacity to elicit the activation, apoptosis and depletion of NOD peripheral T cells in an age-dependent manner. First, we established the optimum conditions to analyze the susceptibility of NOD T cells to anti-CD3-induced apoptosis in vivo by comparing the susceptibility of T cells from 20-wk-old female B6 and NOD mice to depletion induced by treatment for 2–16 h with different doses (10, 20, or 50 μg) of the 2C11 mitogenic anti-CD3 mAb. The 20-μg and 50-μg doses but not the 10-μg dose of anti-CD3 depleted ~50% of B6 spleen $CD4^+$ and $CD8^+$ T cells (our unpublished data). Hence, the 20-μg dose of anti-CD3 was selected to treat NOD mice. Not only did this dose not deplete NOD spleen T cells at 2–16 h after treatment, but anti-CD3 in the 10- to 50-μg dose range also did not deplete B6 or NOD pancreatic draining lymph node-derived T cells. These preliminary data suggested that NOD and B6 spleen T cells may differ in their sensitivity to anti-CD3-induced depletion.

To test whether such a difference exists, and if so, whether this difference varies in an age-dependent manner, the activation, apoptosis, and depletion of spleen T cells from NOD and B6 mice of

different ages was analyzed at 2 h postinjection of 20 μg of anti-CD3. We observed that expression of the CD69 and CD25 early activation markers was elevated on $CD4^+$ (Fig. 1*A*) and $CD8^+$ T cells (Fig. 1*B*) from 10- and 20-wk-old but not 4- and 30-wk-old B6 mice. In contrast, $CD4^+$ and $CD8^+$ T cells from 4- to 30-wk-old NOD mice did not up-regulate their surface CD69 or CD25 expression at any of these ages. These differences in CD69 and CD25 expression between B6 and NOD T cells was paralleled by a difference in susceptibility of NOD and B6 T cells to anti-CD3-induced apoptosis. The susceptibility of NOD and B6 T cells to anti-CD3-induced apoptosis was examined at 20 wk of age, the age at which B6 T cells were maximally activated by anti-CD3 (Fig. 1, *A* and *B*). The percent of annexin V^+CD4^+ and annexin V^+CD8^+ apoptotic T cells was significantly increased ($p < 0.05$) above control IgG values in anti-CD3-treated B6 mice, whereas this was not the case for anti-CD3 vs IgG-stimulated NOD $CD4^+$ and $CD8^+$ T cells (Fig. 1, *C* and *D*). This increased anti-CD3-induced apoptosis of B6 T cells was accompanied by the greater sensitivity of B6 spleen $CD4^+$ and $CD8^+$ T cells than NOD spleen T cells to anti-CD3-induced depletion (Fig. 1, *E* and *F*). Whereas T cells from 10- and 20-wk-old but not 4- and 30-wk-old B6 mice were depleted by anti-CD3, T cells from 4- to 30-wk-old NOD mice were refractory to anti-CD3 depletion relative to control IgG. Importantly, this age-dependent sensitivity of B6 and NOD T cells to depletion closely mirrors the age-dependent variation in CD69 and CD25 expression on activated B6 and NOD T cells seen above in Fig. 1, *A* and *B*. Collectively, these findings show that NOD and B6 spleen T cells indeed differ in their sensitivity to anti-CD3-induced depletion, and this difference varies in an age-dependent manner according to the extent of activation and induced apoptosis of these T cells. NOD spleen T cells from 4- to 30-wk-old NOD mice are essentially refractory to activation, apoptosis, and depletion induced by the 20-μg dose of anti-CD3.

Previously, it was reported that anti-CD3 treatment at a 5-μg dose does not prevent T1D in young NOD mice but does reverse T1D in older new-onset diabetic NOD mice (24). This result raised the possibility that NOD T cells may require a stronger stimulus to reach a sufficiently high threshold level of activation to undergo apoptosis at a young age. In support of this notion, we found that a dose of 50 μg but not 20 μg of anti-CD3 induces T cell apoptosis in young (4 wk old) NOD mice (data not shown). To further explore the basis of this dose-dependent susceptibility to apoptosis, nondiabetic NOD mice of different ages (4- to 18-wk-old) and new-onset (<7 day) diabetic NOD mice received 50 μg of anti-CD3 or control IgG, and 16 h later the extent of T cell activation and depletion was assayed. An age-dependent variation in CD69 and CD25 expression on anti-CD3 activated spleen $CD4^+$ and $CD8^+$ T cells was detected in nondiabetic and diabetic NOD mice, with maximal expression obtained in 4- and 18-wk-old nondiabetic and new-onset diabetic mice (Fig. 2, *A* and *B*). A small yet significant ($p < 0.01$) increase or no increase was observed in 12- and 15-wk-old nondiabetic mice, respectively. In contrast to NOD mice, CD69 and CD25 expression on anti-CD3-activated $CD4^+$ and $CD8^+$ T cells was up-regulated at each age in 4- to 18-wk-old diabetes-resistant (31, 32) NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR mice (data not shown). Not surprisingly, $CD4^+$ T cell depletion ($p < 0.05$) and particularly $CD8^+$ T cell depletion ($p < 0.01$) was restricted to 4- and 18-wk-old mice (Fig. 2, *C* and *D*), and similar results were observed not only in new-onset diabetic (15- to 18-wk-old) NOD mice (Fig. 2, *C* and *D*) but also in NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR mice (Fig. 2, *E* and *F*). Thus, the age-dependent increases in NOD T cell activation (CD69 and CD25 expression) again were paralleled by an increase in T cell depletion in nondiabetic and diabetic NOD mice. These observations further suggest that the

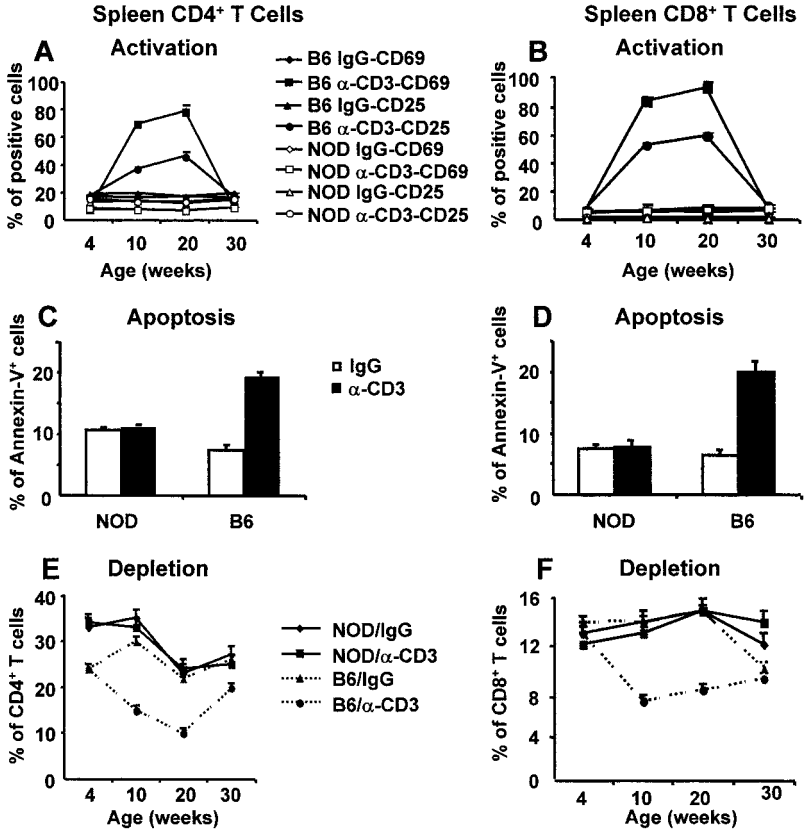

FIGURE 1. Age-dependent anti-CD3 mAb-induced depletion of NOD $CD4^+$ and $CD8^+$ spleen T cells. Nondiabetic NOD mice at different ages were injected i.p. with 20 μg of control IgG or anti-CD3. At 2 h postinjection, spleen cells were stained with the following mAbs: anti-CD4-allophycocyanin plus anti-CD69-FITC plus anti-CD25-PE (*A*); anti-CD8-allophycocyanin plus anti-CD69-FITC plus anti-CD25-PE (*B*); anti-CD4-allophyco cyanin plus annexin V-FITC plus PI to exclude dead cells (*C*); anti-CD8-allophycocyanin plus annexin V-FITC plus PI to exclude dead cells (*D*); anti-CD4-FITC (*E*); and anti-CD8-PE (*F*). In *E*, although the percent of spleen $CD4^+$ T cells in NOD mice exceeded that in B6 mice at 4 wk of age, the number of lymphocytes per spleen in NOD mice ($\sim 50 \times 10^6$) was less than that in B6 mice ($\sim 80 \times 10^6$). Thus, the number of $CD4^+$ T cells per spleen is similar in 4-wk-old NOD and B6 mice. At 10 wk of age, the number of B6 $CD4^+$ and $CD8^+$ T cells was decreased from $\sim 24 \times 10^6$ to $\sim 9 \times 10^6$/spleen and from $\sim 11 \times 10^6$ to $\sim 5 \times 10^6$/spleen after anti-CD3 treatment, respectively. At 20 wk of age, the number of B6 $CD4^+$ and $CD8^+$ T cells was similarly decreased from $\sim 18 \times 10^6$ to $\sim 6 \times 10^6$/spleen and from $\sim 12 \times 10^6$ to $\sim 5 \times 10^6$/spleen after anti-CD3 treatment, respectively. The number of B6 T cells per spleen at 4 and 30 wk of age and number of NOD T cells per spleen at all ages did not change significantly after anti-CD3 treatment. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

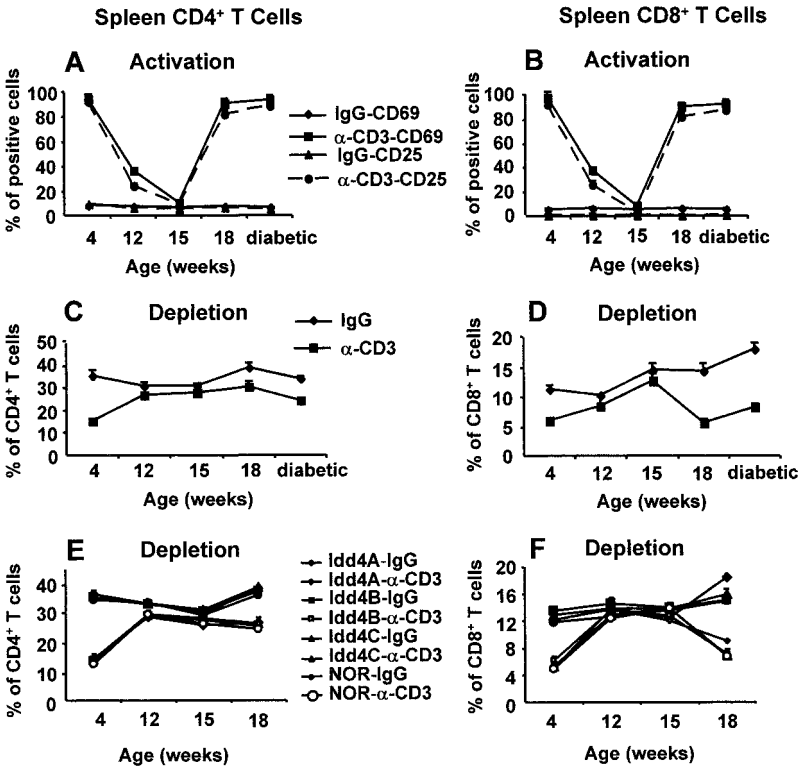

FIGURE 2. Age-dependent anti-CD3-induced T cell depletion. NOD mice of different ages or new-onset diabetic mice (<7 day) were injected i.p. with 50 μg of control IgG or anti-CD3. At 16 h postinjection, spleen cells were stained with anti-CD69-FITC plus anti-CD25-PE plus anti-CD4-allophycocyanin (*A*); anti-CD69-FITC plusanti-CD25-PEplusanti-CD8-allophycocyanin(*B*);anti-CD4-allophycocyanin (*C*); and anti-CD8-allophycocyanin (*D*). In *C* and *D*, the number of NOD $CD4^+$ and $CD8^+$ T cells respectively was decreased from $\sim 23 \times 10^6$ to $\sim 14 \times 10^6$/spleen and from $\sim 9 \times 10^6$ to $\sim 3 \times 10^6$/spleen at 18 wk of age, from $\sim 17 \times 10^6$ to $\sim 6 \times 10^6$/spleen and from $\sim 6 \times 10^6$ to $\sim 2 \times 10^6$/spleen at 4 wk of age, and from $\sim 19 \times 10^6$ to $\sim 11 \times 10^6$/spleen and $\sim 10 \times 10^6$ to $\sim 4 \times 10^6$/spleen in diabetic mice after anti-CD3 treatment. Note that although spleen $CD4^+$ and $CD8^+$ T cells from nondiabetic NOD mice at 12 wk and 15 wk of age were refractory to anti-CD3-induced depletion, T cells from new-onset diabetic NOD mice at 13–17 wk of age were susceptible to this depletion. NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* mice at different ages were treated similarly, and spleen cells were harvested and stained with anti-CD4-allophycocyanin (*E*) or anti-CD8-allophycocyanin (*F*). Age-dependent changes in the number of T cells in NOD.B6*Idd4* and NOR mice were similar to those listed above for NOD T cells in *C* and *D*. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

susceptibility of NOD T cells to anti-CD3-induced activation and depletion varies in a dose- and age-dependent manner.

In an attempt to understand why this age-dependent variation in anti-CD3-induced activation and depletion occur, particularly between 15 and 18 wk of age, we reason as follows. In our NOD mouse colony, islets are infiltrated around the periphery as periinsulitis develops between 5 and 10 wk of age, and exhibit an invasive and then destructive insulitis from 15 to 25 wk of age. The extent of invasive and destructive insulitis detectable histologically is significantly greater at 18 rather than 15 wk of age. Interestingly, this more severe insulitis at 18 wk of age correlates with the increased susceptibility of spleen T cells to anti-CD3-induced activation and AICD at 18 vs 15 wk of age. Thus, spleen T cells from 18-wk-old nondiabetic NOD mice may reach a higher threshold of activation that renders them more susceptible to apoptosis than T cells from 15-wk-old nondiabetic NOD mice. Similarly, activated T cells from 15- to 18-wk-old diabetic NOD mice may reach this higher threshold. This reasoning is compatible with the increased incidence of T1D in female NOD mice in our colony from ~25% at 15 wk of age to 40% at 18 wk of age and >80% by 25 wk of age. Hence, >50% of the female mice in our colony develop T1D at ≥18 wk of age.

It is also important to consider the possibility that the age-dependent variations in susceptibility to anti-CD3-induced activation, apoptosis, and depletion may be associated with, at least in part, the age-dependent variations in insulin levels. In female NOD mice, insulin levels are quite low both at the time of weaning (4 wk) and at an older age just before and immediately after the onset of overt T1D. The latter age-dependent reductions in insulin levels mirror the age-dependent reduced susceptibility of NOD T cells to the anti-CD3-induced effects noted above.

*NOD spleen $CD4^+$ and $CD8^+$ T cells are less activated and less susceptible than NOD.B6*Idd4 *and NOR T cells to anti-CD3 stimulation and depletion at 12 and 15 wk of age*

Because the rank order of T cell susceptibility to anti-CD3 (50 μg) depletion is B6 > NOD.B6*Idd4* = NOR > NOD in 4- to 18-wk-old mice (our unpublished observations), we explored whether this decreased susceptibility of NOD T cells was due to their lower state of activation. We compared the level of CD69 and CD25 expression on T cells from 15-wk-old NOD, NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR mice at 16 h postinjection of anti-CD3 (50 μg). CD69 and CD25 expression was increased on spleen $CD4^+$ (Fig. 3, *A* and *B*) and $CD8^+$ (Fig. 3, *C* and *D*) T cells from NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR mice, but not NOD mice. Anti-CD3-induced T cell activation in NOD.B6*Idd4* and NOR mice suggested that these T cells may be primed for subsequent AICD. Thus, we determined whether T cell depletion occurs at 24 h after anti-CD3 treatment. Spleen $CD4^+$ and $CD8^+$ T cells were depleted from NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR mice, but not from NOD mice (Fig. 3, *E* and *F*). $CD8^+$ T cell depletion ($p < 0.01$) was more significant than $CD4^+$ T cell depletion ($p < 0.05$). Similarly, $CD4^+$ and $CD8^+$ spleen T cells from 12-wk-old NOD mice also displayed decreased susceptibility to anti-CD3 depletion at 24 h compared with T cells from age-matched NOD.B6*Idd4* and NOR mice (our unpublished observations). Thus, at 12 and 15 wk of age, NOD spleen $CD4^+$ and $CD8^+$ T cells are less susceptible than NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR (all diabetes-resistant) (31, 32) T cells to anti-CD3-induced activation and AICD.

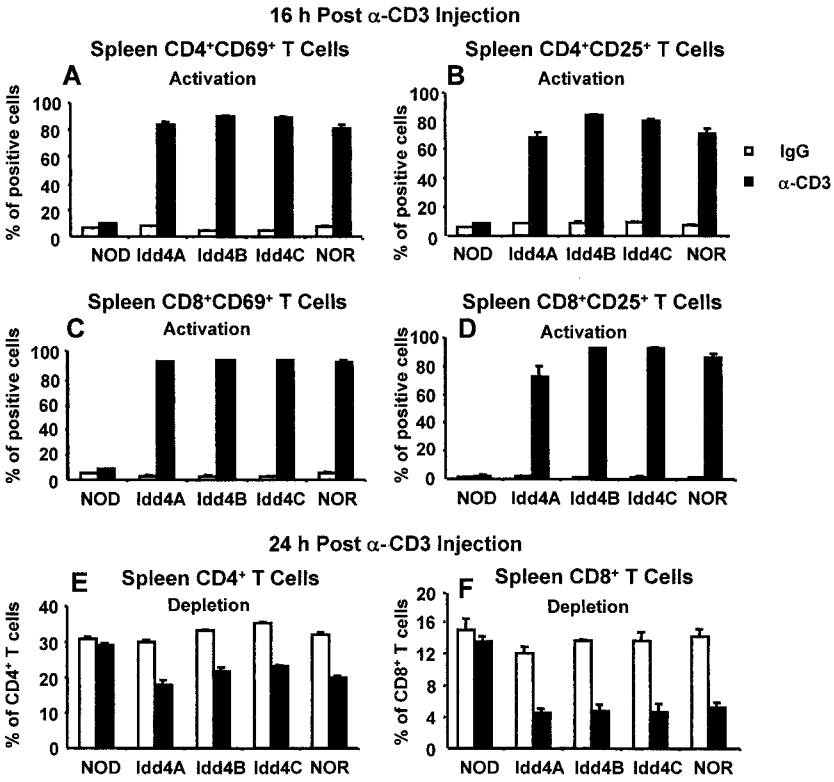

FIGURE 3. Anti-CD3 mAb-induced activation and depletion of $CD4^+$ and $CD8^+$ spleen T cells. Nondiabetic NOD, NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOR mice (15 wk old) were injected i.p. with 50 μg of control IgG or anti-CD3. At 16 h postinjection, splenocytes were stained with anti-CD69-FITC plus anti-CD4-allophycocyanin (*A*), anti-CD25-PE plus anti-CD4-allophycocyanin (*B*), anti-CD69-FITC plus anti-CD8-allophycocyanin (*C*), and anti-CD25-PE plus anti-CD8-allophycocyanin (*D*). At 24 h after injection, splenocytes were stained with anti-CD4-allophycocyanin (*E*) or anti-CD8-allophycocyanin (*F*). In *E* and *F*, the number of $CD4^+$ and $CD8^+$ T cells was decreased respectively from $\sim 18 \times 10^6$ to $\sim 17 \times 10^6$/spleen and from $\sim 9 \times 10^6$ to $\sim 8 \times 10^6$/spleen in NOD mice, from $\sim 18 \times 10^6$ to $\sim 9 \times 10^6$/spleen and from $\sim 7 \times 10^6$ to $\sim 2 \times 10^6$/spleen in NOD.B6*Idd4A* mice, from $\sim 20 \times 10^6$ to $\sim 11 \times 10^6$/spleen and from $\sim 8 \times 10^6$ to $\sim 2 \times 10^6$/spleen in NOD.B6*Idd4B* mice, from $\sim 21 \times 10^6$ to $\sim 11 \times 10^6$/spleen and from $\sim 8 \times 10^6$ to $\sim 2 \times 10^6$/spleen in NOD.B6*Idd4C* mice, and from $\sim 19 \times 10^6$ to $\sim 10 \times 10^6$/spleen and $\sim 8 \times 10^6$ to $\sim 2 \times 10^6$/spleen in NOR mice. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

Activated NOD spleen T cells display a decreased susceptibility to AICD compared with T cells from B6 mice

We next analyzed whether activated $CD4^+$ and $CD8^+$ spleen T cells from 20-wk-old NOD mice are less susceptible to anti-CD3-induced AICD than T cells from age-matched B6 mice. At 16 h after injection of a mitogenic anti-CD3 (50 μg), CD69 and CD25 expression was elevated on NOD and B6 $CD4^+$ and $CD8^+$ spleen T cells (data not shown). After 72 and 96 h of restimulation by plate-bound mitogenic anti-CD3, $CD4^+$ and $CD8^+$ spleen T cells from anti-CD3-treated NOD mice were less susceptible to AICD than T cells from similarly treated B6 mice (Fig. 4, *A* and *B*). In control IgG-treated mice, while B6 $CD4^+$ T cells displayed more AICD than NOD T cells (Fig. 4*A*), NOD and B6 $CD8^+$ T cells were equally susceptible to AICD (Fig. 4*B*). In addition, despite

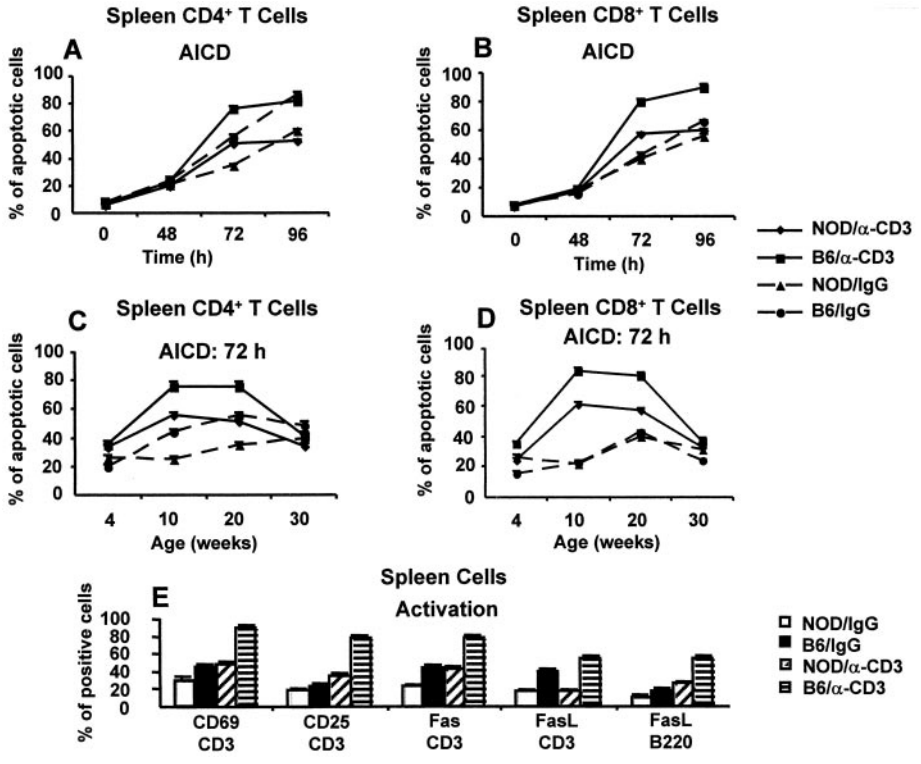

FIGURE 4. Reduced susceptibility of activated NOD $CD4^+$ and $CD8^+$ spleen T cells to AICD. Nondiabetic NOD mice and B6 mice (20 wk old) were injected i.p. with 50 μg of control IgG or anti-CD3. At 16 postinjection, spleen cells were cultured in anti-CD3 precoated 12-well plates. At different time points after culture, spleen cells were stained with annexin V-FITC plus PI plus (*A*) anti-CD4-allophycocyanin or (*B*) anti-CD8-allophycocyanin. At 72 h after culture, splenocytes were stained with annexin V-FITC plus PI plus (*C*) anti-CD4-allophycocyanin or (*D*) anti-CD8-allophycocyanin. *E*, Spleen cells were harvested after 48 h of culture and stained with anti-CD3-allophycocyanin plus the following mAbs: anti-CD69-FITC, anti-CD25-PE, anti-Fas-FITC, anti-FasL-PE, or B220-FITC plus anti-FasL-PE. Nondiabetic NOD and B6 mice at different ages were treated similarly. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

the greater AICD observed for $CD4^+$ and $CD8^+$ T cells from anti-CD3-treated mice compared with that seen in control IgG-treated mice at 72 h poststimulation, these differences were much less significant at 96 h poststimulation (Fig. 4, *A* and *B*).

Thus, we determined the percentage of apoptotic T cells from mice of different ages at 72 h after in vitro stimulation. The differences in the percentages of apoptotic $CD4^+$ and $CD8^+$ T cells found between anti-CD3-treated NOD and B6 mice at 10 and 20 wk of age were not detected in mice at 4 and 30 wk of age (Fig. 4, *C* and *D*). In control IgG-treated 10- and 20-wk-old mice, the percentages of apoptotic NOD and B6 $CD4^+$ but not $CD8^+$ T cells also differed significantly (Fig. 4*C*). T cells from 20-wk-old anti-CD3-treated NOD mice did not up-regulate CD69, CD25, Fas, or FasL expression relative to that detected on B6 T cells (Fig. 4*E*). NOD T cells from IgG-treated mice also displayed decreased surface expression of CD69, Fas, and FasL, but not CD25. T cells from diabetic NOD mice at all ages were similarly resistant to AICD compared with T cells from nondiabetic NOD mice at 20 wk of age (our unpublished data). Hence, in vivo activated $CD4^+$ and $CD8^+$ T cells from NOD mice at 10 and 20 wk of age display a decreased susceptibility to AICD upon in vitro restimulation compared with T cells from age- and sex-matched B6 mice. The diminished AICD of NOD T cells is accompanied by defective up-regulation of FasL on B cells (Fig. 4*E*). LPS-activated B cells trigger the apoptosis of Th1 cells by expressing FasL and prevent the onset of T1D in NOD mice (33). Therefore, defective up-regulation of FasL on B cells may contribute to the decreased susceptibility of NOD T cells to AICD.

Decreased susceptibility of NOD T cells to AICD is accompanied by reduced secretion of TNF-α, IFN-γ, IL-4, IL-10, and TGF-β

Spleen cells from anti-CD3 or control IgG-treated NOD and B6 mice were restimulated in vitro with anti-CD3 for 48 h, and culture supernatants were assayed for their cytokine content by ELISA. Small but significant increases in TNF-α, IL-4, IL-10, and TGF-β secretion were noted for spleen cells from anti-CD3-treated NOD mice (Fig. 5). However, note that these increases were significantly greater in supernatants of activated B6 spleen cells. The largest increases in anti-CD3-induced secretion by NOD T cells were detected for IL-10 and TGF-β. Anti-CD3 treatment also increased IFN-γ secretion by B6 but not NOD-activated spleen cells. These results suggest that the secretion of Th2 cytokines is increased in NOD spleen T cells after anti-CD3 stimulation in vivo.

Anti-CD3 treatment increases the proportion of $CD4^+CD25^+CD62L^+$ spleen T cells in NOD mice

To investigate whether regulatory $CD4^+$ T cells mediate anti-CD3-induced protection from T1D, we determined the CD25 and CD62L surface Ag phenotype of $CD4^+$ spleen T cells from anti-CD3 or control IgG-treated 20-wk-old NOD mice. The proportion of $CD4^+CD25^+$ T cells in splenic $CD4^+$ T cell and whole spleen cell populations of nondiabetic NOD mice increased ~3-fold at 16 h postinjection of anti-CD3 (Fig. 6, *A* and *C*). Similar increases were also found in 20-wk-old NOR, NOD.B6*Idd4A*, NOD.B6*Idd4C*, and BALB/c mice (Fig. 6*C*). These numerical increases in $CD4^+CD25^+$ T cells may result from the expansion of $CD4^+CD25^+$ T cells and/or activation of $CD4^+CD25^-$ T cells. Because CD62L expression did not change appreciably on NOD $CD4^+CD25^+$ T cells after anti-CD3 treatment (data not shown), the increase in $CD4^+CD25^+$ T cells observed may result from the expansion of $CD4^+CD25^+$ T cells. To test this possibility, we examined CD25 expression on $CD4^+CD62L^+$ T cells and found that anti-CD3 treatment depleted the $CD4^+CD25^-$ but not

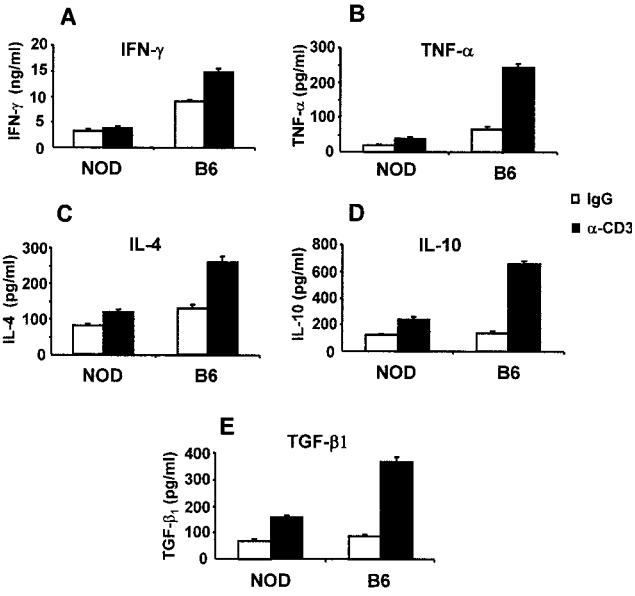

FIGURE 5. Anti-CD3 activated NOD spleen cells secrete lower levels of cytokines than activated B6 spleen cells. NOD and B6 mice were treated with 50 μg of anti-CD3 or control IgG, and 16 h later their spleen cells were restimulated in vitro with anti-CD3 for 48 h, as described in Fig. 4. The concentration of TNF-α, IL-4, IFN-γ, IL-10, and TGF-β in culture supernatants was assayed by ELISA. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

$CD4^+CD25^+$ T cell subset of NOD spleen $CD4^+CD62L^+$ T cells (Fig. 6*B*). Accordingly, the proportion of $CD4^+CD25^+$ T cells in the $CD4^+CD62L^+$ subset was increased in the spleens of anti-CD3-treated nondiabetic NOD mice (Fig. 6*D*). Similar increases in $CD4^+CD25^+$ spleen T cells were also detected in anti-CD3-treated NOR, NOD.B6*Idd4A*, NOD.B6*Idd4B*, NOD.B6*Idd4C*, and BALB/c mice (Fig. 6*D*) as well as in diabetic NOD mice (our unpublished observations). These results suggest that an increase in the number of $CD4^+CD25^+CD62L^+$ T cells, a portion of which may be $CD4^+$ regulatory T cells, may contribute to anti-CD3-mediated protection from T1D.

Spleen T cells from nondiabetic NOD mice at 4 or 18 wk of age and from diabetic NOD mice are depleted after treatment with a nonmitogenic anti-CD3

Conventional anti-CD3 treatment of mice can induce acute toxicity by triggering the systemic release of many cytokines (27). This cellular activation may be a consequence of the cross-linking between T cells and FcγR-bearing cells that is mediated by the high affinity of a hamster anti-CD3 mAb for murine FcγRs (28). However, administration of a chimeric IgG3 isotype with a very low affinity for murine FcγRs does not result in a burst of cytokine secretion, a humoral response against the mAb or TCR desensitization (29). As shown above, the susceptibility of NOD T cells to depletion induced by treatment with a conventional anti-CD3 changes in an age-dependent manner. Thus, we examined whether T cell depletion also occurs after treatment in vivo with a nonmitogenic anti-CD3 mAb.

In the following experiments, a higher dose (100 μg) of a nonmitogenic anti-CD3 was used, because in pilot studies we found that this dose was required to elicit T cell AICD in NOD mice. At 16 h postinjection of a nonmitogenic anti-CD3-IgG3-Fc, $CD4^+$- and $CD8^+$-activated T cells acquired an age-dependent change in

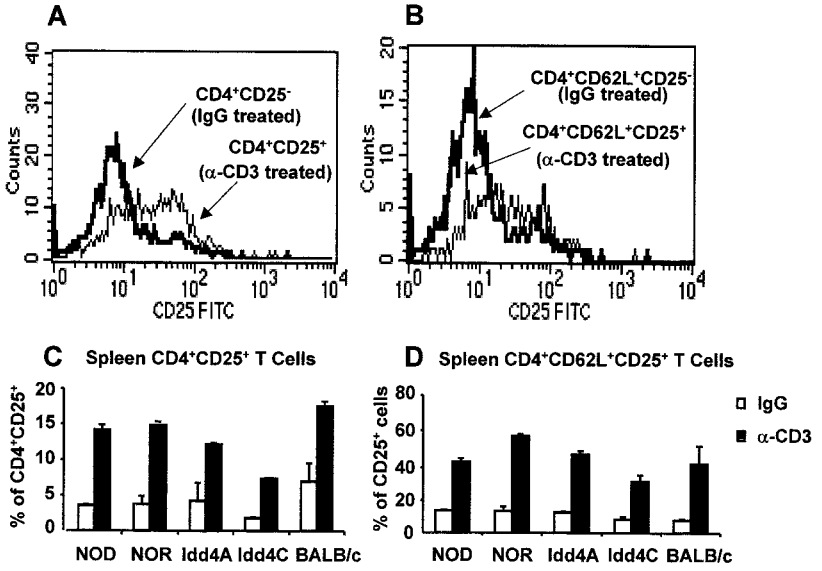

FIGURE 6. Anti-CD3 treatment induces an increase in the proportion of NOD $CD25^+$ spleen T cells in the $CD4^+CD62L^+$ T cell subset. Nondiabetic NOD, NOR, NOD.B6*Idd4A*, and NOD.B6*Idd4C* mice (20 wk old) were injected i.p. with 50 μg of control IgG or anti-CD3. At 16 h postinjection, spleen cells were stained with anti-CD4-allophycocyanin, anti-CD25-FITC, and anti-CD62L-PE. *A*, CD25 expression by NOD spleen $CD4^+$ T cells. *B*, CD25 expression by $CD4^+CD62L^+$ NOD spleen T cells. *C*, Percentage of $CD4^+CD25^+$ spleen T cells from different stains. *D*, CD25 expression by $CD4^+CD62L^+$ spleen T cells from different strains. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

their surface phenotype following anti-CD3-IgG3-Fc treatment. This treatment increased the expression of CD69 on $CD4^+$ and $CD8^+$ T cells from 4- and 18-wk-old nondiabetic and diabetic NOD mice, but this increase did not occur on T cells from NOD mice at 12 and 15 wk of age (Fig. 7, *A* and *B*). In addition, the increase in CD69 expression on T cells from 18-wk-old NOD mice was less than that obtained in 4-wk-old or diabetic mice. In contrast, anti-CD3-IgG3-Fc treatment did not elicit an increase in CD25 expression on NOD $CD4^+$ T cells at all ages examined, but this treatment did result in a significant increase (from 1% to 11%) on $CD8^+$ T cells from 4-wk-old nondiabetic and diabetic NOD mice. Consistent with these profiles of T cell activation, the percentage of $CD4^+$ and $CD8^+$ spleen T cells was decreased significantly not only in 4- and 18-wk-old nondiabetic but also in diabetic NOD mice relative to that observed in control IgG-treated mice (Fig. 7, *C* and *D*) ($p < 0.05$). Interestingly, the extent of $CD8^+$ T cell depletion in 4-wk-old nondiabetic and in diabetic NOD mice ($p < 0.01$) exceeded that observed at 18 wk of age ($p < 0.05$). Thus, even though a nonmitogenic anti-CD3 depletes T cells, this depletion occurs in an age-dependent manner as the amount of $CD4^+$ and $CD8^+$ T cell depletion detected was not significant in NOD mice at 12 and 15 wk of age. Spleen $CD4^+$ and $CD8^+$ T cells are most susceptible to nonmitogenic anti-CD3 depletion in diabetic NOD mice and nondiabetic NOD mice at 4 and 18 wk of age.

Anti-CD3 treatment delays the onset of T1D in 8.3 TCR$\alpha\beta$ Tg NOD mice

8.3 TCR$\alpha\beta$ Tg NOD mice (7 wk old) were injected i.p. on day 0 and day 3 with 100 μg of either control IgG or nonmitogenic anti-CD3. At 16 h after the first injection, anti-CD3 induced the depletion of ~23% of spleen $CD4^+$ T cells ($p < 0.05$) and 55% of spleen $CD8^+$ T cells ($p < 0.01$) in comparison to that observed for the control IgG-treated mice (Fig. 8*A*). 8.3 TCR$\alpha\beta$ Tg NOD mice at 7 wk of age show a severe invasive insulitis due to the infiltration of islets by $CD8^+$ T cells, as detected by H&E (Fig. 8*Ba*) and anti-CD8 (Fig. 8*Bb*) staining. Treatment of these mice

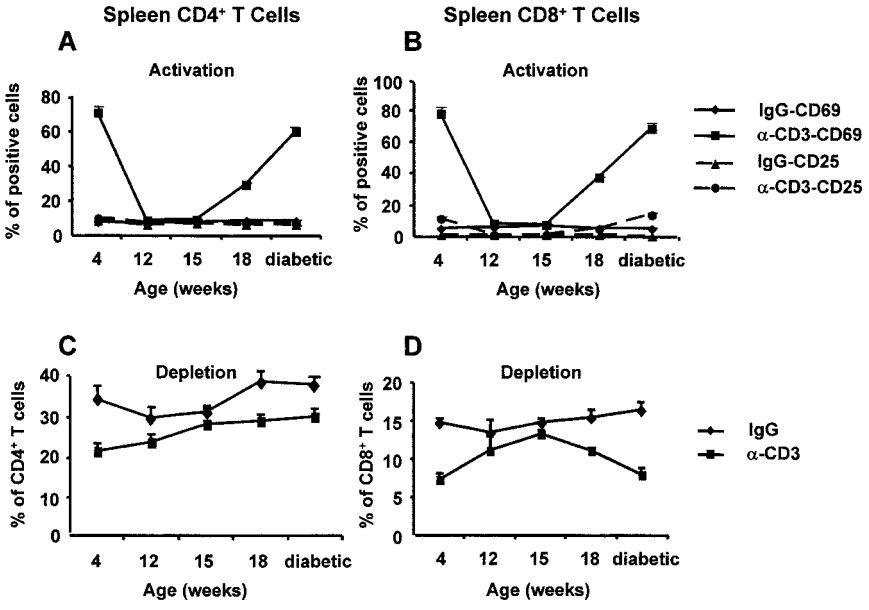

FIGURE 7. Age-dependent nonmitogenic anti-CD3-induced depletion of NOD $CD4^+$ and $CD8^+$ spleen T cells. NOD mice at different ages or new-onset diabetic mice (<7 day) were injected i.p. with 100 μg of anti-CD3 IgG3-Fc or control IgG. At 16 h postinjection, spleen cells were stained with anti-CD4-allophycocyanin plus anti-CD69-FITC plus anti-CD25-PE (*A*); anti-CD8-allophycocyanin plus anti-CD69-FITC plus anti-CD25-PE (*B*); anti-CD4-allophycocyanin (*C*); and anti-CD8-allophycocyanin (*D*). The number of NOD $CD4^+$ and $CD8^+$ T cells respectively was decreased from ~16×10^6 to ~8×10^6/spleen and from ~8×10^6 to ~3×10^6/spleen at 4 wk of age, from ~21×10^6 to ~13×10^6/spleen and from ~9×10^6 to ~5×10^6/spleen at 18 wk of age, and from ~20×10^6 to ~14×10^6/spleen and ~10×10^6 to ~4×10^6/spleen in diabetic mice after anti-CD3 treatment. Results from three independent reproducible experiments are presented. Groups of six mice per group were pooled and used per experiment, and the error bars represent the mean values of three experiments.

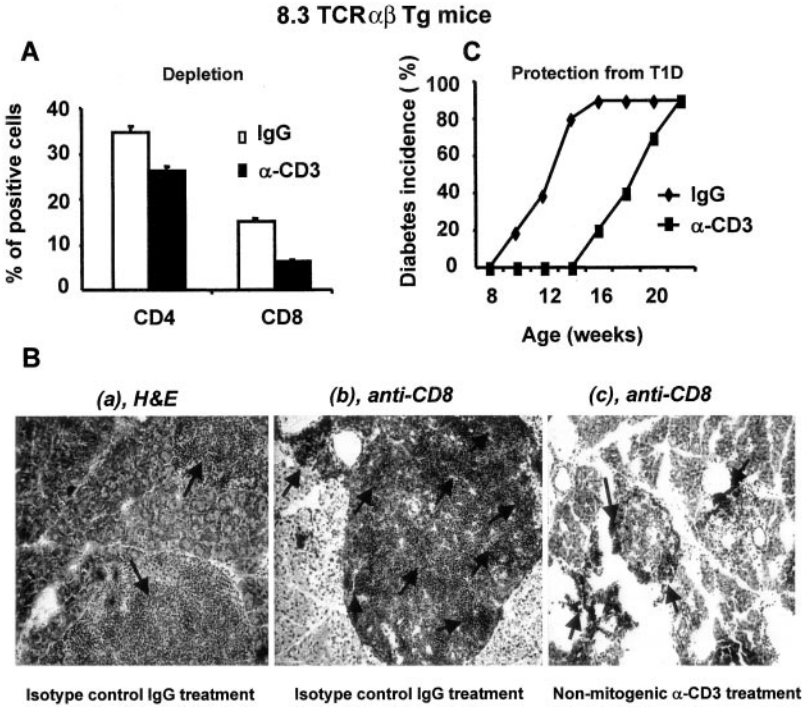

FIGURE 8. Anti-CD3 treatment induces the protection of 8.3 TCR$\alpha\beta$ Tg NOD mice from diabetes. 8.3 TCR$\alpha\beta$ Tg NOD mice (7 wk old) were injected i.p. on day 0 and day 3 with 100 μg of either control IgG or anti-CD3-IgG3-Fc. *A*, At 16 h after the first injection, spleen cells were stained with anti-CD4-FITC or anti-CD8-PE. The number of NOD $CD4^+$ and $CD8^+$ T cells was decreased from $\sim 21 \times 10^6$ to $\sim 12 \times 10^6$/spleen and from $\sim 9 \times 10^6$ to $\sim 3 \times 10^6$/spleen, respectively. Groups of 10 mice per group were used, and the error bars represent the mean values of 10 mice. *B*, Histological and immunohistochemical analyses demonstrate that 8.3 TCR$\alpha\beta$ Tg NOD mice ($n = 3$ mice/group) at 7 wk of age show severe invasive insulitis detected by H&E (*a*) and anti-CD8 (*b*) staining. In comparison to islets of isotype control IgG-treated mice (*a* and *b*), islets from nonmitogenic anti-CD3-treated mice showed a decreased invasive insulitis as most of the anti-CD8 staining was localized to the periphery of the islets (*c*). Areas depicted by arrows show severe insulitis (H&E staining) and intense anti-CD8 staining. The results shown are representative of 10 islets observed in each section. *C*, The incidence of T1D was determined by examining glycosuria and BGL. Groups of 10 mice per group were used.

with nonmitogenic anti-CD3 decreased the amount of invasive insulitis and converted more to a peri-insulitis, as most of the anti-CD8 staining was localized to the periphery of the islets compared with islets from isotype IgG-treated mice (Fig. 8*B*, *b* and *c*). Anti-CD3 treatment delayed the onset of T1D by 6 wk and yielded a significantly reduced incidence of T1D (0%) compared with that in control-treated mice (80%) at 14 wk of age (Fig. 8*C*). However, at 20–24 wk of age, >80% of anti-CD3-treated mice developed T1D. Thus, anti-CD3 treatment slows the kinetics of onset of T1D in 8.3 TCR$\alpha\beta$ Tg mice.

Discussion

In this report, we addressed the questions of why anti-CD3 treatment protects NOD mice against T1D preferentially if administered shortly after the onset of T1D and whether this age-dependent protection mediated by anti-CD3 correlates with its ability to induce the activation, AICD, and depletion of peripheral T cells at a given age. The effects of a mitogenic and nonmitogenic anti-CD3 were compared because recent evidence suggests that a nonmitogenic anti-CD3 may halt disease progression and improve metabolic control in patients with new-onset T1D (26). Several interesting findings emerge from our studies. First, we demonstrate that anti-CD3 treatment can induce the activation, apoptosis, and depletion of NOD and B6 spleen $CD4^+$ and $CD8^+$ T cells, but that this occurs with different levels of susceptibility in these mice and in a dose- and age-dependent manner.

Second, the susceptibility of T cells to anti-CD3-induced apoptosis and depletion was found to correlate directly with the ability of the T cells to be activated. We observed that T cell depletion induced by a nonmitogenic anti-CD3-IgG3-Fc mAb is accompanied by the up-regulation of CD69 but not CD25 expression, whereas activation by a mitogenic anti-CD3 leads to the increased expression of both CD69 and CD25. Thus, a nonmitogenic anti-CD3 appears to elicit less T cell activation than a mitogenic anti-CD3, which may explain why we found that a higher dose of the nonmitogenic anti-CD3 was required to induce a significant level of T cell apoptosis. The latter result may also be attributable in part to the fact that a nonmitogenic anti-CD3 delivers only a partial TCR activation signal (33, 34). Hence, the use of nonmitogenic anti-CD3 that leads to less T cell activation, apoptosis, and depletion may make it a more attractive agent than a mitogenic anti-CD3 to treat T1D patients in clinical trials.

Third, NOD $CD4^+$ T cells and $CD8^+$ T cells, and particularly NOD $CD8^+$ T cells, were shown to be less susceptible to anti-CD3 stimulated activation, apoptosis and depletion than T cells from diabetes-resistant B6, NOR and NOD.B6*Idd4* mice. Treatment of 8.3 TCR$\alpha\beta$ Tg NOD mice with a nonmitogenic anti-CD3 significantly depletes $CD8^+$ T cells from the spleen, modulates their capacity to invade islets, and delays the onset of T1D. This result provides evidence that a nonmitogenic anti-CD3 partially protects 8.3 TCR$\alpha\beta$ Tg NOD mice against T1D by depletion of islet β cell-autoreactive $CD8^+$ T cells from the spleen and modulation of their capacity to infiltrate and destroy islets. Thus, it is conceivable that the restoration of euglycemia and more normal insulin levels in new-onset T1D patients treated with a nonmitogenic anti-CD3 may occur in part as a result of the depletion of a critical number of islet β cell-autoreactive $CD8^+$ T cells.

Fourth, our data show that treatment of NOD mice with a mitogenic anti-CD3 depletes $CD4^+CD25^-CD62L^+$ T cells but not $CD4^+CD25^+CD62L^+$ T cells and increases the proportion of $CD25^+$ T cells in the $CD4^+CD62L^+$ subpopulation in the spleen. These results are of interest because $CD4^+CD25^+CD62L^+$ T cells function as regulatory T cells in NOD mice (35, 36) and inhibit the transfer of T1D into immune-compromised NOD.*Scid* mice (37, 40). These regulatory T cells are more resistant to TCR-mediated activation and AICD than $CD4^+CD25^-$ T cells (38, 39). We also found that this anti-CD3 treatment increases the proportion of $CD4^+CD25^+$ cells in spleen $CD4^+$ T cell and whole splenocyte populations, and also stimulates TGF-β and IL-10 production by splenocytes. These findings are similar to those reported for a nonmitogenic anti-CD3 that restores self-tolerance in NOD mice by increasing the number of TGF-β-producing regulatory $CD4^+CD25^+$ T cells (40). In addition, these findings are in agreement with the report that T cell activation is associated

with increased IL-10 secretion in T1D patients treated with an FcR nonbinding humanized anti-CD3 mAb hOKT3γ1(Ala-Ala) (34). Taken together with the results presented above, our results raise the possibility that anti-CD3 may protect from T1D by inducing an increase in the number of $CD4^+CD25^+$ regulatory T cells and a decrease in the number of effector islet β cell-autoreactive $CD8^+$ T cells.

Fifth, we presented evidence that the susceptibility of NOD $CD4^+$ and $CD8^+$ T cells, and particularly $CD8^+$ T cells, to AICD induced by both mitogenic and nonmitogenic anti-CD3 mAbs varies with age. T cells from diabetic NOD mice and nondiabetic NOD mice at 18 wk of age are more susceptible to anti-CD3 (mitogenic and nonmitogenic)-induced AICD than T cells from 12- and 15-wk-old NOD mice. This may arise due to defective TCR signal transduction (41, 42) that leads to decreased susceptibility to TCR-mediated activation and AICD in T cells from prediabetic NOD mice at 12 and 15 wk of age. These defects in NOD T cell signaling may also explain why a higher dose of anti-CD3 was required for the activation of NOD T cells than B6 T cells (43). The age-dependent variations in NOD T cell activation and AICD detected are in agreement with a previous report that anti-CD3 induces the long-term remission of T1D when administered to newly diabetic NOD mice but protects from T1D only transiently when treatment begins at 12 wk of age (24). Although Chatenoud et al. (24) used lower doses (5–20 μg) of anti-CD3 to treat newly diabetic NOD mice than we used in this study, the protocol of continuous injection of anti-CD3 every day for 5 days used by these investigators may elicit a higher activation of NOD T cells and render them more susceptible to anti-CD3-induced AICD and depletion. Thus, it appears that the age-dependent variability in the susceptibility of NOD T cells to TCR-mediated AICD influences the outcome of anti-CD3 treatment on the development of T1D. The ability of anti-CD3 treatment to increase the susceptibility of NOD T cells to AICD may be one factor that mediates anti-CD3 protection mice from T1D. However, it remains somewhat enigmatic that although T cells from NOD mice at 4 wk of age and new-onset diabetic mice possess a similar level of susceptibility to TCR-mediated depletion, anti-CD3 (mitogenic) treatment protects NOD mice from T1D only when treatment is initiated immediately after the onset of disease but not at 4 wk of age (23). This may be due in part to the fact that T cells from 4-wk-old NOD mice are not diabetogenic upon cell transfer and that little or no invasive insulitis is detectable at this age (1).

Finally, we found that the decreased susceptibility of anti-CD3-activated NOD T cells to AICD is accompanied by a significant decrease in their level of secretion of several cytokines, including TNF-α, IL-4, IL-10, IFN-γ, and TGF-β1. This may not be surprising as each of these cytokines is known to stimulate T cell AICD (44–52), and a decrease in their level of production would be expected to mediate the decreased susceptibility of NOD T cells to AICD in vivo. Moreover, administration of each of these cytokines to NOD mice protects them from T1D, which may occur in part by restoring the susceptibility of $CD8^+$ T cells to AICD and depletion, as described for IFN-γ (50).

In conclusion, we demonstrated that NOD T cells, especially $CD8^+$ T cells, are refractory to anti-CD3-induced AICD and depletion in vivo. This refractoriness may reduce the ability of NOD mice to delete islet autoreactive $CD8^+$ T cells from the periphery and lead to increased islet β cell destruction. We also show that the age- and dose-dependent capacity of anti-CD3 treatment to protect NOD mice from T1D correlates closely with its ability to induce $CD8^+$ T cell AICD and depletion as well as increase the number of $CD4^+CD25^+CD62L^+$ T cells. Our observations suggest that agents that augment anti-CD3-induced $CD8^+$ T cell AICD and depletion and enhance the number of $CD4^+CD25^+CD62L^+$ regulatory T cells may be used in combination with anti-CD3 to therapeutically treat new-onset diabetic patients and arrest disease progression.

Acknowledgments

We thank all members of our laboratory for their valuable advice and encouragement, and Drs. Qizhi Tang and Jeffery A. Bluestone for their kind gift of a nonmitogenic anti-CD3 mAb.

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