Expression of Diabetes-Associated Genes by Dendritic Cells and CD4 T Cells Drives the Loss of Tolerance in Nonobese Diabetic Mice

Emma E. Hamilton-Williams, Xavier Martinez, Jan Clark, Sarah Howlett, Kara M. Hunter, Daniel B. Rainbow, Li Wen, Mark J. Shlomchik, Jonathan D. Katz, Georg F. Beilhack, Linda S. Wicker and Linda A. Sherman

*J Immunol* published online 10 July 2009
http://www.jimmunol.org/content/early/2009/07/10/jimmunol.1.0900428
Expression of Diabetes-Associated Genes by Dendritic Cells and CD4 T Cells Drives the Loss of Tolerance in Nonobese Diabetic Mice

Emma E. Hamilton-Williams,2* Xavier Martinez,2* Jan Clark,4 Sarah Howlett,3 Kara M. Hunter,† Daniel B. Rainbow,† Li Wen,‡ Mark J. Shlomchik,‡ Jonathan D. Katz,§ Georg F. Beilhack,¶ Linda S. Wicker,3§ and Linda A. Sherman3,4*

In humans and NOD mice, defects in immune tolerance result in the spontaneous development of type-1-diabetes. Recent studies have ascribed a breakdown in tolerance to dysfunction in regulatory T cells that is secondary to reduced IL-2 production by T cells carrying the NOD diabetes susceptibility region insulin-dependent diabetes 3 (Idd3). In this study, we demonstrate a peripheral tolerance defect in the dendritic cells of NOD mice that is independent of regulatory T cells. NOD CD8 T cells specific for islet Ags fail to undergo deletion in the pancreatic lymph nodes. Deletion was promoted by expression of the protective alleles of both Idd3 (I2) and Idd5 in dendritic cells. We further identify a second tolerance defect that involves endogenous CD4 T cell expression of the disease-promoting NOD alleles of these genetic regions. Pervasive insulitis can be reduced by expression of both Idd3 and Idd5 protective alleles by either the Ag-presenting cell or lymphocytes. The Journal of Immunology, 2009, 183: 1533–1541.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

acknowledged in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by National Institute of Allergy and Infectious Disease (NIAID) Grant AI 070351. E.H.W. and X.M. supported by postdoctoral fellowships from the Juvenile Diabetes Research Foundation (JDRF). L.S.W. is supported by grants from the JDRF and the Wellcome Trust and L.S.W. is a Wellcome Trust Principal Research Fellow. The Cambridge Institute for Medical Research is the recipient of a Wellcome Trust Strategic Award (079895). The availability of NOD congenic mice through the Taconic Farms Emerging Models Program has been supported by grants from the Merck Genome Research Institute, NIAID, and the JDRF.

© 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00
that are primarily responsible for defective deletion of islet-specific CD8 T cells in the PclNs.

Materials and Methods

Mice

Experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. NOD/MrkTac and NOD-SCID mice were purchased from Taconic Farms. NOD-InsHA mice were previously described (4). The NOD Idd3/5, Idd3, and Idd5 congenic strains have been described previously (8, 10). Idd3/5, Idd3, and Idd5 congenic mice were backcrossed for 12 generations onto NOD-InsHA transgenic mice, and CD4 TCR mice as described (7). CD8 T cells were depleted in vivo by injecting 3×10^6 purified CD8 cells i.v. on day 0. Four days after the transfer PcLN and nondraining LNs from individual mice or pooled recipient mice were harvested and mature T cells depleted by incubation with anti-Thy-1.2 (12.1;20) supernatant followed by complement-mediated lysis. Recipient mice were lethally irradiated with a single 1100 or 1200 rad dose and provided with antibiotic (neomycin sulfate, 2 mg/ml) supplemented water for 10 days. At day 0, 1×10^7 T cell-depleted donor BM cells were administered i.v. Recipient mice were treated with depleting doses of either anti-CD4 (GK.1.5) and anti-CD8 (53-6.2) or anti-Thy-1.2 (30H12) mAbs at day +1 to deplete residual T cells. In some experiments, recipients were depleted of NK cells by two doses of 300 μg of rabbit anti-asialo GM1 polyonal Ab (days −3 and −1) to prevent rejection of non-MHC-I-expressing cells. Mice were allowed to reconstitute for a period of at least 7 wk before examination for T cell chimerism as assessed by Thy-1/L1.2 staining was at least 80% and CD11c chimerism as assessed by CD11c/2 staining was >98% donor origin. MHC-I expression on lymphocytes was determined by staining with anti-H-2K^b^ (SFI-1.1). SCID mice were reconstituted with total spleen and lymph node cells prepared from 5-wk-old donor mice. DCs were depleted with Pan-DC microbeads (Miltenyi Biotec) according to the manufacturer’s instructions before transfer of 2–3×10^6 cells i.v. Mice were rested for 6–8 wk before adoptive transfer of CD8 Thyl.1^+^ 8.3 TCR cells. Pancreata were fixed in 10% neutral buffered formalin and paraffin embedded. H&E stained sections were scored for insulitis, with at least 30 islets scored per mouse.

BM-derived DC and macrophages: growth and stimulation conditions

DCs were derived by culturing 2×10^6 BM cells in RPMI 1640 plus 10% FCS plus 15 ng/ml GM-CSF (PeproTech). Macrophages were derived from BM using a similar protocol, but with 10 ng/ml M-CSF (PeproTech) replacing the GM-CSF. After 8 days, the DCs or macrophages were stimulated with 75 U/ml IFN-γ (BD Pharmingen) and 0.1 μg/ml LPS (Escherichia coli, Sigma-Aldrich) for 24 or 48 h. DCs were stimulated with zymosan (InvivoGen) 10 μg/ml for 5 h. All cells were harvested and prepared for FACS and RNA or protein analyses. The DCs were 81–91% CD11c^+^ and the macrophages were 85–96% CD11b^+^ as analyzed by flow cytometry.

Western blots, quantitative PCR, and ELISA

For the analysis of SLc11a1 (NRAMP1) protein expression, BM-derived DCs or macrophages were harvested and then washed in medium containing 2.7 Kunitz U/μl DNase (Qiagen DNase set no. 79254), then lysed with protein lysis buffer containing 1% Nonidet P-40 and 1% Sigma protease inhibitor mixture (Sigma-Aldrich no. P8340). The samples were run on a 10% acrylamide gel, transferred to a Protran nitrocellulose membrane, and stained with rabbit anti-nice-1 monoclonal primary Ab at 3 μg/ml (Alpha Diagnostic International, no. NAMR027) and goat anti-rabbit IgG HRP secondary Ab (Santa Cruz Biotechnology, no. sc-2004) at 1.6 ng/ml. Bands were detected using the ECL Advance Western Blotting Detection kit (GE Healthcare, Amersham. no. RPNI2135). RNA was extracted from DCs or macrophages in TRIzol (Invitrogen) and 1000 ng of total RNA were used to make cDNA with Superscript II reverse transcriptase (Invitrogen). TaqMan-based QPCR assays including primers and probes have been described previously for detecting acyl-coenzyme A dehydrogenase long chain (ACADL) (12), CTLA-4, and β2M (normalization control) (14) mRNAs. IL-2 was measured in the supernatants by an Ab-capture assay using purified anti-mouse IL-2 (clone JES6-1A12, BD Pharmingen) bound to 96-well plates to immobilize the secreted IL-2 and biotinylated anti-mouse IL-2 (clone JES6-5H4, BD Pharmingen) and europium-streptavidin (DELFI) to detect the captured IL-2. A standard curve of rIL-2 was used to calculate IL-2 protein levels in the supernatants.

Results

CD4 T cells are not required for survival of islet-specific CD8 T cells in NOD PcLN

NOD clone-4 TCR transgenic mice express a Kd-restricted TCR with specificity for influenza hemagglutinin (HA) that is expressed under the control of the insulin promoter in NOD-InsHA mice (4). In comparing the fate of CFSE-labeled clone-4 T cells in NOD-InsHA mice, diabetes resistant Idd3/5-InsHA mice, and conventional mice, we found that deletion of islet-Ag specific T cells in the PclNs was defective in NOD mice, yet restored to normal levels in Idd3/5-InsHA recipients (7). Survival was evident as a

Bone marrow (BM) chimeras, reconstituted SCID mice, and histology

BM cells were harvested and mature T cells depleted by incubation with anti-Thy-1.2 (12.1;20) supernatant followed by complement-mediated lysis. Recipient mice were lethally irradiated with a single 1100 or 1200 rad dose and provided with antibiotic (neomycin sulfate, 2 mg/ml) supplemented water for 10 days. At day 0, 1×10^7 T cell-depleted donor BM cells were administered i.v. Recipient mice were treated with depleting doses of either anti-CD4 (GK.1.5) and anti-CD8 (53-6.2) or anti-Thy-1.2 (30H12) mAbs at day +1 to deplete residual T cells. In some experiments, recipients were depleted of NK cells by two doses of 300 μg of rabbit anti-asialo GM1 polyonal Ab (days −3 and −1) to prevent rejection of non-MHC-I-expressing cells. Mice were allowed to reconstitute for a period of at least 7 wk before examination for T cell chimerism as assessed by Thy-1/L1.2 staining was at least 80% and CD11c chimerism as assessed by CD11c/2 staining was >98% donor origin. MHC-I expression on lymphocytes was determined by staining with anti-H-2K^b^ (SFI-1.1). SCID mice were reconstituted with total spleen and lymph node cells prepared from 5-wk-old donor mice. DCs were depleted with Pan-DC microbeads (Miltenyi Biotec) according to the manufacturer’s instructions before transfer of 2–3×10^6 cells i.v. Mice were rested for 6–8 wk before adoptive transfer of CD8 Thyl.1^+^ 8.3 TCR cells. Pancreata were fixed in 10% neutral buffered formalin and paraffin embedded. H&E stained sections were scored for insulitis, with at least 30 islets scored per mouse.

BM-derived DC and macrophages: growth and stimulation conditions

DCs were derived by culturing 2×10^6 BM cells in RPMI 1640 plus 10% FCS plus 15 ng/ml GM-CSF (PeproTech). Macrophages were derived from BM using a similar protocol, but with 10 ng/ml M-CSF (PeproTech) replacing the GM-CSF. After 8 days, the DCs or macrophages were stimulated with 75 U/ml IFN-γ (BD Pharmingen) and 0.1 μg/ml LPS (Escherichia coli, Sigma-Aldrich) for 24 or 48 h. DCs were stimulated with zymosan (InvivoGen) 10 μg/ml for 5 h. All cells were harvested and prepared for FACS and RNA or protein analyses. The DCs were 81–91% CD11c^+^ and the macrophages were 85–96% CD11b^+^ as analyzed by flow cytometry.

Western blots, quantitative PCR, and ELISA

For the analysis of SLc11a1 (NRAMP1) protein expression, BM-derived DCs or macrophages were harvested and then washed in medium containing 2.7 Kunitz U/μl DNase (Qiagen DNase set no. 79254), then lysed with protein lysis buffer containing 1% Nonidet P-40 and 1% Sigma protease inhibitor mixture (Sigma-Aldrich no. P8340). The samples were run on a 10% acrylamide gel, transferred to a Protran nitrocellulose membrane, and stained with rabbit anti-nice-1 monoclonal primary Ab at 3 μg/ml (Alpha Diagnostic International, no. NAMR027) and goat anti-rabbit IgG HRP secondary Ab (Santa Cruz Biotechnology, no. sc-2004) at 1.6 ng/ml. Bands were detected using the ECL Advance Western Blotting Detection kit (GE Healthcare, Amersham. no. RPNI2135). RNA was extracted from DCs or macrophages in TRIzol (Invitrogen) and 1000 ng of total RNA were used to make cDNA with Superscript II reverse transcriptase (Invitrogen). TaqMan-based QPCR assays including primers and probes have been described previously for detecting acyl-coenzyme A dehydrogenase long chain (ACADL) (12), CTLA-4, and β2M (normalization control) (14) mRNAs. IL-2 was measured in the supernatants by an Ab-capture assay using purified anti-mouse IL-2 (clone JES6-1A12, BD Pharmingen) bound to 96-well plates to immobilize the secreted IL-2 and biotinylated anti-mouse IL-2 (clone JES6-5H4, BD Pharmingen) and europium-streptavidin (DELFI) to detect the captured IL-2. A standard curve of rIL-2 was used to calculate IL-2 protein levels in the supernatants.

Results

CD4 T cells are not required for survival of islet-specific CD8 T cells in NOD PcLN

NOD clone-4 TCR transgenic mice express a Kd-restricted TCR with specificity for influenza hemagglutinin (HA) that is expressed under the control of the insulin promoter in NOD-InsHA mice (4). In comparing the fate of CFSE-labeled clone-4 T cells in NOD-InsHA mice, diabetes resistant Idd3/5-InsHA mice, and conventional mice, we found that deletion of islet-Ag specific T cells in the PclNs was defective in NOD mice, yet restored to normal levels in Idd3/5-InsHA recipients (7). Survival was evident as a

Downloaded from http://www.jimmunol.org/ by guest on April 14, 2017
higher percentage of activated clone-4 cells accumulating in the PcLN (~70%) as compared with mice in which deletion occurred, such as BALB/c, B10.D2, and Idd3/5 (25–35%). The surviving cells then infiltrated the islets of NOD-InsHA mice but not Idd3/5-InsHA mice (7). To confirm that clone-4 T cells transferred into Idd3/5-InsHA recipients are indeed deleted, a small number of Thy1.1 clone-4 cells (1 × 10⁶) were transferred into NOD, Idd3/5, NOD-InsHA, and Idd3/5-InsHA mice. The recipients were rested for 5 wk to allow time for activation and possible deletion of the transferred cells in the PcLNs. After 5 wk, any remaining responsive clone-4 cells were expanded by infection with recombinant vaccinia virus expressing the H-2Kd restricted epitope of HA to facilitate detection. One week after infection, the frequency of Thy1.1⁺ NOD clone-4 cells in the spleen was determined (Fig. 1A). As expected in the absence of HA Ag, a high frequency of clone-4 cells were recovered from both NOD and Idd3/5 mice. Clone-4 cells were also present at high frequency in NOD-InsHA mice, however, in Idd3/5-InsHA mice they were absent. A somewhat reduced frequency of clone-4 cells was observed in NOD-InsHA mice compared with nontransgenic NOD mice, similar to what we have previously observed in the endogenous HA-specific repertoire (4). This reduction suggests that partial tolerance does result from InsHA expression in NOD mice, however a large population of HA-specific CD8 T cells still remained when compared with Idd3/5-InsHA mice. InsHA-specific CD8 T cells do not expand following infection with wild-type vaccinia (data not shown). We conclude that activation of clone-4 cells in the PcLN of Idd3/5-InsHA mice results in their deletion, a process that is defective in NOD-InsHA mice.

Cognate CD4 T cells activate DCs through CD40L-CD40 interaction, enhancing DC expression of B7 costimulatory molecules that promote survival of clone-4 cells (17–20). We hypothesized that effector CD4 T cells undermine CD8 T cell tolerance and that removal of CD4 T cells would restore deletion. To test this, CD4 T cells were depleted by at least 95% in vivo with anti-CD4 mAb for 10–14 days before injection of CFSE-labeled clone-4 cells, to allow time for replacement of DCs previously activated by CD4 T cells (21). Surprisingly, the frequency of divided clone-4 cells was similarly high in CD4 depleted and nondepleted animals (Fig. 1B). Furthermore when CD4 T cells were depleted in Idd3/5-InsHA mice, clone-4 cells still underwent abortive activation, indicating that CD4 T cells are not required to prevent deletion of CD8 T cells, CD4 depletion was begun in 2-wk-old neonates, before the onset of insulitis and generation of autoreactive CD4 T cells (22). Again, a high level of accumulation of clone-4 cells was observed in CD4-depleted NOD neonates (p > 0.05, Fig. 1C). To confirm further that autoreactive CD4 T cells are not required to prevent deletion of CD8 T cells, CD4 depletion was begun in 2-wk-old neonates, before the onset of insulitis and generation of autoreactive CD4 T cells (22). Again, a high level of accumulation of clone-4 cells was observed in CD4-depleted NOD neonates (p > 0.05, Fig. 1C). Finally, blocking CD40-CD40L interactions with anti-CD40L mAb did not reduce accumulation of clone-4 cells in the PcLN of NOD-InsHA mice (p > 0.05, Fig. 1C). Thus, survival of clone-4 CD8 T cells in the PcLN of NOD-InsHA mice, and abortive activation in Idd3/5 mice, occurs independently of CD4 T cells.
FIGURE 2. Accumulation of clone-4 CD8 T cells in NOD-InsHA hosts is not dependent on B or NK cells. A, CFSE-labeled Thy1.1+ clone-4 T cells (3 x 10^6) were injected into either NOD-InsHA mice (controls) or Human CD20 Tg NOD-InsHA mice that had been injected with Rituxan or 2H7 mAb with or without anti-CD4 on days −10 and −3. Four days after clone-4 cell transfer, PCLN were analyzed by FACS (three pooled experiments). B, NOD-InsHA recipients were injected with anti-Asialo GM1 Ab with or without anti-CD4 on days −10 and −3 before transfer of CFSE-labeled clone-4 cells and analysis of PCLN on day 4 (two pooled experiments).

Both removal of CD4 T cells and costimulation blockade is required to restore deletion of clone-4 cells in NOD PCLNs

An important survival signal for newly activated CD8 T cells is provided by B7.1/2 expression on activated DCs (23). Surprisingly, blocking CD28-B7 interaction with a mixture of anti-B7.1 and B7.2 mAbs did not reduce the accumulation of activated clone-4 cells in the PCLN (Fig. 1D). However, activated clone-4 cells were significantly reduced by such treatment if the mice were first depleted of CD4 T cells (p < 0.0001; Fig. 1D). These results suggest the NOD DCs provide B7 costimulatory signals to clone-4 cells, but when NOD CD4 T cells are present, such costimulation is either not required or another CD4 T cell-mediated costimulatory pathway becomes available. Because clone-4 cells are deleted in Idd3/5 PCLNs, we conclude that protective alleles at Idd3 and Idd5 restore the ability of both CD4 T cells and DCs to promote deletion.

It remained possible that expression of B7 by another cell type (e.g., B-cells) is involved and/or that activation of the CD8 T cell is downstream of a B7 dependent effect on another cell type in which endogenous expression of differential alleles of Idd3/5 genes primarily acts. Consequently, we continued to investigate possible effects from other cell types present in the PCLN.

Neither B cells, nor NK cells, are needed to prevent CD8 T cell tolerance in the PCLN

To determine whether B cells contribute to CD8 T cell tolerance in the PCLNs, NOD InsHA mice were mated with huCD20TgNOD mice expressing the human CD20 molecule on all B cells (15). Following treatment with anti-huCD20mAb, which depleted 85–95% of B cells, no reduction was observed in the accumulation of transferred clone-4 cells in NOD-InsHA huCD20Tg mice (p > 0.05; Fig. 2A). Simultaneous depletion of both B cells and CD4 T cells also did not reduce the frequency of divided clone-4 cells (p > 0.05; Fig. 2A).

Depletion of NK cells in BDC2.5/NOD mice prevents the occurrence of diabetes and may play a role in the loss of T cell tolerance (24). To assess the role of NK cells in promoting accumulation of clone-4 cells in the PCLN, NK cells were depleted from NOD-InsHA mice with anti-asialo ganglio-N-tetraosylceramide for 10 days before clone-4 transfer. This resulted in greater than 87% depletion of NK cells, but accumulation of clone-4 cells in the PCLN was not decreased as compared with controls either with or without simultaneous depletion of CD4 T cells (p > 0.05; Fig. 2B). Collectively, these results suggest that neither B cells nor NK cells contribute to the accumulation of islet-specific CD8 T cells in the PCLN of NOD mice.

Expression of protective Idd3/5 genes by DCs is necessary to promote deletion of autoreactive CD8 T cells

We next sought to test directly whether expression of protective Idd3/5 alleles by APCs is able to promote deletion of CD8 T cells in the PCLN. Mixed BM chimeric mice were constructed in which NOD APCs, but not Idd3/5 APCs, were rendered dysfunctional. Idd3/5-InsHA mice were lethally irradiated and reconstituted with a mixture of BM from Idd3/5 mice and β2M-deficient NOD mice. In such chimeras, only MHC class-I-expressing Idd3/5 APCs can present islet-derived Ags to CD8 T cells. To prevent NK cell-mediated cytotoxicity toward the β2M−/− BM, NK cells were depleted with asialo GM1 for 2 days before BM transfer. After 7 wk, between 35 and 75% (mean 48%) of the lymphocytes in the chimeras were derived from the β2M deficient NOD BM cells. CD4 T cells were depleted for 10 days before adoptive transfer of CFSE-labeled clone-4 cells because NOD CD4 T cells can prevent deletion of islet-specific CD8 T cells (Fig. 1D). In control chimeras, a mixture of class I-sufficient NOD BM and Idd3/5 BM resulted in high accumulation of divided clone-4 cells relative to the abortive activation observed when only Idd3/5 bone marrow was present (Fig. 3A). This occurred even when the proportion of Idd3/5 lymphocytes to NOD lymphocytes ranged between 26 and 68% (mean 52%), indicating that when both NOD and Idd3/5 APCs are present, NOD APCs are dominant and cause accumulation of clone-4 cells. When a mixture of NOD β2M−/− and Idd3/5 BM was used for reconstitution, clone-4 cells underwent abortive activation (Fig. 3A). The presence of β2M−/− BM per se did not reduce accumulation as when a mixture of NOD β2M−/− BM and NOD BM was used for reconstitution, a high percentage of activated clone-4 cells survived (Fig. 3A).

We next sought to determine whether the relevant APC population was a CD11c+ DC. We made use of BM cells from mice that express the diphtheria toxin receptor (DTR) on CD11c+ DCs (16) to construct mixed BM radiation chimeras. Treatment of such mice with diphtheria toxin (DT) causes selective depletion of CD11c+ DCs, lasting ~3 days. Additionally, NOD-CD11c-DTR derived DCs and non-DTR DCs could be distinguished by the expression of GFP that is included in the CD11c-DTR transgene. We also made use of NOD CD45.2 hosts, which allowed confirmation that all CD11c+ DCs in the resultant chimeras expressed CD45.1 and were of donor origin. NOD CD45.2 congeneric mice were lethally irradiated and reconstituted with a mixture of BM from Idd3/5 or NOD mice and NOD-CD11c-DTR mice or NOD-CD11c-DTR BM only. After 6 wk reconstitution, CD4 T cells were depleted for 10 days before adoptive transfer of CFSE-labeled 8.3 cells. Transgenic 8.3 cells are CD8 T cells that recognize an endogenous islet Ag IGRP (25) and like clone-4 cells, also undergo high-level accumulation in NOD PCLN but low-level accumulation in Idd3/5 PCLN (26). The genotype of the remaining
DC was determined in the spleen based on GFP expression and CD45 allotype (Fig. 3B). When mice were reconstituted with NOD-CD11c-DTR BM only, transferred 8.3 cells accumulated following activation (Fig. 3B). When these mice were treated with DT, CD11c<sup>HI</sup> DC were >95% depleted and proliferation of 8.3 cells was completely abolished (Fig. 3B). Therefore CD11c<sup>+</sup> DC are absolutely required to activate islet-specific CD8 T cells in the PDLN. Mice reconstituted with a mixture of either NOD-CD11c-DTR plus NOD BM or NOD-CD11c-DTR plus Idd3/5 BM resulted in high proliferation of transferred 8.3 cells (Fig. 3B). Treatment with DT specifically depleted GFP<sup>+</sup> CD11c<sup>+</sup> cells, but did not reduce accumulation in chimeras reconstituted with NOD-CD11c-DTR and NOD BM, however treatment of NOD-CD11c-DTR plus Idd3/5 BM chimeras with DT reduced proliferation (Fig. 3B). Thus, when naive CD8 T cells are first exposed to islet Ags in the periphery, the allelic form of Idd3/5 genes expressed by the cross-presenting DCs determines deletion vs survival of the activated cells.

Both Idd3 and Idd5 expression in DCs is required for optimal deletion of islet-Ag specific CD8 T cells in the PDLN

We next asked whether deletion in the PDLNs could be restored if DCs expressed only one protective region, Idd3 or Idd5. CFSE-labeled clone-4 cells were transferred into NOD, Idd3/5, Idd5, or Idd3 Ins-HA mice, and the percent of divided clone-4 cells in the PDLN were assessed. The proportion of activated clone-4 cells in Idd5-InsHA was significantly higher than that seen in Idd3/5-InsHA recipients (p<0.0001; Fig. 4) and slightly lower than that in NOD-InsHA mice (p<0.05; Fig. 4). In Idd3-InsHA mice, the frequency of divided clone-4 cells was intermediate between NOD-InsHA mice (p = 0.0001; Fig. 4) and Idd3/5-InsHA mice (p = 0.01; Fig. 4). Thus, neither the Idd3 nor the Idd5 region alone could recapitulate the degree of deletion achieved by expression of protective alleles at both regions. We next depleted CD4 T cells from Idd3 and Idd5 InsHA mice to assess the contributions of these genes on the DCs alone. Removal of CD4 T cells did not reduce clone-4 accumulation in either Idd3 or Idd5 InsHA mice (Fig. 4), which would be expected if only one of the regions was required for correcting DC function. These data support the hypothesis that the DCs must express protective alleles of both Idd3 and Idd5 genes to fully restore deletion of autoreactive CD8 T cells in the PDLN.

Differential expression of candidate genes in NOD and Idd3/5 DCs

We next sought to determine which of the candidate genes in the Idd3/5 regions exhibit allelic differences in expression in DCs. Differential expression of the Idd3/5 candidate genes Il2, Cltu4,
and Acadl have previously been described between NOD and Idd3 or Idd5 T cells and therefore we did not perform additional expression studies in CD4 T cells (10, 12, 14). BM-derived DCs from NOD and Idd3/5 mice were assessed for their expression levels of CTLA-4 (Idd5.1), SLC11a1 (Idd3/5), ACADL (Idd3/5.3), and IL-2 (Idd3) either directly, or after stimulation with either IFN-γ and LPS or LPS alone or zymosan. CTLA-4 mRNA was not detected in BM-derived DCs under any condition. DCs and control BM-derived macrophages from NOD and Idd3/5 mice were tested for the induction of SLC11a1 protein following 48 h of stimulation with yIFN and LPS (Fig. 5A). Low expression of SLC11a1 protein in Idd3/5 cells is predicted by the presence of the G169D mutation which prevents the proper folding and/or insertion of SLC11a1 into the membrane thereby subjecting the misfolded protein to degradation, especially upon its up-regulation (27). NOD cells demonstrated an induction of protein consistent with the wild-type SLC11a1 allele (Lanes A–D) (13). Without stimulation, NOD and Idd3/5 DC and macrophages had a similar level of SLC11a1 protein (Lanes B vs G and D vs I), but with stimulation, Idd3/5 DCs and macrophages failed to detectably increase SLC11a1 protein (Lanes F–I).

ACADL is an ubiquitously expressed enzyme whose mRNA is differentially expressed in CD4 T cells from NOD mice having B10-derived alleles at Idd5.3 as compared with the NOD parental strain (8, 12). The levels of ACADL mRNA in BM-derived DCs from NOD and Idd3/5 mice were tested (Fig. 5B) and found to be 2- to 6-fold higher in DCs from Idd3/5 mice, which have B10-derived DC alleles, with and without stimulation as compared with DCs from NOD mice. Therefore, differential expression by genotype of ACADL mRNA is observed in DCs as well as in T cells.

Stimulation of BM-derived DCs with zymosan or LPS has been shown to induce IL-2 by this cell population (28). Five hours after zymosan was added, 83 and 832 pg/ml IL-2 had been produced by the NOD and NOD Idd3/5 DCs, respectively (Fig. 5C). To avoid any potential influences from T cells present in the BM-derived DCs, we also used NOD-SCID BM and BM from a NOD-SCID congenic strain expressing a protective allele at Idd3, NOD.B6 Idd3/10/18-SCID. Zymosan-stimulated NOD-SCID-derived DCs produced 400 pg/ml and Idd3/10/18-SCID DCs produced 800 pg/ml IL-2 (data not shown). Higher production of IL-2 by DCs having the B6-derived Il2 allele as compared with DCs having the NOD allele at Il2 mirrors the results obtained with stimulated T cells (10).

Both lymphocytes and nonlymphocytes can provide protection from insulitis

To determine whether DC expression of Idd3/5 genes could potentially protect islet β cells from autoimmune attack, we reconstituted either NOD-SCID or Idd3/5-SCID mice with spleen and lymph node cells depleted of CD11c+ cells, derived from 3-wk-old NOD or Idd3/5 female donors. In these animals, the SCID hosts provide the DCs and the lymphocytes are of donor origin. Six to 8 wk following reconstitution, the mice received 3 × 106 CFSE-labeled NOD 8.3 CD8 T cells. CD4 T cells were not depleted, as they are required to promote insulitis. On day 4 after transfer, a high proportion of activated 8.3 cells survived in PCLNs of both NOD->NOD-SCID and NOD->Idd3/5-SCID mice (Fig. 6A). This confirms that NOD CD4 T cells prevent deletion of 8.3 cells even when DCs express a protective genotype. However, when lymphocytes expressed protective Idd3/5 genes, the genotype of
the host determined the survival of activated 8.3 cells, with significantly greater levels of accumulation of activated 8.3 CD8 T cells in Idd3/5→NOD-SCID as compared with Idd3/5→Idd3/5-SCID mice (p < 0.005; Fig. 6A). Thus, NOD-SCID host DCs were sufficient to drive high-level accumulation of islet-specific CD8 T cells in the PcLN. We next assessed the degree of insulitis in these same mice and mice that did not receive 8.3 cells (Fig. 6B). NOD→NOD-SCID mice had extensive insulitis that was reduced in both NOD→Idd3/5-SCID mice and Idd3/5→NOD-SCID mice. Idd3/5→Idd3/5-SCID mice were highly protected with very few infiltrated islets. Thus, expression of protective Idd3/5 alleles by either lymphocytes or a host cell was able to provide significant protection from the development of insulitis. The greatest protection from insulitis, as well as maximal CD8 T cell tolerance induction was achieved when both lymphocytes and host cells expressed protective Idd3/5 alleles.

**Discussion**

NOD mice exhibit a defective ability to tolerate islet-specific CD8 T cells as compared with nondiabetes-prone strains such as BALB/c, B10.D2, and Idd3/5. We have observed that although transferred islet-specific CD8 T cells undergo a similar number of divisions in NOD and Idd3/5 mice (7), they accumulate to a lower level in Idd3/5 PcLN and are deleted (Fig. 1). Of interest, other than the difference in levels of accumulation, we have found no evidence for a difference in function between CD8 T cells activated in the PcLN of Idd3/5 and NOD. Transferred clone-4 cells produce very little IFN-γ in either NOD or Idd3/5 InsHA mice (E.H.W. and X.M., unpublished observations). The functional capacity of the transferred cells was also assessed by the highly sensitive in vivo CTL assay and we found low levels of specific killing by clone-4 cells in the PcLN of NOD and Idd3/5 InsHA mice, proportional to the frequency of divided cells (X.M., unpublished observations). Consequently, the primary difference between clone-4 cells activated in NOD or Idd3/5 PcLN is survival.

The goal of the current study was to determine which cell(s) are responsible for defective CD8 T cell deletion in the PcLN of NOD mice. Genes within Idd3 and Idd5 regulate this tolerance checkpoint (7). Our initial candidate for a cell type capable of preventing deletion was host CD4 T cells. The presence of islet-Ag specific CD4 T cells prevents deletion of CD8 T cells (17), and NOD mice harbor high levels of islet-Ag specific CD4 T cells (29, 30). Surprisingly, we found that removal of CD4 T cells was insufficient to restore deletion of islet-specific CD8 T cells in the PcLN. In previous experiments, we found that B7 costimulatory signals were critical to achieve high-level accumulation of clone-4 cells in the PcLN of BALB/c InsHA recipients (31). Thus, we hypothesized that NOD DCs may deliver enhanced costimulatory signals to CD8 T cells in the steady state. Indeed, we found that in the absence of CD4 T cells, CD8 T cell accumulation that occurred in the PcLN in NOD-InsHA recipients was inhibited by anti-B7.1/2. As inhibition did not occur in the presence of CD4 T cells, it is possible that additional costimulatory pathways are activated on DCs by CD4 T cells. Alternatively, activated CD4 T cells may provide an inflammatory milieu that either directly, or indirectly, enhances survival of activated CD8 T cells.

Other types of cells that have been reported to play a role in progression of disease in NOD mice included NKT, B, and NK cells. We did not consider further the role of NKT-cells, as the variation in numbers of NKT-cells in NOD and C57BL/6 mice does not map to the Idd3 or Idd5 regions (32). Furthermore, NKT cells do not affect initial activation of T cells but rather, their survival at later time points (33). Idd5 genes have been reported to reduce diabetes incidence in part through effects on B cells (34). Depletion of B cells or NK cells, either alone or in combination with CD4 T cells, did not reduce accumulation of activated CD8 T cells. As depletion of the candidate cells was not complete (87% depletion of NK and 85–95% depletion of B cells), we cannot exclude the possibility that a small number of B cells or NK cells present in the PcLN may be sufficient to prevent deletion. However, the results obtained using radiation BM chimeras (discussed below) makes this possibility unlikely.

A number of laboratories have reported abnormalities in NOD DC populations (35–39). We next sought direct evidence that NOD DCs prevent deletion of activated CD8 T cells in the PcLN observed when CD4 T cells are removed. To this end, we produced mice in which BM-derived cells of both NOD and Idd3/5 origin coexisted, yet only DCs of Idd3/5 origin were functional for Ag presentation to CD8 T cells. Irradiated Idd3/5 mice were reconstituted with a mixture of bone marrow from Idd3/5 and NOD-β2M<sup>−/−</sup> mice. As we could not completely exclude the possibility that loss of β2M expression by APCs other than DCs contributed to the observed decrease in proliferation, we also took a second approach. We used the newly available NOD CD11c-DTR strain (16) to construct mixed BM chimeras. This experiment identified the genotype of the DC as liable for the loss of tolerance within islet-Ag specific CD8 T cells in the PcLN. This is a particularly striking result because in this model bone marrow cells of NOD and Idd3/5 origin develop together and the NOD DCs are dominant with regard to activating CD8 T cells when both genotypic sources of DCs are present. Under these conditions, removal of the NOD DCs is sufficient to promote CD8 T cell deletion in the...
PcLN. Unfortunately in this model depletion of CD11c-DTR ex- pressing cells is only temporary and it is not possible to use this same system to assess the role of DCs in the development of in- sulinis or diabetes. Another limitation of the CD11c-DTR trans- genic model is a small amount of ectopic expression of the trans- gene on other cell types including a small subset of macrophages (40). We think it is unlikely that a macrophage contributes to CD8 tolerance in the PcLN as the Idd3/5 plus NOD-βM-/- mixed chimeras indicated that a cell presenting Ag to the CD8 T cell is critical and evidence in the literature supports a role for DCs, not macrophages, in presentation of islet Ags to CD8 T cells in the PcLN (41, 42).

Although CD11c+ DCs are generally believed to be the only Ag-presenting cell capable of cross-presenting exogenously derived Ag to activate naive CD8 T cells, it has recently been re- ported that lymph node stromal cells endogenously express a range of peripheral Ags and can activate CD8 T cells (43). Depletion of DCs in NOD CD11c-DTR->NOD chimeric mice completely abolished proliferation of transferred 8.3 cells. Thus, we found no evidence that cells other than DCs could activate IGRP-specific CD8 T cells in the PcLN.

To determine whether NOD DCs may potentially support insulinis development in the presence of Idd3/5 T and B-lymphocytes, we reconstituted SCID mice. We found a reduction in insulitis when either donor lymphocytes or the host SCID cells expressed protective Idd3/5 genes and maximal protection when both com- partments were Idd3/5. This was consistent with our findings that both CD4 T cells and DC are involved in CD8 T cell tolerance induction. However, expression of Idd3/5 genes on either CD4 T cells or DC alone did not have a measurable effect on accumulation of CD8 T cells in the PcLN. Collectively, these data suggest there may be protective effects from Idd3/5 genes that prevent insulitis that are distinct from reduced accumulation of islet specific CD8 T cells in the PcLN. For example Idd3/5 genes may have protective effects in the islet as was previously observed for Idd9 genes (7).

Our results suggest that Idd3 (I12) and one or more of the Idd5 region genes (Cita4, Acad1, and Scl11a1), exert an effect on tol- erance through expression in both CD4 T cells and DCs. It is the subject of ongoing investigations to identify exactly which combi- nation of protective genes must be expressed in each cell for maximal tolerogenic effect. Both protective regions were required for optimal tolerance induction and depletion of CD4 T cells in either Idd3 or Idd5 mice did not significantly alter the degree of accumulation observed in each strain. The literature provides evi- dence for expression of I12, Cita4, and Acad1 by NOD CD4 T cells (12, 44, 45), but there is no evidence of expression of Scl11a1 outside of macrophages, polymorphonuclear leukocytes and DCs (46). DC expression of IL-2 has been reported to be of importance in immune responses to pathogens (47). Our results suggest it also plays a role in T cell tolerance. As our expression studies were performed with BM-derived DC using strong stimulatory condi- tions, it is unclear which conditions and locations in vivo such differences would be observed. Ongoing investigations are at- tempting to address this technically challenging issue. It is possible that DC-produced IL-2 has autocrine effects on DC development or function rather than being required by the T cells at the time of tolerance induction. As such, restoration of DC function by in- creased IL-2 may require precise timing during DC maturation.

SLC11a1 protein is involved in resistance to intracellular patho- gens and influences phagosomal acidification, which may alter Ag processing and thereby quantitatively affect cross-presented pep- tide Ag (48). This could contribute to the amount of CD8 T cell proliferation. However, we do not believe that the difference in proliferation of islet-specific CD8 T cells attributable to CD8-DC interaction in the PcLN is principally due to differences in amount of Ag. Differences in the amount of costimulatory molecules and cytokines can also affect the level of T cell activation. In an attempt to remove these additional signals, we eliminated CD4 cells and provided costimulation blockade. This lowered the level of clone-4 accumulation in PcLNs of NOD mice to a level similar to Idd3/5. This suggests that the amount of Ag presented to CD8 T cells is similar between NOD and Idd3/5 DCs, and that they differ in de- livery of costimulation.

Differential expression of the enzyme ACADL by DCs may confer altered fatty acid metabolism and it remains to be investi- gated how this could affect DC function. The DCs were tested for expression of all CTLA-4 mRNA isoforms (14), but none were detected in either stimulated or unstimulated cells, however this locus could influence the function of NOD vs Idd3/5 CD4 T cells. As maximal tolerance was only achieved when both Idd3 and Idd5 genes were expressed, it is possible that epistatic gene interactions occur to enhance protection.

It has recently been reported that the protective allele of Idd3 (I12) correlates with enhanced production of IL-2 (10). In the 8.3 DC8 T cell transgenic model of diabetes, enhanced levels of IL-2 production correlated with protection from diabetes by a mecha- nism involving enhanced homeostasis of Tregs, which are depend- ent on IL-2 for survival. In fact, although we have found the level of accumulation of clone-4 cells does increase somewhat follow- ing removal of CD25+ Tregs in Idd3/5 mice (7), the level of ac- cumulation is never as high as that seen in NOD mice and, im- portantly, the CD8 T cells do not migrate to the islets following removal of Tregs (X.M., unpublished results) suggesting only brief accumulation. Anderson et al. (49) have presented evidence sup- porting the hypothesis that the reduction of Treg function in the NOD mice as compared with mice expressing protective Idd3 genes is in part due to the effects of Idd3 on CD11b+CD11c- APC. We think it is likely that the Idd3/I12 gene acts indirectly on the development or function of CD11b+CD11c- cells, as we have been unable to detect expression of IL-2 by macrophages (L.W., unpublished results) and it has not been reported elsewhere in the literature. Thus, expression of IL-2 by CD4 T cells may in part affect CD8 T cell tolerance through mechanisms involving Tregs. Taken together, these results demonstrate a participation of IL-2 in multiple layers of tolerance.

Due to the complex nature of the Idd3/5 loci it is not surprising that we have identified two cell types that contribute to restoration of CD8 T cell tolerance to islet Ags. It also remains possible that Idd3/5 genes expressed in other cell types may impact CD8 T cell-independent aspects of diabetes progression. For example, the diabetes-protective effect of Idd5 expression in B cells could act by increasing regulatory T cell function (34). In conclusion, our data demonstrate that Idd3 and Idd5 genes establish multiple layers of tolerance. We have identified the islet-Ag presenting DC as a key modulator of autoreactive CD8 T cells. The DC has not previously been identified as a cell type in which Idd-mediated gene variation influences the disease state. Dissection of complex, multigenetic disease susceptibility pathways could lead to identification of novel targets for potentially modulating tolerance.

Acknowledgments

We thank Dr. Judith A. Shizuru (Stanford University) for her contribution to developing the NOD-CD45.2 congenic strain. We thank all members of the Sherman laboratory for helpful discussion; Kristi Marquardt, Rebecca Trenney, and Judith Biggs for excellent technical assistance; and Shelly Gassert for excellent secretarial assistance.
Disclosures

The authors have no financial interest of conflict.

References


