

Type 1 Diabetes Development Requires Both CD4⁺ and CD8⁺ T cells and Can Be Reversed by Non-Depleting Antibodies Targeting Both T Cell Populations

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■ Abstract

Type 1 diabetes development in NOD mice appears to require both CD4⁺ and CD8⁺ T cells. However, there are some situations where it has been suggested that either CD4⁺ or CD8⁺ T cells are able to mediate diabetes in the absence of the other population. In the case of transgenic mice, this may reflect the numbers of antigen-specific T cells able to access the pancreas and recruit other cell types such as macrophages leading to a release of high concentrations of damaging cytokines. Previous studies examining the requirement for CD8⁺ T cells have used antibodies specific for CD8 α . It is known that CD8 α is expressed not only on $\alpha\beta$ T cells, but also on other cell types, including a DC population that may be critical for presenting islet antigen in the pancreatic draining lymph nodes. Therefore, we have re-examined the need for both CD4⁺ and CD8⁺ T cell populations in diabetes development in NOD mice using an antibody to CD8 β . Our studies indicate that by using highly pu-

rified populations of T cells and antibodies specific for CD8⁺ T cells, there is indeed a need for both cell types. In accordance with some other reports, we found that CD4⁺ T cells appeared to be able to access the pancreas more readily than CD8⁺ T cells. Despite the ability of CD4⁺ T cells to recruit CD11b class II positive cells, diabetes did not develop in the absence of CD8⁺ T cells. These studies support the observation that CD8⁺ T cells may be final effector cells. As both T cell populations are clearly implicated in diabetes development, we have used a combination of non-depleting antibodies to target both CD4-positive and CD8-positive cells and found that this antibody combination was able to reverse diabetes onset in NOD mice as effectively as anti-CD3 antibodies.

Keywords: type 1 diabetes • T cell • CD4⁺ • CD8⁺ • NOD • NOD.scid • pancreas infiltration • CD8 alpha chain • dendritic cell • IgG2 antibody • aCD3 antibody

Introduction

here is much evidence to suggest that CD8⁺ T cells play a role in the development of diabetes. Early studies in NOD mice showed that the transfer of diabetes by spleen cells from diabetic donors into immuno-compromised recipients required the presence of both CD4⁺ and CD8⁺ T cells [1-3]. Further studies in NOD mice showed

that CD8⁺ T cells were required for cyclophosphamide-induced diabetes [4] and also that MHC class I expression was required in NOD mice for diabetes to spontaneously develop [5, 6]. Depletion of CD8⁺ T cells has been shown to afford protection from disease and an overall reduction in islet infiltration [2, 7], which has led to the proposition that CD8⁺ T cells may facilitate recruitment of lymphocytes to the pancreas. This interpretation is sup-

ported by the findings of Wang *et al.* who showed that early administration of an IgG2a rat anti-mouse CD8 monoclonal antibody prevents insulinitis in NOD mice, with no effect of such treatment at later time points [8]. However, the requirement for CD8⁺ T cells in the development of diabetes in NOD mice was not a universal observation. It was suggested that CD4⁺ T cells alone could transfer diabetes to NOD.*scid* recipients, if they were derived from a diabetic donor, but that CD8⁺ T cells were required if the CD4 population was derived from pre-diabetic mice [9].

Every *in vivo* antibody study targeting CD8⁺ T cells has used antibodies directed at CD8 α . The antibodies used to deplete CD8⁺ T cells *in vitro* have also been directed against the CD8 α chain. There are several cell types apart from $\alpha\beta$ T cells that express CD8 α including $\gamma\delta$ T cells, NKT cells, and some dendritic cells (DCs). In these cases the $\alpha\alpha$ homodimer is expressed. This means that all previous studies could not distinguish between effects on $\alpha\beta$ T cells and on other cell types. As CD8 α -expressing DCs have been shown to play a role in cross presentation, a process of particular importance in the presentation of islet antigens and T cell activation in the pancreatic draining lymph node [10], we felt it important to clearly establish that $\alpha\beta$ T cell depletion alone influenced diabetes onset. We have used an antibody to the CD8 β chain to show that depletion of CD8⁺ cells with this antibody prevents diabetes development in a transfer model of T1D in the NOD mouse. This confirmed that CD8⁺ T cells are indeed required for diabetes development in NOD mice.

We have previously shown that administration of a short course of non-depleting anti-CD4 antibody to 6 week old NOD mice provides long term prevention from diabetes development [11]. However, this antibody was unable to reverse diabetes onset once it was established; unlike anti-CD3 which had been shown to reverse diabetes onset in NOD mice [12]. As the anti-CD3 antibody would be able to target both CD4⁺ T cells and CD8⁺ T cells, we carried out a series of experiments to establish whether the use of anti-CD4 antibodies together with anti-CD8 antibodies could reverse diabetes onset. For

these studies we used both a non-depleting anti-CD4 as well as a non-depleting anti-CD8 antibody. The latter antibody recognized CD8 α . For therapeutic purposes, using *in vivo* antibody treatment with anti-CD8 antibodies, there may be a significant advantage in using an antibody that also targets other cell types such as CD8 α DCs. Such an antibody may be able to target not only the T cells, but also the cells involved in cross-presenting islet antigens in the pancreatic draining lymph nodes.

Materials and methods

Mice

NOD mice were housed and bred under specific pathogen-free conditions in the Pathology Department, University of Cambridge animal facilities. NOD.*scid* mice were maintained in microisolator cages with filtered air and handled under sterile conditions in a laminar flow hood. All animal work was carried out under UK Home Office project licence regulations after approval by the Ethical Review Committee of the University of Cambridge.

Antibodies and *in vivo* treatment

The following hybridomas were a gift from Herman Waldmann (Oxford, UK): YTS 177.9.6.1 (rat IgG2a, anti-CD4), YTS 105.18.10 (rat IgG2a, anti-CD8 α), YTS 191.1.2 (rat IgG2b, anti-CD4), YTS 169.4.2 (rat IgG2b, anti-CD8 α), YTS 156.7.7

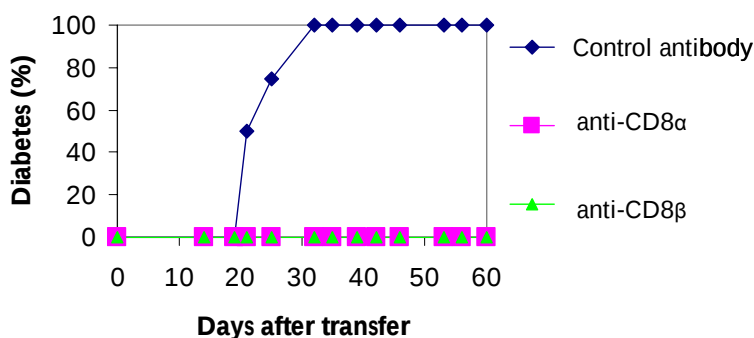


Figure 1. Diabetes transfer is inhibited by anti-CD8 α and anti-CD8 β . Spleen cells from a pool of diabetic NOD donors (2×10^7) were transferred intravenously to NOD.*scid* recipients. The recipient mice (4 per group) were treated with 2 mg of the appropriate antibody i.p. at the same time as the cell transfer, and also on days 2 and 5 after the transfer. Mice were tested every 2-3 days for the presence of urinary glucose and considered diabetic when positive on at least two occasions.

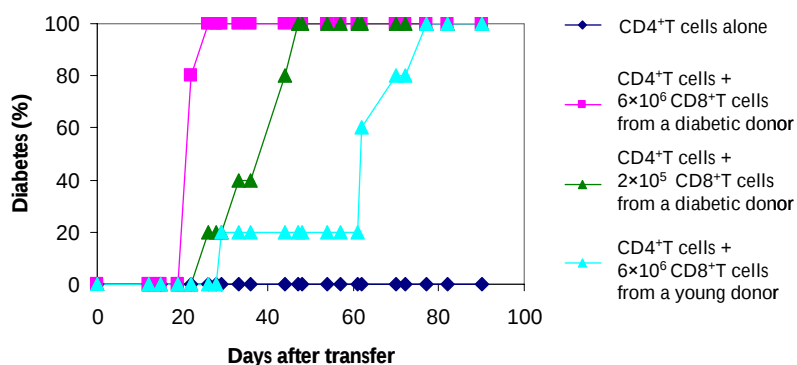


Figure 2. Purified CD4 T cells only transfer diabetes to NOD.scid recipients when CD8 cells are present. Purified CD4 cells (1×10^7) were injected i.v. into NOD.scid recipients alone ($n = 5$), together with a high number of purified CD8 cells from diabetic donors (6×10^6 , $n = 5$), together with a low number of purified CD8 cells from diabetic donors (2×10^5 , $n = 5$), or together with purified CD8 cells from 4 week old female donors (6×10^6 , $n = 5$). Mice were tested every 2-3 days for the presence of urinary glucose and considered diabetic when positive on at least two occasions.

(rat IgG2b, anti-CD8 β), and the isotype control hybridoma YFC 51 (rat IgG2b). The isotype control hybridoma MAC 219 (rat IgG2a) was a gift from Geoff Butcher (Babraham, UK). All hybridomas were grown in our own laboratory in hollow fibre cartridges. Antibodies were purified by precipitation with 50% saturated ammonium sulphate and dialyzed extensively against PBS. An estimate of total protein was determined from the OD₂₈₀. Antibody concentrations were determined by an anti-rat immunoglobulin ELISA. The endotoxin levels were <1 EU/mg protein and the preparations were stored at -20°C until use. Groups of mice were treated intra-peritoneally (i.p.) with the test antibodies or isotype controls. Timings of antibody administrations are given in the results section or figure legends. Mice were tested for the presence of urinary glucose using Diastix (Bayer plc, Newbury, UK) test strips. Mice showing positive urinary glucose on at least two occasions were considered diabetic and this was confirmed using a Glucometer Ascensia Esprit 2 (Bayer plc, Newbury, UK).

The incidence of diabetes in females in our NOD colony is currently 80-90% by 30 weeks of age. Diabetic female mice which were treated with antibodies were regularly tested for blood glucose in whole blood from the tail vein.

T cell isolation

CD4 and CD8 T cells were isolated using anti-CD4 or anti-CD8 magnetic beads (Miltenyi Biotec, Surrey UK) used according to the manufacturer's instructions. NOD donors of the T cells were pretreated with 3 intraperitoneal injections on days -7, -5 and -3 with either a depleting anti-CD4 (YTS 191), for the CD8 cells or a depleting anti-CD8 (YTS 169), for the CD4 cells. This method gave populations of CD4 or CD8 cells, which had less than 0.5% contamination with the other cell population (data not shown). T cells were injected intravenously into NOD.scid recipients.

Immunohistochemistry

Pancreases were removed after sacrifice and snap frozen in isopentane. Five-micrometer cryostat sections were air-dried and fixed in acetone for 10 minutes. Air-dried sections were stored at -80°C . Pancreatic β -cells were detected by pre-blocking sections with 20% NMS followed by incubation with guinea pig anti-porcine insulin (Dako, High Wycombe, GB) in 10% NMS and detected by rhodaminated goat anti-guinea-pig IgG (ICN Pharmaceuticals, Thame, GB) in 10% NMS. CD4 was detected using monoclonal antibody supernatant KT4 from Prof. K. Tomonari, (Japan), CD8 β with antibody supernatant YTS 156, and CD11b with M 1/70 (BD Pharmingen) in 10% NMS and visualized with FITC goat anti-rat Ig (Serotec, Kidlington, GB). Sections were photographed using a Zeiss Axiophot photomicroscope at magnification $\times 40$.

Results

Diabetes transfer into immuno-compromised recipients was inhibited by anti-CD8 β

When spleen cells from diabetic NOD donors were transferred into immuno-compromised recipient mice, diabetes developed usually within 2-6 weeks. From Figure 1 it can be seen that when

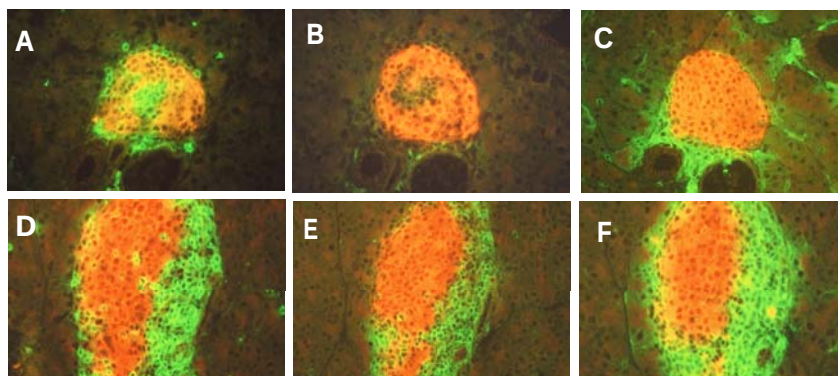


Figure 3. Purified CD4 T cells transferred to NOD.scid recipients do not cause diabetes but they do enter the pancreas and recruit CD11b cells. Purified CD4 cells (1×10^7) or purified CD8 cells (6×10^6) were injected i.v. into NOD.scid recipients either individually or combined. After 14 days, sequential sections of pancreas were examined (top row received CD4 cells alone and bottom row received CD4 and CD8 cells). **A** and **D**: Sections were stained for insulin (red) together with CD4 (green). **B** and **E**: Sections stained for CD8 β (green). **C** and **F**: Sections stained for CD11b (green).

NOD.scid recipients of 2×10^7 spleen cells from a pool of diabetic donors were treated with either anti-CD8 α or anti-CD8 β , diabetes development was completely prevented. Control antibody treatment had no effect with 100% of recipient mice developing diabetes within 3 weeks of cell transfer. This shows that it is indeed the CD8 $^+$ T cells that are required together with CD4 $^+$ T cells for diabetes transfer.

Transfer of 1×10^7 highly purified CD4 $^+$ T cells from diabetic donor mice failed to transfer diabetes, while addition of 6×10^6 purified CD8 $^+$ T cells from a diabetic donor to this CD4 $^+$ T cell population resulted in 100% transfer of disease within 3 weeks. When fewer purified CD8 $^+$ T cells (2×10^5) from a diabetic donor were co-transferred with the highly pure CD4 $^+$ T cells, disease transfer was slightly delayed. This suggests that if a CD4 $^+$ T cell preparation was transferred with a small contaminant of CD8 $^+$ T cells these could expand up over time and mediate diabetes transfer.

If 6×10^6 purified CD8 $^+$ T cells were derived from a 4 week old non-diabetic NOD mouse, their addition to the CD4 $^+$ T cell population resulted in a much delayed diabetes transfer (Figure 2). These studies suggest that the frequency of islet-reactive CD8 $^+$ T cells is reduced in non-diabetic younger mice and that CD8 $^+$ T cells are primed to islet antigen and expand during diabetes development in NOD mice.

Immunohistochemical analysis of the recipient pancreas 14 days after transfer of the highly purified T cell populations showed that following transfer of CD4 $^+$ T cells, the transferred cells could be found at islet sites in the pancreas. There was also recruitment of CD11b-positive cells (macrophages and dendritic cells) with class-II-positive and F4/80-positive cells (Figure 3 A, C and data not shown). As expected with this transfer, there were no CD8 β^+ T cells (Figure 3B).

In contrast to the results obtained with the purified CD4 $^+$ T cell transfers, none of the trans-

ferred T cells could be seen in the pancreas following purified CD8 $^+$ T cell transfers. This is an interesting result given that the transferred T cells were clearly present in the pancreatic lymph nodes and there was no recruitment of class II positive cells around the islet area (data not shown). When both purified populations were transferred together, CD4, CD8 and CD11b cells, were all seen at the islet sites (Figure 3D-F).

Combined treatment with anti-CD4 and anti-CD8 can reverse diabetes in NOD mice

As both CD4 $^+$ T cells and CD8 $^+$ T cell populations are involved in the development of diabetes, we examined whether targeting both of these can reverse established diabetes at onset. We have previously shown that a non-depleting anti-CD4 antibody was able to prevent the spontaneous development of diabetes in NOD mice, if administered for two weeks when mice were 6 weeks of age; but was unable to reverse ongoing diabetes [11]. One interpretation of this result is that at this stage of the disease it becomes necessary to additionally target the CD8 population. An advantage of the non-depleting rat monoclonal anti-CD4 antibody is that the antibody induces tolerance to its own rat Fc region and does not generate an anti-globulin response. When this antibody was used in combination with the IgG2a anti-CD8 α an-

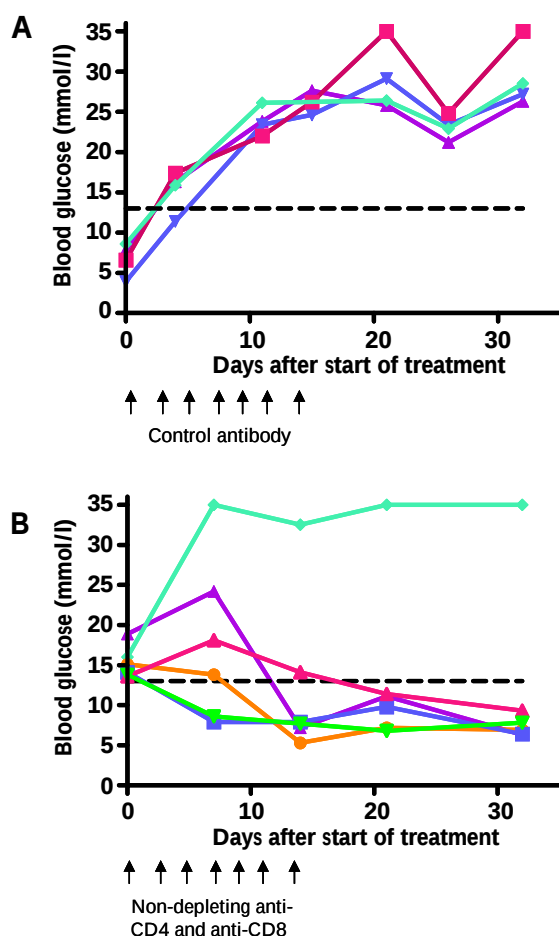


Figure 4. Remission of diabetes by treatment of NOD mice with non depleting anti-CD4 and anti-CD8. At the time of diagnosis of diabetes by high urinary glucose, NOD mice were treated with either the IgG2a isotype control (MAC 219) (A), or a combination of non-depleting antibodies to CD4 (YTS 177) and CD8 (YTS 105) (B). Antibodies (2 mg of MAC 219, or 1 mg each of YTS 177 and YTS 105) were given i.p. every other day for 2 weeks. The glucose levels in the blood of individual mice treated with the antibodies are shown. Mice with glucose values above the dotted black line (13 mmol/l glucose) are considered diabetic.

tibody, YTS105, CD8⁺ T cells were not depleted, but were functionally inactivated. These antibodies have previously been used in combination to induce alloantigen-specific tolerance of skin, cardiac and bone marrow allografts [13-16].

Female NOD mice were tested for diabetes onset and once tested positively they were administered a combination of two non-depleting antibodies, an anti-CD4 and an anti-CD8 α . In Figure 4, it

can be seen that the combination therapy was able to reverse diabetes onset. In contrast, diabetes progressed inexorably in mice given isotype control antibody. The reversal of diabetes observed with combined anti-CD4 and anti-CD8 is comparable to that obtained following treatment with anti-CD3 [12, 17].

Discussion

There are many studies suggesting that diabetes development in NOD mice requires the presence of both CD4⁺ and CD8⁺ T cells. Some of these data suggest that there is a requirement for both populations to be present to transfer diabetes into immuno-compromised recipients [2, 7]. On the other hand, there are also data suggesting that CD4⁺ T cells from diabetic NOD donors may be able to transfer disease on their own [9].

We and others using monoclonal anti-CD8 antibodies have used antibodies that target CD8 α . However, as CD8 α is also expressed on DCs and $\gamma\delta$ T cells, we have carried out a series of experiments using a monoclonal antibody to CD8 β to fully clarify the role of CD8⁺ T cells in diabetes development in NOD mice. Our studies indicated firstly that CD8⁺ T cells are required together with CD4⁺ T cells, for diabetes development. Secondly, our studies showed that in the absence of CD8⁺ T cells, CD4⁺ T cells infiltrate the pancreas and recruit CD11b⁺ cells to the islet area, but that diabetes nevertheless does not develop. In the absence of CD4⁺ T cells, there is no significant influx of cells into the islet area.

The data presented here is strongly supportive of the view that CD8⁺ T cells play an effector cell role [18, 19]. Our previous studies have shown that CD11b-positive cells are also required for the transfer of diabetes by spleen cells from diabetic donors [20], indicating a dependency on all three types of cells for optimal diabetes induction in NOD mice. There are several studies showing that diabetes can be transferred by either CD4⁺ or CD8⁺ T clones alone or by transgenic T cells generated using the clone TCRs [21-24]. However, in these cases a large bolus of islet-specific T cells were transferred. Once activated, these were capable of recruiting large numbers of CD11b-positive cells and mediating substantial aggressive inflammation in the pancreas. The development of diabetes in the NOD mouse is more measured. CD4⁺ T cells may play another role in facilitating the survival and activity of islet antigen-specific CD8⁺ T cells, possibly following activation in the pancreatic draining lymph node. Such an effect has been

nicely demonstrated in a model system using transgenic mice expressing ovalbumin in the pancreas together with TCR transgenic T cells specific for ovalbumin [25].

Monoclonal antibodies have been employed with considerable success to treat a range of pathological conditions including autoimmunity. Such therapeutic antibodies have included those directed against T cell surface antigens as well as antibodies against co-stimulatory molecules and cytokines. The observation that anti-CD3 antibodies were able to reverse diabetes onset in NOD mice [12] was the basis upon which antibodies targeting human anti-CD3 were used to treat newly diagnosed patients [26, 27]. It had been hoped that, following tolerance induction, β -cell repair and/or regeneration might be able to ensue and replace the destroyed and impaired β -cell mass [28]. However, this has thus far not been realized, as anti-CD3 antibodies alone do not seem to facilitate this process, possibly because this agent inhibits the elaboration of molecules important to drive regeneration [17, 29]. Recent studies have shown improved β -cell recovery in diabetic NOD mice when they are given anti-CD3 combined with exendin-4 [30].

There are also data suggesting that anti-CD3, and the combined anti-CD4 and anti-CD8, might function in different ways. Despite Fc modification, the anti-CD3 antibody causes some transient

side effects in patients, which may relate to cytokine release [27]. Comparable studies using an aglycosyl anti-CD3 in the NOD mouse have not been reported, but it might be anticipated that this would also cause some cytokine release, albeit less than the conventional anti-CD3 antibody. Whether such cytokine release is also seen with anti-CD4 and anti-CD8 antibody treatments remains to be clarified. Both anti-CD3 and non-depleting anti-CD4 have been shown to induce regulatory T cells, which may play a key role in maintaining tolerance.

Tolerance induced by non-depleting anti-CD8 antibodies is dependent on IL-10 [11], while that induced by anti-CD3 and anti-CD4 has been reported to be TGF β -dependent [31, 32]. A more detailed understanding of the mechanism by which these therapeutic approaches mediate diabetes reversal, should lead to an identification of key pathways that could be targeted. This could lead to the development of small molecule inhibitors that could favor both inhibition of the autoreactive process and β -cell recovery, and might circumvent the development of the side effects associated thus far with some of the antibody treatments.

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Conflict of interest statement: The authors declare that they have no conflict of interests.

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