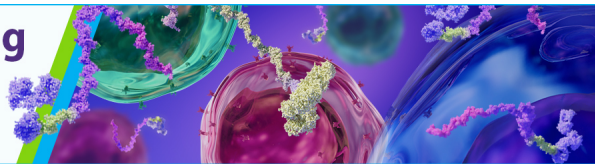


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Dynamics of Pathogenic and Suppressor T Cells in Autoimmune Diabetes Development

Silvia Gregori,¹ Nadia Giarratana, Simona Smiroldo, and Luciano Adorini²

In the nonobese diabetic (NOD) mouse, pathogenic and suppressor CD4⁺ T cells can be distinguished by the constitutive expression of CD25. In this study, we demonstrate that the progression of autoimmune diabetes in NOD mice reflects modifications in both T cell subsets. CD4⁺CD25⁺ suppressor T cells from 8-, but not 16-wk-old NOD mice delayed the onset of diabetes transferred by 16-wk-old CD25-depleted spleen cells. These results were paralleled by the inhibition of alloantigen-induced proliferation of CD4⁺CD25⁻ cells, indicating an age-dependent decrease in suppressive activity. In addition, CD4⁺CD25⁻ pathogenic T cells became progressively less sensitive to immunoregulation by CD4⁺CD25⁺ T cells during diabetes development. CD4⁺CD25⁻ T cells showed a higher proliferation and produced more IFN- γ , but less IL-4 and IL-10, whereas CD4⁺CD25⁺ T suppressor cells produced significantly lower levels of IL-10 in 16- compared with 8-wk-old NOD mice. Consistent with these findings, a higher frequency of Th1 cells was observed in the pancreas of 16-wk-old compared with 8-wk-old NOD mice. An increased percentage of CD4⁺CD25⁻ T cells expressing CD54 was present in 16-wk-old and in diabetic NOD, but not in BALB/c mice. Costimulation via CD54 increased the proliferation of CD4⁺CD25⁻ T cells from 16-, but not 8-wk-old NOD mice, and blocking CD54 prevented their proliferation, consistent with the role of CD54 in diabetes development. Thus, the pathogenesis of autoimmune diabetes in NOD mice is correlated with both an enhanced pathogenicity of CD4⁺CD25⁻ T cells and a decreased suppressive activity of CD4⁺CD25⁺ T cells. *The Journal of Immunology*, 2003, 171: 4040–4047.

The nonobese diabetic (NOD)³ mouse spontaneously develops autoimmune diabetes, and represents a good model for human type 1 diabetes (T1D) (1–3). Several cellular mechanisms have been implicated in T1D development in the NOD mouse, including IL-12-dependent Th1 cells (4), cytotoxic CD8⁺ lymphocytes and macrophages (5), and defective immunoregulation (6). The activation of pathogenic Th1 cells specific for pancreatic autoantigens could reflect defective thymic deletion of autoreactive T cell clones (7), inefficient mechanisms of peripheral tolerance (6), alteration in IL-12 expression (8), or impaired suppressor T cells (9).

CD4⁺CD25⁺ suppressor T cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion (10). They fail to proliferate and secrete cytokines in response to polyclonal or Ag-specific stimulation, and are not only anergic, but also inhibit the activation of responsive T cells (11). These suppressor T cells play an important role in preventing the induction of several autoimmune diseases, such as the autoimmune syndrome induced by day 3 thymectomy in genetically susceptible mice (12), inflammatory bowel disease (13), T1D in thymectomized rats (14), and in NOD mice (9, 15). CD4⁺CD25⁺ suppressor T cells are reduced in NOD compared with other mouse strains, and this reduction could be a factor in their susceptibility to T1D (9). A defect in peripheral regulatory cells affecting both

CD4⁺CD25⁺ and NK cells has recently been described also in T1D patients (16).

The progression of T1D in NOD mice is highly regulated, and different phases are characterized by specific features. Insulinitis starts to appear at 3–4 wk and is well established by 6–8 wk of age. Progression to overt diabetes occurs in ~80% of females between 12 and 30 wk of age. Two major checkpoints have been defined in the pathogenesis of T1D in the NOD mouse. The first regulates the onset of insulinitis and is active before 3 wk of age, while the second exerts its activity at ~8–12 wk of age by controlling the switch from nondestructive to destructive insulinitis and the development of overt disease (17). The first checkpoint is controlled by mechanisms such as the composition of APC populations (18, 19) and the expression of integrins and adhesion molecules (20). Among the adhesion molecules involved in leukocyte trafficking into inflammatory sites and in the delivery of costimulatory signals during Ag presentation to T cells, CD54 appears to be critically important in the development of T1D. Administration of anti-CD54 mAb protects from disease (21, 22), and a CD54-dependent pathway has been implicated in the pathogenesis of IL-10-mediated diabetes (23). In addition, NOD mice with a disrupted CD54 gene are completely protected from T1D (24), demonstrating the dominant and nonredundant role of this molecule in diabetes development. The second checkpoint relies on several non-exclusive mechanisms, including APCs; cytokines; Th1/Th2 balance; modulation of surface molecules such as CD152, CD25, and NK-like receptors; recruitment of pathogenic cells; and number and function of suppressor cells (17). However, the relative contribution of these factors to the balance of pathogenic and protective T cells during T1D development is still poorly understood.

Results in this study indicate a dynamic interplay between CD4⁺CD25⁺ suppressor T cells and CD4⁺CD25⁻ pathogenic T cells that correlates with diabetes progression in the NOD mouse. T1D development reflects both an age-dependent decrease in the capacity of CD4⁺CD25⁺ T cells to exert suppressive activity and

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³ Abbreviations used in this paper: NOD, nonobese diabetic; T1D, type 1 diabetes.

a progressively enhanced pathogenicity of CD4⁺CD25⁻ cells, expressing up-regulated CD54, which became less sensitive to suppressive signals.

Materials and Methods

Mice

NOD/Lt and NOD-SCID mice from The Jackson Laboratory (Bar Harbor, ME), as well as BALB/c and C57BL/6 (B6) mice were obtained from Charles River Laboratories (Calco, Italy). All mice were kept under specific pathogen-free conditions. Glucose levels in the tail venous blood were quantified using a EUROFlash (Lifescan, Issy les Moulineaux, France). A diagnosis of diabetes was made after two sequential glucose measurements higher than 200 mg/dl.

Cell transfer

Spleen cells, depleted of red cells, were incubated with anti-CD25 mAb (ATCC 7D4; American Type Culture Collection, Manassas, VA), and cells expressing CD25 were negatively selected, after incubation with streptavidin beads, using miniMACS columns (Miltenyi Biotec, Bergish Gladbach, Germany). CD4⁺CD25⁺ and CD4⁺CD25⁻ spleen cells were sorted with MultiSort Kit (Miltenyi Biotec). The purity of CD4⁺CD25⁺ cells was routinely ~85–90%. Purified cells were mixed as indicated and adoptively transferred by i.v. injection into NOD-SCID mice. Glucose levels in the tail venous blood were quantified at the indicated time points.

Cell cultures

CD4⁺CD25⁺ and CD4⁺CD25⁻ spleen cells were sorted with MultiSort Kit (Miltenyi Biotec). Purified CD4⁺CD25⁻ cells (2×10^5 /well) were cultured for 5 days in round-bottom 96-well plates (Costar, Cambridge, MA) with T cell-depleted C57BL/6 spleen cells (10^5 /well) in the presence of the indicated numbers of CD4⁺CD25⁺ cells. Cultures were performed in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% of fetal clone serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 50 μ g/ml gentamicin (Sigma-Aldrich). In addition, cultures were performed in the presence or absence of neutralizing anti-IL-10 mAb (JES5-2A5; BD PharMingen, San Diego, CA). To measure cell proliferation, cultures were pulsed 8 h before harvesting with 1 μ Ci [³H]TdR (40 μ Ci/nmol; Radiochemical Center, Amersham, U.K.). Incorporation of [³H]TdR was measured by liquid scintillation spectrometry. For quantification of secreted cytokines, purified CD4⁺CD25⁺ and CD4⁺CD25⁻ spleen cells were cultured for 24, 48, or 96 h in round-bottom 96-well plates (Costar) precoated with 10 μ g/ml purified anti-CD3 mAb (17A2; BD PharMingen) with 5 μ g/ml anti-CD28 mAb. For proliferation assays, purified CD4⁺CD25⁻ spleen cells were cultured for 48 h in round-bottom 96-well plates (Costar) precoated with 0.01 μ g/ml purified anti-CD3 mAb (17A2; BD PharMingen) and 10 μ g/ml purified anti-CD54 mAb (3E2; BD PharMingen), or 10 μ g/ml purified anti-CD28 mAb or 10 μ g/ml purified isotype control mAb. Cultures were performed in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM L-glutamine and 50 μ g/ml gentamicin (Sigma-Aldrich). Cell proliferation was measured, as described above.

Quantification of secreted cytokines

Secreted IFN- γ , IL-4, IL-2, and IL-10 were quantified by two-site ELISA, as described (25, 26). For IFN- γ , polyvinyl microtiter plates (Falcon 3012) were coated with 100 μ l AN-18.17.24 in carbonate buffer. Samples were titrated in test solution (PBS containing 5% FCS and 1 g/L phenol) and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated with biotinylated goat anti-mouse XMG1.2 mAb in test solution. After washing, the bound biotinylated Abs were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Avondale, PA) diluted 1/5000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma-Aldrich) in diethanolamine buffer (pH 9.6; 100 μ l/well). For IL-2, IL-4, and IL-10 determination, two-site ELISA were performed with paired mAbs from BD PharMingen. For capture, JES6-1A12 (anti-IL-2), BVD4-ID11 or 11B11 (anti-IL-4), and JES5-2A5 (anti-IL-10) were used. Samples were titrated in test solution and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated with the biotinylated mAb JES6-5H4 (anti-IL-2), BVD6-24G2 (anti-IL-4), or SXC-1 (anti-IL-10) in test solution. After washing, the bound biotinylated mAbs were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Chester, PA) diluted 1/5000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma-Aldrich) in diethanolamine buffer (pH 9.6; 100 μ l/well). The reaction was stopped by adding 50 μ l/well NaOH 3N, and absorbance was read at 405 nm. Cytokines were quantified from two to three titration points using standard curves generated by purified recombinant mouse cytokines, and results were expressed as cytokine concentration in pg/ml. The detection limit for all cytokines was 15 pg/ml.

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Isolation of pancreas-infiltrating T cells

After removal of all visible pancreatic lymph nodes, pancreata were individually digested in 3 ml HBSS containing 1 mg/ml collagenase IV (Sigma-Aldrich), by shaking (200 rpm) at 37°C for 15 min. Single cell suspensions were collected after diluting the enzyme with ice-cold HBSS containing 5% FCS and removal of the aggregates by settling for 2 min on ice. Aggregates were further digested with collagenase IV at 0.5 mg/ml for 10 min, and at 0.25 mg/ml for 6 min. Single cell suspensions were washed three times, and passed over a MASC Preseparation filter (Miltenyi Biotec) to remove cell aggregates and clumps. Single cell suspensions were incubated with anti-CD4 mAb-coated Microbeads and applied onto MiniMACS columns (Miltenyi Biotec). This procedure yielded a positive fraction containing CD4⁺ cells. Immediately after purification, cells were stimulated with PMA and ionomycin in complete medium, fixed, and stained the next day for intracellular production of cytokines.

Intracellular staining for cytokine production

Cells were stained for IFN- γ and IL-4, as previously described (25, 26). Reagents for intracytoplasmic staining contained 1% FCS, 0.5% saponin (Sigma-Aldrich), and 0.1% sodium azide. All incubations were performed at room temperature. Cells were washed, preincubated for 10 min with PBS/FCS/saponin, and then incubated with FITC-labeled rat anti-mouse IFN- γ (XMG1.2) and PE-labeled rat anti-mouse IL-4 (11B11). Isotype controls were FITC- and PE-labeled rat IgG1 (R3-34). After 30 min, cells were washed twice with PBS/FCS/saponin and then with PBS containing 5% of FCS without saponin to allow membrane closure. The cell surface was then stained with CyChrome-labeled anti-CD4 (L3T4) for 15 min at room temperature. Analysis was performed with a FACScan flow cytometer (BD Biosciences, Mountain View, CA) equipped with CellQuest software, and ~10,000 events were acquired.

Flow cytometric analysis

Stainings were performed in the presence of 100 μ g/ml mouse IgG using the following mAbs, all from BD PharMingen: CyChrome-labeled streptavidin, FITC-labeled anti-CD4 (L3T4), PE-labeled anti-CD4 (L3T4), FITC-labeled anti-CD69 (H1.2F3), PE-labeled anti-CD62L (MEL-14), PE-labeled anti-ICAM-1 (3E2), and biotinylated anti-CD25 (7D4). For CD132 staining, cells were incubated with purified anti-CD132 (4G3) (BD PharMingen), followed by PE-labeled anti-rat IgG (Jackson ImmunoResearch Laboratories). Cells were analyzed with a FACScan flow cytometer equipped with CellQuest software.

Results

CD4⁺CD25⁺ suppressor T cells from 8-, but not from 16-wk-old NOD mice inhibit diabetes development

To evaluate the role of NOD CD4⁺CD25⁺ suppressor T cells in controlling T1D development, we cotransferred them, together with splenocytes depleted of CD4⁺CD25⁺ T cells, from early diabetic NOD mice, ~16–18 wk of age, into NOD-SCID recipients. As shown in Fig. 1A, transfer of 10^7 CD25-depleted spleen cells from prediabetic NOD mice induced a significantly accelerated diabetes compared with 10^7 total spleen cells, indicating an enrichment in diabetogenic cells. Cotransfer of 9.5×10^6 CD25-depleted spleen cells and 5×10^5 CD4⁺CD25⁺ spleen cells isolated from 8-wk-old NOD mice induced diabetes with kinetic comparable to total spleen cells, but cotransfer with 10^6 CD4⁺CD25⁺ T cells significantly delayed diabetes onset, indicating that a sufficient number of CD4⁺CD25⁺ regulatory T cells is required to control T1D development. Interestingly, CD4⁺CD25⁺ spleen cells could transfer diabetes to NOD-SCID recipients, although with a significant delay, indicating that CD4⁺CD25⁺ spleen cells contain not only regulatory, but also pathogenic T cells. In contrast to CD4⁺CD25⁺ cells from 8-wk-old NOD mice, the same cell population isolated from 16-wk-old NOD mice could

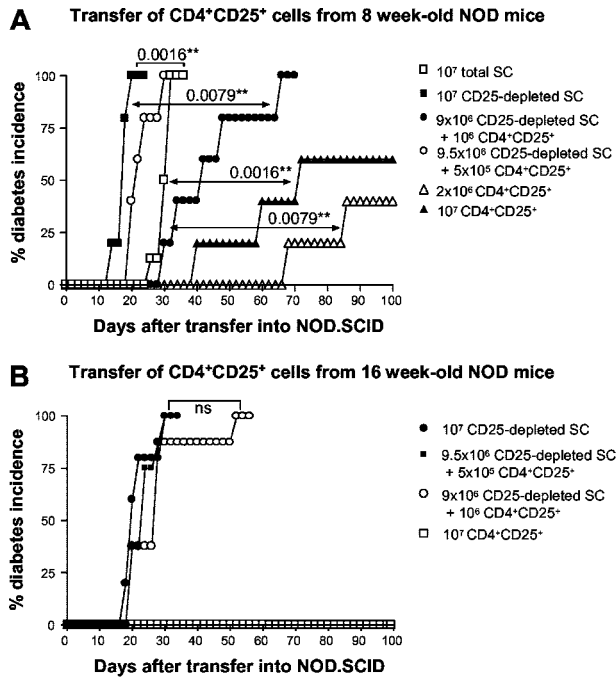


FIGURE 1. $CD4^+CD25^+$ T cells from 8- but not 16-wk-old NOD mice inhibit diabetes transfer. **A**, Eight- to ten-week-old NOD-SCID mice were injected with 10^7 splenocytes from 16-wk-old prediabetic NOD mice (\square , $n = 10$), or 10^7 CD25-depleted splenocytes (\blacksquare , $n = 10$), or coinjected with 9×10^6 or 9.5×10^6 CD25-depleted splenocytes and 10^6 (\bullet , $n = 8$) and 5×10^5 (\circ , $n = 8$) $CD4^+CD25^+$ splenocytes from 8-wk-old NOD mice. In addition, NOD-SCID recipients were injected with 2×10^6 (\triangle , $n = 5$), or 10^7 (\blacktriangle , $n = 5$) $CD4^+CD25^+$ splenocytes from 8-wk-old NOD mice. **B**, Eight- to ten-week-old NOD-SCID mice were injected with 10^7 CD25-depleted splenocytes from 16-wk-old prediabetic NOD mice (\bullet , $n = 8$), or coinjected with 9×10^6 or 9.5×10^6 CD25-depleted splenocytes and 10^6 (\circ , $n = 8$) or 5×10^5 (\blacksquare , $n = 8$) $CD4^+CD25^+$ splenocytes from 16-wk-old NOD mice. In addition, 10^7 (\square , $n = 5$) $CD4^+CD25^+$ splenocytes from 16-wk-old NOD mice. Blood glucose levels were determined twice weekly. Values of p were calculated by Mann-Whitney U test.

not inhibit diabetes development and could not transfer disease either (Fig. 1B). The inability of $CD4^+CD25^+$ T cells from 16-wk-old NOD mice to transfer diabetes in NOD-SCID recipients could be explained by the heterogeneity of $CD4^+CD25^+$ T cells (27) and by the lower frequency of pathogenic T cells contained in this population. These results indicate an age-dependent decrease in the capacity of NOD $CD4^+CD25^+$ cells to control diabetes development. In addition, a decrease in the pathogenic potential of $CD4^+CD25^+$ T cells from 16-wk-old NOD mice is also apparent.

CD4⁺CD25⁻ T cells isolated from 16-wk-old NOD mice are less susceptible to regulation by CD4⁺CD25⁺ cells

We next compared the suppressive activity of $CD4^+CD25^+$ T cells and the sensitivity to suppression of $CD4^+CD25^-$ cells from 8- and 16-wk-old NOD mice. No differences were observed in the frequency and in the total number of $CD4^+CD25^+$ or $CD4^+CD25^-$ cells in either spleen or pancreatic lymph nodes (data not shown). To determine the regulatory properties of $CD4^+CD25^+$ cells, we tested their ability to suppress the proliferative response of $CD4^+CD25^-$ cells to alloantigens. The proliferation of $CD4^+CD25^-$ cells from 8-wk-old NOD mice induced by C57BL/6 splenocytes was inhibited equally well by $CD4^+CD25^+$ cells from 8- or 16-wk-old NOD mice (Fig. 2). In contrast, $CD4^+CD25^-$ cells from 16-wk-old NOD mice were less sensitive to suppression, and only $CD4^+CD25^+$ cells from 8-wk-

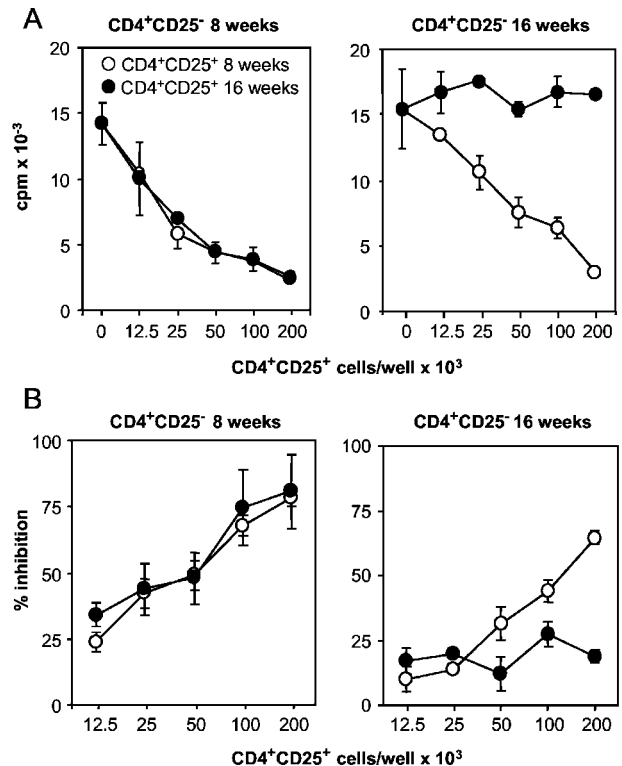


FIGURE 2. $CD4^+CD25^+$ T cells from 8- but not 16-wk-old NOD mice suppress the alloantigen-induced proliferation of $CD4^+CD25^-$ T cells from 16-wk-old NOD mice. $CD4^+CD25^-$ T cells from 8- or 16-wk-old NOD mice (2×10^5 /well) were incubated with T cell-depleted C57BL/6 cells (10^5 /well) in the presence of increasing numbers of $CD4^+CD25^+$ T cells isolated from 8- or 16-wk-old NOD mice. **A**, Proliferation of alloreactive $CD4^+CD25^-$ T cells from 8- or 16-wk-old NOD mice in the presence of increasing numbers of $CD4^+CD25^+$ T cells. Results represent mean cpm \pm SE of triplicate cultures from a representative experiment of five performed. Background proliferation of $CD4^+CD25^-$ T cells from 8- and 16-wk-old NOD mice was 1204 ± 173 and 1007 ± 78 cpm, and of $CD4^+CD25^+$ T cells from 8- and 16-wk-old NOD mice was 554 ± 34 and 909 ± 153 cpm, respectively. **B**, Percent inhibition of the proliferative response induced in $CD4^+CD25^-$ T cells from 8- or 16-wk-old NOD mice (2×10^5 /well) by T cell-depleted B6 cells (10^5 /well) in the presence of increasing numbers of $CD4^+CD25^+$ T cells isolated from 8- or 16-wk-old NOD mice. Results are expressed as mean \pm SE from five independent experiments.

old NOD mice could inhibit their proliferation, albeit less efficiently compared with the inhibition exerted on $CD4^+CD25^-$ cells from 8-wk-old NOD mice (Fig. 2). Even a higher ratio of $CD4^+CD25^+$ cells (3×10^5 /well) from 8-wk-old mice was not able to inhibit the proliferation of 2×10^5 $CD4^+CD25^-$ cells/well from 16-wk-old NOD mice ($16,225 \pm 477$ vs $15,446 \pm 1,302$ cpm obtained from $CD4^+CD25^-$ cells cultured alone). These results confirm the age-dependent impairment in the regulatory activity of $CD4^+CD25^+$ cells observed in vivo. In addition, they indicate that $CD4^+CD25^-$ T cells become progressively less sensitive to the control exerted by the $CD4^+CD25^+$ T regulatory cells, suggesting an intrinsic increase in their pathogenic potential.

Modulation of cytokine production by CD4⁺CD25⁺ and CD25⁻ cells during diabetes development

To determine whether the age-dependent modification in the function of $CD4^+CD25^+$ and $CD25^-$ cells could reflect a modification of their cytokine profile, we analyzed the ability to proliferate and the cytokine production of splenic $CD4^+CD25^+$ and $CD4^+$

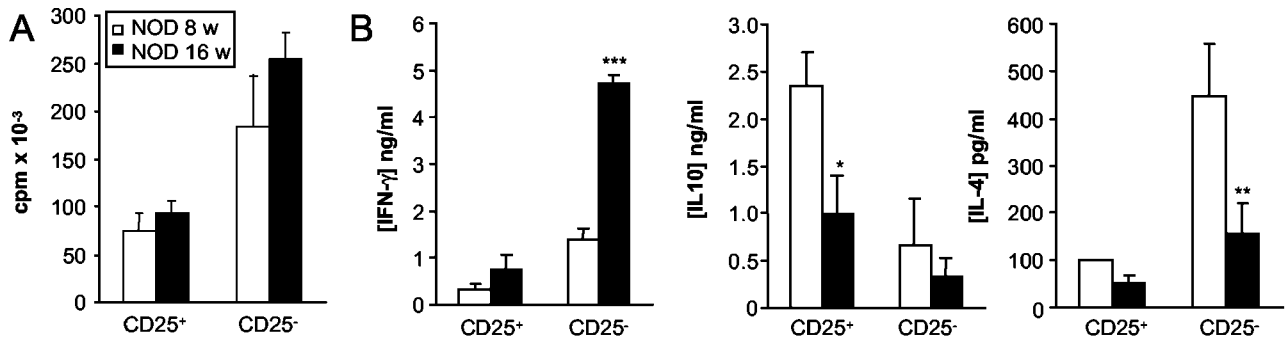


FIGURE 3. Cytokines secreted by CD4⁺CD25⁺ and CD4⁺CD25⁻ splenic T cells from 8- or 16-wk-old NOD mice. CD4⁺CD25⁺ and CD4⁺CD25⁻ splenic T cells were stimulated with plate-bound anti-CD3 mAb and 5 μg/ml soluble anti-CD28 mAb. After 48 h of culture, IFN-γ, IL-10, and IL-4 were quantified in culture supernatants by two-site ELISA. Cell proliferation was measured, as described in *Materials and Methods*. Bars represent mean ± SE of cell proliferation and cytokine production from four independent experiments. The *p* values were calculated by two-tailed Student's *t* test (*, *p* ≤ 0.05; **, *p* ≤ 0.005; ***, *p* ≤ 0.0005).

CD25⁻ T cells in response to immobilized anti-CD3 and soluble anti-CD28 stimulation. CD4⁺CD25⁺ regulatory T cells either from 8- or 16-wk-old mice proliferate, compared with CD4⁺CD25⁻ cells, less in response to polyclonal stimulation (Fig. 3A). In addition, CD4⁺CD25⁺ regulatory T cells from either 8- or 16-wk-old mice produced, compared with CD4⁺CD25⁻ cells, lower levels of IFN-γ and IL-4, but higher levels of IL-10 (Fig. 3B). Age-dependent effects in cytokine production were clearly observed in both T cell subsets. CD4⁺CD25⁺ T cells from 16-wk-old mice displayed a significant reduction in IL-10 production compared with cells from 8-wk-old mice. In contrast, the relatively low levels of IL-10 remained constant in CD4⁺CD25⁻ cells. However, this T cell subset displayed a striking age-dependent increase in IFN-γ production (4719 ± 175 pg/ml at 16 wk vs 1387 ± 239 pg/ml at 8 wk, *p* = 0.0005) and significantly lower levels of IL-4. To determine the role of IL-10 in the suppression mediated by CD4⁺CD25⁺ T cells from 8-wk-old mice, we tested their ability to suppress the alloantigen-induced proliferative response of CD4⁺CD25⁻ T cells from 8- or 16-wk-old NOD mice in the presence or absence of a neutralizing anti-IL-10 mAb (28). The addition of anti-IL-10 mAb did not affect the suppression mediated by CD4⁺CD25⁺ T cells (Fig. 4). Thus, although CD4⁺CD25⁺ cells from 8-wk-old NOD mice produce higher levels of IL-10 compared

with CD4⁺CD25⁺ cells from 16-wk-old NOD mice, their suppressive effect does not appear to be mediated by IL-10. These results support the observation that CD4⁺CD25⁺ T regulatory cells exert their suppressive activity through cell-cell contact rather than cytokine secretion (29).

Therefore, these data, demonstrating enhanced production of IFN-γ by CD4⁺CD25⁻ cells from 16- compared with 8-wk-old NOD mice, are consistent with the enhanced pathogenic activity of CD4⁺CD25⁻ cells during T1D progression. To determine whether the enhanced IFN-γ production by splenic CD4⁺CD25⁻ cells was paralleled by an age-dependent increase of Th1 cells in the target organ, pancreas-infiltrating CD4⁺ cells were analyzed for intracytoplasmic production of IFN-γ and IL-4. Results in Fig. 5 demonstrate that the percentage of Th1 cells infiltrating the pancreas was significantly higher in 16- compared with 8-wk-old NOD mice.

Age-dependent up-regulation of CD54 expression by CD4⁺CD25⁻ cells in NOD mice

To identify phenotypic modifications of CD4⁺ spleen cells, we investigated the expression of surface markers on cells isolated from 4-, 8-, and 16-wk-old and diabetic NOD mice as well as from 4-, 8-, and 16-wk-old BALB/c mice (Table I). The expression of CD69, CD62L, and CD132 was comparable on CD4⁺CD25⁻ spleen cells from mice at different ages in both NOD and BALB/c strains. The percentage of CD4⁺CD25⁻ cells expressing CD62L was significantly lower in NOD compared with BALB/c mice, as expected from the lower expression of CD62L in memory compared with naive T cells (30). The percentage of CD28⁺ cells was

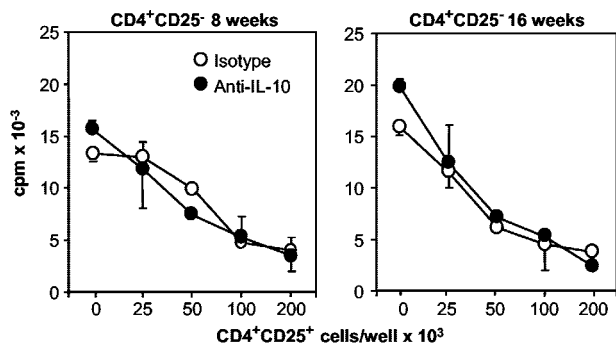


FIGURE 4. The suppression mediated by CD4⁺CD25⁺ T cells from 8-wk-old NOD mice is IL-10 independent. CD4⁺CD25⁻ T cells from 8- or 16-wk-old NOD mice (2 × 10⁵/well) were incubated with T cell-depleted C57BL/6 cells (10⁵/well) and with increasing numbers of CD4⁺CD25⁺ T cells isolated from 8-wk-old NOD mice in the presence of 10 μg/ml of anti-IL-10 mAb (●) or 10 μg/ml isotype control (○). Results represent mean cpm ± SE from triplicate cultures. Background proliferation of CD4⁺CD25⁻ T cells from 8- and 16-wk-old NOD mice was 1307 ± 73 and 1003 ± 104 cpm, and of CD4⁺CD25⁺ T cells was 177 ± 23 cpm, respectively.

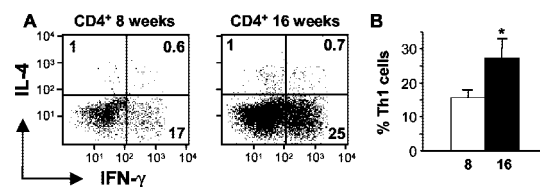


FIGURE 5. Age-dependent increase in the percentage of pancreas-infiltrating Th1 cells. *A*, Pancreas-infiltrating cells were isolated from 8- and 16-wk-old NOD mice. Positively selected CD4⁺ cells (2 × 10⁵ cells/well) were stimulated with PMA and ionomycin and analyzed flow cytometry for IFN-γ and IL-4 production. Acquisition was performed on CD4⁺ cells. Percentage of positive cells, set according to the isotype-matched controls (not shown), is shown in the *top corner* of each quadrant. *B*, Bar graphs represent the mean of two independent experiments, 8-wk-old (*n* = 4) and 16-wk-old (*n* = 6) NOD mice. The *p* value was calculated by Mann-Whitney *U* test.

Table I. Surface marker expression by splenic CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells^a

Surface Markers	NOD				BALB/c		
	4	8	16	Diabetic	4	8	16
% of positive cells in CD4 ⁺ CD25 ⁻ T splenocytes at the indicated ages (wk)							
CD69	1.7 ± 0.6	1.0 ± 0.3	1.5 ± 0.5	1.2 ± 0.2	1 ± 0.3	1.5 ± 0.5	0.7 ± 0.1
CD28	7 ± 1.6	11 ± 1	15 ± 6	28 ± 0.8**	5 ± 1.7	12 ± 4	17 ± 3
CD62L	42 ± 10	41 ± 9	40 ± 7	39 ± 8	86 ± 3 [†]	82 ± 8 [†]	85 ± 2 [‡]
CD54	13 ± 1	23 ± 5	35 ± 5*	32 ± 3*	45 ± 6	42 ± 16	36 ± 3
CD132	69 ± 15	73 ± 13	82 ± 10	80 ± 12	77 ± 18	89 ± 0.1	89 ± 0.1
% of positive cells in CD4 ⁺ CD25 ⁺ T splenocytes at the indicated ages (wk)							
CD69	5.6 ± 0.6	7 ± 0.6	8 ± 1.1	5.7 ± 1.2	3.2 ± 1.3	4.7 ± 1.2	3.3 ± 0.4
CD28	10.8 ± 1.6	11.4 ± 1.9	13.5 ± 2.6	21.7 ± 2.8	n.t. ^b	n.t.	n.t.
CD62L	47.2 ± 11	50.8 ± 8.7	50.9 ± 6.4	52.9 ± 8.5	64.2 ± 13	78.7 ± 4.3	81.6 ± 0.4
CD54	74.7 ± 2.3**	87 ± 2.8	89.8 ± 3.0	90.5 ± 1.3	91.7 ± 5.7	94.4 ± 3.6	90 ± 3.0
CD132	76.6 ± 5.9	79.2 ± 7.1	84.3 ± 4.8	83.9 ± 3.1	79 ± 27.2	97 ± 2.6	95.5 ± 0.8

^a Spleen cells were stained with mAbs specific for the indicated surface markers and CD25, and analyzed by flow cytometry. Acquisition was performed on CD4⁺ T cells. Results are mean ± SE from 12 individual NOD mice at each age and from 6 individual BALB/c mice at each age. The *p* values were calculated by two-tailed Student's *t* test between 4- and 16-wk-old and diabetic NOD mice vs 8-wk-old NOD mice (*, *p* < 0.05; **, *p* < 0.005), or between BALB/c vs NOD mice of the corresponding age (†, *p* < 0.05; ‡, *p* < 0.005).

^b n.t., not tested.

significantly increased in diabetic compared with prediabetic NOD mice, suggesting an involvement of this costimulatory pathway in the effector phase of T1D development. In addition, the percentage of CD4⁺CD25⁻ expressing CD54⁺ was significantly higher in NOD at 16 wk of age (*p* = 0.035) and in diabetic NOD mice (*p* = 0.048) compared with 4- and 8-wk-old NOD mice. Conversely, the frequency of CD4⁺CD25⁺ cells expressing CD54 was comparable between NOD and BALB/c mice, but was considerably higher compared with CD4⁺CD25⁻ cells, consistent with the higher expression of CD54 on CD4⁺CD25⁺ regulatory T cells (31).

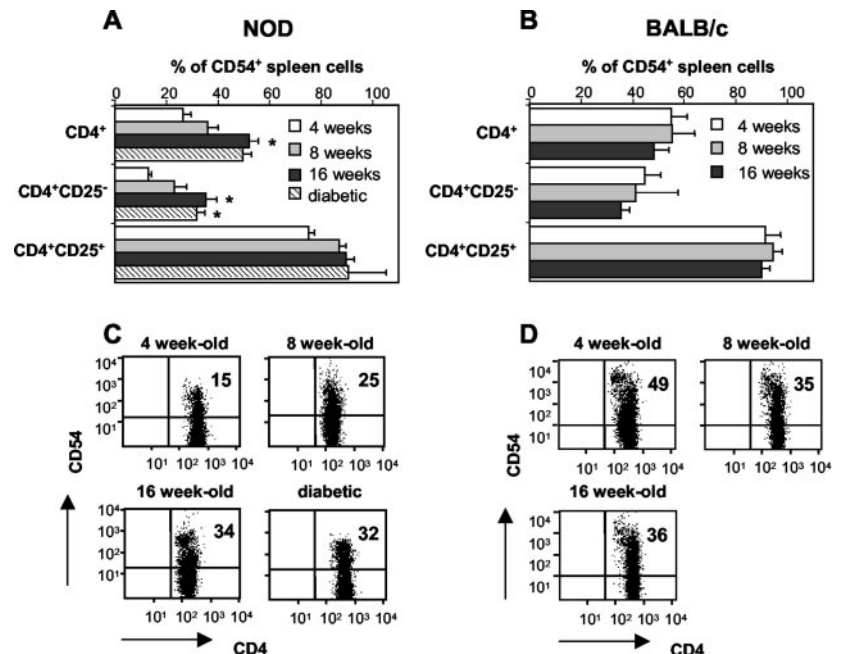
Results in Fig. 6, A and C, confirm the significantly higher percentage of CD25⁻CD54⁺ cells in 16-wk-old and diabetic NOD mice compared with 4- and 8-wk-old NOD mice. In contrast, the percentage of CD25⁺CD54⁺ T cells was similar in NOD mice of different ages. Conversely, the percentage of both CD25⁻CD54⁺ and CD25⁺CD54⁺ cells was comparable among cells isolated

from BALB/c mice of different ages (Fig. 6, B and D). These results suggest a possible role of CD54 in the pathogenicity of CD4⁺CD25⁻ cells in NOD mice.

CD54 blockade inhibits the proliferation of CD4⁺CD25⁻ T cells

Ligation of CD54 expressed on naive T cells delivers a costimulatory signal leading to T cell proliferation (32). Following costimulation with anti-CD3 and anti-CD54, a significantly higher proliferation was observed in CD4⁺CD25⁻ cells from 16- compared with 8-wk-old NOD mice, in agreement with their higher frequency of CD54-expressing cells (Fig. 7). Conversely, as predicted by the similar percentage of CD28⁺ T cells, no difference was observed in the T cell proliferation induced by costimulation with anti-CD3 and anti-CD28 (Fig. 7). To evaluate the role of CD54 expressed by CD4⁺CD25⁻ T cells, we analyzed their proliferative response to alloantigen in the presence of blocking anti-

FIGURE 6. Selective age-dependent increase in the percentage of splenic CD4⁺CD25⁻CD54⁺ cells in NOD mice. Spleen cells isolated from 4-, 8-, and 16-wk-old or diabetic NOD mice (A), or from 4-, 8-, and 16-wk-old BALB/c mice (B) were stained with mAbs specific for CD54 and CD25 and analyzed by flow cytometry. Acquisition was performed on CD4⁺ T cells. Results are mean ± SE from 12 individual mice at each age. Values of *p* were calculated by two-tailed Student's *t* test (*, *p* ≤ 0.05). Lower panels, Show the results of a representative experiment performed with NOD (C) or BALB/c (D) spleen cells stained with anti-CD54 mAb. Acquisition was performed on CD4⁺ T cells.



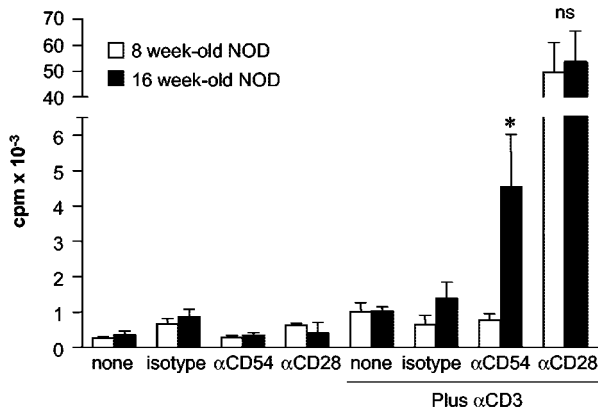


FIGURE 7. Increased CD4⁺CD25⁻ T cell proliferation by costimulation via CD54. CD4⁺CD25⁻ T cells from 8- or 16-wk-old NOD mice (2×10^5 /well) were stimulated by plate-bound anti-CD3 (0.01 μ g/ml) in the presence of plate-bound anti-CD54, anti-CD28, or isotype control, all coated at 10 μ g/ml. Bars represent mean cpm \pm SE of triplicate cultures from six individual mice/group from two separate experiments. Values of p were calculated by Mann-Whitney U test (*, $p \leq 0.05$).

CD54 mAb (Fig. 8). CD4⁺CD25⁻ T cells from 16-wk-old and diabetic NOD mice proliferated more to B6 stimulators compared with CD4⁺CD25⁻ T cells from 8-wk-old NOD mice, consistent with their increased pathogenicity and decreased sensitivity to immunosuppression. Addition of anti-CD54 mAb to the cultures inhibited the proliferative response of CD4⁺CD25⁻ pathogenic T cells from 8- and 16-wk-old and diabetic NOD mice, indicating that interaction of CD54 with CD11a (LFA-1) is required for their increased proliferative activity.

Discussion

In the present study, we have examined the dynamics of suppressor and pathogenic T cells during the development of autoimmune diabetes in the NOD mouse. CD4⁺CD25⁺ T suppressor cells from young, but not from older NOD mice inhibit, when cotransferred with pathogenic T cells into NOD-SCID recipients, T1D development. The pathogenesis of T1D in NOD mice is associated with modifications not only in CD4⁺CD25⁺ T suppressor cells that progressively produce less IL-10 and became less effective in modulating the proliferative response of pathogenic T cells, but also in the pathogenic T cells themselves. During T1D progression, there is an enhanced Th1 cell accumulation in the pancreas, and

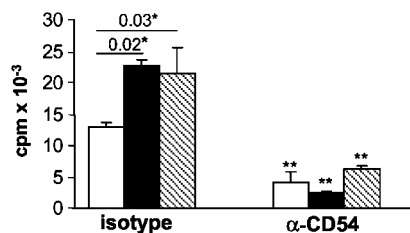


FIGURE 8. Inhibition of CD4⁺CD25⁻ T cell proliferation by anti-CD54 mAb. CD4⁺CD25⁻ T cells from 8 (open bars)- and 16-wk-old (filled bars) and diabetic (striped bars) NOD mice (2×10^5 /well) were incubated with T cell-depleted B6 cells (10^5 /well) in the presence of 10 μ g/ml of soluble anti-CD54 mAb or an isotype control. Bars represent mean of cpm \pm SE from triplicate cultures. Background proliferation of CD4⁺CD25⁻ T cells from 8- or 16-wk-old, and diabetic NOD mice was 554 ± 173 , 908 ± 107 , and 1460 ± 184 cpm, respectively. Values of p were calculated by Mann-Whitney U test (*, $p \leq 0.05$; **, $p \leq 0.005$). A representative experiment of three performed is shown.

CD4⁺CD25⁻ T cells produce higher levels of IFN- γ . In addition, CD4⁺CD25⁻ T cells expressing CD54, an important costimulatory/adhesion molecule in T1D development, progressively increase with age.

The presence of suppressor T cells in the NOD mouse is well established. CD4⁺ T cells from nondiabetic young mice could prevent the transfer of T1D by splenocytes from diabetic mice (33). Conversely, splenocytes from prediabetic mice depleted of CD4⁺CD25⁺ T cells induced a rapid T1D onset after transfer into NOD-SCID recipients (9). A subpopulation of CD4⁺ T cells expressing high levels of CD62L has been described in the thymus (34) and in the periphery (35, 36) of NOD mice, and has been found to protect against diabetes when cotransferred with pathogenic T cells into NOD-SCID recipients (34–36). CD4⁺CD62L^{high} cells have been suggested to play a role in regulating the final effector phase of T1D in NOD mice (37), and are similar to CD4⁺CD25⁺ suppressor T cells, because both subsets derive from the thymus and express high levels of CD62L (38, 39).

Our data show that CD4⁺CD25⁺ suppressor T cells are major players in modulating the outcome of T1D. Doubling their percentage delayed the onset of T1D in cotransfer experiments, consistent with the 50% reduction of this cell subset observed in NOD compared with other mouse strains (9). However, the CD4⁺CD25⁺ cell population does not contain only suppressor, but also pathogenic T cells, as shown by the induction of T1D in NOD-SCID recipients transferred with CD4⁺CD25⁺ NOD spleen cells. These results most likely reflect the presence within the CD4⁺CD25⁺ T cell population of both suppressive and nonsuppressive clones (27). Interestingly, the CD4⁺CD25⁺ T cell subset shows an age-dependent decrease in the capacity to either suppress or induce T1D, indicating that aging reduces, in the CD4⁺CD25⁺ T cell population, both suppressive and pathogenic properties.

Although the suppressive mechanisms mediated by CD4⁺CD25⁺ T cells are not yet fully characterized, a major role appears to be played by cell-cell interactions rather than soluble molecules (11, 40). Our data show that CD4⁺CD25⁺ T regulatory cells can suppress the proliferative response of NOD CD4⁺CD25⁻ T cells and inhibit their capacity to transfer diabetes, and this was associated with secretion of high levels of IL-10. However, the suppressive activity mediated in vitro by CD4⁺CD25⁺ T cells was, in our experimental model, independent of IL-10. Failure to reverse suppression mediated by CD4⁺CD25⁺ T cells by in vitro addition of neutralizing Abs specific for IL-10 has already been reported (38). In addition, CD4⁺CD25⁺ T cells from IL-10-deficient mice have been shown to possess a fully competent suppressive activity in vitro (38). Therefore, in vitro IL-10 production appears to be a marker, rather than an effector molecule, of CD4⁺CD25⁺ suppressor T cells. In vivo studies using blocking mAbs have suggested a key role for IL-10 produced by CD4⁺CD25⁺ T cells in the suppression of pathogenic responses in inflammatory bowel disease (41) and allograft rejection (42) models, but IL-10 absence fails to accelerate T1D development in the NOD mouse (43). Thus, the loss of suppressive activity by CD4⁺CD25⁺ T cells isolated from 16- compared with 8-wk-old NOD mice could be unrelated to the significant reduction of IL-10 secretion observed in vitro.

In addition to the progressive decrease in the suppressive activity of CD4⁺CD25⁺ cells, our studies have evidenced a progressive increase in pathogenic features of CD4⁺CD25⁻ cells. Th1 cells play a dominant role in the pathogenesis of T1D in NOD mice (3). In agreement with these data, the present results show an enhanced frequency of pancreas-infiltrating Th1 cells and a strongly increased IFN- γ production by CD4⁺CD25⁻ cells during T1D progression. Our data also indicate an important role for CD54 in the

increased pathogenicity of CD4⁺CD25⁻ T cells during T1D progression, as evidenced by the increased frequency, selectively in NOD mice, of CD4⁺CD25⁻ T cells expressing this adhesion/co-stimulatory molecule. CD54 plays a role in T cell proliferation (44) and Ag presentation (45), and induces the development of Th1 cells characterized by enhanced secretion of IL-2 and IFN- γ (46). Transgenic expression of pancreatic IL-10 has been shown to accelerate NOD diabetes via a CD54-dependent pathway (23), and several other studies have also suggested an important role of CD54 in the pathogenesis of T1D. Administration of anti-CD54 mAbs reduces insulinitis and protects from diabetes (21). In addition, autoreactive T cell responses to β cell Ag are completely blocked by anti-CD54 mAbs (22). The key role of CD54 in T1D development has been conclusively shown by the complete protection from disease in CD54-deficient NOD mice (24). Stimulation of T cells with CD54-deficient APCs induced significant amounts of IL-4 and IL-10, whereas IFN- γ production was reduced (47, 48), and T cell priming with CD54-deficient APCs led to the generation of effector T cells that migrated to the pancreas, but did not cause T1D, in contrast to T cells primed by CD54-expressing APCs that could induce rapid disease onset (49).

As evidenced above, the CD11a:CD18-CD54 receptor pair has been most widely studied focusing on the role of CD54 expressed by APCs interacting with CD11a⁺ T cells. However, a signaling role has been identified for CD54 expressed by T cells (50), and triggering CD54 by CD11a⁺ APCs has been shown to deliver a potent costimulatory signal leading to T cell activation, cytokine production, and proliferation comparable to costimulation via CD11a or CD28 (32). Our findings, showing that a significantly higher percentage of pathogenic T cells expresses CD54 during T1D development, support the important role of CD54 in disease pathogenesis, which could contribute to T cell interaction with APCs and T cell trafficking to the target tissue. Thus, the higher frequency of CD4⁺CD25⁻CD54⁺ NOD T cells can explain the increased Th1 infiltration into the pancreatic islets and the enhanced IFN- γ production associated with T1D progression. Compared with CD4⁺CD25⁻ cells, the percentage of CD4⁺CD25⁺ cells expressing CD54 is 2- to 4-fold higher both in NOD and in BALB/c mice, in agreement with recent data documenting the higher expression of CD54 in regulatory compared with nonregulatory T cells (31). Interestingly, the frequency of CD4⁺CD25⁺ cells expressing CD54 does not change during T1D development, suggesting a role for this adhesion/co-stimulatory molecule selectively in CD4⁺CD25⁻ cells.

In conclusion, we have demonstrated that progression of diabetes in NOD mice depends on both reduced suppressor activity of CD4⁺CD25⁺ T cells and increased pathogenicity of CD4⁺CD25⁻ T cells. The CD4⁺CD25⁺ T cells are characterized by a progressive reduction in the capacity to inhibit the proliferation of CD4⁺CD25⁻ T cells, associated with a progressively lower IL-10 secretion. Conversely, CD4⁺CD25⁻ pathogenic T cells progressively accumulate into the pancreas and produce higher levels of IFN- γ associated with enhanced frequency of CD54 expression. Therefore, these correlative data indicate that T1D in the NOD mouse is the result of a complex, progressive remodelling of suppressor and pathogenic T cells that underlies the disease process. Rebalancing these two cell subsets might be the key to successful immunointervention in autoimmune diabetes.

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