

# The Role of Lymphocyte Subsets in Accelerated Diabetes in Nonobese Diabetic–Rat Insulin Promoter–B7-1 (NOD-RIP-B7-1) Mice

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## Summary

B7-1 transgene expression on the pancreatic islets in nonobese diabetic (NOD) mice leads to accelerated diabetes, with >50% of animals developing diabetes before 12 wk of age. The expression of B7-1 directly on the pancreatic  $\beta$  cells, which do not normally express costimulator molecules, converts the cells into effective antigen-presenting cells leading to an intensified autoimmune attack. The pancreatic islet infiltrate in diabetic mice consists of CD8 T cells, CD4 T cells, and B cells, similar to diabetic nontransgenic NOD mice. To elucidate the relative importance of each of the subsets of cells, the NOD–rat insulin promoter (RIP)-B7-1 animals were crossed with NOD. $\beta$ 2microglobulin  $-/-$  mice which lack major histocompatibility complex class I molecules and are deficient in peripheral CD8 T cells, NOD.CD4  $-/-$  mice which lack T cells expressing CD4, and NOD. $\mu$ MT  $-/-$  mice which lack B220-positive B cells. These experiments showed that both CD4 and CD8 T cells were necessary for the accelerated onset of diabetes, but that B cells, which are needed for diabetes to occur in normal NOD mice, are not required. It is possible that B lymphocytes play an important role in the provision of costimulation in NOD mice which is unnecessary in the NOD-RIP-B7-1 transgenic mice.

Key words: nonobese diabetic mice • rat insulin promoter–B7-1 transgene • accelerated diabetes • T cells • B cells

Insulin-dependent diabetes in the nonobese diabetic (NOD)<sup>1</sup> mouse occurs as a result of an autoimmune attack on pancreatic  $\beta$  cells. Although T cells play a very important role in the process, and both CD4 and CD8 T cells are required to adoptively transfer diabetes (1, 2), other cell types are clearly also important in the pathogenesis of disease.

Compelling evidence for the role of CD8 T cells in the initiation of diabetes comes from studies where the  $\beta$ 2microglobulin ( $\beta_2m$ )  $-/-$  mutation was bred onto the NOD background (NOD $\beta_2m^{null}$ ), generating mice in which MHC class I expression is deficient (3, 4); consequently, these mice have very poor development of CD8 T cells. These NOD $\beta_2m^{null}$  mice do not develop either insulinitis or diabetes (3–6). In addition, treatment of NOD mice with antibodies against CD8 T cells, when given before 5 wk of age, prevents both insulinitis and diabetes (7). Further support for the role of CD8 T cells in initiation of disease has come from studies that demonstrate that T cells from young pre-

diabetic NOD mice can transfer diabetes to MHC class I–positive NOD.SCID mice but not NOD.SCID mice with the  $\beta_2m^{null}$  mutation lacking MHC class I (8). It is clear that CD8 T cells also play a role in the final effector phases of diabetes. This is shown by the fact that development of diabetes is delayed when diabetic spleen cells are adoptively transferred into NOD $\beta_2m^{null}$  mice, but there was no delay in disease transfer into NOD $\beta_2m^{null}$  mice bearing  $\beta_2m$  on the rat insulin promoter (RIP), which express MHC class I exclusively on the pancreatic  $\beta$  cells (9).

CD4 T cells are also clearly important in the evolution of insulinitis and for diabetes to occur, as it has also been shown that both insulinitis and diabetes are prevented using antibodies against CD4 (10). Although adoptive transfer experiments have shown, for the most part, that both CD4 and CD8 T cells are required for diabetes to occur (1, 2), both CD8 T cells (11) and CD4 T cells (12) are able to transfer disease when given alone to NOD.SCID animals, which lack lymphocytes. T cell receptor transgenic mice that bear a single specificity of CD4 T cells, which are reactive to pancreatic  $\beta$  cells, having been crossed with recom-

<sup>1</sup>Abbreviations used in this paper:  $\beta_2m$ ,  $\beta$ 2microglobulin; NOD, nonobese diabetic; RIP, rat insulin promoter.

binase activating gene (RAG)-2  $-/-$  mice on the NOD background, develop accelerated diabetes (13). In addition, NOD mice crossed with mice that lack MHC class II molecules and are therefore deficient in CD4 T cells do not develop insulinitis (5). However, this experiment is not easily interpreted, since these mice also have altered MHC class I molecules. This is because the MHC class II knockout mutation is made directly into I-A<sup>b</sup>, and therefore, when NOD mice are crossed with I-A<sup>b</sup>  $-/-$  mice, they will have the MHC class I molecules K<sup>b</sup> and D<sup>b</sup> (rather than K<sup>d</sup> and D<sup>b</sup> in NOD). CD4 T cells are clearly involved in the development of disease, as adoptive transfer of diabetic CD4 T cells alone into NOD.SCID animals will eventually cause disease, whereas isolated CD8 T cells do not (14). However, when cells from younger NOD donors are used, both CD4 and CD8 T cells are required.

B cells also play a role in the development of diabetes (15–17), the lack of B cells preventing development of diabetes. Although B cells are not required for adoptive transfer of diabetes (18), unable to cause disease when transferred alone to NOD.SCID mice (our unpublished observations), and serum does not transfer disease, B cells clearly influence the development of diabetes in a manner that probably relates to their antigen-presenting function. However, the precise role these cells play is still to be elucidated.

We have previously described NOD mice that express the costimulatory molecule B7-1 using the RIP on the pancreatic  $\beta$  cells, which develop clearly accelerated diabetes, with >50% of mice developing diabetes before the age of 12 wk when their nontransgenic littermates are just beginning to develop disease (19). This phenomenon is seen as early as the first backcross generation in mice that are homozygous for H-2<sup>g7</sup> and B7-1 transgene positive. Diabetes in such mice occurs as early as 3 wk of age, and when examined histologically, the infiltrate is very similar to that seen in NOD mice, with large numbers of CD4 and CD8 T cells as well as a substantial number of B220-positive B cells (19). This study aimed at examining the role of CD4 and CD8 T cells, as well as B cells, in the accelerated model of diabetes of the NOD-RIP-B7-1 transgenic mice and comparing them to the role of these cells in the nontransgenic NOD mouse. We postulate that the B7-1-expressing  $\beta$  cells are a potent stimulus for CD8 T cells that are specific for  $\beta$  cell peptides presented by MHC class I molecules. When  $\beta$  cells are attacked by CD8 T cells, they release soluble antigens that are taken up by antigen-presenting cells which then present peptide to CD4 T cells in a similar manner to that found in the nontransgenic NOD mouse.

## Materials and Methods

**Mice.** NOD-RIP-B7-1 transgenic mice were generated by crossing C57BL/6 mice transgenic for the human B7-1 molecule with NOD/Caj mice from our colony (19). Mice used in the experiments were all homozygous for H-2<sup>g7</sup>, ascertained at the first backcross, and were used at the fourth backcross generation to NOD. NOD $\beta_2$ m<sup>null</sup> mice at the ninth backcross generation were provided by Linda Wicker (Merck, Rahway, NJ) and David Ser-

reze (The Jackson Laboratory, Bar Harbor, ME). CD4  $-/-$  mice on H-2<sup>b</sup> background (20) were bred with NOD/Caj mice and then backcrossed for four generations.  $\mu$ MT  $-/-$  mice lacking B cells (21) were obtained originally from Klaus Rajewsky (University of Cologne, Germany) and backcrossed for nine generations onto NOD/Caj mice. The mice were all housed in specific pathogen-free conditions. NOD.SCID animals were obtained originally from The Jackson Laboratory, and NOD.SCID-RIP-B7-1 mice were generated by crossing the NOD-RIP-B7-1 transgenic mice with NOD.SCID mice at The Jackson Laboratory (by David Serreze) and backcrossed to NOD.SCID mice. All the NOD.SCID-RIP-B7-1 mice used in the experiments were heterozygous for the RIP-B7-1 transgene. All the animal studies were performed under protocols approved by the Yale University Animal Care and Use Committee.

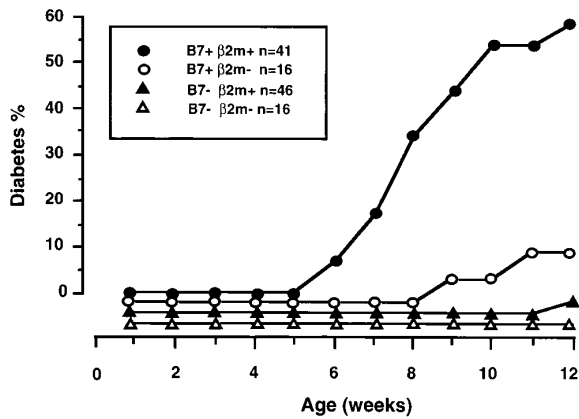
**Breeding Scheme.** The mice bearing the knockout mutations were bred initially onto the NOD-RIP-B7-1 transgenic mice. The F1 mice are all heterozygous for the knockout mutation and are either transgene positive (heterozygous) or transgene negative. F1 mice positive for the B7-1 transgene were then intercrossed with F1 mice which were B7-1 transgene negative, in order not to generate mice that were homozygous for the B7-1 transgene. The frequency of mice from this cross that were homozygous for the knockout mutation, were homozygous for H-2<sup>g7</sup>, and had the B7-1 transgene was 2 out of 32.

**Diabetes Screening.** Animals were tested weekly for glycosuria using Diastix (Bayer Corp., Elkhart, IN), and if present, diabetes was confirmed by a blood glucose measurement using One Touch test strips (LifeScan, Inc., Milpitas, CA) of >250 mg/dl (13.9 mmol/liter).

**Genotyping.** The presence or absence of the hB7-1 transgene was determined by PCR on tail DNA using the following primers, made in the Keck Facility (Yale University): 5' TGA AGC CAT GGG CCA CAC and 5' GAC ACT GTT ATA CAG GGC.

Typing for the various null mutations was carried out using PCR for neomycin to identify the presence of the mutation, and then staining of peripheral blood with mAbs to identify homozygous mice as follows. Heterozygous carriers of the  $\beta_2$ m<sup>null</sup> allele, CD4  $-/-$  allele, and  $\mu$ MT  $-/-$  allele were identified using the following primers specific for the neomycin in the knockout mutation: 5' GGC ACA ACA GAC AAT CGG CT and 5' CCT GAT GCA CTT CGT CCA GA. Homozygosity for the  $\beta_2$ m<sup>null</sup> gene was tested for by staining peripheral blood lymphocytes with FITC-conjugated anti-CD8 (GIBCO BRL, Gaithersburg, MD). Homozygosity for the CD4  $-/-$  mutation was tested for by staining with FITC-conjugated anti-CD4 (GIBCO BRL). Homozygosity for the  $\mu$ MT  $-/-$  mutation was tested for by staining of peripheral blood lymphocytes with FITC-conjugated anti-mouse Ig (Sigma Chemical Co., St. Louis, MO). The mice were screened for homozygosity for H-2<sup>g7</sup> by testing for the absence of K<sup>b</sup> using the mAb Y-25 and FITC-conjugated anti-mouse IgG (Sigma Chemical Co.) followed by flow cytometric analysis as described previously (19).

**Histology.** Pancreatic tissue was fixed in formalin, paraffin embedded, and stained with hematoxylin and eosin. The sections were examined microscopically, and insulinitis of individual islets was assessed by two independent observers according to the scale of 0 to 4 as follows: 0, no insulinitis; 1, periinsulinitis; 2, periinsulinitis with some insulinitis; 3, >50% of the islet infiltrated; and 4, complete islet destruction. Additionally, some of the pancreata were fixed in periodate-lysine-paraformaldehyde, sucrose infused, and then frozen in Tissue-Tek OCT (Bayer Corp.). Sections (7 mm thick) were stained with biotinylated YT4.3 antibody recognizing



**Figure 1.** Mice were observed and screened for glycosuria. All mice which had glycosuria had a blood glucose measurement taken using One Touch test strips (LifeScan, Inc., Milpitas, CA), and diabetes was diagnosed if the blood glucose was >250 mg/dl. The graph shows the percentage of diabetes in mice that were RIP-B7-1 transgene positive and  $\beta_2m$  sufficient (filled circles),  $n = 41$ ; RIP-B7-1 transgene positive and  $\beta_2m$  deficient (open circles),  $n = 16$ ; RIP-B7-1 transgene negative and  $\beta_2m$  sufficient (filled triangles),  $n = 46$ ; and RIP-B7-1 transgene negative and  $\beta_2m$  deficient (open triangles),  $n = 16$ . The numbers indicate the total population of both male and female mice.

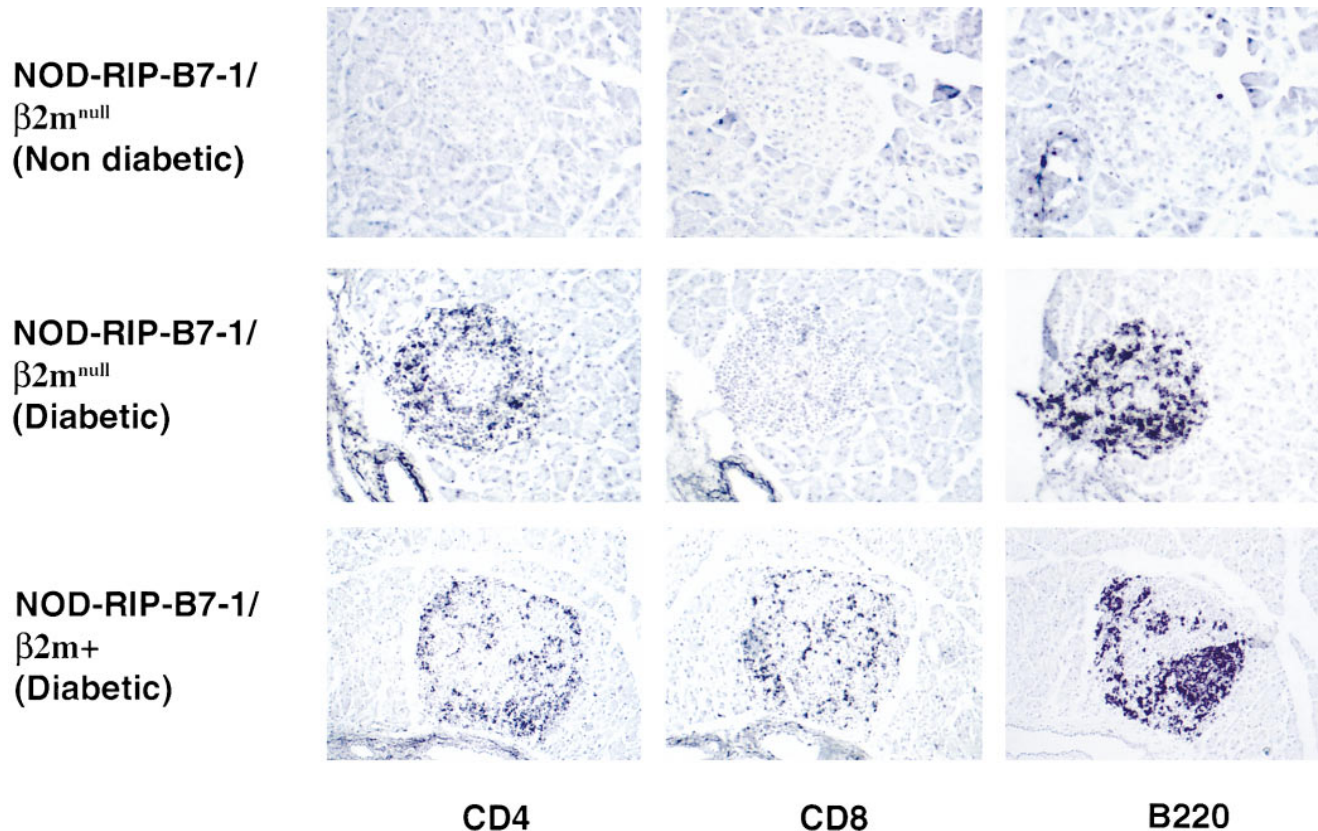
CD4, TIB 105 antibody recognizing CD8, and B220 antibody which stains the majority of B cells. The color was developed using diaminobenzidine tetrahydrochloride and nickel ammonium sulphate. The sections were then counterstained with hematoxylin.

**Adoptive Transfer Studies.** Splensens were removed from 6- and 12-wk-old female NOD mice that were nondiabetic (ascertained by blood glucose measurement of <250 mg/dl), and recently diabetic NOD mice (ascertained by a blood glucose measurement of >250 mg/dl).

Splenocytes ( $2 \times 10^7$  cells) were adoptively transferred to female NOD.SCID and NOD.SCID-RIP-B7-1 mice, and the onset of diabetes was monitored, initially by checking for glycosuria and confirmed by measurement of blood glucose. At the onset of diabetes, the animals were killed, and the pancreas was removed for immunohistochemistry. In addition,  $10^7$  CD8 NOD-derived islet-reactive cloned T cells (11) were also adoptively transferred to these groups of mice.

## Results

**NOD-RIP-B7-1/ $\beta_2m^{null}$  Mice Develop a Low Incidence of Accelerated Diabetes.** NOD $\beta_2m^{null}$  mice have <1% peripheral CD8 T cells but do not develop diabetes, as reported previously (3, 4, 6). NOD-RIP-B7-1/ $\beta_2m^{null}$  also had



**Figure 2.** Pancreatic section from an NOD-RIP-B7-1/ $\beta_2m^{null}$  mouse that was nondiabetic is shown (top), illustrating staining with anti-CD4, anti-CD8, and anti-B220 antibodies at 24 wk, and showing that when diabetes did not occur, there was no insulinitis. (Middle) Staining from one of the two NOD-RIP-B7-1/ $\beta_2m^{null}$  mice that became diabetic, showing the presence of CD4 T cells and B cells. (Bottom) Staining of a pancreatic section from a diabetic NOD-RIP-B7-1/ $\beta_2m$ -sufficient mouse.

**Table 1.** Insulinitis Scores in NOD-RIP-B7-1/ $\beta_2m^{null}$  and NOD-RIP-B7-1/CD4  $-/-$  Mice

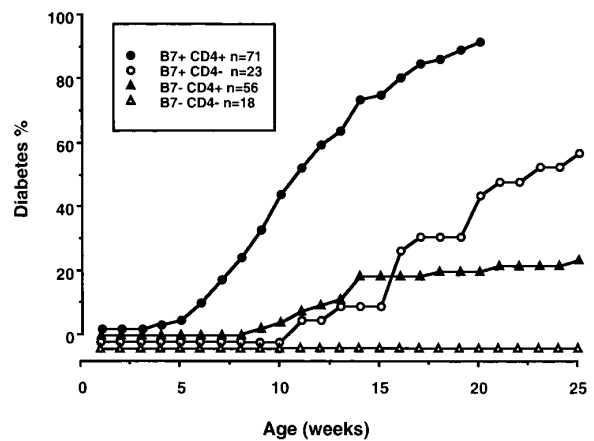
Mice	Insulinitis score				
	0	1	2	3	4
NOD-RIP-B7-1/ $\beta_2m^{null}$	86				
NOD-RIP-B7-1/CD4 $-/-$		6	23	13	35

Insulinitis of individual islets was assessed according to the scale of 0 to 4 as follows: 0, no insulinitis; 1, periinsulinitis; 2, periinsulinitis with some insulinitis; 3, >50% of the islet infiltrated; 4, complete islet destruction. Insulinitis in NOD-RIP-B7-1/ $\beta_2m^{null}$  mice was assessed in 86 islets from 10 nondiabetic mice. Insulinitis in NOD-RIP-B7-1/CD4  $-/-$  mice was assessed in 77 islets from 9 nondiabetic mice.

<1% CD8 T cells in peripheral blood, compared with 5–10% total lymphocytes in the  $\beta_2m$ -sufficient mice (data not shown), and 14 out of 16 mice failed to develop diabetes (Fig. 1), even when followed until 24 wk of age. Histology showed that these mice also did not develop insulinitis (Table 1, and Fig. 2). However, surprisingly, 2 out of 16 mice that were NOD-RIP-B7-1/ $\beta_2m^{null}$  developed accelerated diabetes (Fig. 1). In the two animals that developed diabetes, the islet infiltrate was made up of CD4 T cells and B cells (Fig. 2), compared with NOD-RIP-B7-1 animals, which develop diabetes in which the infiltrate consists of CD8 and CD4 T cells as well as B cells.

**NOD-RIP-B7-1/CD4  $-/-$  Mice Do Not Develop Accelerated Diabetes.** NOD mice crossed with CD4  $-/-$  to the fourth backcross generation do not develop either insulinitis or diabetes (Fig. 3). When NOD.CD4  $-/-$  mice were crossed with NOD-RIP-B7-1 mice, no diabetes occurred before the age of 12 wk. After 12 wk, some mice (mainly males) did develop diabetes (Fig. 3). In contrast to the NOD.CD4  $-/-$  mice that are RIP-B7-1 transgene negative, where no insulinitis is seen, even in the NOD-RIP-B7-1/CD4  $-/-$  mice that did not develop diabetes, insulinitis was seen, as shown in Fig. 4. The insulinitis scores are shown in Table 1. Although these mice lack the CD4 coreceptor, it has been reported previously that there is an increased number of double-negative CD3+ T cells in CD4  $-/-$  mice that may play the role normally taken by CD4 T cells. In NOD-RIP-B7-1/CD4  $-/-$  mice, many of the islet-infiltrating CD3+ cells stained with anti-CD8.

**NOD-RIP-B7-1/ $\mu$ MT  $-/-$  Mice Develop Accelerated Diabetes.** We have shown that NOD. $\mu$ MT  $-/-$  mice at the ninth and tenth backcross generation to NOD, which are homozygous for all the NOD susceptibility markers described (15), do not develop diabetes (our unpublished observations). In contrast, when the mice are crossed with the NOD-RIP-B7-1 mice, they develop diabetes very rapidly, and absence of B cells in this model does not impair the development of the accelerated diabetes (Fig. 5). Histology shows that NOD. $\mu$ MT  $-/-$  mice develop some insulinitis



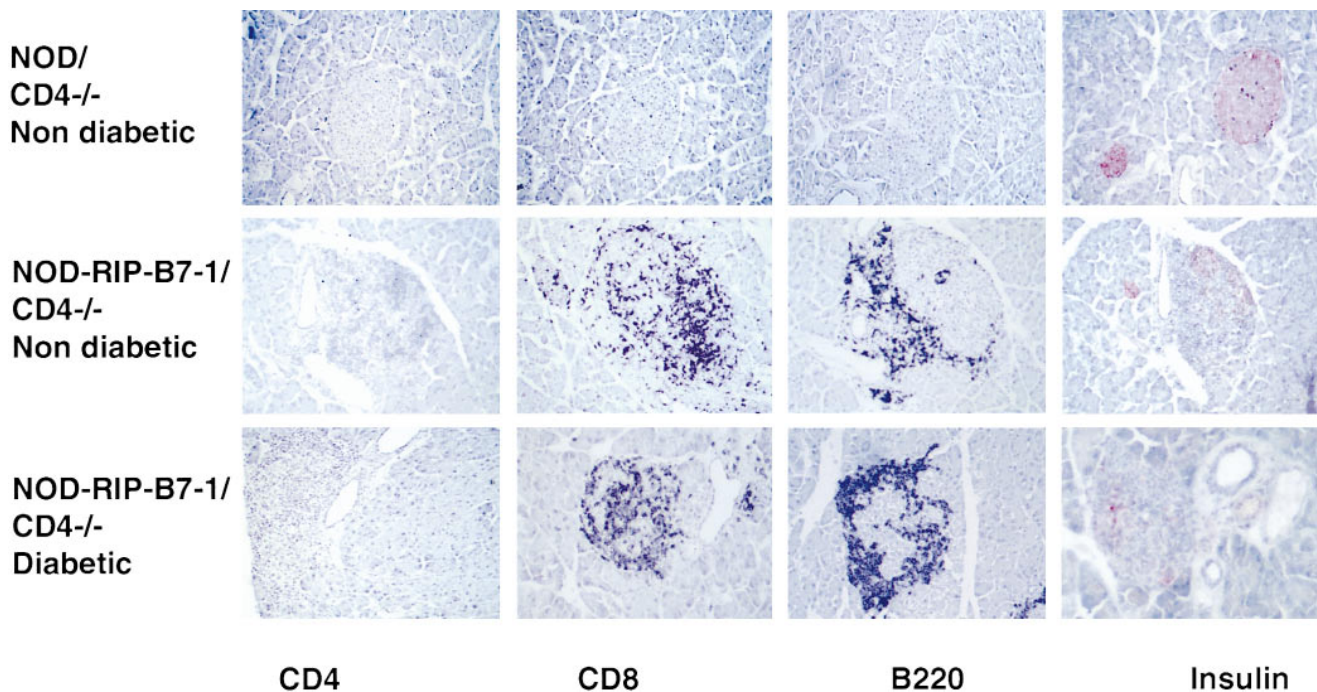
**Figure 3.** The graph shows the percentage of diabetes in mice that were RIP-B7-1 transgene positive and CD4 sufficient (filled circles),  $n = 71$ ; RIP-B7-1 transgene positive and CD4 deficient (open circles)  $n = 23$ ; RIP-B7-1 transgene negative and CD4 sufficient (filled triangles),  $n = 56$ ; and RIP-B7-1 transgene negative and CD4 deficient (open triangles),  $n = 18$ . The numbers indicate the total population of both male and female mice.

by the age of 30 wk, although they do not develop diabetes (Fig. 6). In the NOD-RIP-B7-1 mice lacking B cells, histology at the time of diabetes shows that there is a large number of CD4 and CD8 T cells present. There are a few B220-positive cells that are not B cells but rather B220T cells, such as those seen in MRL/lpr/lpr mice, as shown by the absence of staining for Ig (Fig. 6).

**NOD.SCID-RIP-B7-1 Mice Develop Diabetes More Rapidly after Adoptive Transfer of Splenocytes and CD8 Cloned T Cells Than NOD.SCID Mice.** Splenocytes from 6-wk-old prediabetic NOD mice are able to transfer diabetes to NOD.SCID mice in 6–10 wk. This time course is accelerated by  $\sim 3$  wk, when the cells are transferred into NOD.SCID-RIP-B7-1 mice (Fig. 7 a). A similar result is seen when just prediabetic or diabetic spleen cells are transferred into NOD.SCID mice compared with NOD.SCID-RIP-B7-1 mice, in that diabetes is accelerated in the latter recipients (Fig. 7, b and d). When CD8 T cell clones (11), which respond to an undefined  $\beta$  cell antigen, are transferred, diabetes is also accelerated in the NOD.SCID-RIP-B7-1 mice, showing that these cells can be costimulated in vivo (Fig. 7 d). Histology indicates that there is increased presence of CD8 T cells bearing V $\beta$ 6 in the infiltrate of the diabetic NOD.SCID-RIP-B7-1 mice (Fig. 8). Staining with anti-V $\beta$ 8 did not show any excess of these cells in either the diabetic NOD.SCID-RIP-B7-1 mice or the diabetic NOD.SCID mice (Fig. 8).

## Discussion

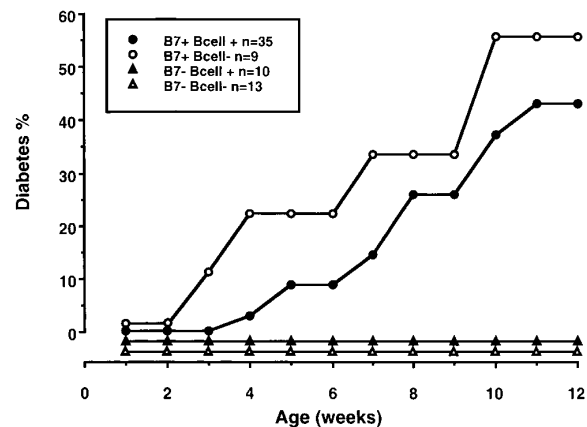
We have shown that pancreatic islets of NOD-RIP-B7-1 mice are very potent stimulators of CD8 T cells, and the use of these islets has allowed us to clone CD8 T cells that are able to very rapidly transfer diabetes in the absence of CD4 T cells (11). We had noted that in the original NOD-



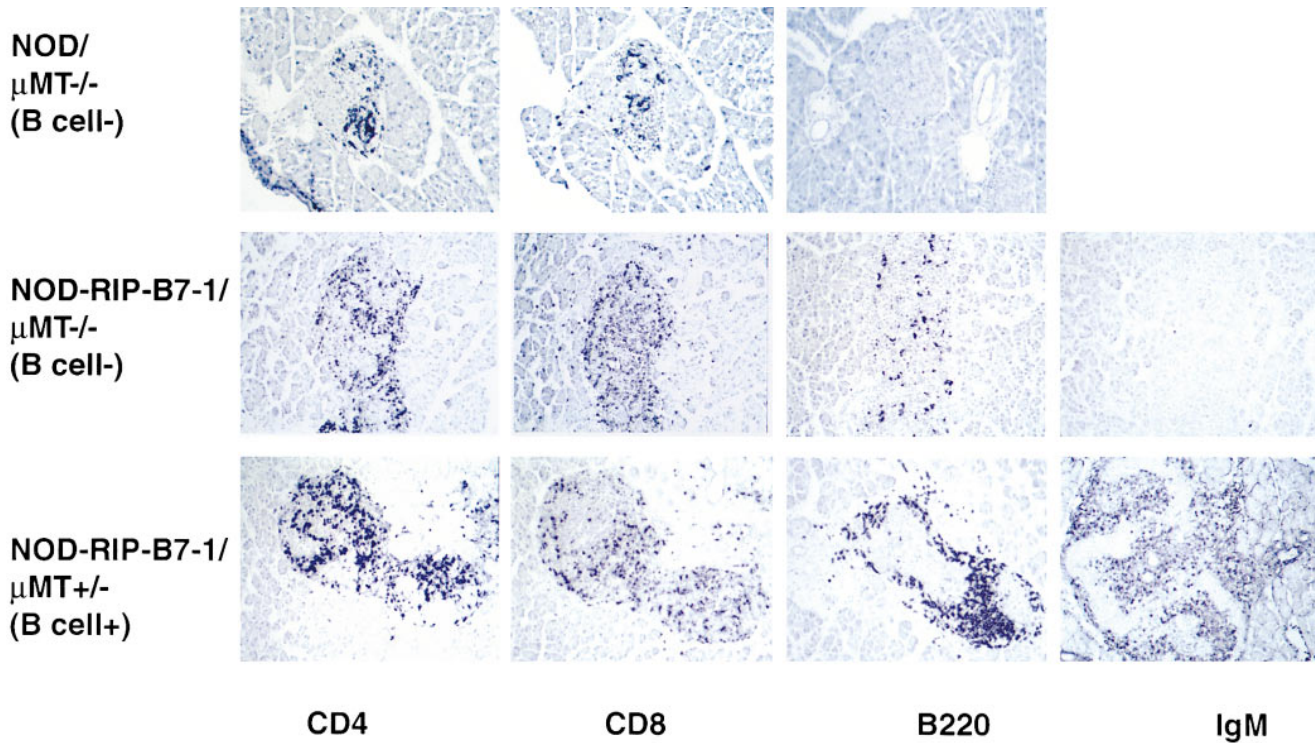
**Figure 4.** (Top) Pancreatic sections from a non-RIP-B7-1 transgenic mouse that was CD4 deficient. All these mice were nondiabetic, and there was no insulinitis seen, as illustrated by staining with anti-CD4, anti-CD8, and anti-B220 antibodies at 24 wk. The islets are intact, as shown by staining with insulin (red). (Middle) Staining from an NOD-RIP-B7-1/CD4<sup>-/-</sup> mouse that did not become diabetic. (Bottom) Staining of a pancreatic section from a diabetic NOD-RIP-B7-1/CD4-sufficient mouse.

RIP-B7-1 mice, there was no difference in the incidence of diabetes in male and female animals, unlike the female preponderance seen in NOD mice (19). This lack of sex difference was also seen in the NOD-RIP-B7-1 mice that were homozygous for the CD4<sup>-/-</sup> and  $\mu$ MT<sup>-/-</sup> mutations. In the NOD-RIP-B7-1 accelerated model of diabetes, we postulate that on  $\beta$  cells, which normally do not express costimulatory molecules even when exposed to cytokines (reference 22, and our unpublished data), the provision of the B7-1 molecule has increased the capacity of CD8 T cells to initiate damage to the islets, accelerating the process that normally takes 12 wk to develop in the NOD mouse. Indeed, under normal circumstances, as  $\beta$  cells do not express costimulatory molecules, the initiation process would be expected to occur outside the islets, perhaps in the peripancreatic lymph nodes, with antigens shed from the islets and presented by dendritic cells (23). As cells are stimulated most optimally when the antigen is presented together with the costimulatory molecules on the same cell (24), the B7-1-expressing  $\beta$  cells are a potent stimulus for CD8 T cells that are specific for  $\beta$  cell peptides presented by MHC class I molecules. This may allow more T cells to become activated or provoke local clonal expansion of these autoaggressive T cells, or alternatively become activated earlier than in the NOD mouse. There are several possible explanations for the fact that diabetes was seen in 2 out of 16 NOD-RIP-B7-1/ $\beta_2m^{null}$  mice, but has not been found in NOD $\beta_2m^{null}$  mice. It has been documented that H-2D<sup>b</sup> is less dependent on  $\beta_2m$  than other MHC class I

molecules, and that although reduced, the D<sup>b</sup> molecule is expressed even in the absence of endogenous  $\beta_2m$  (25). The presence of B7-1 on the islets could stimulate the few CD8 T cells that have been selected on D<sup>b</sup> to initiate the process whereby antigens are released, leading to the production of diabetes by the increased population of CD4 T



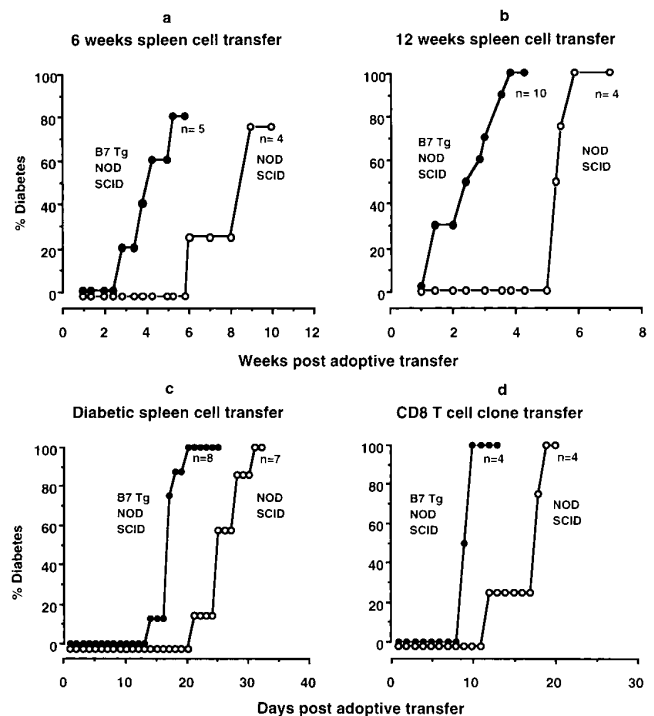
**Figure 5.** The graph shows the percentage of diabetes in mice that were RIP-B7-1 transgene positive and B cell sufficient (filled circles),  $n = 35$ ; RIP-B7-1 transgene positive and B cell deficient (open circles),  $n = 9$ ; RIP-B7-1 transgene negative and B cell sufficient (filled triangles),  $n = 10$ ; and RIP-B7-1 transgene negative and B cell deficient (open triangles),  $n = 13$ . The numbers indicate the total population of both male and female mice.



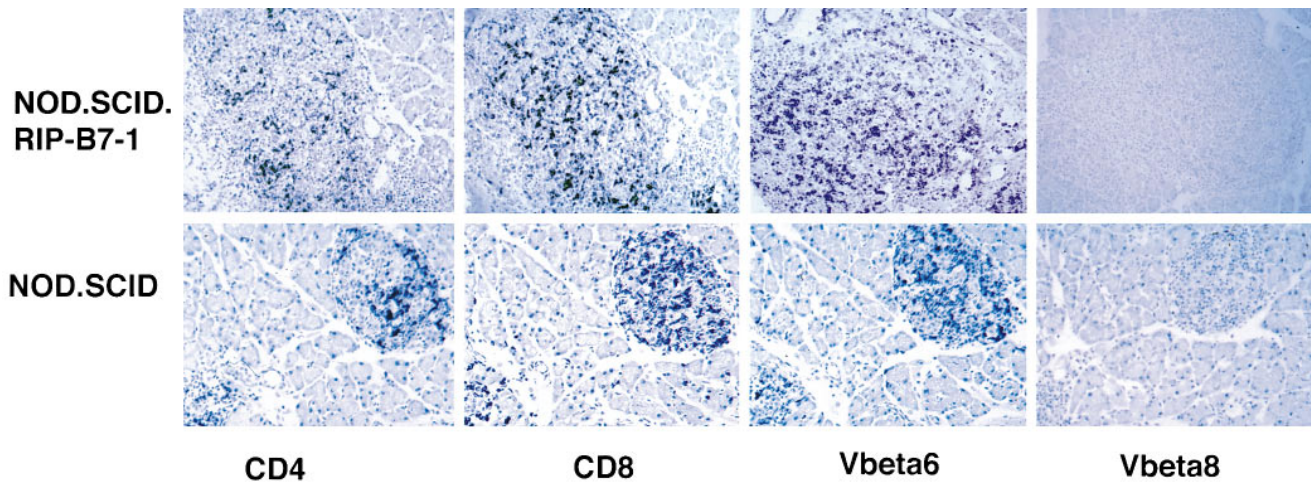
**Figure 6.** (Top) Pancreatic sections from a nontransgenic mouse that was B cell deficient taken at 30 wk. Mild insulinitis is seen, as illustrated by staining with anti-CD4, anti-CD8, and anti-B220 antibodies. (Middle) Staining from an NOD-RIP-B7-1/ $\mu$ MT $^{-/-}$  mouse that became diabetic, showing intense insulinitis. There are a few B220-positive cells seen, but these are negative for staining with anti-Ig antibody and are likely to be T cells. (Bottom) Staining of a pancreatic section from a diabetic NOD-RIP-B7-1, B cell-sufficient mouse.

cells. In any event, the result does show that CD8 T cells do, indeed, play an important role in the accelerated diabetes of this model, as they do in diabetes in the NOD mouse.

It is clear from these results, and from those of other investigators, that CD4 T cells are required for the development of diabetes in the NOD mouse. The fact that accelerated diabetes does not occur in the absence of cells bearing the CD4 coreceptor indicates that these cells are also important for the development of the accelerated diabetes seen in the NOD-RIP-B7-1 transgenic mice. It has been reported that CD4 $^{-/-}$  mice have CD4-CD8 $^{-}$  T cells that are able to perform the function of CD4 T cells (26). In the current experiments, clearly any CD4-CD8 $^{-}$  T cells present do not function sufficiently to induce early diabetes in the NOD-RIP-B7-1 transgenic mice. However, the fact that the NOD-RIP-B7-1 transgenic mice lacking CD4 T cells can develop diabetes at a slower rate indicates either that the CD8 T cells which have been activated can kill the  $\beta$  cells alone, although less efficiently, or alternatively, that the CD4-CD8 $^{-}$  T cells can help the process of  $\beta$  cell damage, but not unless more CD8 T cells have been activated to perform the final effector function. Certainly, these mice had massive infiltration of CD8 T cells, which suggests that the CD8 T cells can cause diabetes under the conditions where they can be maximally costimulated, but that the process is very much slower in the absence of CD4 T cells. However, the reduction of diabetes that occurs



**Figure 7.** Incidence of diabetes after adoptive transfer of 6-wk-old NOD spleen cells (a), 12-wk-old NOD spleen cells (b), diabetic spleen cells (c), and CD8 cloned T cells (d) into NOD.SCID-RIP-B7-1 mice (filled circles) and NOD.SCID mice (open circles).



**Figure 8.** Immunohistochemistry showing staining with anti-CD4, anti-CD8, anti-V $\beta$ 6, and anti-V $\beta$ 8 in NOD.SCID-RIP-B7-1 mice (*top*) and NOD.SCID mice (*bottom*) that have become diabetic after adoptive transfer of spleen cells from 12-wk-old NOD mice.

without CD4 T cells implies that the process whereby both CD4 and CD8 T cells in the NOD mouse are required to develop diabetes also applies to this model, and that even in the presence of optimal stimulation of CD8 T cells, these alone cannot cause diabetes in an accelerated fashion, although diabetes does occur after many weeks. This suggests that soluble antigens released after damage of the islets are taken up by professional antigen-presenting cells, such as dendritic cells, which then perhaps present peptide to MHC class II-restricted CD4-CD8- T cells in a similar manner to that found in the nontransgenic NOD mouse. It would appear that CD4 T cells are ultimately required for the generation of accelerated diabetes, and any CD4-CD8- T cells that are present are not sufficient to perform this function. Ultimately, the role of CD4 T cells selected on MHC class II could be tested by crossing the NOD-RIP-B7-1 mice with class II transactivator (CIITA)  $-/-$  mice (which lack MHC class II without changing MHC class I) (27) crossed onto the NOD background.

The presence of B cells within the islet in diabetic NOD-RIP-B7-1 mice suggests either that B cells are present because of bystander recruitment, or that B cells play a role in antigen presentation to the CD4 T cells in this accelerated model of diabetes, as in the native NOD mouse. However, the NOD-RIP-B7-1 mice that lack B cells are just as able to develop accelerated diabetes as those that are B cell sufficient. This suggests that the dendritic cells and macrophages are sufficient to present antigens to the CD4 T cells in this model, and that B cells are not required for this function. It perhaps also suggests that under normal circumstances, the B cells are required to maximally costimulate cells to develop diabetes. Alternatively, it is also possible that in addition to the costimulation of autoreactive cells, the loss of costimulation of regulatory cells also plays a role in the accelerated diabetes seen in this model. This suggests that in addition to the role that B cells play in antigen presentation to CD4 T cells, an important function

of B cells in diabetes in NOD mice is to provide costimulation.

It has been demonstrated previously that diabetes could be adoptively transferred to NOD.SCID animals using young spleen cells (28). The current study shows that there is a delay of 3-6 wk when young spleen cells are used compared with diabetic spleen cells. There appears to be a proportional delay in the onset of diabetes, presumably related to the presence of cells that have already been primed to damage the  $\beta$  cells, taken from the older NOD animals. It is of interest that there is a predominant population of CD8 T cells expressing V $\beta$ 6 in the islets of adoptively transferred NOD.SCID-RIP-B7-1 mice. CD8 T cells expressing V $\beta$ 6 are also found in the diabetic nontransgenic NOD.SCID animals. In this study, T cells expressing V $\beta$ 8, postulated to be important in early initiating events (29), were not seen. We have previously isolated very potent CD8 T cell clones that also express V $\beta$ 6 from the islets of young, prediabetic NOD mice (11), which are maintained in culture on nontransgenic NOD islets. These are able to cause rapid diabetes in irradiated NOD mice and NOD.SCID mice. This suggests that these cells are present in the islets of NOD mice, but in the presence of the B7-1 transgene, this particularly pathogenic population is increased and is therefore able to accelerate the onset of diabetes in the NOD.SCID mice bearing the RIP-B7-1 transgene.

In conclusion, the results presented here suggest that in the NOD-RIP-B7-1 mice, CD8 T cells are required for the accelerated diabetes seen, and in addition, CD4 T cells are also required, although diabetes can occur later in the absence of T cells bearing the CD4 coreceptor. Whether this result would be obtained in the absence of cells selected on MHC class II remains to be tested. However, unlike the nontransgenic NOD mouse, where B cells are required for the development of disease, these cells are not required in the RIP-B7-1 transgenic  $\mu$ MT  $-/-$  mouse. The mechanism by which the accelerated diabetes occurs is

likely to be the increase in a potent population of CD8 T cells, which are able to initiate damage and destroy the islets, but this clearly requires the presence of CD4 T cells for the maximum effect to be seen. Although costimulatory molecules have not been shown to be expressed on the pancreatic islets under normal circumstances, this model

has many features similar to that seen in NOD mice, and may allow us to elucidate the nature of the interaction between CD4 and CD8 T cells in the production of diabetes. In addition, as both CD4 and CD8 T cells appear to be required for this accelerated diabetes, the model will also be useful for testing preventative strategies.

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