Further, the expression of protective or pathogenic properties correlated with cytokine production profiles in vitro.

RESEARCH DESIGN AND METHODS

Mice. NOD, NOD-*scid*, BALB/C, and CB-17–*scid* mice were maintained in a breeding colony in the conventional animal facility in the Department of Laboratory Animal Medicine, Stanford University. The NOD-*scid* breeding pairs were provided by Dr. Leonard D. Shultz (The Jackson Laboratory, Bar Harbor, ME). The incidence of diabetes in NOD mice by 24 weeks of age is ~70% in females and <20% in males. No insulitis or diabetes is detectable in the NOD-*scid* mice. Cells were taken from diabetic mice (>20 weeks of age) within 2 weeks of the onset of hyperglycemia. NOD mice aged 7–12 weeks (young mice) or 20–24 weeks (older mice) were used as nondiabetic donors. NOD-*scid* mice aged 4–6 weeks were used as cell transfer recipients.

Preparation of splenocyte subsets. The spleen was removed aseptically and minced. After lysing of red blood cells, cells were resuspended in RPMI-1640 containing 2% fetal calf serum (FCS) (medium). CD4$^+$ or CD8$^+$ subsets were prepared by magnetic separation using the MiniMACS system (Miltenyi Biotec, Auburn, CA)(27). Briefly, splenocytes were incubated with anti-CD4 or anti-CD8 magnetic microbeads (Miltenyi) for 15 min at 4°C, washed, and collected on a magnetic flow-through column. Purified cells (>95% purity) were then resuspended in medium. CD45RB^{high} and CD45RB^{low} subsets of CD4$^+$ cells were prepared by cell sorting. Purified CD4$^+$ cells were stained with *R*-phycoerythrin (PE)-conjugated anti-CD4 (YTS191.1; Caltag, South San Francisco, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD45RB (23G2; Pharmingen, San Diego, CA) antibodies. After washing with medium, the stained cells were resuspended in phosphate-buffered saline (PBS) containing 2% FCS for cell sorting. CD4$^+$ splenocytes were sorted into high and low CD45RB–expressing fractions (brightest 65–75% and dullest 10–15%) on a FACStar (Becton-Dickinson, San Jose, CA). Cells expressing intermediate levels of CD45RB were excluded. Sorting procedures were done aseptically.

Cell transfer procedure. Splenocytes and splenocyte subsets were resuspended in PBS at appropriate concentrations for transfer. Cell suspension (200 μl) was injected intraperitoneally into each NOD-*scid* mouse. NOD-*scid* recipients were then checked for glucosuria by Tes Tape (Eli Lilly, Indianapolis, IN) weekly. If glucosuria was observed, the blood glucose was measured with a One Touch II meter (Johnson and Johnson, Milpitas, CA). Mice with glucosuria and blood glucose >250 mg/dl were considered diabetic. Mice were examined macroscopically at time of killing and were excluded from the study if a thymoma was found.

Histological examination. Some of the NOD-*scid* recipients were used for pancreatic and bowel histology. Each pancreas was removed, fixed with 10% formaldehyde, and embedded in paraffin. At least three levels, 50 μm apart, were cut for staining with hematoxylin-eosin. The insulitis severity of all observable islets was assessed (>20 per mouse). Large and small bowels were similarly treated for histological examination.

Fluorescence-activated cell sorter (FACS) analysis. Splenocytes or peripheral blood lymphocytes were stained with PE-conjugated anti-CD4, FITC-conjugated anti-CD8 (Caltag), FITC-conjugated anti-CD45RB, FITC-conjugated anti-CD44 (pgp-1), or biotinylated anti-Mel-14 (Pharmingen) antibodies at 4°C for 20 min. Streptavidin allophycocyanin (Caltag) was used as a second step with anti-Mel-14 antibodies. After incubation at 4°C for another 20 min, stained cells were washed and resuspended in PBS containing 2% FCS and 10^4 cells were analyzed on a FACScan.

Cytokine assay. Sorted CD45RB^{low} CD4$^+$ splenocytes were resuspended in RPMI-1640 containing 10% FCS and 1% penicillin-streptomycin (culture medium). CD4-depleted splenocytes (CD4 <1%) were prepared using the MiniMACS system, irradiated (3,000 rad), resuspended in culture medium, and used as antigen-presenting cells (APCs). A total of 3×10^5 CD45RB^{low} CD4$^+$ cells and 6×10^5 APCs were transferred to each well on a round-bottom 96-well plate precoated with anti-CD3 antibody (2C11;10 μg/ml) and were cultured for 40–48 h at 37°C in 5% CO_2 (final volume, 250 μl per well). Supernatants were collected at the end of the culture and frozen until assay. Cytokine assays were done by enzyme-linked immunosorbent assay (ELISA). A flat-bottom 96-well plate was coated with anti–γ-interferon (IFN-γ) (R46A2) or anti–interleukin-4 (IL-4) (BVD-1D11) antibodies in PBS by overnight incubation at 4°C. Collected supernatants and standards (purified recombinant IFN-γ and IL-4 were a gift from DNAX) were added and incubated for 2 h. After washing with 0.1% Tween 20 PBS, biotinylated anti–IFN-γ (AN18) or IL-4 (BVD6–24G2) was added and incubated for another 1 h. AB solution (Vectastain ABC kit, Vector Laboratories, CA) was then added and incubated for 30 min. Finally, the color reagent 2,2′-azinobis(3-ethylbenz-thiazolinesulfonic acid) (ABTS; Sigma, St. Louis, MO) was added and the optical density was measured by ELISA reader (Titertek Multiskan) at 405 nm. The amount of cytokine present was determined from the standard curves from purified recombinant cytokines.

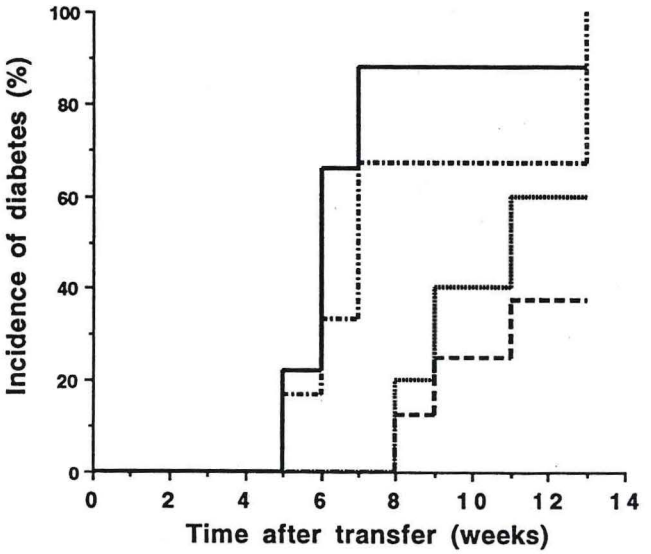

FIG. 1. CD4$^+$ splenocytes from young nondiabetic NOD mice protect from diabetes transfer in NOD-*scid* recipients. Splenocytes from diabetic NOD mice were transferred into NOD-*scid* recipients with subpopulations of splenocytes from young (7- to 12-week-old) nondiabetic NOD mice. Cotransferred cell populations were *1*) total splenocytes (n = 5, ····), *2*) CD4$^+$ splenocytes (n = 8, – – – –), *3*) CD8$^+$ splenocytes (n = 6, – · – · –), or *4*) PBS only (n = 9, ▬▬).

Statistical analysis. For comparison of disease onset times, the Mann-Whitney U test was used. Fisher's exact test was used for comparison of disease incidence. For other statistical analysis, Student's t test was used.

RESULTS

CD4$^+$ splenocytes from young nondiabetic NOD mice inhibit diabetes transfer to NOD-*scid* recipients. Splenocytes from recently diabetic NOD females (6×10^6, 37% CD4$^+$) were transferred into NOD-*scid* males in combination with splenocyte subsets from young (7- to 12-week-old) nondiabetic NOD females. The cotransferred cells were total splenocytes (6.4×10^6, 25% CD4$^+$), CD4$^+$ splenocytes (4.4×10^6), CD8$^+$ splenocytes (4.3×10^6), or no cells (PBS). By 13 weeks posttransfer, all of the mice in the PBS (9 of 9) and CD8$^+$ (6 of 6) cotransfer groups became diabetic. Cotransfer of total splenocytes from young nondiabetic NOD mice significantly inhibited disease transfer. Cotransfer of young nondiabetic CD4$^+$ splenocytes also significantly delayed ($P < 0.001$) the onset of diabetes, and the incidence was significantly reduced (3 of 8, $P < 0.05$) (Fig. 1). By 30 weeks posttransfer, only 50% of the CD4$^+$ young nondiabetic splenocyte cotransfer recipients had developed diabetes, although intra-islet insulitis was evident in all mice examined. Thus, CD4$^+$ splenocytes from young nondiabetic NOD mice inhibited the transfer of diabetes to NOD-*scid* recipients using splenocytes from diabetic NOD mice.

CD45RB^{low} and CD45RB^{high} subsets of CD4$^+$ splenocytes from young nondiabetic mice inhibit diabetes transfer. CD4$^+$ splenocytes from young nondiabetic NOD mice were further sorted into those expressing high or low

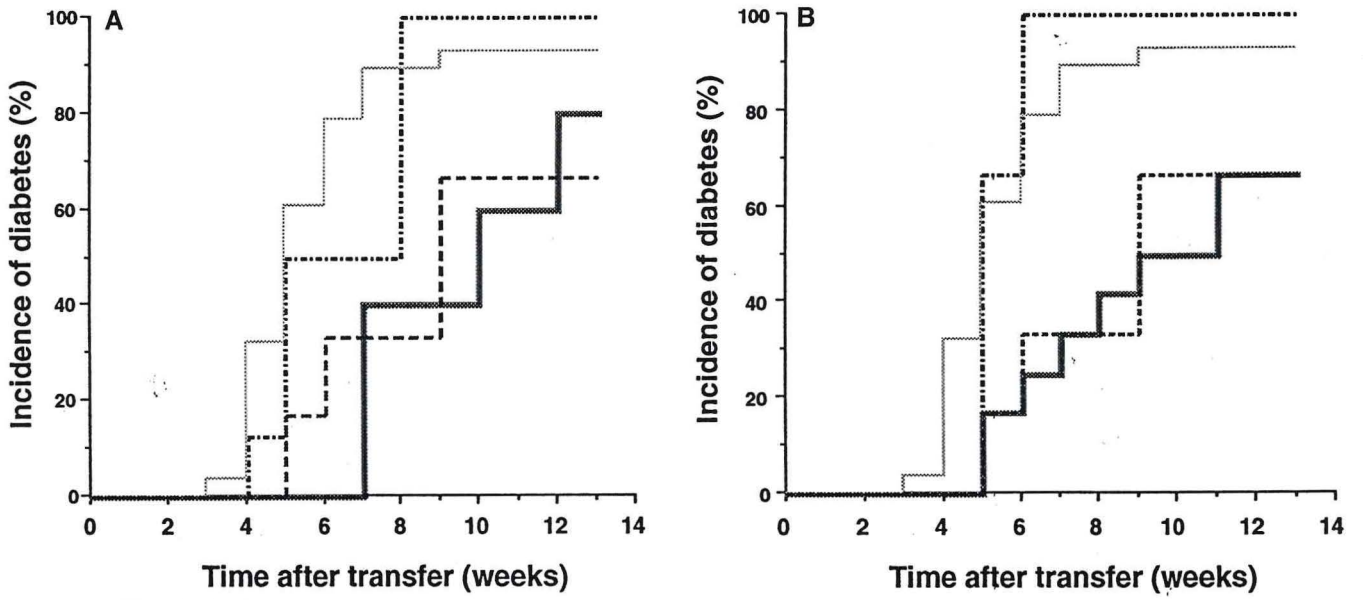

FIG. 2. CD45RB^{low} CD4$^+$ (*A*) and CD45RB^{high} CD4$^+$ (*B*) splenocytes from young nondiabetic NOD mice are protective and inhibit the transfer of diabetes to NOD-*scid* recipients. *A:* splenocytes from diabetic NOD mice were transferred into NOD-*scid* recipients in combination with CD45RB^{low} CD4$^+$ (ratio of diabetic CD4$^+$ to nondiabetic CD45RB^{low} CD4$^+$ cells 2:1 [n = 8, –·–·–] or 1:1 [n = 5, ▬▬]) or CD45RB^{high} plus CD45RB^{low} (n = 6, – – – –) CD4$^+$ splenocytes from young (7- to 12-week-old) nondiabetic mice or PBS only (n = 28, ····). *B:* splenocytes from diabetic NOD mice were transferred into NOD-*scid* recipients in combination with CD45RB^{high} CD4$^+$ (ratio of diabetic CD4$^+$ to nondiabetic CD45RB^{high} CD4$^+$ cells 2:1 [n = 3, –·–·–] or 1:1 [n = 12, ▬▬]) or CD45RB^{high} plus CD45RB^{low} (n = 6, – – – –) CD4$^+$ splenocytes from young (7- to 12-week-old) nondiabetic mice or PBS only (n = 28, ····).

levels of CD45RB (brightest 65–75% and dullest 10–15%). The CD45RB^{high} population was 33% Mel-14^{low}, whereas the CD45RB^{low} population was 79% Mel-14^{low}. Thus, phenotypically, the majority of CD45RB^{low} CD4$^+$ cells were activated/memory cells, while activated/memory cells constituted only a minority of CD45RB^{high} CD4$^+$ cells.

In agreement with a previous report (26), transfer of CD45RB^{low} CD4$^+$ or CD45RB^{high} CD4$^+$ cells from young nondiabetic NOD mice alone into NOD-*scid* recipients did not result in diabetes within 2–4 months after transfer (data not shown). Splenocytes from diabetic NOD mice (4–8 × 10^6, 43% CD4$^+$) were then transferred into NOD-*scid* mice in combination with CD45RB^{high} (2–3 × 10^6), CD45RB^{low} (1–2 × 10^6), or CD45RB^{high} (2 × 10^6) plus CD45RB^{low} (1.5 × 10^6) CD4$^+$ splenocytes from young (7- to 12-week-old) nondiabetic mice or no cells (PBS). The ratio of CD4$^+$ cells from nondiabetic mice to those from diabetic mice was varied by changing the cell numbers of CD4$^+$ cells from nondiabetic mice cotransferred. When the ratio of CD4$^+$ cells from diabetic versus nondiabetic mice was 1:1, the onset of diabetes was significantly delayed in recipients of both CD45RB^{low} and CD45RB^{high} CD4$^+$ cells compared with the PBS group (mean >10.0 and >9.8 vs. 5.8 weeks, $P < 0.05$); there was no significant difference in protection between CD45RB^{high} CD4$^+$, CD45RB^{low} CD4$^+$, and CD45RB^{high} plus CD45RB^{low} CD4$^+$ cells (Fig. 2). When the ratio was 2:1 (diabetic versus nondiabetic), neither subset showed any protective effect, which suggested a dose-response effect. Pancreatic histology revealed a similar degree of insulitis (peri-insulitis, intra-islet insulitis) in all nondiabetic recipients examined (data not shown). Thus, cotransfer of either CD45RB^{high} or CD45RB^{low} CD4$^+$ splenocytes from young nondiabetic NOD mice inhibited the transfer of diabetes.

Rapid change of CD45RB^{high} to CD45RB^{low} CD4$^+$ cells correlates with diabetes development in NOD-*scid* recipients. Five weeks after the transfer of splenocytes (6–12 × 10^6) from diabetic NOD mice with or without splenocytes from young nondiabetic NOD mice (Fig. 1), peripheral blood lymphocytes from one of the two experimental groups of NOD-*scid* recipients were analyzed by FACS. As shown in Fig. 3, in this group, all of the mice (11 of 11) that developed diabetes by 9 weeks posttransfer had a high proportion of CD45RB^{low} CD4$^+$ cells (57.4 ± 8.8%), whereas all of those (4 of 4) that did not develop diabetes by 9 weeks posttransfer had a low proportion of CD45RB^{low} CD4$^+$ cells (19.2 ± 2.0%,

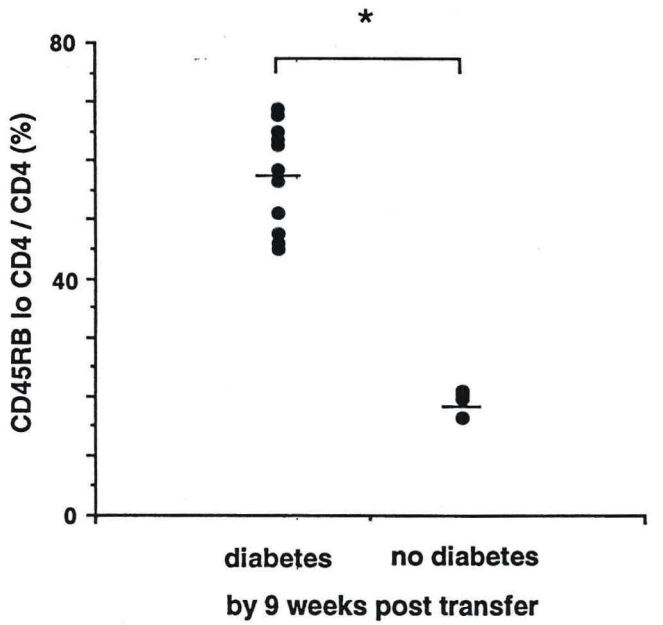

FIG. 3. The proportion of CD45RB^{low} CD4$^+$ cells correlates with diabetes development in NOD-*scid* recipients. The peripheral blood lymphocytes of one experimental group of NOD-*scid* recipients were analyzed by FACS at 5 weeks posttransfer (n = 15). By 9 weeks posttransfer, 11 mice had developed diabetes (diabetes group) and 4 mice had not (no-diabetes group). *$P < 0.0001$.

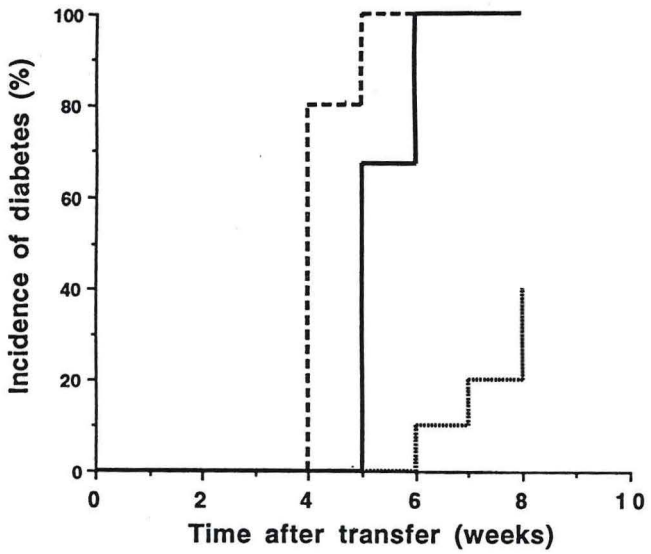

FIG. 4. CD45RB^{low} CD4$^+$ splenocytes from diabetic NOD mice are pathogenic and rapidly transfer diabetes to NOD-*scid* recipients when combined with CD8$^+$ splenocytes from diabetic mice. A total of 10^6 CD45RB^{high} CD4$^+$ (n = 10, ····), CD45RB^{low} CD4$^+$ (n = 5, – – – –), or total CD4$^+$ (n = 3, ▬▬) splenocytes from diabetic NOD mice were transferred into NOD-*scid* recipients in combination with 5 × 10^5 CD8$^+$ splenocytes from diabetic mice. Reconstitution of lymphoid tissue was more extensive with CD45RB^{high} cells than with CD45RB^{low} cells, cell recovery being approximately twofold greater.

$P < 0.0001$) at 5 weeks posttransfer. Following these recipient mice over time, diabetic NOD-*scid* recipients still showed a high proportion of CD45RB^{low} CD4$^+$ cells (~55%) at the time of killing (5–9 weeks posttransfer). However, nondiabetic NOD-*scid* recipients also developed a high proportion of CD45RB^{low} CD4$^+$ cells (~65%) over time (30 weeks posttransfer), suggesting that CD45RB^{high} CD4$^+$ cells changed to CD45RB^{low} slowly in these nondiabetic recipients. Thus, the majority of CD4$^+$ cells in recipient NOD-*scid* mice eventually became CD45RB^{low} regardless of the development of diabetes. These results suggested that progression to diabetes correlated with a rapid increase in the proportion of CD45RB^{low} CD4$^+$ cells. The observation that the time to onset in NOD-*scid* recipient mice that developed diabetes was significantly correlated with the proportion of CD45RB^{low} CD4$^+$ cells present at 5 weeks posttransfer was further evidence in support of this hypothesis (r^2 = 0.9).

CD45RB^{low} CD4$^+$ splenocytes from diabetic mice rapidly transfer diabetes to NOD-*scid* recipients. The correlation between the appearance of CD45RB^{low} CD4$^+$ cells and the progression to diabetes suggested that these cells might have pathogenic potential. We therefore transferred CD45RB^{low} CD4$^+$ (1 × 10^6), CD45RB^{high} CD4$^+$ (1 × 10^6), or total CD4$^+$ (1 × 10^6) splenocytes, combined with 5 × 10^5 CD8$^+$ splenocytes from diabetic mice, into NOD-*scid* recipients. All recipients of CD45RB^{low} CD4$^+$ plus CD8$^+$ cells developed diabetes within 5 weeks posttransfer (5 of 5), whereas no recipients of CD45RB^{high} CD4$^+$ plus CD8$^+$ cells developed diabetes within this period (0 of 10, $P < 0.001$) (Fig. 4). In CD45RB^{high} CD4$^+$ plus CD8$^+$ cell recipients, the diabetes incidence at 8 weeks after transfer was still lower (4 of 10, $P < 0.05$) and the time to diabetes much longer (mean >8.2 vs. 4.2 weeks, $P < 0.005$) than in recipients of CD45RB^{low} CD4$^+$ plus CD8$^+$ cells. As few as 4 × 10^5 CD45RB^{low} CD4$^+$ plus 4 × 10^5 CD8$^+$ splenocytes from diabetic mice were capable of transferring diabetes by 6 weeks posttransfer.

Thus, in terms of diabetes transfer, CD45RB^{low} CD4$^+$ splenocytes from diabetic NOD mice represent a pathogenic rather than a protective population; CD45RB^{high} CD4$^+$ cells from diabetic mice appear to be only weakly pathogenic.

Inflammatory bowel disease results from transfer of CD45RB^{high} CD4$^+$ cells. Although they did not transfer diabetes effectively, CD45RB^{high} CD4$^+$ cells produced inflammatory bowel disease in NOD-*scid* recipients (5 of 5), consistent with observations in other immunodeficient recipients (22,23). Mice lost weight and eventually died (after 10 weeks), and inflammatory bowel disease was uniformly present, as evidenced by obvious macroscopic and microscopic inflammation and thickening (Fig. 5). No NOD-*scid* recipients of CD45RB^{low} CD4$^+$ cells (0 of 4) developed inflammatory bowel disease within the period of observation.

Cytokine profiles of CD45RB^{low} CD4$^+$ splenocytes correlate with protective or pathogenic role in diabetes. Since CD45RB^{low} CD4$^+$ cells from young nondiabetic mice protected against the transfer of diabetes, whereas the same subset from diabetic mice was pathogenic, the cytokine profiles of these cells were assessed to see if any correlation existed. As shown in Fig. 6, CD45RB^{low} CD4$^+$ cells from diabetic NOD mice produced a significantly higher amount of IFN-γ than the same subset examined from either young (8- to 12-week-old) or older (20- to 24-week-old) nondiabetic NOD mice (57.6 ± 10.2 vs. 10.7 ± 5.9, 21.7 ± 4.3 U/ml; $P < 0.01$). CD45RB^{low} CD4$^+$ cells from diabetic NOD mice showed a significantly higher ratio of IFN-γ to IL-4 than did CD45RB^{low} CD4$^+$ cells from young or older nondiabetic NOD mice or BALB/C mice (18.5 ± 3.8, 2.3 ± 0.4, 3.7 ± 0.9, 0.7 ± 0.1; $P < 0.001$). Thus, the pathogenic potential of CD45RB^{low} CD4$^+$ cells from NOD mice correlated with an increased production of IFN-γ and reduced production of IL-4 after stimulation in vitro.

CD45RB^{low} CD4$^+$ splenocytes from older nondiabetic NOD mice cannot transfer diabetes. While the IFN-γ–to–IL-4 ratio increased with age in NOD mice, there was a dramatic (approximately fivefold) increase in this ratio after the onset of diabetes when compared with age-matched older nondiabetic mice (Fig. 6).

Because of the lower IFN-γ–to–IL-4 ratio of older nondiabetic NOD mice and the lack of overt disease, we compared transfer of CD45RB^{low} CD4$^+$ splenocytes (5 × 10^5) from diabetic mice with transfer of the same subset from older nondiabetic (20- to 24-week-old) NOD mice into NOD-*scid* recipients along with 5 × 10^5 CD8$^+$ splenocytes from diabetic mice. Transfer of age-matched nondiabetic CD45RB^{low} CD4$^+$ cells with CD8$^+$ cells from diabetic mice did not result in diabetes development in recipient NOD-*scid* mice whereas CD45RB^{low} CD4$^+$ cells from diabetic mice cotransferred with CD8$^+$ cells from diabetic mice allowed diabetes to develop in all recipients (Fig. 7).

DISCUSSION

Previous studies of the adoptive transfer of diabetes into irradiated NOD mice showed that cotransfer of CD4$^+$ cells from young NOD mice inhibited diabetes transfer (17,18). More recently, it was shown that depletion of CD4$^+$ cells in NOD mice facilitated transfer of diabetes into those mice

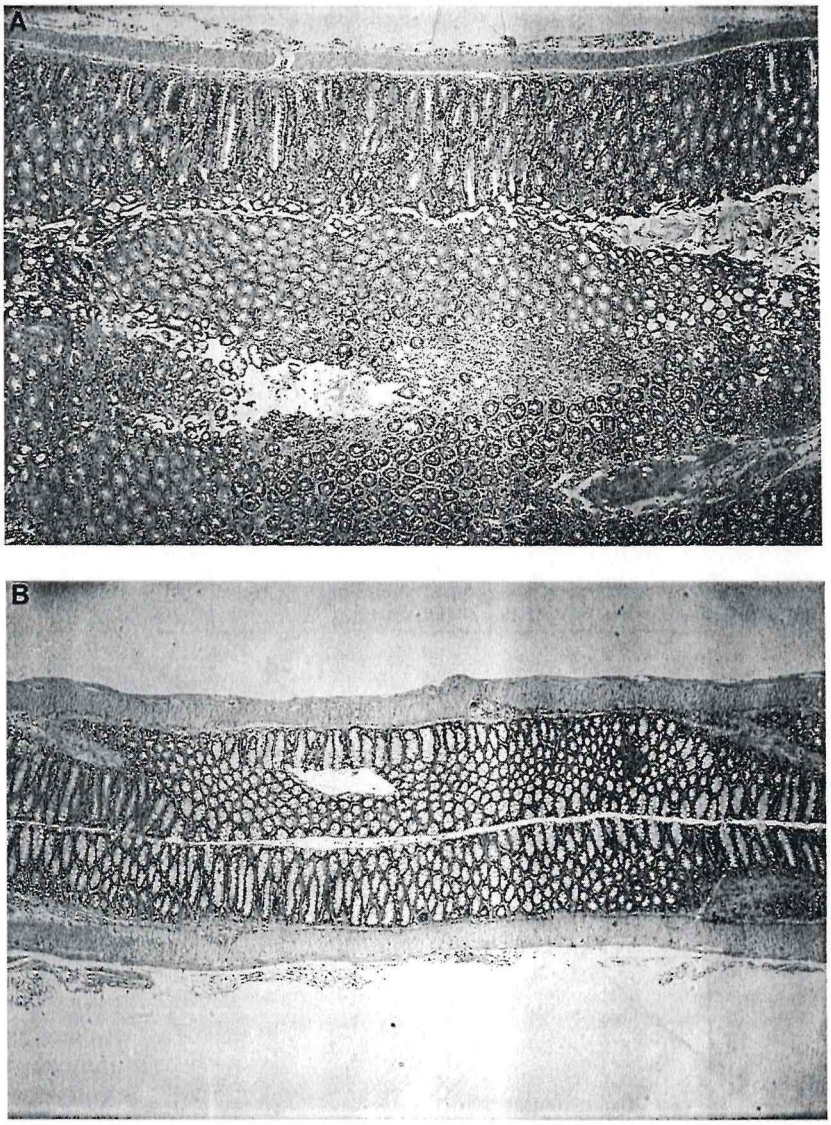

FIG. 5. Inflammatory bowel disease develops in NOD-*scid* recipients of CD45RB^{high} CD4^{+} cells. Histological appearance of a section of the large intestine from NOD-*scid* recipients of CD45RB ^{high} CD4^{+} (*A*) and CD45RB^{low} CD4^{+} (*B*) cells. Both sections are presented at the same magnification (40×), showing mucosal hyperplasia in recipients of CD45RB^{high} CD4^{+} cells. The bowel of untreated NOD-*scid* mice appeared similar to that of recipients of CD45RB^{low} CD4^{+} cells.

with adoptively transferred cells from diabetic NOD mice; untreated mice were resistant to diabetes development after adoptive transfer of diabetic cells (28). Thus, in two different models, CD4^{+} T-cells have been shown to control the development of transfer of diabetes in NOD mice. However, it has been shown that CD4^{+} T-cells are also necessary for the development of diabetes in NOD mice. Depletion of CD4^{+} cells prevented the development of insulitis (29), the progression from insulitis to hyperglycemia (1), and the recurrence of disease in islet grafts (30). Adoptive transfer of diabetes is dependent on transfer of at least CD4^{+} cells (31,32). Thus, in the NOD mouse, it appears that the CD4^{+} T-cell subset contains both pathogenic and protective cell populations, the balance of which changes as diabetes develops.

CD4^{+} T-cells can be further categorized phenotypically according to the level of expression of several surface molecules. One of these, CD45RB, has recently been shown to correlate with pathogenic potential of CD4^{+} cells in some animal models (20,22,23,33). The transfer of CD4^{+} cells expressing CD45RB (RC in rats) to immunodeficient mice or rats resulted in a multiple-organ inflammatory disease. The small and large bowels were particularly affected by the inflammatory response (20,22,23). Cotransfer of CD45RB^{low} CD4^{+} cells or transfer of total CD4^{+} cells prevented the development of pathological changes in the bowel. The response was thought to be the result of an autoaggressive potential of the CD45RB^{high} subset of CD4^{+} cells manifest as a cell-mediated immune response (33). Thus, in this model, CD45RB^{high} CD4^{+} cells were pathogenic, while CD45RB^{low} CD4^{+} cells were protective and regulated the CD45RB^{high} CD4^{+} cell activity (23).

This scenario was also described in an organ-specific model of autoimmune disease. In a model of islet-specific autoimmunity induced in thymectomized, sublethally irradiated rats, the development of diabetes could be prevented by the transfer of CD45RC^{low} CD4^{+} cells after irradiation (21). The authors concluded that the irradiation protocol uncovered potential islet autoreactivity and that the pathogenic autoimmune response could be prevented by the protective CD45RC^{low} CD4^{+} subset.

A relationship between the expression of CD45RB and production of particular cytokines by CD4^{+} cells has been reported; CD45RB^{high} CD4^{+} cells tend to produce IL-2 and IFN-γ, while CD45RB^{low} CD4^{+} cells produce IL-4 and IL-10 (23,34). Based on current knowledge of the interactions

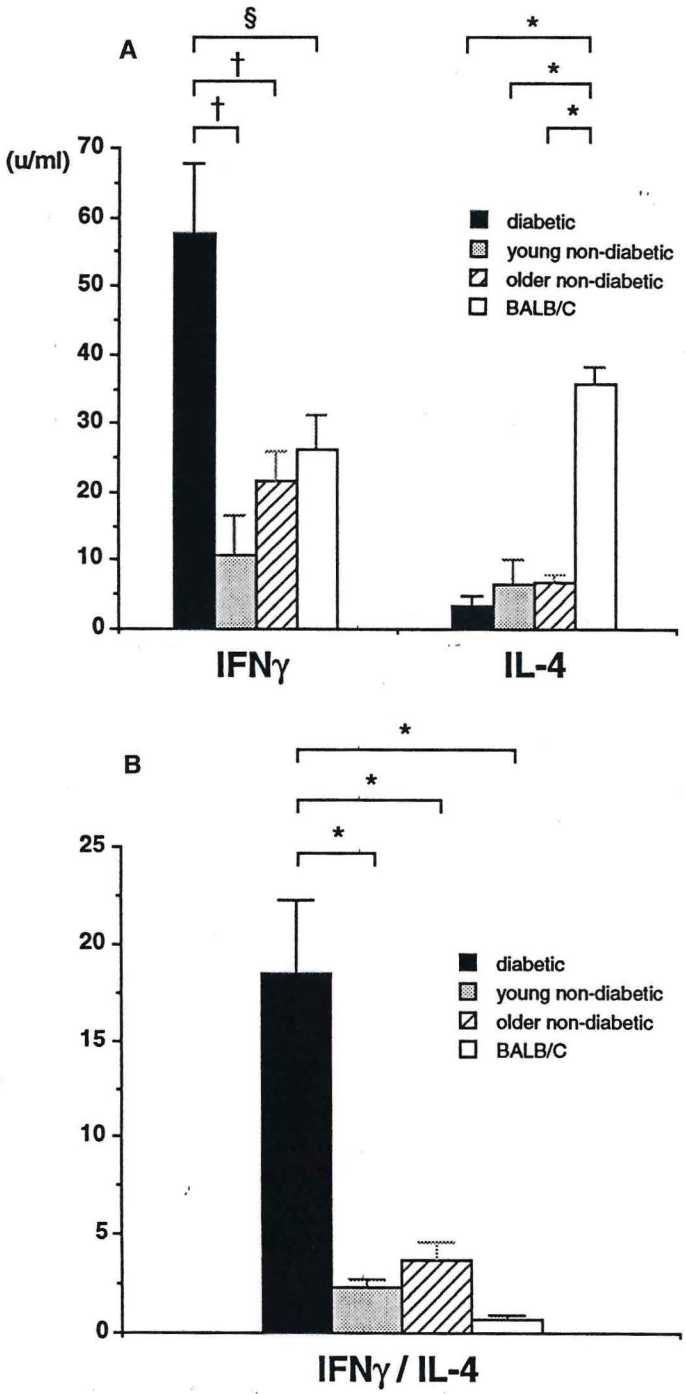

FIG. 6. CD45RB^{low} CD4^{+} splenocytes from diabetic NOD mice have a higher ratio of IFN-γ to IL-4. CD45RB^{low} CD4^{+} splenocytes (3 × 10^{5}) from diabetic (n = 6), young (8- to 12-week-old, n = 12), or older (20- to 24-week-old, n = 8) nondiabetic NOD or BALB/C (13- to 14-week-old, n = 4) mice were stimulated with anti-CD3 antibodies for 40–48 h with APCs (6 × 10^{5} 3,000-rad irradiated CD4-depleted splenocytes). Supernatants were analyzed by ELISA. *P < 0.001; †P < 0.01; §P < 0.05.

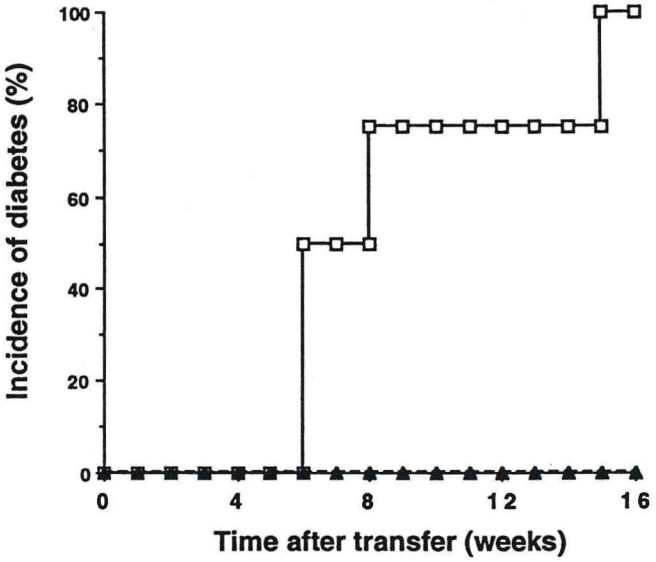

FIG. 7. CD45RB^{low} CD4^{+} splenocytes from older nondiabetic NOD mice cannot transfer diabetes. CD45RB^{low} CD4^{+} splenocytes (5 × 10^{5}) from diabetic (n = 4, □) or older nondiabetic (20- to 24-week-old, n = 4, ▲) NOD mice were transferred into NOD-*scid* recipients with 5 × 10^{5} CD8^{+} splenocytes from diabetic mice.

between Th1- and Th2-type cells, it could be expected that Th2-type CD45RB^{low} cells (IL-4/IL-10) might suppress the activity of Th1-type CD45RB^{high} cells (IFN-γ). Some evidence has been produced for this in the inflammatory bowel disease model (33). This paradigm for the control of cell-mediated autoimmune responses is attractive because of its potential application to human autoimmune diseases including IDDM. The role of CD45RB^{low} CD4^{+} cells in the control of disease in the NOD mouse, a model of spontaneous autoimmune diabetes in humans, was therefore examined.

To characterize regulatory cells in the disease process of the NOD mouse, we transferred diabetic NOD splenocytes into NOD-*scid* recipients with splenocyte subsets from young nondiabetic NOD mice. The CD45RB^{low} subset of CD4^{+} cells from young nondiabetic NOD mice was found to be protective and inhibited diabetes transfer to NOD-*scid* recipients. However, in contrast to other models, the CD45RB^{high} subset was also protective and inhibited diabetes transfer to a similar extent. Thus, CD45RB^{low} CD4^{+} cells do not appear to be the only subset capable of transferring protective function in NOD mice.

Most CD4^{+} cells in recipient mice, regardless of whether or not diabetes developed, were activated/memory cells as determined by surface phenotype (Mel-14^{low}, CD44^{high}, CD45RB^{low}). This finding seems similar to that reported after other cell transfers into immunodeficient mice and rats (20,22,23). FACS analysis of islet-infiltrating cells in some recipient mice showed that the CD4^{+} cells within the islets were also largely CD45RB^{low} and Mel-14^{low} (70–80% and 80–90%, respectively, n = 3; data not shown) regardless of whether diabetes developed. Moreover, analysis of peripheral blood lymphocytes from NOD-*scid* recipients of NOD splenocytes clearly showed that the rate of increase in the acquisition of the CD45RB^{low} phenotype of CD4^{+} cells correlated with the development of diabetes. These observations suggested that CD45RB^{low} CD4^{+} cells might have a pathogenic role in disease transfer. This was tested by cotransferring CD45RB^{low} CD4^{+} cells from diabetic mice to NOD-*scid* recipients in combination with CD8^{+} splenocytes from diabetic mice.

The CD45RB^{low} CD4^{+} cells from diabetic mice combined with CD8^{+} cells from diabetic mice (26) rapidly transferred diabetes into NOD-*scid* recipients, whereas CD45RB^{high} CD4^{+} cells were very inefficient. Thus, in a diabetic NOD mouse, the pathogenic cells are mainly contained in the CD45RB^{low} population. While this appears inconsistent with

the regulatory mechanisms suggested by previous studies (22,23), it is not surprising that a population of cells that express an activated/memory phenotype should be able to transfer diabetes. It suggests that in the NOD mouse, the $CD45RB^{low}$ $CD4^+$ cell population changes in function according to the disease status of the mouse, from protective in nondiabetic status to pathogenic in diabetic status.

While the $CD45RB^{high}$ subset of $CD4^+$ cells in diabetic NOD mice was ineffectual at diabetes transfer, this subset was very potent as a mediator of inflammatory bowel disease. This is not different from other studies in immunodeficient mice (22,23) and shows that diabetes and inflammatory bowel disease are pathophysiologically different.

Since $CD45RB^{low}$ $CD4^+$ cells from diabetic and nondiabetic mice behaved so differently in the NOD-*scid* transfer model, a correlate in vitro of their function in vivo was sought. Because of the evidence favoring cytokine involvement in the pathogenesis of inflammatory bowel disease (23, 33), an examination of cytokine production by $CD45RB^{low}$ $CD4^+$ cells in relevant NOD mice was performed. After stimulation with anti-CD3 antibodies, $CD45RB^{low}$ $CD4^+$ cells from diabetic mice produced significantly more IFN-γ (and less IL-4) and therefore a higher ratio of IFN-γ to IL-4 than those from nondiabetic mice. This difference was not age related, since the cytokine profiles of age-matched older nondiabetic mice were similar to those of young nondiabetic mice. Not only did $CD45RB^{low}$ $CD4^+$ cells from older nondiabetic NOD mice produce less IFN-γ than cells from diabetic mice, but they were also unable to transfer diabetes to NOD-*scid* recipients. An association between increased IFN-γ production by $CD4^+$ cells and the presence of diabetes was also observed in the NOD-*scid* recipients. Purified $CD4^+$ cells from diabetic NOD-*scid* recipients produced a higher ratio of IFN-γ to IL-4 than those from nondiabetic NOD-*scid* recipients (data not shown).

The $CD45RB^{high}$ subset of $CD4^+$ cells was not extensively studied because of the more obvious dichotomy of function of the $CD45RB^{low}$ population. It is not clear whether the $CD45RB^{high}$ subset directly affords protection from diabetes or whether the $CD45RB^{low}$ derivatives actually exert the effect. It is, however, of note that mitogenic stimulation of the $CD45RB^{high}$ population results in less IFN-γ than that produced by $CD45RB^{low}$ cells from diabetic mice, consistent with the established association (data not shown).

The correlation between cytokine profiles in vitro and pathogenic or protective function in vivo suggested that Th1-like $CD45RB^{low}$ $CD4^+$ cells were involved in the disease processes, whereas Th0- or Th2-like $CD45RB^{low}$ $CD4^+$ cells were associated with protection from diabetes. This has been suggested to be the case for diabetes induced in double transgenic mice (35). However, this will need to be more directly demonstrated using cells from both peripheral lymphoid organs and islet lesions and measuring responses to appropriate islet antigens.

We conclude that there exist functional differences between the $CD45RB^{low}$ $CD4^+$ populations of cells from diabetic and nondiabetic NOD mice and that both pathogenic and protective cells reside in this subset. The pathogenic or protective function correlates with production of IFN-γ and IL-4 in vitro. Since the majority of $CD45RB^{low}$ $CD4^+$ cells have an activated/memory phenotype, it is hypothesized that regulatory or nonpathogenic autoantigen-reactive cells dominate in nondiabetic mice but that with disease progression there is a change and the majority of activated/memory cells become pathogenic.

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