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Pancreatic Infiltration But Not Diabetes Occurs in the Relative Absence of MHC Class II-Restricted CD4 T Cells: Studies Using NOD/CIITA-Deficient Mice

Conchi Mora,* F. Susan Wong,* Cheong-Hee Chang,‡ and Richard A. Flavell²*†

The NOD (nonobese diabetic) mouse is a good animal model for human IDDM. MHC class II-restricted CD4 T cells are necessary for the onset of diabetes in NOD mice. Here, we demonstrate that NOD mice lacking the CIITA (class II transactivator) molecule, and hence deficient in MHC class II expression and peripheral CD4 T cells, show significant pancreatic infiltration but do not develop diabetes. CD4 T cell deficiency, then, does not prevent initial pancreatic infiltration, but does stop progression to insulitis. Adoptive transfer studies show that the paucity of CD4 T cells in NOD-CIITA knockout mice is responsible for the absence of diabetes, since the CD8 T cell and B cell compartments are functional. An autoaggressive CD8+ T cell clone can, however, transfer diabetes in CIITA knockout recipient mice without CD4 T cell help, albeit with some delay compared with that in CIITA-sufficient recipients. This highlights the fact that a high number of in vitro activated autoaggressive CD8 T cells can over-ride the requirement for CD4 T cell help for the onset of diabetes. The Journal of Immunology, 1999, 162: 4576–4588.

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which selective destruction of pancreatic islet β-cells leads to a deficiency in insulin secretion. The NOD (nonobese diabetic) mouse is one of the best models to study the mechanisms involved in IDDM due to its similarity to the human disease (1). Type I diabetes in both humans and NOD mice has a strong genetic component. Specific MHC class II alleles are associated with the disease (HLA-DQA1*0301 and DQB1*0302 in humans and I-Aβ7 in NOD mice), and other susceptibility loci (IDDM in humans and idd in NOD mice) have been mapped (1–3). In NOD mice similar susceptibility loci have been associated with different features of the progression of diabetes (peri-insulitis, insulitis, and diabetes) (2, 3).

In diabetic NOD mice, pancreatic islets are infiltrated by CD4+ T cells and at lower levels by CD8+ T cells, B lymphocytes, monocytes, macrophages, dendritic cells, and NK cells (1, 4). Studies with athymic NOD nude mice (5, 6) have clearly documented the importance of T lymphocytes in the onset of diabetes in NOD mice; however, the precise role of T cell subsets remains unclear. Insulitis and β-cell destruction are clearly mediated by these invading T cells (7). The relative importance of CD4+ and CD8+ T cells in the pathogenesis of diabetes has been widely discussed (6–15). Some authors have proposed that CD8+ T cells are only required for effective priming and expansion of autoreactive CD4+ T cells (12), while others have shown that cloned autoaggressive CD8+ T cells are able to cause diabetes by themselves (13). It is critical, then, to determine which population triggers the diabetogenic process to design effective therapeutic approaches.

CIITA (16) is a non-DNA-binding transcription factor that is required for the constitutive and IFN-γ-induced expression of conventional MHC class II molecules. CIITA is also involved in the expression of molecules related to Ag presentation, such as the invariant chain and H2-M molecules (18, 24). CIITA KO mice have markedly decreased numbers of peripheral CD4+ T cells due to a defect in thymic positive selection because of the relative absence of MHC class II molecules (25–27). The repertoire of CD8+ T cells remains intact (18).

To elucidate the role of MHC class II-restricted CD4+ T cells in the pathogenesis of IDDM we have studied the development of diabetes in CIITA-deficient mice on the NOD background. CIITA KO/NOD mice, then, were superficially similar to MHC class II KO/NOD mice. However, in NOD/MHC class II KO mice both diabetes and pancreatic infiltration were abrogated (28), and the same phenotype was exhibited by the heterozygous littermates (28). Since the MHC class II genes are located on chromosome 17, closely linked to MHC class I, the MHC class I and other MHC alleles linked to the disrupted β-chain in the MHC class II gene (originally gene targeted in 129/Sv embryonic stem cells and backcrossed onto the C57BL/6 background), also derive from the 129/Sv-C57BL/6 strains whose haplotype for MHC class I is H-2Kb and H-2Dβ. Due to the tight linkage between both MHC class I and class II, backcrossing onto NOD does not substitute the MHC class I diabetes-resistant alleles (i.e., H-2Kb) for the NOD-susceptible...
alleles (i.e., H-2Kd). Thus, effects observed in the MHC class II KO mice cannot readily be interpreted (28). The CIITA gene, unlike MHC class I and II genes, is not located on chromosome 17, so NOD class I molecules (H-2Kd and H-2Dd) are expressed normally instead of being replaced by MHC class I genes of the MHC class II KO mice originally derived from the 129/Sv strain. NOD/CIITA KO mice, then, provide a cleaner model to study the role of CD4 T cells in the pathogenesis of IDDM. In contrast to MHC class II KO mice, NOD/CIITA KO mice exhibit pancreatic infiltration, although seldom insulinitis, and they do not develop diabetes. A normal CD4 T cell repertoire is required for severe insulinitis and diabetes.

Materials and Methods

Generation and genotyping of NOD/CIITA-deficient mice

The CIITA KO mice were previously generated in our laboratory by gene-targeted disruption, and homozygosity for the mutation was obtained by intercrossing on the C57BL/6 background (18). The CIITA KO mice were backcrossed five times onto the NOD background, selecting from the third generation those mice homozygous for the highest number of NOD Idd alleles. In the fourth generation one female was already homozygous for all 15 Idd NOD alleles. This female was backcrossed once again onto the NOD background, and the progeny was intercrossed to provide experimental mice. Screening for the CIITA mutation was performed using Southern analysis as previously described (18). Unless otherwise stated all NOD/CIITA mice used correspond to the fifth backcross. We have further backcrossed onto the NOD background up to 10 generations (N11) for some of the experiments performed.

Genotyping for the Idd loci was performed by using PCR primers specific for the different loci that are polymorphic for the 129/Sv, C57BL/6, and NOD strains. The primers used were: Idd1 (H-2g); for Idd1 screening two sets of PCR primers were used, a and b: a, D17 Mit34; b, 5'-TGT CTT TTC CAG ACC AGA ACA-3', 3'-TAC ACC TCG TAG GTG CGG ACT CTT-5'; the PCR product generated by b primers (282 bp) was digested using MspI; the NOD allele is resistant to cleavage (29); Idd2, D9 Mit25; Idd3, D3Nds36; Idd4, D11 Mit12, D11 Mit320; Idd5, D1 Mit24, D1 Mit26; Idd6, D6 Mit52; Idd7, D7 Mit20; Idd8, D14 Mit11; Idd9, D4 Mit59; Idd10, D3 Mit103; Idd11, D4 Mit202; Idd12, D14Nds3; Idd13, D2 Mit395; Idd14, D13 Mit61; and Idd15, D5 Mit48. The PCR products were run in 4% agarose gels.

Assessment of diabetes and immunohistochemical analysis

Female NOD/CIITA N6 (fifth backcross) KO (−/−) or their CIITA-sufficient (+/+ or +/−) littermates were monitored weekly for the development of glycosuria with Diastix (Ames, Elkhart, IN), starting at 7 wk of age. Diabetes was confirmed by measuring glucose levels in the blood. Glycerina was measured using One Touch test strips (Lifescan, Johnson & Johnson, Palmitas, CA), and values over 250 mg/dl (>13.9 mM) were considered positive for diabetes. Pancreata were either fixed in 10% buff- ered formaldehyde or processed for immunohistochemistry by fixation in paraformaldehyde-lysine-periodate buffer. In the former case the tissue was embedded in paraffin, sectioned, and stained with hematoxylin-eosin to assess the presence of mononuclear infiltrate in the pancreatic islets (insulinitis). For immunohistochemistry, after 16 h in paraformaldehyde-lysine-periodate buffer at 4°C the tissue was incubated in increasing concentrations of sucrose dissolved in phosphate buffer (10, 20, and 30%), embedded in Tissue-Tek OCT (Miles, Elkhart, IN), and frozen in 2-methylbutane (isopentane). Five- to seven-micron-thick frozen sections were taken and stained for insulin and glucagon (alkaline-phosphatase kit, BioGenex, San Ramon, CA); for mouse CD4, CD8, B220, and Gr-1 (PharMingen, San Diego, CA); and for F4/80 (Serotec, Raleigh, NC). The sections were incubated in the presence of alkaline-phosphatase-conjugated streptavidin and subsequently in the presence of the substrate HistoMark Red (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for the development of color.

To assess the degree of pancreatic infiltration, sections were taken, and islets were counted. To estimate the percentage of peri-insulitis or infiltrated islets, at least 10 islets were counted in each field, and at least two different fields were observed. In each estimation no fewer than 3 mice were included. For quantitative analysis of the infiltrates similar criteria were followed.

Isolation of PBL

Mice were bled through the retro-orbital vein using heparinized tubes. Blood mononuclear cells were isolated by centrifuging total blood in a Ficoll gradient (Organon Teknika, Durham, NC) following the manufacturer’s instructions.

Adaptive transfer experiments

Recipient mice (older than 4 wk) were irradiated using 725 rad from a cesium source 24–48 h before adoptive transfer. The source of the transferred cells varied in each experiment. If they were originally isolated from the spleen, total splenocytes were isolated in sterile conditions by physical disruption of the spleen using frosted glass slides, then RBC were lysed by hypotonic shock with distilled water. For adoptive transfer in which the CD4 or CD8 T cell subsets were purified from splenocytes, IsoCell (CD4 or CD8) isolation columns were used (Pierce, Rockford, IL), following the manufacturer’s instructions. The purity of the fraction obtained in the desired T cell population was >90%. TGNF87 cloned CD8 T cells were activated in vitro as previously described (13) and adoptively transferred subsequently. In all adoptive transfer experiments cells were transfected in 200 µl of physiological saline (0.9% NaCl) by i.v. injection.

Flow cytometric analysis of spleen cells

Splenocytes were isolated as described above and stained following the standard procedure in PBS supplemented with 1% FBS (for directly conjugated Abs) or 2% BSA in PBS (for biotinylated Abs). The Abs used for flow cytometric analysis (CD4, CD8, CD3, B220, CD23, CD40, CD62L, CD69, B7-1, and B7-2) were directly conjugated to one of the following fluorochromes: FITC, phycoerythrin, or Cyochrome (PharMingen). Only the Abs used for MHC class II detection (the clones 10.2.16 (American Type Culture Collection, Manassas, VA) and AMS-32.1 (PharMingen)) were biotinylated. Incubation with directly conjugated Abs or biotinylated Abs was conducted at 4°C for 30 min. Biotinylated Abs were detected using streptavidin-FITC, -phycoerythrin, or -Cy-Chrome. After the staining procedure the cells were analyzed on FACS IV (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA). For FACS cell sorting, Ab solutions were filtered (0.2 µm pore size), and the staining process was conducted under sterile conditions.

Depletion of CD4 T cells with magnetic beads

Total spleen cells were isolated as described above and incubated at 4°C for 30 min in the presence of rat anti-mouse CD4 Ab (GK1.5, American Type Culture Collection); they were then incubated in the presence of magnetic beads conjugated to goat anti-mouse IgG (BioMagsé, PerSeptive Biosystems, Framingham, MA) for two rounds to deplete CD4-positive cells. As a negative control of depletion half the initial spleen cells were incubated only in the presence of the magnetic beads (nondepleted group). The degree of depletion was ascertained by staining for the presence of CD4 before and after depletion. The depletion of CD4 T cells achieved following this protocol was remarkably satisfactory; from 96–99% of CD4 cells were eliminated (see Fig. 3A, a and b).

In vitro activation of CD8 T cells

On day 0 CD8 T cells from 3- or 18-wk-old NOD/CIITA KO or wt males were FACs sorted using anti-mouse Cy-Chrome-conjugated CD8 Ab. Sorted cells were cultured in Clicks (Bruff’s) medium supplemented with 10% FBS and seed in U-bottom 96-well plates at 105 cells/well in the presence or the absence of 2 × 105 irradiated (3000 rad from a cesium source) total spleen cells from nondiabetic 4-wk-old NOD females as APCs. Con A was used for in vitro stimulation at 2.5 µg/ml on day 0. On day 1 the activation state of the cells was assessed by staining for CD69 and CD62L expression on the cell surface. On day 4 cells were restimulated with Con A (2.5 µg/ml; Boehringer Mannheim, Indianapolis, IN), and supernatants were collected on day 5 to assess cytokine production (IFN-γ and IL-4) by ELISA (the unconjugated capture and biotinylated detection Abs were purchased from PharMingen). On day 3 wells were pulsed with 1 µCi of [3H]thymidine (New England Nuclear, Boston, MA) and were harvested 16–24 h later.

In vitro activation of B cells

On day 0 B220+ cells were sorted and then cultured in Clicks (Bruff’s) medium supplemented with 5% FBS at 107 cells/well in U-bottom 96-well plates. LPS alone (10 µg/ml; Salmonella typhimurium; Sigma, St. Louis, MO) or LPS and 20 U/ml of recombinant mouse IL-4 (DNAX, Palo Alto, CA) were added to activate B cells. On day 1 or 2 cells were harvested to

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assess the expression of the activation markers on the cell surface by FACS analysis (CD23, B7.1, B7.2, and CD40 (day 1); MHC class II (day 2)). On day 2 cells were pulsed with 1 μCi of [3H]thymidine and were harvested after 16–24 h. For the detection of Ig production supernatants were collected on day 7 and assayed for the presence of IgM, IgG1, IgG2a, and IgG2b by ELISA (the unconjugated capture and biotinylated detection Abs were purified). The percentage of MHC class II-positive cells in spleen (0.8% in the KO vs 31% in CIITA-sufficient mouse) may correlate with observations in the nonautoimmune-prone C57/BL6 background (18). The low percentage of MHC class II-positive cells in spleen (0.8% in the KO vs 31% in CIITA-sufficient mouse) may correspond to residual CIITA-independent MHC class II expression (24) and some background staining.

Natural history of the development of diabetes in the NOD/CIITA KO mice

NOD/CIITA-deficient (−/−), heterozygous (+/−), and wt (+/+ ) mice were monitored up to 35 wk of age. While in the CIITA-sufficient group (+/+ or +/−) the incidence of diabetes was approximately 40% by 35 wk of age, none of the CIITA-deficient (−/−) mice had developed IDDM up to that age (Fig. 2A) or even when observed at later times (data not shown). To assess the possibility that the absence of diabetes in the CIITA KO may be due to an impairment in the infiltration of the islets, we examined histological sections taken in NOD/CIITA KO females and prediabetic or diabetic CIITA-sufficient littersmates (Fig. 2B). Surprisingly, the NOD/CIITA-deficient mice showed pancreatic infiltration at 15 wk of age. Infiltrates were located predominantly in the parenchymal tissue and around the vessels (perivascular), and surrounding the islets (peri-insulitis), seldom infiltrating the islets (insulitis). Two percent of the islets exhibited some degree of infiltration, mostly peri-insulitis. In wt NOD mice the infiltrates were localized mainly around (peri-insulitis) and inside (insulitis) the islets (75% in total) in both prediabetic and diabetic mice (Fig. 2B). The composition of the infiltrate, as shown by immunohistochemical analysis of frozen sections, was qualitatively similar, with the exception of CD4 T cells; CD3-positive T cells, CD8 T cells, B cells, and macrophages were also present in the pancreatic infiltrate in CIITA KO mice (Fig. 2C). Thus, the absence of diabetes in CIITA-deficient mice is not due to an intrinsic defect in homing and infiltration of the pancreas, although they show much lower levels of insulitis and a delay in the initiation of infiltration (data not shown).

Splenocytes from NOD/CIITA-deficient mice cannot transfer diabetes to NOD/SCID recipients

Adoptive transfer experiments were performed to determine whether the main defect was the near absence of MHC class II-restricted CD4+ T cells in the periphery and/or the impaired expression of MHC class II by peripheral APCs. Total spleen cells from 15-wk-old NOD/CIITA-deficient females or prediabetic CIITA wild-type (wt) littersmates were isolated and transferred (15 × 10⁶ cells/recipient) into NOD/SCID recipient females, which have MHC class II-positive APC, with the exception of B cells. Diabetes was monitored on a weekly basis up to 56 days posttransfer, and we observed that while all mice that received splenocytes from prediabetic wt female mice developed diabetes after the transfer, none of the recipients of the NOD CIITA KO spleen cells did (Fig. 2A). This experiment confirmed the inability of total spleen cells from NOD/CIITA-deficient mice to cause disease. In contrast, adoptive transfer of prediabetic total NOD spleen cells into NOD/SCID was able to cause diabetes within approximately 60 days after the transfer (32). Interestingly, pancreata from NOD/SCID females, which received NOD/CIITA KO total spleen cells, did show a small degree of infiltration of the pancreas (data not shown). This later observation added to the fact that NOD/CIITA KO mice exhibit pancreatic infiltration strongly suggest that the low percentage of CD4 cells remaining in the periphery of NOD/CIITA KO mice is able to promote the incipient infiltration. To address the question of whether the low levels of CD4 T cells present in the periphery of NOD/CIITA KO mice are responsible for the pancreatic infiltration observed in these mice or in NOD/SCID mice, recipients of total spleen cells from NOD/CIITA KO...
FIGURE 2. NOD/CIITA-deficient mice develop pancreatic infiltrates but not diabetes. A, NOD/CIITA wt (+/+; empty squares; n = 16); heterozygous for the mutation (+/-, empty circles; n = 15); combined wt and heterozygous (+/+ and +/-, crosses; n = 31), or CIITA KO (filled squares, n = 12) females were monitored for the development of diabetes on a weekly basis starting at 7 wk of age until 35 wk of age. Once glycosuria was found, diabetes was confirmed by analysis of the levels of glucose in the blood. Histological analysis confirmed the autoimmune condition. B, Pancreata from a 15-wk-old prediabetic NOD/CIITA N6 +/- female (a), a NOD/CIITA -/- littermate female (b), a 16-wk-old diabetic NOD/CIITA N6 heterozygote (c), and a non-diabetic NOD/CIITA N6 +/- littermate (d) were formalin fixed and paraffin embedded. Sections were stained with hematoxylin-eosin. C, Pancreata extracted from a 16-wk-old diabetic NOD/CIITA N6 heterozygous female (+/-; a, b, c, d, i, and k) or from the NOD/CIITA N6 KO littermate female (-/-; e, f, g, h, j, and l) were fixed in paraformaldehyde, OCT embedded, and frozen. Sections were stained for insulin (a and e), glucagon (b and f), CD3 (c and g), and CD8 (d and h) B220 as markers for B cells (i and j), CD4 (k), and F4/80 for detection of macrophages (see small arrows pointing to positive cells; l). The sections shown are representative of at least three independent mice for each group (NOD/CIITA sufficient or NOD/CIITA deficient).
mice, we performed adoptive transfer experiments in which NOD/SCID were recipients of total spleen cells or CD4-depleted spleen cells from NOD/CIITA KO donors (see Fig. 3D, a and b) to monitor CD4 depletion. As a positive control of the adoptive transfer, NOD/SCID mice were transferred with total spleen cells from NOD/CIITA-sufficient mice (positive control recipients). As soon as infiltration was detected in the positive control recipients (70 days after adoptive transfer), the other two experimental groups (nondepleted or CD4-depleted NOD/CIITA KO spleen cells) were bled (to assess the presence of CD4 cells in the periphery) and sacrificed for histological analysis (see Fig. 3Dd). Surprisingly we observed that in the CD4-depleted experimental group the degree of pancreatic infiltration (parenchymal, perivascular, and perinsular infiltration) was comparable to that in the nondepleted group (50 and 32%, respectively; see Fig. 3Dd). This result can be potentially explained by the expansion of some residual CD4 T cells.
that could not be depleted and that expand in the presence of non-lymphoid MHC class II⁺ APCs in NOD/SCID mice. This hypothesis seems to be supported by the fact that peripheral blood cells isolated from NOD/SCID mice recipients for nondepleted or CD4-depleted NOD/CIITA KO splenocytes contain a low percentage of CD4<sup>high</sup> cells, apparently higher than that observed in regular NOD/SCID mice (Fig. 3B). These results, although not conclusive, point out a potential role of very low numbers of CD4 T cells in the initiation of infiltration.

Furthermore, in a complementary approach (Fig. 3B), when total spleen cells (15 × 10<sup>6</sup>/recipient) from recently diagnosed diabetic NOD female mice were transferred into 9- to 10-wk-old irradiated NOD/CIITA KO or wt recipients, diabetes was induced, but the onset of the disease was significantly delayed (9 days; by t test, p < 0.005) in the CIITA KO recipients compared with that in the wild-type mice. This indicated that although spleen cells from diabetic mice are already activated, further local activation in the recipient is required for the development of disease. Last, but not least, providing NOD/CIITA KO mice with competent (activated) NOD splenocytes (lymphocytes) rescued the diabetic phenotype, albeit with retarded kinetics, suggesting that a deficiency in local activation of the transferred cells causes a delay in the onset of the disease.

Moreover, further backcrossing of CIITA KO mice onto NOD up to 10 generations (N11) increased the incidence of diabetes of NOD/CIITA-sufficient mice from 40 to 80%. To exclude the possibility that further backcrossing would also promote the NOD/CIITA KO mice to develop diabetes due to unknown Idd susceptibility loci added after more backcrosses, we performed adoptive transfer experiments in which total spleen cells from N11 NOD/CIITA KO females or NOD/CIITA wt littermates were transferred into NOD/SCID recipients. As shown in Fig. 3C, total spleen cells from N11 NOD/CIITA KO mice were not able to transfer disease, while all recipients for NOD/CIITA wt spleen cells developed diabetes by 70 days after the adoptive transfer. This result clearly eliminates the possibility that unknown resistant alleles and not the lack of CIITA expression are responsible for the absence of diabetes in NOD/CIITA KO mice.
CD8 T cells from NOD/CIITA KO mice are able to transfer disease to NOD/SCID mice when cotransferred with wt NOD CD4 T cells from diabetic donors. A, CD4 T cells from diabetic NOD mice, CD8 T cells from diabetic NOD mice, and CD8 T cells from NOD/CIITA KO N6 were isolated and adoptively transferred into 5-wk-old NOD/SCID females as follows: NOD CD4 T cells alone from diabetic mice (6.5 ± 10^6 cells/mouse; solid triangles; n = 6), NOD/CIITA KO CD8 T cells alone (10.5 ± 10^6 cells/mouse; crosses; n = 4), NOD CD4 T cells from diabetic mice (6.5 ± 10^6 cells/mouse) and NOD CD8 T cells from diabetic mice (6.5 ± 10^6 cells/mouse; empty circles; n = 4), NOD CD4 T cells from diabetic mice (6.5 ± 10^6 cells/mouse) and NOD/CIITA KO CD8 T cells (10.5 ± 10^6 cells/mouse; solid squares; n = 7). The delay in the onset of diabetes between the group transferred with NOD CD4 and CIITA KO CD8 cells and that transferred with NOD CD4 and NOD CD8 is statistically significant (by t test, p < 0.001). B, Immunohistochemistry analysis of the NOD/SCID mice recipients for different T cell subsets. a, Islet of diabetic NOD/SCID mouse recipient for NOD CD4 and NOD CD8 T cells stained for CD8 T cells. b and c, Diabetic NOD/SCID mouse recipient for NOD/CIITA KO CD8 T cells and NOD CD4. Infiltrates were stained for CD8 T cells (b) and CD4 T cells (c). d and e, Nondiabetic NOD/SCID mouse recipient for NOD CD4 T cells alone showing CD4 infiltration (d) and no CD8 infiltration (e). f, Pancreas from NOD/SCID female recipient for NOD/CIITA KO CD8 T cells alone, stained for CD8 T cells. There was no infiltration. The data shown in A correspond to two grouped experiments.
The main lymphocyte defect in the NOD/CIITA KO is in the CD4 T cell subset

To ascertain whether CD8 T cells from the NOD/CIITA KO mice are functional and, that when provided with competent CD4 T cell help, both CD4 and CD8 together can cause diabetes, we performed adoptive transfer experiments in which NOD/SCID females were used as recipients for the following: CD4⁺ T cells from diabetic NOD female mice, diabetic NOD female CD4⁺ T cells plus CD8⁺ T cells, diabetic NOD female CD4⁻ T cells plus NOD/CIITA KO female CD8⁺ T cells, and NOD/CIITA-deficient female CD8⁺ T cells. The results (Fig. 4A) clearly showed that CD8 T cells from NOD/CIITA KO mice are able to cause disease in the presence of NOD CD4 T cells, but not in their absence, which again demonstrates the importance of CD4 T cells in the pathogenesis of diabetes. The near absence of peripheral MHC class II-restricted CD4 T cells in the NOD/CIITA KO mice may lead to an impairment in the activation of the different effector populations, like that in CD8 T cells. Interestingly, when pancreata from NOD/SCID recipients of NOD/CIITA KO CD8 T cells were examined, no infiltration was observed (Fig. 4B), indicating that CD4 T cells are also necessary for the initiation of infiltration in adoptive transfer. This hypothesis is supported by the fact that the recipients of NOD CD4 T cells alone showed a high degree of infiltration (see Fig. 4B), almost comparable to that observed in the recipients of both CD4 and CD8 T cells, although no diabetes was observed when transferring CD4 T cells alone. The slight delay observed in the onset of diabetes when a mixture of NOD CD4 T cells and NOD/CIITA KO CD8 T cells was transferred was statistically significant (by t test, p < 0.001). This delay may be due to the fact that CD8 cells obtained from diabetic NOD females as donors are already activated, while those from NOD/CIITA KO mice have to be activated upon cotransfer with the NOD CD4 T cells into the host.

NOD/CIITA KO CD8 T cells show normal function

To confirm that NOD/CIITA KO CD8 T cells are functional and to assess whether they show a Tc2-like phenotype that might protect these mice from diabetes we performed in vitro activation studies on FACS-sorted CD3⁺CD8⁺ T cells from spleen either from NOD/CIITA-sufficient (+/+; 18 wk old, on the average) or KO (19 wk old, on the average) mice. Cells were stimulated in vitro with Con A on day 0 and at 20 h (day 1) were tested for surface expression of CD69 and CD62L (Fig. 5A) as markers to assess the level of activation. On day 3 cells were pulsed with [³H]thymidine and harvested on day 4 (data not shown). In parallel, a separate batch of cells was restimulated with Con A, and supernatants were collected on day 5 to analyze cytokine production (IFN-γ and IL-4). The degree of up-regulation of CD69 (expressed as the increase over the nonstimulated control value) upon activation with Con A was similar in both populations (wt and KO) of CD8 T cells (between 1.6-fold in the KO and 2.3-fold in the wt; Fig. 5A). Similarly, CD62L expression was down-regulated to the same extent in CD8 T cells from both wt and KO mice (1.5- and 1.3-fold, respectively; Fig. 5A). Both populations proliferated equally in response to Con A stimulation (data not shown), and interestingly, the cytokine profile was the same: high production of IFN-γ (almost 400 U/ml; Fig. 5B) was seen, while no IL-4 production was detected (data not shown). This observation suggested that in vivo CD8 T cells in the NOD/CIITA KO probably have a Tc1 phenotype comparable to that seen in the CIITA-sufficient mice. There does not, therefore, appear to be an intrinsic defect in CD8 T cells that prevents them from being activated in NOD/CIITA-deficient mice. The results shown in Fig. 4A clearly indicate that CD8 T cells in NOD/CIITA KO mice need CD4 T cell help to be activated and to be able to promote diabetes.

In vitro B cell activation is similar in NOD/CIITA KO and NOD/CIITA wt mice

Another possible deficiency in NOD/CIITA KO mice could be that B cell function, which is also involved in the pathogenesis of diabetes (20), could be impaired due to the lack of MHC class II expression. We isolated B220⁺ cells by FACS and stimulated them in vitro with LPS in the presence or the absence of IL-4 on day 0. On day 1, some of the cells were harvested to check the expression of activation markers on the surface (CD23, B7.1, B7.2, MHC class II). Up-regulation of CD23 and B7.2 was observed in B cells from both CIITA-sufficient and -deficient mice only when IL-4 (20 U/ml) was also present in the culture medium (Fig. 5C). No up-regulation of CD40 (data not shown) and B7.1 was observed in either B cells of CIITA-sufficient or -deficient mice (Fig. 5C), and only in the NOD/CIITA-sufficient mice was up-regulation of MHC class II observed upon stimulation (Fig. 5C). The level of proliferation upon LPS stimulation was the same in both B cell populations (data not shown). We also measured Ig production in the presence or the absence of LPS, with or without IL-4 in the culture medium. Supernatants were collected after 7 days and were assayed by ELISA for the presence of IgM, IgG1, IgG2a, IgG2b, and IgG3. The values obtained for all the lgs tested are similar in the CIITA-sufficient and -deficient mice (data not shown). There is, therefore, no intrinsic defect in B cell function in the NOD CIITA-deficient mice.

The autoaggressive CD8⁺ T cell clone TGNFC8 is able to cause IDDM when transferred into NOD/CIITA KO recipients

The TGNFC8 CD8 T clone exhibits an autoaggressive phenotype, since it can cause disease when transferred in the absence of CD4 T cells into NOD/SCID or irradiated NOD recipients (13, 33). We questioned whether this would be the case in NOD/CIITA KO mice, which have a deficiency in CD4 T cell help. CD8 T cells were adoptively transferred into nonirradiated 3- to 4-wk-old NOD recipients (10⁷ cells/mouse). The CD8⁺ T cell clone evoked diabetes in NOD/CIITA KO recipients, although with a significant delay (by t test, p < 0.05) compared with that in NOD/CIITA-sufficient (+/-) recipients (Fig. 6A). This outcome highlights the fact that high numbers of activated autoaggressive CD8 T cells can over-ride the need for CD4 T cell help. The delay in the onset of diabetes in NOD/CIITA KO recipients may be explained by the requirement of CIITA for IFN-γ-induced MHC class I expression (22, 23). This delay is also observed when irradiated N11 NOD/CIITA KO mice were recipients of the TGNFC8 CD8 cloned T cells (Fig. 6B) and compared with irradiated N11 NOD/CIITA-sufficient recipients.

Discussion

In the present study we demonstrate that CD4 T cells are required in normal numbers to cause insulitis and diabetes but not for the initiation of pancreatic infiltration. This result is in conflict with that observed previously in the NOD/MHC class II KO mouse (28), which also retains about 5% CD4 T cells. It is important to observe that on chromosome 17 of the mouse, the class II genes are flanked by the class I and TNF family genes, which are putative Id susceptibility loci. In the gene-targeting disruption technique, embryonic stem cells are obtained from the nonautoimmune prone strain 129/Sv. Thus, even after backcrossing several generations onto the NOD background, the class I-resistant alleles closely linked to the 129/Sv class II-disrupted I-Aβ gene will still remain. The absence of infiltration in the MHC class II-deficient/NOD
Figure 5. CD8 T cells from NOD/CIITA KO mice are indistinguishable from their analogous populations in NOD/CIITA-sufficient mice in vitro. A, Activation markers. CD8+CD3+ T cells were sorted on day 0 from pooled splenocytes from NOD/CIITA-deficient males (19 wk old, on the average; upper three panels) and NOD/CIITA-sufficient males (18 wk old, on the average; lower three panels). The same day they were stimulated (thick line) or not (thinner line) with Con A (2.5 μg/ml). After 16 h they were stained for the expression on the surface of CD69 (middle panel) or CD62L (right panel). The numbers show the level of stimulation by Con A in unstimulated samples. B, IFN-γ production. On day 4 cells were restimulated with Con A (2.5 μg/ml), and on day 5 supernatants were collected to compare the production of IFN-γ by NOD/CIITA KO (solid columns) and NOD/CIITA-sufficient (dashed columns) CD8 T cells. These data correspond to a representative experiment of two performed. C, B cells from NOD/CIITA KO mice are indistinguishable from B cells from NOD/CIITA-sufficient mice upon in vitro stimulation. B220+CD4+CD8+CD3+ cells were sorted on day 0 from splenocytes from NOD/CIITA-deficient (+/-) male (10-wk-old; right panels) or NOD/CIITA-sufficient (-/-) male (10-wk-old) mice (left panels). On the same day cells were stimulated or not (dashed line) with LPS (10 μg/ml; thinner line) or LPS plus IL-4 (20 U/ml; thicker line). After 20 h they were stained for expression on the surface of CD23, B7.2, and CD69. After 48 h they were stained for the expression of MHC class II. The numbers represent the stimulation level of LPS and IL-4 over the unstimulated samples. These data correspond to a representative experiment of two performed.
work, NOD/CIITA-deficient mice were backcrossed five times and genotyped for 15 of the Idd susceptibility alleles (3, 29, 30). Among these, Idd1 (H-2g7; the unique haplotype corresponding to the NOD strain, which is required for the onset of diabetes and insulitis) and Idd3 and Idd10 (both strongly related to insulitis and diabetes and located on chromosome 3; they act in an epistatic way), the three major Idd loci in NOD mice, have been fixed as important NOD markers in the first generations of backcrosses (2, 34, 35). Because of the absence of known Idd loci on chromosome 16, where the CIITA gene is located, and the intensive selection based on Idd genotyping performed before the intercrossing phase, we conclude that the phenotype observed is due to the absence of the CIITA gene and is probably not due to the presence of unknown resistant Idd alleles closely linked to the disrupted CIITA gene.

Moreover, adoptive transfer experiments using CD4 T cells alone isolated from diabetic NOD females show that activated CD4 T cells alone (Fig. 4B) are able to infiltrate the pancreas. On the other hand, $\beta_{2}$m KO mice on the NOD background exhibit no infiltration (11, 14), which clearly suggests that CD8 T cells are required at the beginning for the priming of CD4 T cells. Interestingly, recent work shows that there are residual class II $^+$ cells in the thymus and in s.c. lymph nodes in CIITA-deficient mice (dendritic cells in inguinal s.c. lymph nodes express MHC class II, although at fivefold reduced levels compared with those from mice heterozygous for the mutation) (24). These class II $^+$ cells may account for the 5% CD4-positive cells observed in the periphery (by providing some degree of positive selection in the thymus) and the pancreatic infiltration observed in the NOD/CIITA KO mice (Ag presentation by class II $^+$ APCs in the periphery). However, those low levels of MHC class II expression are not enough to promote insulinitis and diabetes. This is also supported by the significant delay observed in the development of disease following adoptive transfer of total spleen cells from diabetic NOD mice into irradiated NOD/CIITA KO mice compared with irradiated NOD/CIITA-sufficient mice (Fig. 3B). The deficiency in MHC class II itself may be detrimental for the local activation of the different lymphocytic populations. IFN-γ induces MHC class II expression in a variety of different cell types, for instance cells of the macrophage-monocyte lineage, endothelial cells, epithelial cells, fibroblasts, and muscle cells (19, 36, 37). As a consequence, endothelial cells can become competent APCs, as has been reported for human vascular endothelium, being more efficient in presenting Ags to resting memory T cells than to naive T cells (37). Thus, the lack of CIITA may prevent IFN-γ-induced MHC class II expression in pancreatic endothelial cells, causing a delay in the transfer of disease by the activated CD4 $^+$ T cells existing in the spleen from the diabetic donor.

While the paucity of CD4 T cells in the CIITA KO mice probably explains the phenotype, these results do not exclude a role for the residual CD4 T cells that are found in both these mice and class II KO mice. In fact, our results are consistent with a role for the remaining CD4 T cells in promoting pancreatic infiltration. Specifically, when CD8 T cells from NOD/CIITA KO mice are transferred into NOD/SCID mice no infiltration is observed, while CD4 T cells from diabetic NOD females infiltrate the pancreata from NOD/SCID recipients without causing disease. Moreover, we have also observed that total spleen cells from NOD/CIITA KO mice promote infiltration, with no diabetes induction in NOD/SCID recipients (data are shown for the N11 generation in Fig. 3D). On the other hand, the results obtained in adoptive transfer experiments of CD4-depleted spleen cells from NOD/CIITA-deficient donors.
into NOD/SCID mice are not conclusive, since pancreatic infiltration remained, but a compensatory increase in CD4 T cells occurred posttransfer. These studies may indicate 1) an important potential role for low numbers of CD4 T cells in the initiation of pancreatic infiltration; and 2) that caution should be taken in the interpretation of CD4 depletion experiments, since there is no absolute guarantee of complete blocking or removal (depletion) of CD4 cells, and very few residual cells may be able to undergo compensatory proliferation in the presence of MHC class IIβ cells.

The infiltration observed in the NOD/CIITA-deficient mouse is consistent with the major postulated role of the CD8 T cell subset in these mice in promoting insulitis (12, 28). Reconciling these observations, we propose that CD4 T cells are involved in the initiation of the infiltration together with CD8 T cells. This is consistent with the less severe degree of infiltration (mostly perivascular and parenchymal) observed in NOD/CIITA KO mice compared with that in wild-type littersmates. This may be seen in terms of CD4 T cells facilitating the initial entry of CD8 T cells into the islet to cause early damage to the β-cells. CD4 T cells are also necessary later in the final effector phases. Furthermore, in the absence of MHC class II expression, B cells cannot be activated by CD4 T cells, whose numbers, in turn, are very low in NOD/CIITA-deficient mice. The constitutive expression of MHC class II in B cells is dependent on CIITA, whose expression in these cells is driven by the CIITA promoter called PIII (18, 20, 38). However, the up-regulation of MHC class II depends not on B cells on IFN-γ but, rather, on other cytokines, such as IL-4, IL-10, or Abs against surface Ags (i.e., αIL2, αIL2G, αIL2D, αLy2, or αB220) (39–44). IFN-γ has a down-regulating effect on MHC class II expression by B cells (45). B cells may play a role in the precipitation of disease, since total spleen cells from NOD mice transferred into NOD/SCID cause disease more rapidly that isolated T cell populations (32).

CD4 T cell help can be over-ridden when high numbers (10 × 10⁶ cells/mouse) of in vitro activated cloned autoaggressive CD8 T cells are transferred into NOD/CIITA KO recipients. This fact emphasizes that numbers of cells are of importance in the pathogenesis of diabetes. However, the delay in the kinetics of onset of the disease suggest that there is also an impairment in the local activation of autoaggressive transferred CD8 T cells in NOD/CIITA KO mice. This delay, also observed in further backcrossed NOD/CIITA KO mice (N11), may be due to impaired IFN-γ induction of MHC class I in the NOD/CIITA KO recipients’ pancreata. This seems, however, to be in conflict with a recent report showing that peritoneal macrophages obtained from CIITA KO mice do not exhibit impaired MHC class I up-regulation upon IFN-γ treatment. Since all nucleated cells express MHC class I, however, it is possible that regulation of MHC class I expression by these cell types may be different and in some cases CIITA dependent.

The cumulative incidence of diabetes in CIITA-sufficient littersmates (+/+ or +/−) was 40% (at the fifth backcross with 15 NOD alleles fixed), and a slight delay in the time of onset of the disease was observed. In our NOD colony the incidence in females is about 90% by 30 wk of age. After nine backcrosses onto the NOD background, the cumulative incidence observed in NOD/CIITA-sufficient mice was 80% by 30 wk of age, with no significant delay compared with our NOD colony (data not shown). After further backcrossing, total spleen cells from N11 NOD/CIITA KO mice are still unable to transfer diabetes into NOD/SCID recipients. This excludes the possibility that unknown resistant Idl loci, and not the CIITA gene, are the responsible for the absence of diabetes in N6 NOD/CIITA KO mice (Fig. 3C).

It has been postulated that the functional deficiency of NK1-like T cells in the NOD mouse leads to an impairment in Th2 cell function, leading to a Th1 phenotype (46, 47). According to this model these NK T cells would be the main producers of IL-4 upon initial stimulation (48). One could hypothesize that in NOD/CIITA-deficient mice this NK T cell population would be enriched in the periphery due to the absence of MHC class II-restricted CD4 T cells and that this would protect from diabetes. The remaining CD4⁺ T cells in the periphery of MHC class II-deficient mice (5%) are, however, also present in β2m KO MHC/class II KO double-deficient mice (31). This indicates that those residual cells are not CD1 restricted NK T cells, which are poorly selected in the absence of β2m. In NOD/CIITA-deficient mice we have observed that in the spleen the remaining CD4⁺ cells are mostly CD3β⁺. The low number of this T cell subset makes their study difficult. CD8 T cells obtained from NOD/CIITA-deficient mice appear to be normal and do not inhibit the activated NOD CD4 T cells when co-transferred onto NOD/SCID to cause diabetes. This argues against a protective Tc2 phenotype conferred by increased IL-4 production. In addition, CD8 T cells, when activated in vitro, show a clear Tc1 cytokine production pattern: large amounts of IFN-γ are produced, while IL-4 is not detectable. Moreover, we have performed experiments in which total spleen cells from NOD/CIITA KO mice have been adoptively transferred into 3-wk-old NOD females and compared with controls in which total spleen cells from NOD mice were transferred into NOD recipients. We did not observe any delay in the onset of diabetes in recipients of NOD/CIITA KO total spleen cells compared with that in recipients of NOD total spleen cells. This observation clearly suggests that there is no protective (IL-4-related?) phenotype for those cells coming from NOD/CIITA KO when transferred into NOD recipients (data not shown).

In addition, the interaction between CD40L on T cells and CD40 on APCs has been shown to play a crucial role in the activation of both APCs and T cells (49). CD40 is expressed in B cells, monocytes, dendritic cells, hemopoietic progenitors, and epithelial cells; CD40L is expressed in activated T cells, mostly CD4 T cells, but also some CD8 T cells (50, 51). For APCs CD40 ligation constitutes a survival signal (52) and up-regulates the expression of costimulatory molecules such as B7.1 and B7.2, adhesion molecules (ICAM-1), and MHC class II (53, 54). Upon CD40 ligation endothelial cells up-regulate ICAM-1, E-selectin, and VCAM-1 (55). On the other hand, CD40L-CD40 interactions enhance T cell priming (56), and are necessary to provide T cell help to B cells (57–59). It is generally assumed that the main source of CD40L is activated CD4 T cells, although CD8 T cells may be also primed indirectly through CD40 signaling to the APC (60). Then in NOD/CIITA KO mice, the near absence of CD4 T cells, and therefore the main source of CD40L, may lead to a scenario in which activation of APCs (absence of MHC class II and CD40 ligation) and endothelial cells (failure of up-regulation of MHC class II and adhesion molecules) and indirect priming of CD8 T cells (60–62) (through either activated APCs and/or CD4 T cells) are severely impaired. That these effects are likely to be important contributions of CD4 T cells is supported by the recent observation that anti-CD40L Abs block diabetes and insulitis (63).

From the present study we conclude that the main defect in the development of diabetes in NOD/CIITA-deficient mice is the practical absence of functional MHC class II-restricted CD4 T cells, which would provide help for the initial infiltration into the islets (insulitis) and for the different effector mechanisms involved in β-cell destruction. The precise nature of these mechanisms remains a topic for further study.
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