A crucial role of CD4 T cells as a functional source of CD154 in the initiation of insulin-dependent diabetes mellitus in the non-obese diabetic mouse

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Abstract

Although the critical requirement of CD4 T cells in type I (insulin-dependent) diabetes mellitus (T1DM) has been well documented, information on the exact role(s) of CD4 T cells in T1DM development is still limited. Here, utilizing non-obese diabetic (NOD) mice deficient for CD154 (CD154-KO/NOD), we have identified a mandatory role of CD4 T cells as the functional source of CD154 in the initiation of T1DM. Without CD154, CD4 T cells were not capable of mediating help in disease development in NOD mice. In fact, full expression of CD154 on the CD4 T cells seems to be essential in the normal spontaneous development of T1DM, since no diabetes was observed in CD154+/− mice in which around half of CD4 T cells do not express CD154 at all, at least by the time they were 40 weeks old. It was also shown that transgenic expression of CD80 on β cells of pancreatic islets, which is believed to provide β cells with the ability to prime cytotoxic T lymphocytes specific for islet antigens, did not restore insulitis in CD154-KO/NOD mice. Taken collectively, these results indicated that CD4 T cells play a crucial role in T1DM as a source of CD154, and that the role of CD154 on CD4 T cells in insulitis may not be just to facilitate priming and expanding of auto-reactive CD8 T cells by activating antigen-presenting cells bearing islet antigens.

Introduction

The non-obese diabetic (NOD) mouse, which develops spontaneous diabetes as a result of a complex autoimmune response, is known to share many characteristics with human type I diabetes (1,2) and, thus, has long been used as a murine model system for this disease. A number of studies have demonstrated that T lymphocytes play critical roles in insulin-dependent diabetes mellitus (T1DM) development in the NOD mouse (3–13). Analyses of NOD mice deficient for β2-microglobulin (7–10) or NOD mice depleted of CD8 T cells by in vivo antibody treatment (11) demonstrated an essential role for CD8 T cells in T1DM development. The importance of CD4 T cells was recently highlighted by the analysis of NOD mice
deficient for the MHC class II transactivator (CIITA), in which the number of CD4 T cells is greatly reduced (12). However, although the necessity of CD4 T cells and CD8 T cells in the development of the disease in NOD mice has been well documented, their precise role(s) in disease development still remains unclear.

In immune responses, CD4 T cells play an important role as a source of various lymphokines, which induce proliferation, activation and/or differentiation of various leukocytes. Activation and differentiation of CD8 T cells also requires CD4 T cell-mediated help. In the induction of the CD8+ cytotoxic T lymphocyte (CTL) response, the role of CD4 T cells was originally believed to be the provision of cytokines, such as IL-2, to promote activation and survival of CD8+ CTL. Recent studies, however, have suggested that CD4 T cell help in CTL induction could be mediated by CD40 through activating antigen-presenting cells (APC) (14–16). In priming of CD4 T cells, the CD154–CD40 interaction is also important probably because CD40-mediated induction or up-regulation of co-stimulatory molecules such as CD80 or CD86 on APC is critical for naive T cells to be activated (17,18).

CD154 and its receptor, CD40, are expressed on multiple types of cells and the interaction of CD154 with CD40 induces many different events, depending on the cell types involved. The CD154–CD40 interaction has been demonstrated to be important in regulating various phases of immune responses and for the effector functions of various APC such as B cells, macrophages or dendritic cells [for review, see (19)].

In a previous study, we reported that CD154-deficient NOD mice failed to develop insulitis, indicating that the CD154–CD40 interaction is critical for the onset of the disease (20). However, given that many types of cells are now known to express CD154 and/or CD40, the source of CD154 required in T1DM remains to be determined. Moreover, almost no information has been available on the mechanism by which CD154 would contribute to initiate insulitis.

In the current study, we demonstrate that CD4 T cells play a critical role in the onset of diabetes as the functional source of CD154. Furthermore, utilizing transgenic mice expressing CD80 under the control of rat insulin promoter (RIP), the possibility was examined that the role of CD154 on CD4 T cells is to mediate help in priming islet antigen-specific CD8 T cells by generating auto-antigen-bearing APC possessing co-stimulatory activity in the islets.

Methods

Mice

CD154+/− mice on the C57BL/6 background were backcrossed 11 times with NOD mice to generate CD154-deficient NOD mice, in which 15 idd genes were confirmed to be homozygous for the corresponding NOD allele (20). BDC2.5 transgenic mice, bearing class II MHC-restricted, islet antigen-specific TCR, on the NOD background were provided by J. D. Katz (21). RIP-CD80 transgenic CD154-deficient NOD mice were prepared by crossing N11 CD154+/− NOD mice with N4 RIP-CD80 transgenic mice (22). Genotyping was performed by genomic PCR as described previously (20,22). All NOD mice and SCID/NOD mice used in this study were maintained in our animal facility.

Adaptive transfer experiments

CD4+ or CD8+ T cells were purified from spleens using an IsoCell column (Pierce, Rockford, MD). Depletion of CD4 or CD8 T cell subsets from spleen was performed as described previously (12). Briefly, splenocytes were incubated with 10 μg/ml of anti-CD4 antibody (GK1.5, rat IgG; ATCC, Manassas, VA) or anti-CD8 antibody (53-6.72, rat IgG; ATCC) on ice for 30 min. After washing, cells were then incubated with magnetic beads conjugated to goat anti-rat Ig (BioMags; PerSeptive Biosystems, Framingham, MA). The degree of purification or depletion was always confirmed by flow cytometry and >90% purity or >95% depletion was achieved. Cells to be transferred were suspended in physiological saline (0.9% NaCl) and 200 μl of cell suspensions was injected i.v.

Assessment of diabetes development and histological analysis

Diabetes was monitored by measuring urine glucose level with Diastix (Bayer, Elkhart, IN), and confirmed by measuring blood glucose levels with One Touch test strips (LifeScan, Milpitas, CA). Animals were considered diabetic after two consecutive readings of blood glucose levels >250 mg/dl. To assess insulitis, pancreata were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin & eosin.

Measurement of in vitro recall response to keyhole limpet hemocyanin (KLH)

KLH (Calbiochem-Novabiochem, La Jolla, CA), either emulsified in complete Freund’s adjuvant or precipitated with alum, at 100 μg per mouse were injected into hind foot pads. Between 7 and 9 days later, 5 × 10⁵/well popliteal lymph node cells were incubated with the indicated concentration of KLH for 3 days. To assess proliferative response, incorporation of [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) for the last 16 h was measured. Concentrations of IFN-γ or IL4 in the supernatant of 3-day cultures were determined by ELISA as described previously (20).

Results

Incidence of spontaneous diabetes in CD154+/−, CD154+/− and CD154−/− mice

In order to define the role of CD154–CD40 interaction in T1DM, we developed CD154-deficient NOD mice by intercrossing CD154-KO male+/− mice and CD154−/− female mice, both of which had been back-crossed with NOD mice for 10 generations (N10). To obtain CD154−/− NOD mice as control mice, N11 CD154+/− males were crossed with CD154−/− females. Before inter-crossing, microsatellites Idd1 to 15 were examined, and all of these loci were confirmed to be derived from the NOD strain (data not shown). Among progeny from inter-crossing, female offspring were used to determine diabetes incidence. As we showed in a previous study (20), CD154−/− mice did not develop either insulin or diabetes until
40 weeks of age, by which time ~70% of CD154+/± mice developed disease (Fig. 1A). Unexpectedly, none of the CD154+/± mice developed diabetes, although most of them manifested moderate insulitis to a certain degree (Fig. 1B).

**CD154 on CD4 T cells is important for the initiation of the disease**

Since many types of cells have been demonstrated to be able to express CD154, various cells could be the functional source of CD154 in T1DM development. In the first attempt to determine the CD154-expressing cell type required for the disease, we investigated the ability of CD154-deficient CD4 T cells to induce disease in SCID/NOD mice. CD4 T cells from CD154-/- or CD154+/+ mice together with wild-type CD8 T cells were transferred into SCID/NOD mice. As shown in Fig. 2(A), CD154-deficient CD4 T cells, but not CD154-deficient CD4 T cells, could induce diabetes in SCID recipients together with wild-type CD8 T cells, suggesting that CD154 on CD4 T cells is essential for the onset of disease.

Since it was shown that T cell priming is impaired in CD154-KO mice (23), it was possible that there might be a difference in repertoire formation between CD154-sufficient and -deficient mice. In other words, it was possible that generation

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**Fig. 1.** (A) Incidence of spontaneous diabetes in CD154+/+ NOD (square, n = 9), CD154+/- NOD (triangles, n = 8) and CD154+/- NOD (circles, n = 10) mice. Female offspring obtained by intercrossing N11 CD154+/- NOD and CD154+/+ or CD154+/- NOD and CD154+/- were monitored for diabetes development as described in Methods. (B) Hematoxylin & eosin staining of formalin-fixed paraffin sections of pancreas from CD154+/+(right), CD154+/- (middle) and CD154-/- (left) NOD mice.

**Fig. 2.** Critical requirement of CD154 on CD4 T cells in the development of diabetes in NOD mice. (A) Incidence of diabetes in SCID.NOD mice that received wild-type CD4 and wild-type CD8 T cells (circles, n = 5), CD154-KO CD4 T cells and wild-type CD8 T cells (crosses, n = 6), wild-type CD4 T cells alone (triangles, n = 2) or wild-type CD8 T cells alone (squares, n = 2). (B) Incidence of diabetes in SCID.NOD recipients of spleen cells from BDC2.5-Tg wild-type males (squares, n = 3), BDC2.5-Tg CD154+/- females (triangles, n = 4) and BDC2.5-Tg CD154-KO males (circles, n = 3). (C) Paraffin sections of pancreas from CD154-KO NOD female recipients of CD4-depleted splenocytes (a and b) or CD8-depleted splenocytes (c and d) stained with hematoxylin & eosin.
or enrichment of auto-antigen-specific T cells is impaired in CD154+/− mice, thereby resulting in their inability to cause disease in SCID/NOD recipients. To examine this possibility, BDC2.5 TCR-Tg/NOD mice, in which most CD4 T cells have a transgenic TCR specific for an unidentified islet antigen (21), were crossed with CD154-KO mice to prepare CD4+ BDC2.5 T cells with or without CD154. As shown in Fig. 2(B), CD154−/− BDC2.5 T cells could also not induce diabetes when transferred to the recipient SCID/NOD mice, whereas CD154+ T cells could transfer diabetes, indicating that CD T cells cannot initiate disease in the absence of CD154 even when their TCR is essentially directed against an islet antigen.

In order to examine whether CD4 T cells are the only functional source of CD154 among several cell types in the spleen, we performed another adoptive transfer experiment in which spleen cells from CD154-sufficient diabetic mice, depleted of either CD4 T cells or CD8 T cells, were transferred into CD154+/−/NOD mice. As shown in Fig. 2(C) and Table 1, CD154−/− recipients of CD8-depleted spleen cells or total spleen cells (not shown), but not the recipients of CD4-depleted spleen cells, manifested insulin to indicate that CD154 expressed on CD4 T cells alone may be sufficient to provoke insulin.

No apparent protective activity of CD154-deficient CD4 T cells in T1DM development

As a result of the random inactivation of X chromosome in females, about half of CD4 T cells in CD154+/− mice do not express CD154 at all, while the other half can express this fully (data not shown). The lack of diabetes in CD154+/− mice might indicate that CD154+ T cells are protective against T1DM development. In an attempt to investigate this possibility, CD154+/+ spleen cells with or without CD154+/− spleen cells were co-transferred into NOD/SCID mice to compare their ability to induce diabetes in the recipient mice. As shown in Fig. 3(A), both recipients developed diabetes, although a slight delay in the progress of the disease was observed in the recipients of wild-type and CD154-KO spleen cells as compared with the recipients of wild-type cells alone. In another adoptive transfer experiment, we examined the effect of CD154-deficient CD4 T cells, purified from CD154-KO mice on the development of the disease in NOD/SCID mice which received total spleen cells from CD154-sufficient NOD mice (Fig. 3B). The result demonstrated no protective effect of CD154-KO CD4 T cells, indicating that failure of diabetes development in CD154+/− mice is unlikely to be due to the protective ability of the CD154-deficient CD4 T cells which constitute half of the CD4 T cells in these mice.

No effects of RIP-CD80 transgenics on pathogenesis in CD154-KO mice

Since CD8 T cells are crucial in the onset of T1DM and priming of CD8 T cells normally requires CD4 T cell help which, in turn, may consist of CD154-mediated activation of APC (14–16) to acquire co-stimulatory activity, we next investigated the effect of transgenic CD80 expression on pancreatic islet cells on the development of the disease in CD154+/−/NOD mice. As demonstrated previously, β cells constitutively expressing CD80 were capable of activating naive CD8 T cells in the absence of CD4 T cell help (26). In addition, the RIP-CD80 transgene could indeed restore disease development in CD4-deficient NOD mice in which the number of class II MHC-restricted Tp cells is significantly reduced and no insulitis was observed (27). To examine if disease could be restored in CD154+/−/NOD mice by constitutive CD80 expression on islet β cells, we crossed CD154+/− mice with RIP-CD80 transgenic
mice which had been back-crossed to NOD for four generations. Surprisingly, although CD154 heterozygous and wild-type mice developed diabetes, none of the CD154-KO mice developed diabetes (Fig. 4). Histological analyses demonstrated that no insulitis occurred in these mice (Table 2), indicating that the generation of APC which potentially have the ability to stimulate islet antigen-specific CD8 T cells without CD4 T cell help is not sufficient to induce insulitis without the CD154–CD40 interaction.

Discussion

Although several studies demonstrated that the CD154–CD40 interaction plays important roles in many murine models of organ-specific autoimmune diseases (28–31,32), its mechanism still remains unclear, mainly due to the fact that CD154–CD40 interaction regulates many phases of immune responses. CD40 is expressed on a variety of APC, and CD154 has also been shown to be expressed on multiple cell types, including, but not limited to, CD4 T cells, a small subset of CD8 T cells, NK cells, mast cells, basophils and dendritic cells (19). The CD154–CD40 interaction by various cell combinations would thus induce various responses.

Recently, Henn et al. demonstrated that platelets could express CD154 upon activation and that CD154 on activated platelets would stimulate endothelial cells to secrete chemokines and to express adhesion molecules, suggesting that platelet-mediated activation of endothelial cells via CD154 would result in the extravasation of leukocytes and in their recruitment into sites of inflammation (33). During the course of insulitis in T1DM, it was therefore possible that activation of endothelia by CD154 on platelets could be one of the critical earliest steps in T1DM development. This possibility appeared to be supported by the finding that the lack of disease in CD154+/−/NOD mice could be restored by islet-specific expression of tumor necrosis factor (TNF)-α (20), which is similarly capable of activating endothelia. When TNF-α is expressed specifically in islet cells, massive insulin is observed even in C57BL/6 mice (34), indicating that TNF-α secreted from islet β cells would activate vascular endothelium in the pancreas and thereby might recruit mononuclear cells into the islets. At present, it is not clear whether the main role of CD154 in insulitis is to activate vascular endothelial cells in the pancreas. However, even if the endothelium is the target tissue, the source of CD154 may be CD4 T cells, but not platelets, since, as observed in the current study, CD154−/− mice adoptively transferred with total or CD8-depleted spleen cells, but not CD4-depleted spleen cells from diabetic NOD mice, developed insulitis.

Interestingly, we have demonstrated that the presence of CD154 is required on CD4 T cells to transfer diabetes into SCID/NOD recipients. When CD154-sufficient CD4 T cells were co-transferred with wild-type CD8 T cells into SCID/NOD recipients, they transferred diabetes in 100% of cases. In contrast, when CD154-deficient CD4 T cells were co-transferred with wild-type CD8 T cells, they were not able to induce diabetes at all. This finding emphasizes the fact that CD4 T cells are the functional carriers of CD154 in autoimmune diabetes.

The lack of diabetes in CD154 heterozygous mice, in which half of the CD4 T cells do not express CD154 at all, possibly indicates that CD154− T cells might be somehow protective against T1DM, adoptive transfer experiments did not reveal an obvious protective effect of CD154− T cells (Fig. 3). It is of course possible that there might be an idd susceptibility locus close to the Cd154 gene and thereby that the loss of one allele of that gene might lead to dramatically retarded progress of the disease in CD154−/− mice. However, no such susceptibility genes have been so far identified on the X chromosome. Perhaps, the existence of the same number of CD154− CD4 T cells in CD154−/− mice might ‘dilute’ the functional effect of CD154+ CD4 T cells, which are required for the normal development of the disease in NOD mice.

CD154-mediated activation of APC is believed to be important not only in the priming of CD4 T cells (23), but also in the priming of CD8 T cells (14–16). It is now widely accepted that initiation of insulitis in the NOD mouse requires both CD4 and CD8 T cells. As demonstrated in the study of NOD mice depleted of CD8 T cells by in vivo antibody treatment (11) or β2-microglobulin-deficient NOD mice (7–9), naive CD4 T cells

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Table 2. Quantification of pancreatic infiltration in RIP-CD80/CD154-WT and RIP-CD80/CD154-KO mice

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<th>Mice</th>
<th>Infiltration score</th>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>RIP-CD80/CD154-WT</td>
<td>20</td>
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<tr>
<td>RIP-CD80/CD154-KO</td>
<td>124</td>
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Pancreatic infiltration and insulitis in non-diabetic mice were scored as follows: 0, healthy islet (or pancreas); 1, vascular infiltration; 2, peri-insulitis; 3, insulitis and aggressive infiltration.
alone are insufficient for the initiation of insulitis. However, CD4 T cells from diabetic NOD mice could cause insulitis without CD8 T cells when adoptively transferred into NOD/SCID mice, suggesting that lymphocytic infiltration into islets would require CD8 T cells until auto-antigen-specific CD4 T cells have expanded and are at a high frequency. However, since, in most cases, the activation of naive CD8 T cells requires CD4 T cell help and since CD4 help for CTL induction was demonstrated to be mediated by CD154, it was possible that the role of CD154 (or CD4 T cells) in the insulitis in NOD mice is to stimulate CD40 on APC which present islet antigenic peptide on class I MHC and to provide those APC with the capability of activating islet-antigen-specific CD8 T cells efficiently. In an attempt to explore this possibility, we have utilized RIP-CD80 transgenic mice, since it has been previously demonstrated that provision of co-stimulatory activity to islet cells by the RIP-CD80 transgene could overcome the lack of most class II MHC-restricted Tc cells in CD4±/± mice, but not of CD8 T cells in β2-microglobulin−/− mice, in T1DM development. Surprisingly, however, CD154−/−/NOD mice crossed with RIP-CD80 transgenic mice did not develop insulitis, indicating that some function of CD154 other than the generation of activated APC capable of priming auto-reactive CD8 T cells might be necessary for the insulitis. In addition, it was also suggested that in CD4-deficient, RIP-CD80 transgenic NOD mice, CD154 (possibly on class II MHC-restricted, CD8− (resulting in CD4/CD8-double negative)) Tc cells which are observed in substantial numbers in CD4±/± mice (35) play a mandatory role(s) in the insulitis. The results in Fig. 2(C) which show that CD154-deficient CD4 T cells could not induce infiltration of auto-reactive CD8 T cells from diabetic NOD mice into islets may suggest that CD154 on CD4 T cells may be required even after priming of auto-reactive CD8 T cells.

It remains unknown which cell type is required to be activated by CD40 stimulation for the initiation of insulitis. One possible candidate cell is the endothelial cell, as discussed above. In addition, it is also possible that some APC which reside in spleen could be the target cells. In our previous study using CIITA-deficient NOD mice, it was shown that total spleen cells from diabetic NOD mice could transfer disease in irradiated CIITA−/−/NOD mice which rarely develop insulitis. However, our preliminary results demonstrated that transferring CD4 T cells alone purified from spleens of wild-type NOD mice did not induce diabetes in CIITA−/− mice, suggesting that the interaction of CD4 T cells with donor-derived splenic APC would be also important in the initiation of disease. Thus, among splenic APC, B cells, for example, are one of the candidates for APC. It has been shown that many functions of B cells are impaired in the absence of CD40 stimulation (36–39) and that NOD mice lacking B cells do not develop insulitis (40–42). Another likely candidate as the target APC is the macrophage, which has been suggested to be important in the generation of β cell-toxic T cells (43). It is also possible that CD4 T cell-mediated activation of dendritic cells which have captured and carry islet antigen via CD154 in the pancreatic lymph node would be required to initiate insulitis (44).

Further experiments using CD40−/−/NOD mice would be required to identify the target APC or to investigate the role of CD40 on endothelial cells or various APC (by adaptively transferring total spleen cells from wild-type NOD mice or various types of APC purified from wild-type NOD mice into CD40−/−/NOD mice).

After this study was completed, analyses of CD154-deficient NOD mice bearing transgenic, auto-reactive TCR were reported, where a critical requirement of CD154 in T1DM development was established (45). The current study agrees with those results and also reveals a role of CD4 T cells in the onset of T1DM as a functional source of CD154.

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Abbreviations

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<th>Term</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CIITA</td>
<td>MHC class II transactivator</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>T1DM</td>
<td>type I (insulin-dependent) diabetes mellitus</td>
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<td>KHI</td>
<td>keyhole limpet hemocyanin</td>
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<td>NOD</td>
<td>non-obese diabetic</td>
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<td>RIP</td>
<td>rat insulin promoter</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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References


