

Neonatal Tumor Necrosis Factor α Promotes Diabetes in Nonobese Diabetic Mice by CD154-independent Antigen Presentation to CD8⁺ T Cells

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Abstract

Neonatal islet-specific expression of tumor necrosis factor (TNF)- α in nonobese diabetic mice promotes diabetes by provoking islet-infiltrating antigen-presenting cells to present islet peptides to autoreactive T cells. Here we show that TNF- α promotes autoaggression of both effector CD4⁺ and CD8⁺ T cells. Whereas CD8⁺ T cells are critical for diabetes progression, CD4⁺ T cells play a lesser role. TNF- α -mediated diabetes development was not dependent on CD154-CD40 signals or activated CD4⁺ T cells. Instead, it appears that TNF- α can promote cross-presentation of islet antigen to CD8⁺ T cells using a unique CD40-CD154-independent pathway. These data provide new insights into the mechanisms by which inflammatory stimuli can bypass CD154-CD40 immune regulatory signals and cause activation of autoreactive T cells.

Key words: TNF- α • CD154 • diabetes • NOD mice • CD8⁺ cells

Introduction

The differentiation of naive CD8⁺ T cells into effector CTLs normally requires the interaction of three types of cells: APCs bearing peptide-MHC complexes, CD4⁺ T cells, and CD8⁺ T cells. Until recently, our understanding of the importance of CD4⁺ T cells in this three-cell interaction was to provide "help" directly to the naive CD8⁺ T cell, e.g., by the secretion of lymphokines like IFN- γ (1). Now, however, three reports have demonstrated that CD4⁺ T cell help for the differentiation of naive CD8⁺ T cells into effector CTLs is at the level of the APC (2-4), where initial engagement of CD4⁺ T cells with APCs triggers the maturation of the APC into a cell fully competent to prime CD8⁺ T cells.

Deciphering these molecular interactions between CD4⁺ T cells and APCs involved in the generation of humoral and cell-mediated immunity has advanced remarkably (5-7). In particular, interactions between CD40 on APCs and CD154 on activated CD4⁺ T cells play a dominant role in the generation of T and B cell immunity (for review see reference 8). CD40-CD154 signals enhance APC activity by upregulating adhesion and costimulatory molecules (9), as well as

generating antiapoptotic signals that increase APC survival (10) and thus prolong contact between APCs and T cells. The importance of CD40-CD154 signals in the generation of efficient T cell immunity is exemplified by the finding that cross-linking of CD40 on APCs can bypass the need for CD4⁺ T cells in the differentiation of naive CD8⁺ T cells into effector CTLs (3). Indeed, blockade of CD40-CD154 interactions with antisera, or the absence of a functional CD40 or CD154 gene, leads to impaired humoral responses to thymus-dependent antigens (11-13) by preventing efficient activation of APCs (8). Several autoimmune conditions have been shown to be dependent on CD40-CD154 signals (13-15), and in diabetes, blockade of CD40-CD154 signals abrogates both insulinitis and diabetes (14).

In insulin-dependent autoimmune diabetes, where insulin-producing β cells in the islets of Langerhans are destroyed by T cell-mediated mechanisms (for review see reference 16), the importance of CD4⁺ and CD8⁺ T cells for β cell destruction has been strongly debated. For example, several reports in nonobese diabetic (NOD)¹ mice that spontaneously develop diabetes have shown that deficiency in β_2 -microglobulin (and, as a consequence, CD8⁺ T cells) or

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¹Abbreviations used in this paper: CIITA, MHC class II-transactivating; DCs, dendritic cells; GAD, glutamic acid decarboxylase; KLH, keyhole limpet hemagglutinin; NOD, nonobese diabetic.

treatment with anti-CD8 antibodies from birth protects from disease (17–20). Similarly, NOD mice treated with anti-CD4 antibodies or NOD mice deficient in CD4⁺ T cells do not develop diabetes (21, 22). These reports suggest a requirement for both CD4⁺ and CD8⁺ T cells in insulin-dependent autoimmune diabetes, which has been substantiated by adoptive transfer experiments (23, 24). The dual role for CD4⁺ and CD8⁺ T cells is believed to relate in part to the requirement for CD4⁺ T cell help in the generation of β cell-specific CD8⁺ CTLs (21). This suggests that diabetes development in NOD mice involves the cross-presentation (25) of exogenous islet antigen to naive islet-specific CD8⁺ T cells.

Inflammatory cytokines have been implicated in the breakdown of tolerance of self-reactive T cells to host tissue and subsequent autoimmunity (26), although the mechanisms responsible remain elusive. In particular, there has been a strong association between the inflammatory cytokine TNF- α and the development of diabetes in NOD mice. This has been based on studies demonstrating that (a) TNF- α mRNA is produced in inflamed islets in NOD mice (27) and (b) systemic administration of TNF- α in neonatal NOD mice accelerates diabetes progression, whereas (c) neutralization of TNF- α in neonatal NOD mice protects against disease (28). Recently, we demonstrated that localized, islet-specific expression of TNF- α in neonatal NOD (TNF- α -NOD) mice was sufficient to accelerate diabetes onset. This rapid

progression to diabetes was preceded by apoptosis of some β cells, followed by TNF- α -mediated recruitment of APCs, particularly dendritic cells (DCs), and T cells to neonatal islets and a subsequent enhancement in the presentation of islet antigen to autoreactive T cells in situ (29). However, the requirement for CD4⁺ and CD8⁺ T cells in this accelerated model of diabetes was unknown.

In this article, we show that TNF- α enhances autoimmunity in NOD mice by provoking islet-infiltrating APCs to cross-present exogenous islet antigen to CD8⁺ T cells. Furthermore, using a series of knockout models, we establish that effector CD4⁺ T cells are not required for the priming of islet-specific CD8⁺ T cells. Indeed, we provide strong evidence that TNF- α can substitute for CD4⁺ T cell help in the cross-presentation of exogenous islet antigen to CD8⁺ T cells by overcoming the need for CD154 signals in the activation of islet-infiltrating APCs. These findings reveal that inflammatory stimuli may promote autoimmunity by thwarting normal CD4⁺ T cell-dependent CD154 immune regulatory mechanisms.

Materials and Methods

Mice. All knockout and transgenic mice used in this study are on the NOD background and are described in Table I. TNF- α -NOD mice and NOD mice deficient for β_2 -microglobulin (NOD- $\beta_2\mu^{-/-}$) have been described previously (18, 20, 29).

Table I. Classification of the Transgenic and Knockout Models Used in This Study

Mouse	Phenotype	Insulinitis	Diabetes	Reference no.
TNF- α -NOD	Transgenic expression of TNF- α in islets	yes	yes	29
NOD- $\beta_2\mu^{-/-}$	No MHC class I; few CD8 ⁺ T cells	no	no	18, 20
TNF- α - $\beta_2\mu^{-/-}$	No MHC class I; few CD8 ⁺ T cells; transgenic expression of TNF- α in islets	yes	no	This study
NOD-CIITA ^{-/-}	No MHC class II on APCs; few CD4 ⁺ T cells	yes (periinsulinitis)	no	21
TNF- α -CIITA ^{-/-}	No MHC class II on APCs; few CD4 ⁺ T cells; transgenic expression of TNF- α in islets	yes	yes	This study
NOD-CD154 ^{-/-}	No CD154 on T cells	no	no	This study
TNF- α -CD154 ^{-/-}	No CD154 on T cells; transgenic expression of TNF- α in islets	yes	yes	This study

The phenotypes of all of the transgenic and knockout NOD models used in this study and their ability to progress to diabetes.

TNF- α - $\beta_2\mu^{-/-}$ mice were generated by crossing N10 TNF- α -NOD mice to N10 NOD- $\beta_2\mu^{-/-}$ mice. TNF- α^+ progeny were detected by PCR (29) and further screened for the presence of the $\beta_2\mu$ null mutation by measuring CD8 $^+$ T cell numbers and assessing for MHC class I H2-K d and H2-D b molecules on immune cells in the peripheral blood. TNF- α -MHC class II transactivator $^{-/-}$ (TNF- α -CIITA $^{-/-}$) mice were generated by crossing N11 TNF- α -NOD mice to N11 NOD-CIITA $^{-/-}$ mice (21). TNF- α -CIITA $^{-/-}$ mice were detected by Southern blot analysis as previously described (30).

TNF- α -CD154 $^{-/-}$ mice were generated by crossing N11 TNF- α -NOD mice with N11 NOD-CD154 $^{-/-}$ mice and then intercrossing heterozygous populations. All mice were screened for the presence of CD154 by PCR combining primer sequences that detected the wild-type gene (5' \rightarrow 3' CCC AAG TGT ATG AGC ATG TGT GT and GTT CCT CCA CCT AGT CAT TCA TC) and the *neo* cassette (5' \rightarrow 3' GCC CTG AAT GAA CTG CAG GAC G; CAC GGG TAG CCA ACG CTA TGT C). Cycling conditions were 30 cycles of 94°C for 45 s, 57°C for 1 min, and 72°C for 1 min. The CD4 $^+$ islet-specific TCR-transgenic mice, BDC2.5 on the NOD background, have been described elsewhere (31) and were provided by J.D. Katz (Washington University, St. Louis, MO). All NOD mice used for breeding and experimental purposes were bred and maintained in our animal facility, the disease incidence in the colony being 80% for females and 50% for males by 30 wk of age.

Adoptive Transfers. Whole splenocytes were isolated from 6-wk-old BDC2.5 CD4 $^+$ TCR-transgenic mice, and RBCs were lysed by H $_2$ O treatment. Splenocytes were washed twice in Bruff's medium supplemented with 5% FBS (Bruff/5) and then once in sterile saline. For the islet-reactive CD8 $^+$ T cell clone TGNFC8, the cells were removed from culture and washed twice in sterile saline. In both cases, the cells were resuspended in sterile saline and injected into the retroorbital vein at a concentration of 10 7 cells per mouse.

Isolation of Cells. Islets were isolated after collagenase (Boehringer Mannheim) digestion of pancreata. The infiltrating cells were liberated by trypsinization and washed three times in RPMI medium supplemented with 5% FBS (RPMI/FBS). CD4 $^+$ and CD8 $^+$ T cells were isolated from either the pancreatic lymph node or spleen by crushing the tissue through nylon mesh. The RBCs were removed with Tris-buffered NH $_4$ Cl, and the remaining white blood cells were washed three times in RPMI/FBS. For purification of CD4 $^+$ T cells, the cell suspension was incubated with unlabeled rat anti-CD8 (53-6.72) antibodies, followed by incubation with goat anti-rat IgG (H+L chain) and goat anti-mouse IgG (H+L chain) BioMag $^{\text{®}}$ beads (both from PerSeptive Biosystems). Macrophages (M Φ s) and DCs were subsequently removed by incubation on plastic at 37°C for 45 min. Purity of the CD4 $^+$ T cell population was verified by FACS $^{\text{®}}$ analysis. For purification of CD8 $^+$ T cells, the cell suspension was incubated with unlabeled rat anti-CD4 (GK1.5) antibodies, followed by incubation with goat anti-rat IgG (H+L chain) and goat anti-mouse IgG (H+L chain) BioMag $^{\text{®}}$ beads. M Φ s and DCs were subsequently removed as above. Again, purity of the CD8 $^+$ T cell population was verified by FACS $^{\text{®}}$ analysis.

FACS $^{\text{®}}$ Analysis. All antibodies used for FACS $^{\text{®}}$ analysis were obtained from PharMingen. The cells under analysis were prepared as described above and then washed twice in FACS buffer (PBS containing 2% FBS). The cells were incubated with the appropriate fluorochrome-labeled antibody for 30 min at 4°C. After three successive washings in FACS buffer, the cells were analyzed using FACSCalibur $^{\text{TM}}$ (Becton Dickinson).

For measurement of intracellular perforin, after addition of fluorochrome-labeled antibodies to extracellular proteins, the cells were fixed in 1% paraformaldehyde for 30 min at 4°C. After two successive washes in FACS buffer, fixed cells were incubated with biotinylated rat anti-mouse perforin antibody (PharMingen) or isotype control antibody (PharMingen) diluted in PBS supplemented with 2% FBS and 0.03% saponin (PBS/saponin) for 30 min at 4°C. The cells were washed twice in FACS buffer and then incubated with fluorochrome-labeled streptavidin in PBS/saponin buffer for 30 min at 4°C, after which the cells were washed three times in FACS buffer and analyzed as described above.

Immunizations and Measurement of Anti-Keyhole Limpet Hemagglutinin Responses. Keyhole limpet hemagglutinin (KLH; Calbiochem-Novabiochem Corp.) was emulsified in alum and injected into the footpads of 5-wk-old mice at 100 μ g per mouse. 7 d after immunization, the popliteal lymph nodes were removed and the cells isolated as described above. 5 \times 10 5 cells were stimulated with KLH at the concentrations described in the text. After incubation at 37°C in 5% CO $_2$ for 4 d and a 21-h pulse with 1 μ Ci/well [3 H]thymidine (Amersham Pharmacia Biotech Inc.) on the last day of the culture, cells were harvested and [3 H]thymidine incorporation was measured.

Measurement of Glutamic Acid Decarboxylase Responses. Responder CD4 $^+$ T cells were purified as described above. As a source of APCs, 6-wk-old NOD mice were killed, and after removal of red blood cells, total splenocyte populations were irradiated (3,000 rads). Unless otherwise stated in the text, 3 \times 10 5 purified CD4 $^+$ T cells were incubated with 5 \times 10 3 irradiated splenocytes, and 0–20 μ g/ml of glutamic acid decarboxylase (GAD)p35 (amino acids 524–543) was added to the assay. After incubation at 37°C in 5% CO $_2$ for 4 d and a 14-h pulse with 1 μ Ci [3 H]thymidine (Amersham) on the last day of culture, cells were harvested and [3 H]thymidine incorporation was measured.

Antigen Presentation Assays. The ability of islet-infiltrating cells to present islet antigen to responder CD4 $^+$ and CD8 $^+$ T cells was assessed as previously described (29). In brief, islet-infiltrating cells to be used as a source of APCs in the assays were isolated by collagenase digestion of pancreata followed by trypsinization and then irradiated (4,000 rads). Responder CD4 $^+$ or CD8 $^+$ T cells were purified as before. Unless otherwise stated, 5 \times 10 4 irradiated islet-derived cells were incubated with 10 5 purified CD4 $^+$ or CD8 $^+$ T cells for 4 d at 37°C in 5% CO $_2$. The cells were pulsed with 1 μ Ci [3 H]thymidine for the last 16 h of the assay, and proliferative responses were measured as above.

Measurement of IL-4 and IFN- γ . Islet-infiltrating cells were liberated from islets as described above. CD8 $^+$ T cells were removed by magnetic bead separation techniques. The remaining islet cells, including APCs and CD4 $^+$ T cells, were incubated for 48 h in the presence of 2.5 μ g/ml of Con A, after which the culture supernatants were harvested for measurement of IL-4 and IFN- γ concentrations using cytokine-specific ELISAs. In brief, Maxisorp Nunc-immuno plates (Nalge Nunc International) were coated with purified rat anti-IL-4 or anti-IFN- γ antibodies (PharMingen) in carbonate buffer, pH 9.6, overnight at 4°C. After blocking in PBS containing 3% FBS, supernatants or standard controls were added and allowed to incubate overnight at 4°C. Bound IL-4 or IFN- γ was visualized by incubation with biotinylated rat anti-IL-4 or IFN- γ , respectively (PharMingen), followed by horseradish peroxidase-labeled streptavidin (Vector Labs., Inc.) and TMB substrate (Kirkegaard & Perry Labs., Inc.). The concentrations of IL-4 and IFN- γ were calculated using the Microplate Manager III program (Bio-Rad Labs.).

Diabetes Detection. Mice were tested once weekly for glycos-

uria using Diastix® reagent strips for urinalysis (Ames Co.) starting at 4 wk of age. Diabetes was confirmed when nonfasting glucose values in the serum were higher than 250 mg/dl, as determined by One Touch test strips (Lifescan; Johnson & Johnson Co.). Animals that tested positive for hyperglycemia on two consecutive occasions 48 h apart were deemed diabetic.

Results

TNF- α Enhances Effector Activity of Both CD4⁺ and CD8⁺ Islet-specific T Cells. Our prior work showed that islet-specific expression of TNF- α in neonatal NOD mice promotes rapid onset of diabetes (29). In part, this was related to the ability of TNF- α to recruit APCs, particularly DCs and M Φ s, to neonatal islets and promote presentation of islet antigen to T cells. However, whether TNF- α -mediated APC activation enhanced activity of CD4⁺ or CD8⁺ T cells was unknown (29). In this study, we first addressed this issue by adoptively transferring BDC2.5 CD4⁺ TCR-transgenic T cells (31) or CD8⁺ T cell clone TGNFC8 (32), into TNF- α -*scid/scid* mice and monitored diabetes development. As controls, we transferred the cells into nontransgenic (non-tg) NOD-*scid/scid* littermates.

Transfer of splenocytes from 6-wk-old BDC2.5 CD4⁺ TCR-transgenic donor mice into TNF- α -*scid/scid* recipients resulted in diabetes as early as 48 h after transfer (Table II). By 72 h, all recipient mice were diabetic. In contrast, transfer of BDC2.5 CD4⁺ TCR-transgenic T cells into control non-tg NOD-*scid/scid* mice did not induce diabetes before 1 wk after transfer, generally taking 9 d to induce disease. TNF- α -*scid/scid* mice or non-tg NOD-*scid/scid* mice that did not receive the CD4⁺ T cells never developed diabetes.

Similar findings occurred when CD8⁺ TGNFC8 cells were transferred into TNF- α -*scid/scid* recipients, diabetes being detectable 48 h after transfer. However, transfer of CD8⁺ TGNFC8 cells into control non-tg NOD-*scid/scid* mice did not induce disease until 1 wk after transfer (Table II). Verification of β cell destruction was established by histological examination for insulin-producing cells in extracted pancreata from diabetic mice (not shown). Thus, TNF- α expression in neonatal islets enhances effector function of both activated CD4⁺ and CD8⁺ T cells.

CD8⁺ T Cells Are Necessary for TNF- α -mediated Progression to Diabetes in NOD Mice. We further investigated the role of CD8⁺ and CD4⁺ T cells in the progression to diabetes in TNF- α -NOD mice by using a series of knockout models (Table I). First, we addressed the role of CD8⁺ T cells. For this, we crossed TNF- α -NOD mice to NOD mice bearing a null mutation for β_2 -microglobulin ($\beta_2\mu^{-/-}$). Such mice do not express MHC class I molecules, lack CD8⁺ T cells due to defective thymic positive selection, and do not develop insulinitis or diabetes (Table I). We monitored TNF- α - $\beta_2\mu^{+/+}$, TNF- α - $\beta_2\mu^{-/+}$, and TNF- α - $\beta_2\mu^{-/-}$ mice for progression to diabetes.

As shown in Fig. 1 a, neither male nor female TNF- α - $\beta_2\mu^{-/-}$ mice developed diabetes over a 30-wk observation period. In contrast, TNF- α - $\beta_2\mu^{+/+}$ littermates developed diabetes with 100% incidence by 14 wk of age in both males and females. TNF- α - $\beta_2\mu^{-/+}$ mice also developed diabetes, although the kinetics of the disease were slightly retarded, with 100% incidence of disease occurring by 20 wk of age in both sexes. Thus, CD8⁺ T cells are required for TNF- α -mediated diabetes in NOD mice. To confirm that β cell destruction required specific stimulation of CD8⁺ T cells via their TCRs, as a control we transferred the TGNFC8 clone into 6-wk-old TNF- α - $\beta_2\mu^{+/+}$, TNF- α - $\beta_2\mu^{-/-}$, or non-tg NOD- $\beta_2\mu^{-/-}$ mice. Only TNF- α - $\beta_2\mu^{+/+}$ developed diabetes over a 6-wk observation period (not shown).

Protection Against Diabetes in TNF- α - $\beta_2\mu^{-/-}$ Mice Is Not Related to Blockade of Insulinitis. NOD- $\beta_2\mu^{-/-}$ mice and NOD mice treated neonatally with anti-CD8 antibodies are protected from diabetes because they fail to develop insulinitis (18–20). To determine whether insulinitis blockade in TNF- α - $\beta_2\mu^{-/-}$ mice was responsible for protection against diabetes, we performed histologic studies of the pancreas of 15- and 42-d-old TNF- α - $\beta_2\mu^{-/-}$ mice or age-matched control TNF- α - $\beta_2\mu^{+/+}$ mice.

At 15 d of age, both TNF- α - $\beta_2\mu^{+/+}$ and TNF- α - $\beta_2\mu^{-/-}$ mice had similar islet infiltrates composed of DCs, B cells, and some CD4⁺ T cells, which shows that TNF- α can bypass the need for MHC class I molecules for the initiation of insulinitis (not shown; reference 33). In contrast, at 42 d of age there were distinct differences in T cells and DCs that infiltrated the islets of TNF- α - $\beta_2\mu^{+/+}$ and TNF- α - $\beta_2\mu^{-/-}$ mice. First, as expected, CD8⁺ T cells were detectable only in islets

Table II. TNF- α Promotes Autoaggression of Islet-specific T Cells

Donor/cell source	Recipient	Days after transfer																											
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
TGNFC9	NOD- <i>scid/scid</i>	0	0	0	0	0	0	3	3	3	3	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	TNF- α -NOD- <i>scid/scid</i>	0	4	6																									
BDC2.5	NOD- <i>scid/scid</i>	0	0	0	0	0	0	0	2	2	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	TNF- α -NOD- <i>scid/scid</i>	0	2	6																									

10⁷ cloned CD8⁺ T cells (TGNFC8) or 10⁷ splenocytes from BDC2.5 CD4⁺ TCR-transgenic mice were transferred into 6-wk-old female TNF- α -*scid/scid* (n = 6) or non-tg *scid/scid* littermates (n = 6). Diabetes progression was monitored until 28 d after transfer.

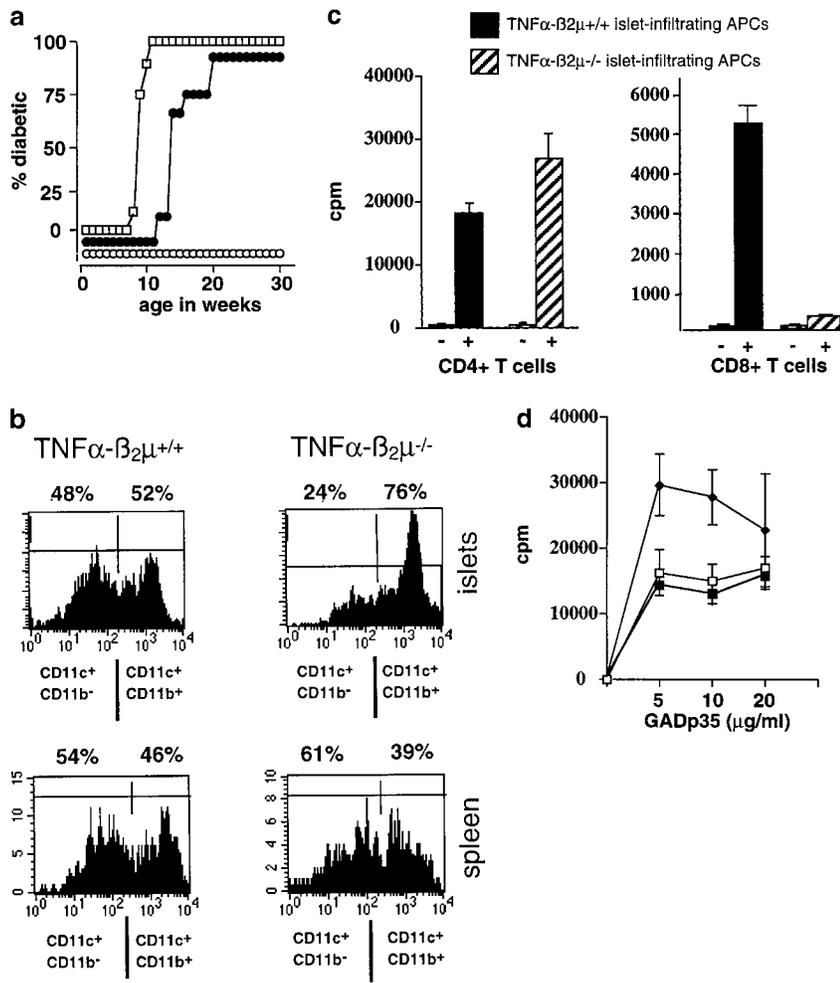


Figure 1. CD8⁺ T cells are required for diabetes development in TNF-α-NOD mice. (a) Graphical representation of diabetes progression in pooled male and female TNF-α-β₂μ^{+/+} (□; n = 25), TNF-α-β₂μ^{+/-} (●; n = 21), and TNF-α-β₂μ^{-/-} (○; n = 26) mice. (b) CD11c⁺CD11b⁻ DCs predominate over CD11c⁺CD11b⁺ DCs in the islets of adult TNF-α-β₂μ^{-/-} mice. Islet-infiltrating cells or splenocytes from 6-wk-old TNF-α-β₂μ^{+/+} or TNF-α-β₂μ^{-/-} mice were incubated with anti-CD3, -CD11c, -CD11b, and -CD45 antibodies. The percentage of CD11b⁺ and CD11b⁻ DCs in the CD45⁺CD3⁺CD11c⁺ fraction was determined by FACS[®]. The data is representative of eight individual animals. (c) Islet-infiltrating APCs from TNF-α-β₂μ^{-/-} mice efficiently present islet antigen to NOD CD4⁺ T cells. Islet-infiltrating cells were isolated from 6-wk-old TNF-α-β₂μ^{+/+} or TNF-α-β₂μ^{-/-} mice, and 5 × 10⁴ irradiated cells were cultured with 10⁵ purified CD4⁺ or CD8⁺ T cells from 6-wk-old NOD mice. After a 16-h pulse with 1 μCi of [³H]thymidine on the last day of a 4-d culture, proliferative responses were measured. The assay was performed in triplicate and the data presented as mean counts per minute (cpm) ± SD. Background cpm for CD4⁺ and CD8⁺ T cells cultured in medium alone was <500. (d) CD4⁺ T cells from TNF-α-β₂μ^{-/-} mice are primed to GADp35. 5 × 10⁵ purified CD4⁺ T cells from 6-wk-old TNF-α-β₂μ^{+/+} (□), TNF-α-β₂μ^{+/-} (●), or non-tg NOD-β₂μ^{-/-} (○) mice were stimulated with GADp35 at the concentrations shown, using 5 × 10⁵ irradiated splenocytes from 6-wk-old NOD mice as a source of APCs. After 4 d of culture, proliferative responses were measured as before. The assay was performed in triplicate and the data presented as mean cpm ± SD. Background cpm for CD4⁺ T cells cultured with APCs alone was <400.

from TNF-α-β₂μ^{+/+} mice (not shown). Second, in TNF-α-β₂μ^{+/+} mice, there was a significant increase in CD11c⁺CD11b⁻ DCs infiltrating the islets, whereas in TNF-α-β₂μ^{-/-} mice, CD11c⁺CD11b⁺ DCs predominated. This latter finding was confirmed by FACS[®] analysis of islet-infiltrating cells from 42-d-old TNF-α-β₂μ^{+/+} and TNF-α-β₂μ^{-/-} mice (Fig. 2 b). This difference in DC populations in the islets between the two groups of mice was not due to a general absence of CD11c⁺CD11b⁻ DCs in TNF-α-β₂μ^{-/-} mice, as CD11c⁺CD11b⁻ DCs were clearly detectable in the spleens of TNF-α-β₂μ^{-/-} mice. Similar histological or FACS[®] analysis of CD4⁺ T and B cells detected no differences between TNF-α-β₂μ^{+/+} and TNF-α-β₂μ^{-/-} mice.

This data suggests that CD8⁺ T cells are required for the progression to diabetes in TNF-α-NOD mice and may also be involved in the recruitment, expansion, or differentiation of CD11c⁺CD11b⁻ DCs in TNF-α-NOD islets. The importance of these emerging CD11c⁺CD11b⁻ DCs in the islets of TNF-α-β₂μ^{+/+} mice is currently under investigation.

Priming of CD4⁺ T Cells to Islet Antigen Is Unaffected in TNF-α-β₂μ^{-/-} Mice. It has been suggested that CD8⁺ T cells are required for effective priming and expansion of autoreactive CD4⁺ T cells in NOD mice (19). Thus, we

tested whether CD4⁺ T cells in TNF-α-β₂μ^{-/-} mice were efficiently primed to islet antigen. First, we asked whether islet-infiltrating APCs from TNF-α-β₂μ^{-/-} could present islet antigen to NOD T cells.

Islet-infiltrating APCs from 6-wk-old TNF-α-β₂μ^{+/+} and TNF-α-β₂μ^{-/-} mice were isolated and cultured with purified CD4⁺ or CD8⁺ T cells from 6-wk-old NOD mice. As shown in Fig. 1 c, islet-infiltrating APCs from TNF-α-β₂μ^{-/-} mice efficiently stimulated purified CD4⁺ T cells as efficiently as APCs extracted from the islets of TNF-α-β₂μ^{+/+} mice. In contrast, only islet-infiltrating APCs from TNF-α-β₂μ^{+/+} mice stimulated purified CD8⁺ T cells, as expected.

Next, we tested the reactivity of purified CD4⁺ T cells from TNF-α-β₂μ^{-/-} mice or TNF-α-β₂μ^{+/+} mice to GADp35, a prominent diabetogenic peptide from glutamic acid decarboxylase GAD. Previous reports have speculated that the generation of GAD-reactive CD4⁺ T cells is a prerequisite for the generation of other antiislet responses (34, 35). As a comparison, we also assayed purified CD4⁺ T cells from non-tg NOD-β₂μ^{-/-} mice. In all cases, anti-GADp35 responses were clearly detectable, with CD4⁺ T cells from TNF-α-β₂μ^{-/-} mice in fact generating the strongest responses (Fig. 1 d).

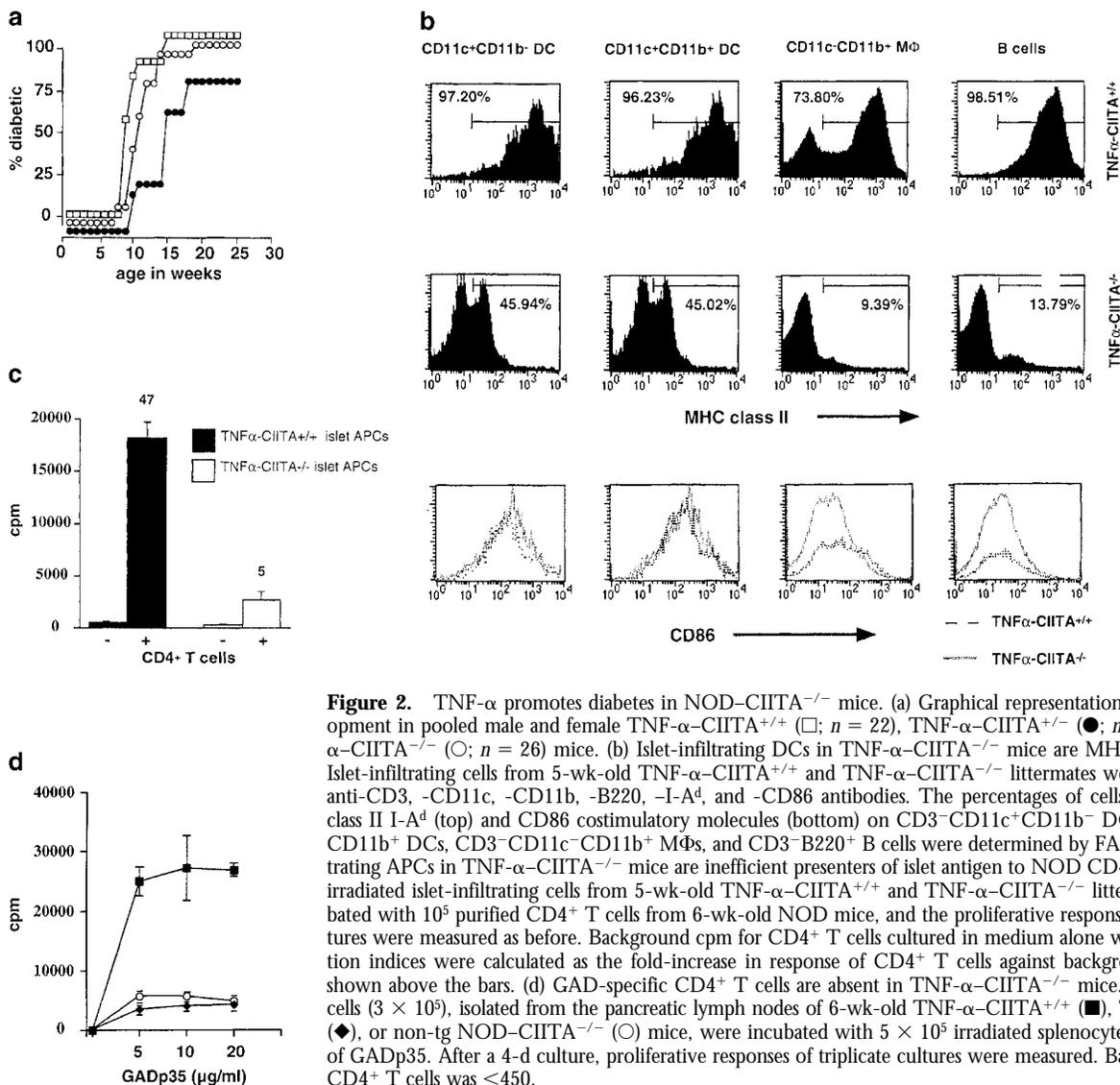


Figure 2. TNF- α promotes diabetes in NOD-CIITA^{-/-} mice. (a) Graphical representation of diabetes development in pooled male and female TNF- α -CIITA^{+/+} (\square ; $n = 22$), TNF- α -CIITA^{+/-} (\bullet ; $n = 16$), and TNF- α -CIITA^{-/-} (\circ ; $n = 26$) mice. (b) Islet-infiltrating DCs in TNF- α -CIITA^{-/-} mice are MHC class II^{hi}CD86^{hi}. Islet-infiltrating cells from 5-wk-old TNF- α -CIITA^{+/+} and TNF- α -CIITA^{-/-} littermates were incubated with anti-CD3, -CD11c, -CD11b, -B220, -I-A^d, and -CD86 antibodies. The percentages of cells expressing MHC class II I-A^d (top) and CD86 costimulatory molecules (bottom) on CD3⁻CD11c⁺CD11b⁻ DCs, CD3⁻CD11c⁺CD11b⁺ DCs, CD3⁻CD11c⁻CD11b⁺ MΦs, and CD3⁻B220⁺ B cells were determined by FACS[®]. (c) Islet-infiltrating APCs in TNF- α -CIITA^{-/-} mice are inefficient presenters of islet antigen to NOD CD4⁺ T cells. 5×10^4 irradiated islet-infiltrating cells from 5-wk-old TNF- α -CIITA^{+/+} and TNF- α -CIITA^{-/-} littermates were incubated with 10^5 purified CD4⁺ T cells from 6-wk-old NOD mice, and the proliferative responses of triplicate cultures were measured as before. Background cpm for CD4⁺ T cells cultured in medium alone was <550. Stimulation indices were calculated as the fold-increase in response of CD4⁺ T cells against background cpm and are shown above the bars. (d) GAD-specific CD4⁺ T cells are absent in TNF- α -CIITA^{-/-} mice. Purified CD4⁺ T cells (3×10^5), isolated from the pancreatic lymph nodes of 6-wk-old TNF- α -CIITA^{+/+} (\blacksquare), TNF- α -CIITA^{-/-} (\blacklozenge), or non-tg NOD-CIITA^{-/-} (\circ) mice, were incubated with 5×10^5 irradiated splenocytes and 0–20 μ g/ml of GADp35. After a 4-d culture, proliferative responses of triplicate cultures were measured. Background cpm for CD4⁺ T cells was <450.

These findings show that CD4⁺ T cells in TNF- α - β 2 μ -/- mice can be efficiently primed to islet antigen in the absence of CD8⁺ T cells. Furthermore, the generation of islet-reactive CD4⁺ T cells is not sufficient to induce diabetes in this TNF- α model. Instead, diabetes progression requires CD8⁺ T cells, perhaps acting through cytolytic attack on the β cells.

TNF- α Bypasses the Need for MHC Class II Signaling to Induce Diabetes in NOD Mice. The generation of cytotoxic CD8⁺ T cells normally requires CD4⁺ T cell help (1). We decided to test whether CD4⁺ T cell help was a prerequisite for progression to diabetes in our TNF- α model. To address this, we crossed TNF- α -NOD mice to NOD mice that do not express MHC class II molecules on their APCs. NOD-CIITA^{-/-} mice, which carry a null mutation for the CIITA molecule, provide a good model system to address this issue (21).

CIITA (36) is a key factor involved in the expression of

MHC class II molecules as well as the upregulation of MHC class I molecules by IFN- γ (37–43). As such, CIITA^{-/-} mice do not express MHC class II on the majority of their APCs and exhibit the same reduction in peripheral CD4⁺ T cell numbers as MHC class II-deficient mice (44, 45). Recently, we showed that NOD-CIITA^{-/-} mice have significant pancreatic infiltration but do not develop diabetes due to CD4⁺ T cell deficiency (21).

Comparative analysis of TNF- α -CIITA^{-/-} and TNF- α -CIITA^{+/+} mice revealed no distinct differences in the kinetics or penetrance of diabetes (Fig. 2 a). In both groups of mice, diabetes started at 10 wk of age, with males and females showing 100% incidence of diabetes by 15 wk of age. In contrast, NOD-CIITA^{-/-} mice never progressed to diabetes over a 50-wk observation period (21). Heterozygous TNF- α -CIITA^{+/-} mice also developed diabetes, albeit with slightly slower kinetics. Thus, islet-specific expression of TNF- α in NOD-CIITA^{-/-} mice restores their susceptibility to diabetes.

APCs in the Islets of TNF- α -CIITA^{-/-} Mice Are Defective in Priming CD4⁺ T Cells to Islet Antigen. A recent report by Williams et al. (46) demonstrated that DCs in CIITA^{-/-} mice express MHC class II, although this was restricted to CD11c⁺ DCs extracted from the inguinal lymph nodes. It was therefore possible that diabetes development in TNF- α -CIITA^{-/-} mice could occur because MHC class II-expressing DCs activated the few CD4⁺ T cells present in these mice to provide help for CD8⁺ T cells. To address these issues, we performed FACS[®] and functional analysis.

First, we extracted islet-infiltrating APCs from 5-wk-old TNF- α -CIITA^{+/+} or TNF- α -CIITA^{-/-} mice and compared expression levels of MHC class II on DCs, M Φ s, and B cells by FACS[®] analysis. As shown in Fig. 2 b, 45% of islet-infiltrating CD11c⁺CD11b⁻ and CD11c⁺CD11b⁺ DCs in TNF- α -CIITA^{-/-} mice expressed MHC class II molecules, but at very low levels. Very few M Φ s and B cells expressed MHC class II (9 and 14%, respectively). Expression levels of MHC class II were \sim 100-fold less than those on islet-derived APCs from TNF- α -CIITA^{+/+} mice.

We next performed functional experiments to determine if these MHC class II^{lo} APCs from TNF- α -CIITA^{-/-} mice could present islet antigen to CD4⁺ T cells from NOD mice. The islet-infiltrating APCs from 5-wk-old TNF- α -CIITA^{+/+} or TNF- α -CIITA^{-/-} mice were cultured with purified NOD CD4⁺ T cells, and the stimulatory capacity of the respective APCs was assessed.

APCs from the islets of TNF- α -CIITA^{+/+} were highly efficient at stimulating purified CD4⁺ T cells, inducing a 47-fold increase in response of CD4⁺ T cells cultured in the presence of medium alone (Fig. 2 c). In contrast, APCs extracted from the islets of TNF- α -CIITA^{-/-} mice were poor stimulators of CD4⁺ T cells, inducing only a fivefold increase in response above background levels of CD4⁺ T cells alone. This inability of APCs from TNF- α -CIITA^{-/-} mice to stimulate CD4⁺ T cells was not due to a defect in the maturation of DCs in the islets of these mice. Indeed, FACS[®] analysis confirmed that DCs from TNF- α -CIITA^{+/+} and TNF- α -CIITA^{-/-} mice expressed identical

high levels of costimulatory molecule CD86, which is a marker for mature DCs (Fig. 2 b). To further examine the MHC class II-dependent activation of CD4⁺ T cells in TNF- α -CIITA^{-/-} mice, we compared the GADp35 responses of purified CD4⁺ T cells from TNF- α -CIITA^{+/+} versus TNF- α -CIITA^{-/-} mice. As shown in Fig. 2 d, only CD4⁺ T cells from TNF- α -CIITA^{+/+} mice were efficiently primed to the autoantigen.

Together, these findings demonstrate that TNF- α can promote diabetes in NOD-CIITA^{-/-} mice that have severely reduced numbers of islet-reactive CD4⁺ T cells, islet-infiltrating APCs incapable of presenting islet antigen to CD4⁺ T cells, and defective CD4⁺ T cell responses to diabetogenic antigens. Nevertheless, we cannot exclude the possibility that TNF- α synergizes with the minimal help provided by the few CD4 T cells in conjunction with the MHC class II^{lo} DCs to promote CD8⁺ T cell responses.

TNF- α Promotes Diabetes in NOD Mice Independent of CD154 Signals. Interaction of CD154 on CD4⁺ T cells and CD40 on immature DCs initiates maturation of DCs and enhances their ability to activate both CD4⁺ and CD8⁺ T cells (5). Indeed, the requirement for CD4⁺ T cell help in the priming of CD8⁺ T cells by DCs can be bypassed if DCs are activated by cross-linking CD40 (2-4).

We determined whether TNF- α , in a way analogous to a viral infection, could induce signaling through a CD40-CD154-independent pathway and induce the activation of CD8⁺ T cells. First, we generated NOD-CD154-deficient (NOD-CD154^{-/-}) mice by backcrossing our previously described C57BL/6 CD154^{-/-} mice (47) to NOD for 11 generations and then intercrossing heterozygous progeny. NOD-CD154^{-/-} mice failed to develop insulinitis and, as a consequence, were protected from diabetes. These findings are consistent with previous reports using anti-CD154 antibodies (14).

We then crossed TNF- α -NOD mice (N11) with non-tg NOD-CD154^{-/-} mice (N11) and monitored diabetes progression in TNF- α -CD154^{+/+}, TNF- α -CD154^{-/-}, and non-tg NOD-CD154^{-/-} littermates. Both TNF- α -

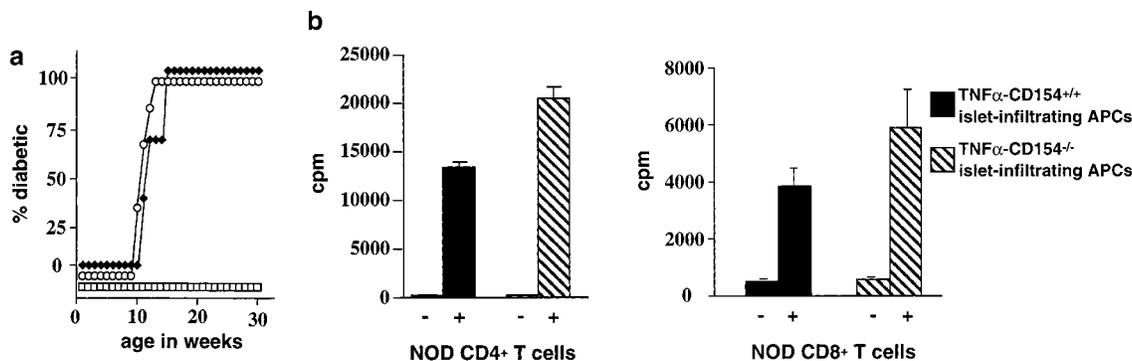


Figure 3. TNF- α promotes diabetes in NOD-CD154^{-/-} mice. (a) Graphical representation of diabetes progression in pooled male and female TNF- α -CD154^{+/+} (O; $n = 25$), TNF- α -CD154^{-/-} (◆; $n = 20$), and non-tg NOD-CD154^{-/-} (□; $n = 15$) mice. (b) Islet-infiltrating APCs from TNF- α -CD154^{-/-} mice can stimulate islet-specific T cells. Irradiated islet-infiltrating cells (5×10^4) from 5-wk-old TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice were incubated with 10^5 purified CD4⁺ or CD8⁺ T cells (left and right panels, respectively) from 6-wk-old NOD mice and proliferative responses for triplicate cultures were determined as before. Background cpm was <500 for CD4⁺ T cells and <300 for CD8⁺ T cells.

CD154^{+/+} and TNF- α -CD154^{-/-} mice developed diabetes with almost identical kinetics (Fig. 3 a); the penetrance of disease was essentially 100% in both sexes by 15 wk of age. In contrast, non-tg NOD-CD154^{-/-} mice never developed disease over the 30-wk observation period.

Histological examination of the islets from 15- and 42-d-old TNF- α -CD154^{+/+} and TNF- α -CD154^{-/-} mice revealed no obvious differences in the kinetics of insulinitis. Nor could we detect any differences in the phenotype of infiltrating cells or their intraislet localization between the two groups of mice (not shown). In contrast, similar analysis of non-tg NOD-CD154^{-/-} mice revealed no evidence of insulinitis (not shown). Thus, TNF- α can bypass the need for CD40-CD154 signals for insulinitis and diabetes in NOD mice.

The Inflammatory Environment Created by TNF- α Can Bypass CD154 Signals to Activate Islet-infiltrating APCs. As TNF- α -CD154^{-/-} mice developed diabetes with kinetics similar to those of TNF- α -CD154^{+/+} mice, we tested whether TNF- α enhanced the presentation of islet antigen to CD4⁺ and/or CD8⁺ T cells by APCs from these mice. Islet-infiltrating APCs were extracted from 5-wk-old TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice and cultured with either purified CD4⁺ or CD8⁺ T cells from 6-wk-old NOD mice. As shown in Fig. 3 b, islet-infiltrating APCs freshly isolated from TNF- α -CD154^{-/-} mice stimulated CD4⁺ and CD8⁺ T cells as efficiently as APCs isolated from the islets of TNF- α -CD154^{+/+} mice. Indeed, proliferative responses for both responder cells were slightly higher after culture with TNF- α -CD154^{-/-} islet-extracted APCs. Thus, TNF- α can promote activation of islet-infiltrating APCs in the absence of CD40-CD154 signals, and these APCs are potent presenters of islet antigen to autoreactive T cells.

CD4⁺ T Cell Responses Are Defective in TNF- α -CD154^{-/-} Mice. We next tested whether CD4⁺ T cells in TNF- α -CD154^{-/-} mice were primed to islet antigen. Purified CD4⁺ T cells were isolated from the pancreatic lymph nodes of 6-wk-old TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice, and their responses to GADp35 were measured in vitro. As a comparison, we included purified CD4⁺ T cells from non-tg NOD-CD154^{-/-} littermates.

Neither CD4⁺ T cells from TNF- α -CD154^{-/-} mice nor non-tg NOD-CD154^{-/-} mice responded to GADp35, which contrasted significantly to the vigorous response by CD4⁺ T cells from TNF- α -CD154^{+/+} mice (Fig. 4 a). To confirm that this lack of response in the latter two groups of mice did not relate to the GAD peptide used, we repeated the experiments with whole GAD protein. Similar to the above findings, only CD4⁺ T cells from TNF- α -CD154^{+/+} mice responded to GAD (not shown).

The generation of GAD-specific responses is dependent on the efficient presentation of GAD by B cells (48). There has been some suggestion in studies with CD154^{-/-} or CD40^{-/-} mice that efficient B cell function is dependent on CD154-CD40 signals, whereas these signals can be bypassed for DC activation after viral infections (49-53). Thus, it was possible that the poor response to GAD in TNF- α -CD154^{-/-} mice simply reflected a defect in B cell presentation. To test this, we assessed whether CD4⁺ T cells from TNF- α -CD154^{-/-} mice could respond to other islet antigens. CD4⁺ T cells were purified from 6-wk-old TNF- α -CD154^{-/-} or age-matched control NOD mice and then incubated with islet-infiltrating APCs from either TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice. CD4⁺ T cells from TNF- α -CD154^{-/-} mice responded poorly to islet antigen irrespective of whether the antigen was presented by TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} islet-

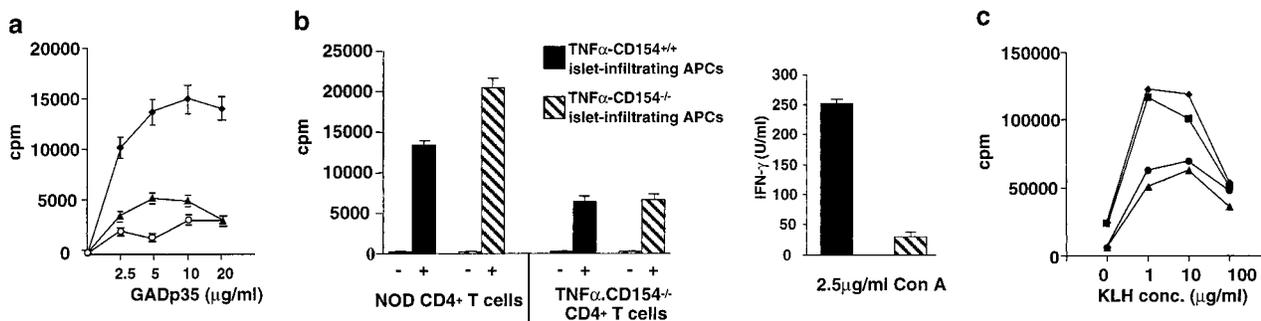


Figure 4. TNF- α -CD154^{-/-} mice cannot mount GAD-specific CD4⁺ T cell responses. (a) CD4⁺ T cells were purified from the pancreatic lymph nodes of 6-wk-old TNF- α -CD154^{+/+} (◆), TNF- α -CD154^{-/-} (○), or non-tg NOD-CD154^{-/-} (▲) mice and cultured with 5×10^5 irradiated splenocytes from 5-wk-old NOD mice and 0–20 μ g of GADp35. After a 4-d culture, proliferative responses of triplicate cultures were determined. Background cpm for CD4⁺ T cells cultured with irradiated splenocytes was <500. (b) CD4⁺ T cells from TNF- α -CD154^{-/-} mice show decreased responses to islet antigen. Left, irradiated islet-infiltrating cells (3×10^4) from 6-wk-old TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice were cultured with 2×10^6 CD4⁺ T cells purified from 6-wk-old TNF- α -CD154^{-/-} mice or age-matched control NOD mice. Proliferative responses of triplicate cultures were measured after 4 d as described previously. Background cpm for CD4⁺ T cells was <520. Right, islet-infiltrating cells from 6-wk-old TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice were depleted of CD8⁺ T cells, and the remaining cells were cultured with 2.5 μ g/ml of Con A for 48 h. Culture supernatants were harvested at the end of the incubation period, and IFN- γ was detected by ELISA. The ELISA assay was performed in triplicate and the data presented as mean \pm SD. (c) CD4⁺ T cells in TNF- α -CD154^{-/-} mice cannot be primed to exogenous protein antigens. Groups of two 6-wk-old TNF- α -CD154^{+/+} (◆), TNF- α -CD154^{-/-} (▲), non-tg NOD-CD154^{+/+} (■), and non-tg NOD-CD154^{-/-} (●) mice were immunized subcutaneously via their footpads with 100 μ g of KLH in alum. 7 d later, 5×10^5 cells isolated from the popliteal lymph nodes were cultured with KLH at the concentrations (conc.) shown. After a 4-d incubation, proliferative responses of duplicate cultures were measured as before. Background cpm values for cells cultured in medium alone have been subtracted from the data.

infiltrating APCs (Fig. 4 b). The response was three- to fourfold less than that seen for NOD CD4⁺ T cells stimulated with the same islet-derived APCs. Further evidence that CD4⁺ T cells in TNF- α -CD154^{-/-} mice were inefficiently primed to islet antigen was suggested by the fact that CD4⁺ T cells infiltrating the islets of TNF- α -CD154^{-/-} mice failed to differentiate into effector Th1 or Th2 cells (Fig. 4 c). Indeed, \sim 250 U/ml of IFN- γ was detectable in culture supernatants after stimulation of CD8⁺ cell-depleted, islet-infiltrating cells from TNF- α -CD154^{+/+} mice with Con A for 48 h. In contrast, $<$ 50 U/ml of IFN- γ was detected after Con A stimulation of CD8⁺ T cell-depleted, islet-infiltrating cells from TNF- α -CD154^{-/-} mice. This reduced ability of TNF- α -CD154^{-/-} islet-derived CD4⁺ T cells to produce IFN- γ was not due to a defect in their capacity to respond to Con A, as both CD4⁺ T cells from TNF- α -CD154^{+/+} and TNF- α -CD154^{-/-} mice proliferated equally well (not shown). Nor was it related to skewing of the CD4⁺ T cells to a Th2 phenotype, as we could detect no IL-4 production by CD4⁺ T cells from either TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice (not shown).

Finally, to establish whether the defect in CD4⁺ T cell responses in TNF- α -CD154^{-/-} mice was specific for islet-derived antigens, we measured recall responses of CD4⁺ T cells from TNF- α -CD154^{-/-} mice to the exogenous protein antigen KLH after primary immunization. 7 d after immunization with KLH, popliteal lymph nodes were removed from TNF- α -CD154^{+/+} and TNF- α -CD154^{-/-} mice, and anti-KLH responses were monitored. As controls, non-tg NOD-CD154^{+/+} and non-tg NOD-CD154^{-/-} mice were included in the assay. As shown in Fig. 4 c, CD4⁺ T cells from both TNF- α -CD154^{-/-} and non-tg NOD-CD154^{-/-} had diminished capacity to respond to KLH in comparison to the response seen for CD4⁺ T cells isolated from TNF- α -CD154^{+/+} and non-tg NOD-CD154^{+/+} mice. Thus, CD4⁺ T cell responses are universally defective in NOD-CD154^{-/-} mice and, islet-specific expression of TNF- α cannot overcome this defect.

CD8⁺ T Cell Responses Are Intact in TNF- α -CD154^{-/-} Mice. As priming of CD4⁺ T cells to antigens was defective in TNF- α -CD154^{-/-} mice, it was important to establish whether CD8⁺ T cell responses to islet antigen were also affected. We had already established that the APCs infiltrating the islets of TNF- α -CD154^{-/-} mice could present antigen to NOD CD8⁺ T cells as efficiently as APCs derived from the islets of TNF- α -CD154^{+/+} mice (Fig. 3 b). To determine whether this led to priming of CD8 T cell responses in vivo, we measured the ability of CD8⁺ T cells from 6-wk-old TNF- α -CD154^{-/-} mice to respond to islet antigen presented by islet-infiltrating APCs from 6-wk-old TNF- α -CD154^{-/-} or TNF- α -CD154^{+/+} mice (Fig. 5 a). As a control, we included CD8⁺ T cells from age-matched NOD mice as responders in the assay. There was no difference in the response of CD8⁺ T cells from TNF- α -CD154^{-/-} mice stimulated with islet-infiltrating APCs from TNF- α -CD154^{-/-} or TNF- α -CD154^{+/+} mice (Fig. 5 a). Importantly, the response was as potent as

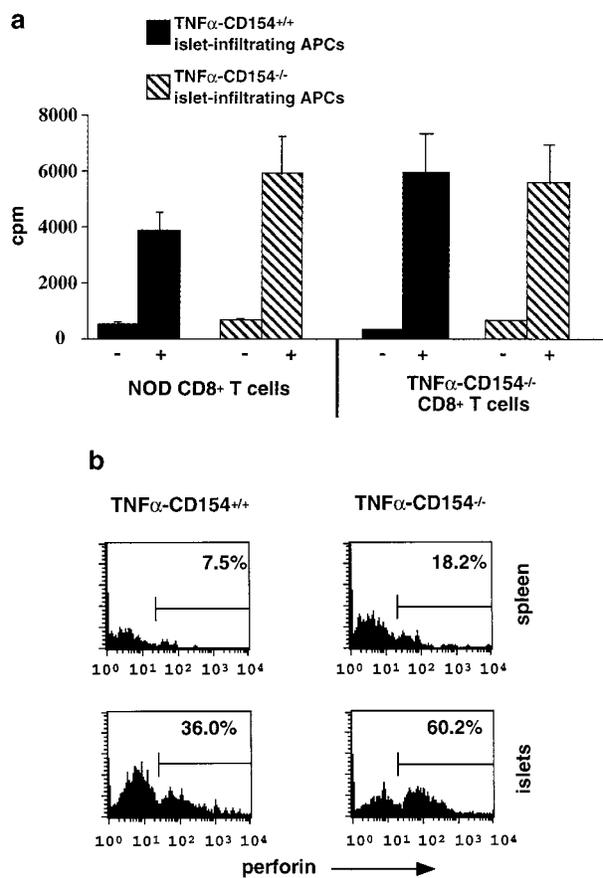


Figure 5. CD8⁺ T cell responses are not impaired in TNF- α -CD154^{-/-} mice. (a) Islet-infiltrating cells (3×10^4) from 6-wk-old TNF- α -CD154^{+/+} or TNF- α -CD154^{-/-} mice were cultured with 2×10^5 purified CD8⁺ T cells from 6-wk-old TNF- α -CD154^{-/-} mice or age-matched control NOD mice. Proliferative responses of triplicate cultures after a 4-d culture were determined as before. Background cpm for CD8⁺ T cells was $<$ 400. (b) Effector CD8⁺ T cells are present in the islets of TNF- α -CD154^{-/-} mice. Islet-infiltrating cells from 5-wk-old TNF- α -CD154^{-/-} or control 8-wk-old TNF- α -CD154^{+/+} mice were incubated with anti-CD45, -CD8, and -CD3 antibodies. After fixation and permeabilization, intracellular levels of perforin in the CD45⁺ CD3⁺CD8⁺ population were determined by FACS[®] using an anti-perforin antibody.

the response of NOD CD8⁺ T cells stimulated with the same respective APCs.

Next, we examined the effector status of the CD8⁺ T cells in the islets of 5-wk-old TNF- α -CD154^{-/-} mice by analyzing the percentage of CD8⁺ T cells that stained positive for perforin compared with the number of perforin-positive CD8⁺ T cells in the spleen. As a control, we also assayed for perforin-positive CD8⁺ T cells in the islets and spleens of 8-wk-old TNF- α -CD154^{+/+} mice. FACS[®] analysis demonstrated that \sim 60% of the CD8⁺ T cells in the islets of TNF- α -CD154^{-/-} mice stained positive for perforin, a strikingly high number, as only 18% of the CD8⁺ T cells in the spleens of these mice were perforin positive (Fig. 5 b). These respective values were even higher than the percentage of CD8⁺ perforin-positive T cells detected in TNF- α -CD154^{+/+} islets and spleens (36

and 7.5%, respectively). Whether this difference in perforin levels between the two groups of mice relates to differences in the age of the mice is unknown. In any event, these data clearly demonstrate that CD8⁺ T cell responses in TNF- α -CD154^{-/-} mice are unaffected by the absence of CD154 and that effector CD8⁺ T cells can be generated in the presence of functionally defective CD4⁺ T cells.

Discussion

In the periphery, activation of self-reactive CD8⁺ T cells usually involves cross-presentation of exogenous self-antigen by APCs bearing MHC self-peptides. Such cross-presentation normally induces expansion followed by deletion of self-reactive CD8⁺ T cells (25, 54). Breakdown in tolerization of self-reactive CD8⁺ T cells can lead to autoimmunity. It is unclear what governs whether APCs promote expansion and survival of activated self-reactive CD8⁺ T cells or their expansion followed by deletion.

Inflammatory cytokines have been implicated in the breakdown of tolerance to self-antigens, yet the mechanisms by which these mediators lead to inappropriate activation of T cells remains elusive (26). Recently, we demonstrated that transgenic expression of TNF- α in the islets of neonatal NOD mice promoted diabetes progression by enhancing DC presentation of islet antigen to T cells *in situ* (29). In this study, we demonstrated the potency of DC presentation of islet antigen to CD4⁺ and CD8⁺ T cells for the progression to diabetes in our TNF- α model. We showed that adoptive transfer of splenocytes from BDC2.5 CD4⁺ TCR-transgenic mice or the CD8⁺ T cell clone, TGNFC8, into TNF- α -*scid/scid* mice (which have extensive DC infiltration in their islets) caused diabetes in all recipients within 72 h after transfer, about two to three times more rapidly than in non-tg *scid/scid* controls. Although this transfer study showed that the inflammatory environment created by TNF- α enhanced autoaggression of previously activated islet-specific CD4⁺ and CD8⁺ T cells, it did not reveal whether diabetes development in TNF- α -NOD mice was dependent on CD4⁺ and/or CD8⁺ T cells. We first addressed this issue by examining the requirement for CD8⁺ T cells. Using the well characterized NOD- $\beta_2\mu^{-/-}$ mice, we demonstrated that TNF- α did not induce diabetes in the absence of CD8⁺ T cells, suggesting that CD8⁺ T cells were vital for diabetes development in TNF- α -NOD mice. However, unlike NOD- $\beta_2\mu^{-/-}$ mice, protection against diabetes in TNF- α - $\beta_2\mu^{-/-}$ mice did not result from a block in insulinitis (17). In fact, recruitment to and activation of APCs in the islets of TNF- α - $\beta_2\mu^{-/-}$ mice occurred in the absence of CD8⁺ T cells. This was evident by comparative FACS[®] analysis of APCs infiltrating the islets of TNF- α - $\beta_2\mu^{+/+}$ and TNF- α - $\beta_2\mu^{-/-}$ mice, where the number of cells carrying high levels of MHC class II and CD86 was equivalent between the two groups of mice (not shown). Furthermore, functional assays demonstrated no difference in the potency of islet-infiltrating APCs from TNF- α - $\beta_2\mu^{+/+}$ and TNF- α - $\beta_2\mu^{-/-}$ mice to stimulate CD4⁺ T cells. This latter finding

suggested that priming of autoreactive CD4⁺ T cells in TNF- α - $\beta_2\mu^{-/-}$ mice should be unaffected by the absence of CD8⁺ T cells, and indeed we showed that CD4⁺ T cell responses to a major diabetogenic antigen, GAD (35), were even more potent in TNF- α - $\beta_2\mu^{-/-}$ mice than in TNF- α - $\beta_2^{+/+}$ mice.

Although the above finding proved that priming of CD4⁺ T cells to islet antigen is not sufficient to induce disease in TNF- α -NOD mice, it did not eliminate the possibility that CD4⁺ T cells were essential for efficient activation of CD8⁺ T cells. CD4⁺ T cells might play a dual role in progression to diabetes in TNF- α -NOD mice, first, by enhancing the capacity of APCs to prime CD8⁺ T cells and second, by promoting CD8⁺ T cell function via the secretion of cytokines like IFN- γ or IL-2 (1). Alternatively, it was possible that the inflammatory environment created by TNF- α could bypass the need for CD4⁺ T cells by promoting APC activation via a CD154-CD40-independent pathway.

To address the role of CD4⁺ T cells in diabetes progression in TNF- α -NOD mice, we monitored diabetes development in mice deficient for either MHC class II or CD154 molecules. The findings presented from these studies indicate that CD4⁺ T cell help can indeed be bypassed in TNF- α -NOD mice. This was first suggested by the observation that TNF- α -CIITA^{-/-} mice progressed to diabetes with kinetics and penetrance similar to TNF- α -CIITA^{+/+} mice, despite an eightfold reduction in the number of peripheral CD4⁺ T cells due to an absence of MHC class II. This finding contrasted remarkably with our recent report that NOD-CIITA^{-/-} mice were protected from developing diabetes due to a paucity of CD4⁺ T cells (21). Although DCs in the islets of TNF- α -CIITA^{-/-} mice did express MHC class II, the levels of expression were so low that these APCs were unable to present antigen to CD4⁺ T cells. Thus, it is unlikely that CD4⁺ T cells are efficiently activated in TNF- α -CIITA^{-/-} mice and are therefore incapable of providing help to CD8⁺ T cells.

Nor does it seem likely that CD4⁺ T cell help is needed for the activation of APCs in TNF- α -NOD mice through the CD154-CD40 pathway. First, TNF- α -CD154^{-/-} mice developed diabetes with similar efficacy to TNF- α -CD154^{+/+} mice. Subsequent support for this hypothesis was provided by comparative FACS[®] analysis of the maturation status of freshly isolated islet-infiltrating APCs from TNF- α -CD154^{-/-} and TNF- α -CD154^{+/+} mice, with similarly high levels of MHC and costimulatory molecules being detectable for both groups of mice. However, it was the demonstration that APCs infiltrating the islets of TNF- α -CD154^{+/+} and TNF- α -CD154^{-/-} mice were similarly efficient at presenting islet antigen to CD4⁺ and CD8⁺ T cells that provided the strongest evidence that the inflammatory environment created by TNF- α can promote APC function in the absence of CD154-CD40 signals. In view of this observation, it is interesting that responses of CD4⁺ T cells from TNF- α -CD154^{-/-} mice to both islet-derived and exogenous protein antigens are as impaired as CD4⁺ T cell responses in non-tg NOD-CD154^{-/-} mice. Indeed,

CD4⁺ T cells present in the islets of TNF- α -CD154^{-/-} mice failed to differentiate into either Th2 or Th1 effector cells, which contrasted with the finding that Th1 cells predominated in the islets of age-matched TNF- α -CD154^{+/+} mice. Taken together, these findings suggest that CD4⁺ T cell activation is dependent on CD154 signals, whereas an inflammatory environment can induce APC maturation independent of the CD154-CD40 pathway.

In contrast to the functional impairment of CD4⁺ T cells in TNF- α -CD154^{-/-} mice, CD8⁺ T cell activation seems to be unaffected by the absence of CD154 signals, as CD8⁺ T cells from TNF- α -CD154^{+/+} and TNF- α -CD154^{-/-} mice responded equally well to islet antigen *in vitro*. Furthermore, >60% of CD8⁺ T cells present in the islets of TNF- α -CD154^{-/-} mice stained positive for perforin, a marker for CD8⁺ effector T cell differentiation. These findings suggest that the inflammatory environment created by TNF- α can promote cross-presentation of exogenous islet antigen by islet-infiltrating APCs to CD8⁺ T cells in the absence of CD4⁺ T cell help, particularly CD154-CD40 signals.

The CD154-CD40-independent molecular pathway used for the activation of APCs in TNF- α -CD154^{-/-} mice is unknown. However, we and our colleagues have shown previously that CD154^{-/-} mice on the C57BL/6 background can mount potent antiviral CD8⁺ T cell responses (4, 50, 51, 53). In these instances, signaling between other members of the TNF and TNFR superfamilies, receptor activator of nuclear factor κ B (RANK) on DCs and TNF-related activation induced cytokine (TRANCE) on T cells, can substitute for the absence of CD154 for efficient activation of CD8⁺ T cells in these mice (49). Whether the environment created by TNF- α indirectly promotes signaling through the RANK-TRANCE pathway or if TNF- α itself directly promotes APC maturation via the TNFRs in TNF- α -CD154^{-/-} mice remains to be established.

How can we correlate the findings presented here with previous reports that islet-specific expression, or systemic administration of TNF- α in adult NOD mice protects against diabetes (55, 56)? TNF- α -mediated protection against diabetes in these latter models is thought to be related to a downregulation of CD4⁺ T cell responses to islet antigens (57). However, this study shows that CD4⁺ T cell responses to islet antigens are not necessary for diabetes in TNF- α -NOD mice. Thus, the differences in pathological outcomes between neonatal and adult expression or systemic administration of TNF- α may relate to specific inhibition of islet-specific CD8⁺ T cell responses in the latter model. How TNF- α can regulate CD8⁺ T cell responses differently depending on the age of NOD mice is open to speculation. It is possible that expression of TNF- α in adult NOD mice enhances presentation of a unique set of peptides that specifically tolerize CD8⁺ T cells. Another possibility is that the signal pathways triggered by the TNF- α -mediated inflammatory environment in neonate versus adult NOD mice are unique. For example, neonatal expression of TNF- α may inhibit signaling through CD30 on

islet-specific CD8⁺ T cells, leading to their expansion and activation (58). In adults, expression of TNF- α may indirectly promote signaling through CD30, thereby preventing CD8⁺ T cell responses to islet antigens (58). Future studies analyzing the ability of TNF- α to manipulate the signaling pathways involved in regulation of CD8⁺ T cell responses should clarify this issue.

Finally, how can we reconcile the findings presented in this study with diabetes development in unmanipulated NOD mice, and presumably humans? We hypothesize that T cell priming to islet antigen in NOD mice also requires cross-presentation of islet antigen. We suggest that apoptosis of β cells, a normal event that occurs in the remodeling of the pancreas, releases islet antigens (59). These antigens are acquired by scavenger APCs, which subsequently traffic to the pancreatic lymph nodes, where they interact with autoreactive CD4⁺ T cells (60). This interaction induces upregulation of CD154 on CD4⁺ T cells and induces APC activation by signaling through CD40, leading to the upregulation of MHC-peptide complexes and costimulatory molecules on APCs. These mature APCs can then efficiently cross-prime CD8⁺ T cells to exogenous islet antigen. Primed T cells then migrate to the islet and promote activation of newly infiltrating APCs, which in turn promote secondary stimulation of the primed T cells. During this secondary stimulation, CD154-CD40 signals are no longer critical, as production of TNF- α *in situ* by activated DCs/M Φ s (61) promotes CD154-CD40-independent activation of CD8⁺ T cells. Thus, in NOD mice, CD154-CD40 signals will be critical only for the initiation phase of diabetes (14). In TNF- α -NOD mice, CD154-CD40 signals are not necessary for either the initiation or effector phases of diabetes, as the secretion of TNF- α *in situ* by transgenic β cells recruits and activates APCs to present antigens from apoptotic β cells to CD8⁺ T cells in neonatal islets.

In conclusion, our data suggests that neonatal expression of TNF- α in NOD islets promotes cross-presentation of islet antigen to CD8⁺ T cells using a unique CD154-CD40-independent pathway. As tolerance of CD4⁺ T cells to self-antigen normally controls activation of self-reactive CD8⁺ T cells, our data also indicate a mechanism by which inflammatory stimuli may precipitate autoimmunity even in the presence of CD4⁺ T cell tolerance.

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