CD4⁺ Type II NKT Cells Mediate ICOS and Programmed Death-1 –Dependent Regulation of Type 1 Diabetes

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CD4\(^+\) Type II NKT Cells Mediate ICOS and Programmed Death-1–Dependent Regulation of Type 1 Diabetes

Nadir Kadri, Eva Korpos, Shashank Gupta, Claire Briet, Linda Löfbom, Agnes Lehuen, Christian Boitard, Dan Holmberg, Lydia Sorokin, and Susanna L. Cardell

Type 1 diabetes (T1D) is a chronic autoimmune disease that results from T cell-mediated destruction of pancreatic β cells. CD1d-restricted NKT lymphocytes have the ability to regulate immunity, including autoimmunity. We previously demonstrated that CD1d-restricted type II NKT cells, which carry diverse TCRs, prevented T1D in the NOD mouse model for the human disease. In this study, we show that CD4\(^+\) 24ββ type II NKT cells, but not CD4/CD8 double-negative NKT cells, were sufficient to down-regulate diabetogenic CD4\(^+\) BDC2.5 NOD T cells in adoptive transfer experiments. CD4\(^+\) 24ββ NKT cells exhibited a memory phenotype including high ICOS expression, increased cytokine production, and limited display of NK cell markers, compared with double-negative 24ββ NKT cells. Blocking of ICOS or the programmed death-1/programmed death ligand 1 pathway was shown to abolish the regulation that occurred in the pancreas draining lymph nodes. To our knowledge, these results provide for the first time cellular and molecular information on how type II CD1d-restricted NKT cells regulate T1D. The Journal of Immunology, 2012, 188: 3138–3149.
BDC2.5 NOD mice express a transgenic TCR isolated from the natural diabetogenic CD4\(^+\) T cell repertoire of NOD mice (18, 19), and CD4\(^+\) BDC transgenic T cells transfer acute diabetes to immunodeficient recipient mice. When the transgenic BDC2.5 TCR is expressed on a wild-type NOD genetic background, the mice accumulate a massive lymphocyte infiltrate in the pancreas that remains innocuous in most animals, with only a 10–20% incidence of diabetes. Regulatory cells expressing endogenous TCRs are necessary to prevent the disease in these mice, as an early aggressive disease develops in a high proportion of BDC2.5 NOD mice lacking rearrangement of TCR chains (20). It has been shown that both regulatory CD4\(^+\) T cells expressing Foxp3 (Tregs) and CD1d-restricted NKT cells are major players in the regulation of BDC cells in BDC2.5 NOD mice (20–23).

To investigate cellular requirements and molecular mechanisms underlying type II NKT cell regulation of T1D, we have explored the BDC2.5 NOD model using adoptive transfer experiments. Our results show that CD4\(^+\) type II NKT cells, but not CD4/CD8 double-negative (DN) NKT cells, were sufficient to regulate diabetogenic T cells. We found a unique phenotype displayed by CD4\(^+\) type II NKT cells, including low expression of NK markers, a phenotype of activated cells, high expression of ICOS, and increased cytokine production upon activation, different from DN type II NKT cells or conventional NOD CD4\(^+\) T cells. Most importantly, we demonstrate that the ICOS/ICOS ligand (ICOS-L) and the programmed death-1 (PD-1)/PD ligand (PD-L1) pathways were required for the regulation to occur. To our knowledge, these results provide, for the first time, cellular and molecular information on the regulation of diabetogenic CD4\(^+\) T cells by a defined population of type II CD1d-restricted NKT cells.

Materials and Methods

**Mice**

Mice were housed under specific pathogen-free conditions. The 24\(\times\)bNOD mice (8), BDC2.5 TCR transgenic NOD mice (BDC2.5 NOD mice) (18), and NOD.scid mice were bred at the Experimental Biomedicine Animal Facility at the University of Gothenburg. BDC2.5 NOD mice deficient for ICOS and its ligand are referred to as BDC2.5 NOD mice (25) were bred at Hôpital Cochin/St. Vincent de Paul (Paris, France). Donor 24\(\times\)bNOD mice were 8–12 wk of age, donor BDC2.5 NOD mice were 4–6 wk of age, and recipient NOD.scid mice were 5–8 wk of age at time of transfer. All experiments involving mice were approved by the Animal Ethics Committee of Gothenburg and performed according to the relevant regulatory standards.

**Flow cytometry**

Cells were stained at 4°C in PBS containing 5% FCS and 0.1% sodium azide after FcR blockade with 2.4G2 Ab. For surface staining, mAbs (clone indicated in parentheses) to V\(\alpha\)3.2 (RR3-16), V\(\beta\)9 (MR 10-2), V\(\gamma\)4 (197.97), IL-4 (JES6-5H4), TCR\(\beta\) (H57-597), CD45R (B220; RA3-6B2), CD4 (GK1.5 and RM4.5), CD8 (56-6.7), CD44 (IM7), CD26 (MEL-14), CD49 (H1.2F3), CD25 (PC61 and 7D7), CD49b (DX5), ICOS (7E.17G9), CD134 (OX-4), LY49G2 (4D11), CD45R5B (16A), CD95 (J02), CXCRI (clone 220803), as well as streptavidin-allophycocyanin and streptavidin-Qdot605 were purchased from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA). The anti-b220 Ab eB220 (22) was provided by D. A. Cooke after permission from Dr. O. Kanagawa. Cells were stimulated in vitro for 4 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 ng/ml) (Sigma-Aldrich). After surface staining, cells were fixed and permeabilized according to the manufacturer’s instructions (BD Biosciences), followed by intracytoplasmic staining using Abs against IL-2 (JES6-5H4), IFN-\(\gamma\) (XMGL2.1), TNF-\(\alpha\) (MP6-XT22), IL-10 (JES5-16D1), IL-13 (eBio13A), or IL-17 (eBio17B7). All reagents were from BD Biosciences, except anti-IL-13 and anti-IL-17, which were from ebioscience. Samples were collected on an LSRII (BD Biosciences) and analyzed by FlowJo (Tree Star).

Preparation of pancreas-infiltrating cells, spleen, and lymph node cells

Spleen, mesenteric lymph nodes, and pancreatic lymph nodes (PLNs) were isolated and single-cell suspensions prepared. Infiltrating immune cells were obtained from lymph node-free pancreas tissue by cell sieving using 40-\(\mu\)m strainers (BD Biosciences). Lymphocytes were subsequently isolated by Percoll (Sigma-Aldrich) gradient centrifugation. For flow cytometry analyses of islet infiltrates, cells from at least three mice were pooled.

Adaptive cell transfer and in vivo blocking

Splenocyte populations from 24\(\times\)bNOD mice were processed to single-cell suspensions. V\(\alpha\)3.2\(^{+}\), B220\(^{-}\), CD11c\(^{-}\), CD11b\(^{-}\), CD8\(^{-}\), and CD25\(^{-}\) cells were depleted as indicated using biotinylated Abs (BD Biosciences) and streptavidin-conjugated microbeads, according to the manufacturer’s instructions (Miltenyi Biotec). Depleted regulatory populations were transferred at the same numbers as control (nondepleted) populations after correction for depleted cells. CD4\(^+\) cells from 24\(\times\)bNOD mice were positively selected to >93% purity using CD4 microbeads (Miltenyi Biotec), and CD4\(^+\) cells containing 7 \(\times\) 10\(^5\) V\(\alpha\)3.2\(^{+}\) cells were transferred to NOD.scid mice unless other numbers are stated. 24\(\times\)bNOD splenocytes were enriched for V\(\alpha\)3.2\(^{+}\) cells or CD4\(^+\) cells using microbeads, followed by sorting to isolate V\(\alpha\)3.2\(^{+}\)V\(\beta\)9\(^{+}\)CD4\(^{+}\) cells (>95% purity) using BD Biosciences. Regulatory cells were injected i.p. simultaneously or 2 d before diabetogenic cells. Diabetogenic CD4\(^+\) cells were obtained from the spleens of 4- to 6-wk-old nondiabetic BDC2.5 NOD mice (or when indicated from Co\(\alpha\)CD2.5 NOD mice) (25) mice using CD4 microbeads (Miltenyi Biotec) (>90% CD4\(^{+}\), of which 80–90% expressed high levels of the BDC clonotype). CD4\(^+\) BDC2.5 NOD cells (referred to as BDC cells) (5 \(\times\) 10\(^5\)) were injected i.p. into NOD.scid mice (referred to as day 0). Abs were injected i.p. into recipient mice (0.5 mg/mouse) on days –2, 2, 4, 7, and 10 relative to transfer of 24\(\times\)bNOD cells. Blocking Abs to PD-L1 (10F9G2), TGF-\(\beta\)1 (1D11), and ICOS (17G9) were from BioXCell. Abs to PD-1 (RMP1-14) and PD-L2 (TY25) were prepared as in Yamazaki et al. (26, 27), and rat IgG (Sigma-Aldrich) served as control. Blocking mAbs to IL-4 and IL-10 were injected i.p. into recipient mice (1 mg/injection) on days –2 and 1 relative to transfer of CD4\(^+\) BDC2.5 NOD cells. Anti-IL-4 (1D11) and anti-IL-10 (JES5-16E3) Abs were purified in our laboratory. The anti-plasmacytoid dendritic cell (pDC) 927 cell line was provided by Dr. M. Colonna. Anti-pDC 927 Ab was purified in our laboratory, and for pDC depletion was injected (1 mg) 2 d before and 1 d after transfer of CD4\(^+\) 24\(\times\)bNOD cells. Depletion efficiency was confirmed by flow cytometry using anti-CD11c, anti-B220, anti-Myeloid class II (I\(\alpha\)a) (BD Pharmingen), and PDCA-1 (Invitrogen) Abs. For inhibition of IDO activity in vivo, 1-methyl-l-tryptophan (>99% pure; Sigma-Aldrich) was supplied in the drinking water, 5 mg/ml, starting 1 d before transfer of CD4\(^+\) 24\(\times\)bNOD cells until day 10 after BDC cell transfer. Water was exchanged every second day. Control mice received normal water.

Immunohistology

Pancreas cryostat sections (5 \(\mu\)m) were air dried and fixed in methanol. Nonspecific staining was blocked by incubation in 1% BSA in PBS. Abs used were rat Abs to CD45 (30G12), V\(\beta\)4 (KT4), F4/80 (A3-1), NIMP-R14 (anti-Ly6b; Abcam), and hamster anti-CD11c (N418), rabbit anti-inducible NO synthase (iNOS) (NOS II (Upstate Cell Signaling Solution), rabbit anti-pan-laminin (455) (28), and guinea pig anti-insulin (Dako). Secondary reagents were Alexa Fluor 488-conjugated goat anti-rat, Cy5-conjugated anti-rabbit, Cy3-conjugated donkey anti-guinea pig, and Alexa Fluor 594-conjugated goat anti-hamster Abs (The Jackson Laboratory and Molecular Probes). Negative controls involved incubation of sections with secondary Abs alone. Sections were examined using a Zeiss AxiosImager microscope equipped with epifluorescent optics and documented using a Hamamatsu ORCA ER camera and Velocity 5.4 software (Improvision).

Optical projection tomography

Pancreatic tissue from NOD.scid mice was isolated, fixed and prepared (29), and stained with rabbit anti-CD3 (C7930; Sigma-Aldrich) followed by anti-rabbit Alexa Fluor 594 (Invitrogen). Optical projection tomography (OPT) scans were carried out using the Bioptions 3001 OPT M scanner with exciter D560/40\(\times\) and emitter E610lpv2 filter (Chroma Technology) (29). Tomographic reconstructions were generated using the NRecon V1.6.1.0 (SkyScan, Kontich, Belgium) software, and reconstructed images were further assessed using a Bioptions viewer v.2.0 (29). The movies and images were constructed using ImageJ 1.43u software.
Diabetes diagnosis

Starting 4 d after transfer of diabetogenic cells, recipient mice were tested every day for glucose in the urine using glucostest strips (Boehringer Mannheim, Mannheim, Germany). Overt diabetes was defined as two consecutive positive tests.

Statistical analysis

GraphPad Prism software was used for statistical comparisons of two groups; p values were calculated by the unpaired Student t test. For comparisons of multiple groups, p values were calculated by one-way ANOVA with GraphPad Prism. A p value of 0.05 or less was considered significant.

Results

A minor CD4+ subset of TCR transgenic type II NKT cells prevented T1D in a transfer model

A first set of experiments was performed to evaluate the capacity of 24αβ type II NKT cells to inhibit the induction of diabetes following transfer of BDC cells to NOD.scid mice. Transfer of 5 × 10^5 BDC cells (day of transfer of BDC cells is regarded as day 0) induced diabetes in 80–100% of recipients (Fig. 1). In contrast, when total spleen cells from 24αβNOD mice were injected at the same time (not shown), or 2 d before BDC cells (Fig. 1A), all mice were protected from disease for several months after transfer (Supplemental Fig. 1A). In all subsequent experiments 24αβNOD cells were transferred 2 d before (or at the same time where indicated) diabetogenic cells, hereafter referred to as cotransfer. Titration of cell numbers showed that 40 × 10^5 total 24αβNOD spleen cells, containing ~5 × 10^6 Vα3.2ββ9+ (TCR transgene-positive) (8, 17, 31) 24αβ NKT cells, were required to prevent disease.

Although most T lymphocytes in 24αβ NOD mice express the transgenic TCR β chain, only 10–15% of T cells also express the transgenic TCR Vα3.2 chain (8). Expression of the full transgenic Vα3.2ββ9 TCR is required for CD1d-dependent development of T cells with the typical NKT cell phenotype (8, 17). Depletion of Vα3.2+ cells from 24αβNOD splenocytes completely abrogated the protection conferred by these splenocytes (Fig. 1A), demonstrating that type II NKT cells were required for regulation of diabetogenic effector T cells. However, depletion of CD11c+, CD11b+, CD8+, and B220− cells did not affect disease incidence (Fig. 1B). Approximately 10–15% of TCR transgenic-positive (Vα3.2ββ9+) 24αβ type II NKT cells were CD4+ (Fig. 1C), and most were CD4+ CD8− (DN). Despite the low proportion of CD4+ cells, their depletion from 24αβNOD splenocytes restored disease induction by BDC cells, demonstrating a pivotal regulatory role for CD4+ 24αβNOD type II NKT cells (Fig. 1D). This was confirmed by cotransfer experiments where total CD4+ spleen cells, containing 5–7 × 10^5 Vα3.2ββ9− cells, were as effective as total 24αβNOD splenocytes in providing long-term protection from BDC cell-mediated disease (Fig. 1D, Supplemental Fig. 1A, 1B). Again, the protection by 24αβNOD CD4+ cells was found to require Vα3.2+ type II NKT cells (Fig. 1E), demonstrating that the CD4+ but not the DN subset of Vα3.2+ 24αβ type II NKT cells was able to regulate T1D induced by BDC cells. Conventional Tregs expressing Foxp3 and CD25 were present among both CD4+ cells from BDC2.5 NOD mice and total CD4+ cells from 24αβNOD mice; however, TCR transgenic Vα3.2ββ9+CD4+ cells did not include cells expressing Foxp3 (Fig. 1C). The regulation of disease in recipient mice was not dependent on transferred CD25+ cells, as depletion of CD25+ cells from one or both populations of CD4+ 24αβNOD and CD4+ BDC cells before transfer did not affect the ability of CD4+ 24αβNOD cells to confer resistance to T1D (Fig. 1F). CD4+− BDC cells, lacking rearrangement of endogenous TCR α-chains (25), were similarly controlled by CD4+ 24αβNOD cells (Supplemental Fig. 1C). Finally, transfer of 7 × 10^5 sorted CD4+Vα3.2+ββ9+ type II NKT cells was sufficient to prevent disease (Fig. 1G), and titration showed that this cell number was necessary to regulate 5 × 10^5 CD4+ BDC cells (Fig. 1H). Therefore, a minor CD4+ subset of type II NKT cells was sufficient for the regulation of T1D induced by CD4+ BDC cells (Fig. 1G), whereas DN type II NKT cells were not able to regulate disease even when transferred at 8- to 9-fold higher numbers (Fig. 1D).

Prevention of diabetes was associated with reduced inflammation in the pancreas

We next investigated whether the clinical observation of disease protection correlated with the extent of infiltration and destruction of islets in the pancreas on days 7–9 after transfer of BDC cells. Three-dimensional imaging using OPT analysis of major portions of pancreases from nondiabetic recipients of BDC cells demonstrated extensive T lymphocyte infiltration of the islets (Fig. 2A, Supplemental Video 1) and markedly reduced T cell infiltration in the case of cotransfer of CD4+ 24αβ spleen cells (Fig. 2A, Supplemental Video 2). Corresponding FACS analysis revealed a >8-fold reduction in infiltrating BDC cell numbers (Fig. 2B) and showed that the numbers of BDC cells and 24αβ Vα3.2ββ9+ type II NKT cells were similar in the pancreas after cotransfer (Fig. 2B). Immunofluorescence analyses of organ sections confirmed an abundance of infiltrating cells positive for the BDC TCR after transfer of these cells, whereas minor, mostly peri-insular, infiltrates containing BDC cells were detected after cotransfer with CD4+ 24αβ spleen cells (Fig. 2C). Further examination revealed an association of F4/80+ macrophages and CD11c+ dendritic cells with pancreatic infiltrates in both recipients of BDC cells alone and after cotransfer of CD4+ 24αβNOD spleen cells (Fig. 2D, 2E). In contrast, only after transfer of BDC cells alone were the infiltrates associated with high numbers of NIMP-R14+ neutrophils and iNOS production (Fig. 2D, 2E). Pancreatic infiltrates were still present 8–10 wk after cotransfer of CD4+ 24αβNOD cells together with BDC cells. BDC cells were present in the infiltrates but were apparently innocuous and under regulation, as suggested by the absence of neutrophils in the infiltrates, positive insulin staining, and the lack of clinical diabetes (Supplemental Fig. 1). Taken together, the data show that cotransfer with CD4+ 24αβNOD spleen cells reduced the extent and composition of inflammatory infiltrates induced by BDC cells in NOD.scid mice. Cotransferred CD4+ 24αβ type II NKT cells accumulated in the PLNs and suppressed activation of CD4+ BDC2.5 cells

The data suggest that CD4+ 24αβNOD type II NKT cells recruited to the pancreas may exert their immunosuppressive function at this site, although the strongly reduced size of infiltrates may also result from regulation of BDC cell activation in the draining PLNs. Before transfer, most BDC cells had a naive phenotype (Supplemental Fig. 2A). Transfer of BDC cells alone resulted in accumulation of a high proportion of activated and cycling BDC cells in the PLN (Fig. 3A–C). In the presence of CD4+ 24αβNOD type II NKT cells, BDC cell numbers in the PLN were unaffected by day 3, but by days 6 and 9 the total cell numbers were 2-fold lower and BDC cell numbers were 5-fold lower in PLN (Fig. 3A, 3B). Analysis of BDC cells showed decreased CD69 levels and reduced cycling days 6–7 after cotransfer with CD4+ 24αβNOD type II NKT cells, and the capacity of BDC cells to produce inflammatory cytokines upon stimulation was significantly decreased (Fig. 3C). At this time, whether transferred alone or in the cotransfer situation, BDC cells preferentially localized to the PLN (Supplemental Fig. 2B).

In contrast, after cotransfer of CD4+ 24αβNOD spleen cells, CD4+Vα3.2ββ9− cells were detected in different lymphoid
organs of recipient mice (Supplemental Fig. 2C), including the PLNs where they were present in similar numbers to BDC cells (Fig. 3B). The CD4+Vα3.2+Vβ9+ type II NKT cells proliferated in the PLN of recipient mice as shown by the expression of Ki67, compared with CD4+Vα3.2+Vβ9+ type II NKT cells in the spleen (Fig. 3D). This indicates that CD4+VαβNOD type II NKT cells accumulated and were activated in the PLN and prevented full activation, differentiation, and cell division of BDC cells at this site. 

Transgenic CD4+ type II NKT cells were ICOS+ and displayed characteristics of increased activation, high cytokine production, and limited NK cell features.

Because CD4+ but not DN VαβNOD type II NKT cells could prevent disease in the transfer model, we first analyzed whether both cell types homed to pancreatic lymph nodes after transfer. Transfer of total spleen cells from VαβNOD mice together with BDC cells resulted in a similar extent of CD4+ and DN type II NKT cell localization to the PLN, as well as an upregulation of the activation

FIGURE 1. CD4+ but not DN type II NKT cells regulated diabetogenic CD4+ T cells. BDC cells (5 × 10⁶) were transferred to NOD.scid mice alone or together with 24αβNOD spleen cells containing 10⁷ CD4+ cells or with depleted or positively selected populations as described. (A) BDC cells were injected alone (n = 24) or cotransferred with total 24αβNOD splenocytes (n = 18, depicted in the upper dot plot) or with 24αβNOD splenocytes depleted of Vα3.2+ type II NKT cells (∆, n = 12, lower dot plot). (B) BDC cells were injected alone (n = 9) or cotransferred with 24αβNOD splenocytes (♦, n = 10, upper dot plot) or with 24αβNOD splenocytes depleted of CD8+, CD11c+, CD11b+, and B220+ cells (APC and CD8 depleted; ○, n = 11, lower dot plot). (C) 24αβNOD splenocytes were gated for Vα3.2+Vβ9+ cells (left dot plot) and displayed for Vα3.2 versus CD4 expression (upper right dot plot) or for Foxp3 versus CD25 staining (lower right dot plot). (D) BDC cells were injected alone (n = 12) or cotransferred with 24αβNOD splenocytes (♦, n = 8), or with 24αβNOD splenocytes depleted of CD4+ cells (▼, n = 6, upper dot plot), or with positively selected CD4+ 24αβNOD cells (★, n = 8, lower dot plot). (E) BDC cells were injected alone (n = 5) or cotransferred with CD4+ 24αβ cells (▲, n = 11, upper dot plot) or with CD4+ 24αβ cells depleted of Vα3.2+ cells (★, n = 5, lower dot plot). (F) BDC cells (n = 7, upper dot plot), or BDC cells depleted of CD25+ cells (▼, n = 5, lower dot plot) were injected alone, or BDC cells were cotransferred with CD4+ 24αβ cells (▲, n = 12), or BDC cells were cotransferred with CD4+ 24αβ cells depleted of CD25+ cells (★, n = 10), or BDC cells depleted of CD25+ cells were cotransferred with CD4+ 24αβ cells depleted of CD25+ cells (★, n = 10). (G) NOD.scid mice received CD4+ BDC2.5 cells alone (♦, n = 6), or cotransferred with CD4+ 24αβ spleen cells (★, n = 6), or cotransferred with sorted CD4+ Vα3.2+Vβ9+ type II NKT cells (▶, n = 6) (95% pure, dot plot) (○, n = 6). (H) BDC cells were injected alone (n = 8) or cotransferred with CD4+ 24αβ cells containing 1.5 × 10⁶ (♦, n = 8), 0.7 × 10⁶ (▲, n = 8), 0.35 × 10⁶ (★, n = 6), or 0.15 × 10⁶ (○, n = 8) Vα3.2+ cells. Data show pooled results from two to three (B–H) or more (A) experiments.
marker CD69 on both subsets in this organ (Supplemental Fig. 2D, 2E). To provide insight on the mechanism of action of the CD4+ 24αβNOD type II NKT cells, we compared the two subsets for previously described characteristics of transgenic type II NKT cells (8, 17, 32): the expression of a panel of surface receptors and their ability to secrete cytokines after stimulation. Conventional CD4+ NOD cells are shown as reference. Several differences were found between the CD4+ and DN 24αβNOD type II NKT cell subsets: CD4+ 24αβNOD type II NKT cells had lower expression of Dx5/CD49b, Ly49G2, and CD122 (Fig. 4A), demonstrating that DN cells but not CD4+ 24αβNOD type II NKT cells had several characteristics of NK cells. CD4+ 24αβNOD type II NKT cells had an expression profile typical of activated cells (CD62Llo and CD45RBlo, and CD44hi and CD69+), whereas in this respect DN 24αβNOD type II NKT cells resembled conventional resting CD4+ NOD T cells (Fig. 4A). DN 24αβNOD type II NKT cells had somewhat higher expression of CXCR3 (Fig. 4A), whereas other chemokine receptors and integrins tested were similarly expressed in both subsets (data not shown). Both type II NKT cell populations expressed a low level of PD-1, with a subset of cells having high levels, whereas PD-L1 but not PD-L2 was found at similar levels on type II NKT cells and NOD CD4+ cells. CD4+ 24αβNOD type II NKT cells, in contrast, displayed increased amounts of CD95 and ICOS. We also found a difference in the production of cytokines upon ex vivo stimulation with PMA and ionomycin (Fig. 4B). The fraction of CD4+ 24αβNOD type II NKT cells producing the Th1 cytokines TNF-α,
IFN-γ, and IL-2 were 3- to 5-fold higher than in DN 24αβNOD type II NKT cells. Both subsets produced low levels of IL-4, IL-13, and IL-17, but negligible amounts of IL-10. Thus, phenotypic analysis demonstrated several differences between CD4+ 24αβNOD type II NKT and DN 24αβNOD type II NKT cells, consistent with distinct functional capacities of the two subsets in the regulation of T1D.

**ICOS/ICOS-L interaction was necessary for type II NKT cell regulation of BDC cells**

In the search for the regulatory mechanism induced by CD4+ 24αβ type II NKT cells, we first investigated a number of known immunoregulatory pathways previously associated with NKT and other regulatory cells (6, 14, 33), including some of the cytokines produced by CD4+ 24αβ type II NKT cells. Using different approaches we could exclude a role for IL-4 and IL-10 (Supplemental Fig. 3A), IL-13 and TGF-β (Supplemental Fig. 3B, 3C), and pDCs and the tryptophan catabolizing enzyme IDO (Supplemental Fig. 3D–F). Next, we turned to ICOS, expressed at high levels on CD4+ 24αβNOD cells (Fig. 4A). ICOS, a T cell costimulatory molecule upregulated on activated T cells, has also been implicated in the regulatory function of Tregs (24, 34, 35). Administration of blocking Ab to ICOS completely abrogated protection by CD4+ 24αβNOD cells (Fig. 5A). Expression of ICOS by BDC cells was not relevant for...
FIGURE 4. CD4+ type II NKT cells were distinct from DN cells in the expression of surface molecules and cytokine production. Spleen cells from 24aβ NOD mice were isolated and stained for surface markers (A) or first stimulated followed by staining for surface markers and intracellular cytokines (B). (A) CD4+Vα3.2+Vβ9+ cells (top panels), CD4−CD8− Vα3.2+Vβ9+ cells (middle panels), and control CD4+ splenocytes from wild-type NOD mice are displayed for the expression of the indicated surface markers (black lines) and negative control stains (gray lines). Data are representative of at least three independent experiments. (B) Spleen cells from 24aβNOD transgenic mice were stimulated and stained for flow cytometry. The data show Vα3.2 versus cytokine expression by CD4+ and CD4−CD8− cells among Vα3.2+Vβ9+ cells and are representative of at least three experiments. Numbers indicate percentage of positive cells.
regulation in this model, as BDC cells lacking ICOS could still transfer diabetes and be downregulated by 24αβNOD type II NKT cells to the same extent as BDC cells expressing ICOS (Fig. 5B). Although not directly demonstrated, this suggests that ICOS expression by CD4+ 24αβNOD type II NKT cells was required for suppression to be observed. Because ICOS has been implicated in the homeostasis and activation of type I NKT cells (36, 37), we tested whether ICOS blockade affected 24αβNOD type II NKT cells in vivo. Injection of 24αβNOD mice with the anti-ICOS Ab did not reduce numbers of Vα3.2+Vβ9+ cells in lymph nodes or in the spleen after 9 d treatment, suggesting that ICOS was not required for 24αβNOD type II NKT cell homeostasis (data not shown). Furthermore, in recipient NOD.scid mice the number of 24αβNOD type II NKT cells and their state of activation (CD44 and CD69 levels) after cotransfer with BDC cells were not altered in the presence of anti-ICOS compared with control mAb (days 3–4 after transfer; Fig. 5C). This implies that ICOS signaling was required for the regulatory function of CD4+ 24αβNOD type II NKT cells.

24αβ Type II NKT cell regulation of T1D was dependent on PD-1/PD-L1 interaction

Expression levels of some surface markers, in particular CD62L and PD-1, divided the CD4+ 24αβ type II NKT cell population into two subsets (Fig. 4). Interestingly, although there was a correlation between high expression of PD-1 and ICOS, CD62L+ cells were low for ICOS and PD-1 expression (Fig. 5D). PD-1 can act as a negative regulator of T cells, but it has also been implied in the mechanism of action of regulatory cells (38–40). We therefore tested whether blocking of the PD-1 receptor would influence the regulation of disease by 24αβ type II NKT cells or diabetes development induced by the transfer of BDC cells. Administration of anti–PD-1 mAb from day −1 to day 10 did not influence the kinetics of disease induced by 5 × 10^5 BDC cells, but it reversed the protective effect of 24αβ type II NKT cells on disease incidence (Fig. 6A). Blocking of the two PD-1 ligands, PD-L1 or PD-L2, demonstrated a role for PD-L1, but not for PD-L2 (Fig. 6B). Reversal of regulation caused by blocking of PD-1/PD-L1 could result from inhibition of the regulatory pathway induced by CD4+ 24αβNOD type II NKT cells but may also result from potentiation of the autoggressive nature of BDC CD4+ T cells. To investigate the latter possibility we titrated the number of BDC cells to identify the minimum number of cells that would induce disease in at least a fraction of the recipient mice and then tested the effect of blocking PD-1 signaling. Transfer of 5 × 10^3 BDC cells resulted in 100% T1D incidence, whereas transfer of 10^4 BDC cells resulted in reduced incidence and delayed kinetics of onset (Fig. 6C). Blocking PD-L1 in mice transferred with 10^3 BDC cells increased disease incidence and resulted in earlier onset of diabetes, but this was still delayed compared with that induced by 5 × 10^5 BDC cells. This suggested that when using 10^5 BDC cells the anti–PD-L1 treatment resulted in a slight increase of diabetogenicity that was less...
severe than that induced by transfer of $5 \times 10^5$ BDC cells and, thus, should readily be regulated by $7 \times 10^5$ CD4$^+$ 24αβ NOD splenocytes. The 24αβ type II NKT cells prevented disease induced by $10^4$ BDC cells in the presence of control mAb, but blocking PD-L1 abolished 24αβ type II NKT-mediated suppression of disease induced by the low number of BDC cells (Fig. 6D). This suggests that anti–PD-L1 resulted in a block of the regulatory pathway, in addition to a slight increase in the diabetogenicity of the BDC cells. Finally, we investigated whether delayed administration of the blocking anti–PD-L1 Ab could release a diabetogenic potential of infiltrated BDC cells that had been cotransferred with 24αβ type II NKT cells, but disease did not occur up to 60 d after transfer in the mice that received mAb treatment starting day 10 (Fig. 6E). This suggested that the regulatory process mediated by 24αβ type II NKT cells required PD-1/PD-L1 interaction for the regulation of BDC cells early after cell transfer, rather than for regulation of autoaggressive BDC cells within the pancreas.

Discussion

In this study we investigated in detail the suppression of T1D by type II NKT cells in a transfer model, seeking to clarify the cellular requirements and the mechanisms underlying their ability to regulate autoaggressive diabetogenic CD4$^+$ T cells. We provide novel and unique information on the characteristics and regulatory capacity of a specific type II NKT cell population with a defined TCR. To our knowledge, our results identify for the first time the PD-1/PD-L1 pathway as an essential component of the mechanism underlying regulation of autoimmunity by CD1d-restricted NKT cells. Furthermore, we also demonstrate a requirement for ICOS for the regulation to occur.

We found that transfer of sorted CD4$^+$ 24αβ type II NKT cells was sufficient to prevent T1D induced by BDC cells in recipient mice. Our data further suggested that CD4$^+$ type II NKT cells were able to regulate diabetogenic CD4$^+$ T cells without the cooperation of other T cells such as Tregs. In contrast, DN 24αβ type II NKT cells, even when transferred at several fold higher

FIGURE 6. The PD-1/PD-L1 pathway played a key role in the regulation of T1D by type II NKT cells. NOD.scid mice were transferred with BDC cells alone or simultaneously with CD4$^+$ 24αβ NOD splenocytes. (A) BDC cells were transferred to NOD.scid mice alone in the presence of rat IgG (●, n = 8) or anti–PD-1 (◆, n = 12) or cotransferred with CD4$^+$ 24αβ NOD cells in the presence of rat IgG (○, n = 12) or anti–PD-1 mAb (◎, n = 12). (B) BDC2.5 cells were injected alone in the presence of rat IgG (●, n = 8), anti–PD-L1 (◆, n = 12), or anti–PD-L2 (▲, n = 12) or cotransferred with CD4$^+$ 24αβ NOD cells in the presence of rat IgG (○, n = 12), anti–PD-L1 (◇, n = 12), or anti–PD-L2 (♯, n = 12). (C) BDC cells (5 $\times$ 10$^5$) (●, n = 6) or 10$^4$ BDC cells were transferred in the presence of rat IgG (○, n = 6) or anti–PD-L1 (◇, n = 6). (D) BDC cells (1 $\times$ 10$^5$) were injected alone in the presence of rat IgG (○, n = 13) or anti–PD-L1 (◇, n = 10) or cotransferred with 24αβ cells in the presence of rat IgG (◆, n = 10) or anti–PD-L1 (♯, n = 6). (E) NOD.scid mice were transferred with BDC cells alone (●, n = 8) or cotransferred with CD4$^+$ 24αβ NOD splenocytes, and cotransferred mice were treated five times with rat IgG (○, n = 8) or anti–PD-L1 (◇, n = 8) (0.5 mg) every 3 d starting on day 10 (indicated by the gray area). The data shown are from one of three similar experiments (C) or a pool from two or three independent experiments.
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number, were not able to regulate disease. Differences in surface markers and cytokine production between CD4+ and DN type I NKT cells in humans and mice have previously been described, suggesting that CD4+ and DN NKT cells may have distinct functions in vivo (41–44); however, none of the studies above has identified features that underlie distinct roles of CD4+ and DN NKT cells in vivo. Our detailed analysis of CD4+ 24αβNOD type II NKT cells revealed the expression of cytokines and surface molecules that have previously been implied in the regulation of immunity by NKT and other cells. IL-4 and/or IL-10 were shown to mediate regulation of T1D after transfer of TCRαβ+ DN thy-mocytes, a population enriched for NKT cells, to young female NOD mice (14). In a different model, type II NKT cells were found to downregulate tumor immunity through the production of IL-13, which in turn induced the secretion of immunomodulatory TGF-β by GR-1+ cells (6). However, administration of blocking Abs to IL-4, IL-10, or both, or to IL-13 or TGF-β could not prevent 24αβNOD type II NKT cell regulation of BDC T cell-induced diabetes. Additionally, a CD4+ β2-microglobulin–dependent NOD T cell subset has been suggested to play a role in regulation diabetogenic BDC2.5 NOD CD4+ T cells (transferred to NOD.scid) via IFN-γ–dependent modulation of host APCs (45). In our model, blocking mAb to IFN-γ completely abolished BDC-induced disease, precluding investigation of the role of IFN-γ in 24αβ type II NKT cell regulation by this approach (data not shown). pDCs have been reported to be necessary for immune regulation by type I NKT cells in a model of virus-induced T1D (33). Comparing DC subsets in pancreatic lymph nodes after transfer of BDC cells alone or together with CD4+ 24αβNOD cells demonstrated a 2-fold increase of pDCs in cotransferred mice but not of other B cell subsets. However, depletion of pDCs in recipient NOD.scid mice before transfer of BDC cells did not alter diabetes development or 24αβ type II NKT cell regulation of the disease, demonstrating that pDCs were not required for either process. Furthermore, chemical blocking of IDO, a tryptophan-catabalizing enzyme associated with suppressive ability of pDCs, did not interfere with disease prevention.

Instead, we found that ICOS, highly expressed on CD4+ 24αβ type II NKT cells, and PD-1 were required for 24αβ type II NKT cell regulation of T1D induced by diabetogenic CD4+ BDC cells. Costimulatory molecules of the CD28 superfamily are central for the proper function of T cells, including regulatory cells. Previous studies demonstrated that ICOS is critical for the control of the nonaggressive pancreatitis infiltrate that accumulates in BDC2.5 transgenic NOD mice. Ab-mediated ICOS blockade or genetic deletion of ICOS in BDC2.5 NOD mice resulted in rapid precipitation of disease (48). We found that regulation of BDC cells by 24αβNOD type II NKT cells was abolished when PD-1 or PD-L1 was blocked by mAb. The blocking mAb reversed regulation by 24αβ type II NKT cells even when cotransferred with a low number (105) of BDC cells, a cell number at which the mAb block only marginally augmented the disease development when BDC cells were transferred alone. We therefore conclude that the PD-L1 mAb blocked immunosuppression by 24αβ type II NKT cells, and that the PD-1/PD-L1 pathway is a necessary component of the 24αβ type II NKT cell-mediated regulatory mechanism. The 24αβ type II NKT cells could potentially mediate PD-1/PD-L1–dependent regulation of BDC cells in different ways. Studies of BDC cells in NOD mice have shown that PD-L1 ligation of PD-1 on BDC cells interferes with TCR signaling, leading to interrupted T cell/DC interaction and aborted T cell activation in the lymph node (49). Thus, PD-L1 expressed on 24αβ type II NKT cells might prevent BDC cell activation by directly interacting with PD-1 on these cells. Furthermore, it was recently found that PD-1 can be expressed on APCs, and engagement of PD-1 on these cells reduced their ability to stimulate T cells through regulation of IL-12 production (50). A second option is therefore that PD-L1 on 24αβ type II NKT cells negatively regulates DCs through interaction with PD-1 on the DCs, leading to decreased activation of BDC cells. Alternatively, PD-1 on 24αβ type II NKT cells may render DCs tolerogenic through reverse signaling through PD-1 ligands on DCs (51). The role of PD-1/PD-L1 in T1D regulation could also be downstream of type II NKT cells. Interaction between 24αβ type II NKT cells and DCs may turn the DCs tolerogenic by increasing the levels of PD-L1 on the DCs, resulting in augmented ligation of PD-1 on BDC cells. Activated DCs upregulate PD-L1 levels, which are further increased upon exposure to IFN-γ (27), a factor abundantly produced by 24αβ type II NKT cells. Additionally, PD-L1 was found to play a role in the peripheral induction of Tregs by CD8+ DCs (40, 52), known to express high levels of CD1d. Therefore, 24αβ type II NKT cells might act through CD8+ DCs and regulate BDC cells by promoting their conversion to induced Tregs in a PD-L1–dependent manner. However, conversion to Tregs was shown to be dependent on TGF-β, and this factor was dispensable for 24αβ type II NKT cell regulation of BDC cells, making this scenario appear unlikely. Interestingly, the PD-1/PD-L1 pathway has also been implied in the suppressive function of Tregs (38–40), suggesting that Tregs and type II NKT cells may share the ability to suppress through this pathway.

In apparent contrast to our findings, PD-1 was found to restrain activation of type II NKT cells by tolerogenic DCs applied in the murine model for multiple sclerosis, experimental autoimmune encephalomyelitis (53). Even if the net outcome of PD-1/PD-L1 block is complete reversal of regulation in the T1D model presented in this study, it is possible that blocking PD-1 selectively on 24αβ type II NKT cells would enhance the regulatory potential of these cells, as they do express PD-1 at intermediate to high levels. If so, this would not be incompatible with our results suggesting...
NKT cells provide long-term protection against T1D by regulating the immunoregulatory potential of these cells in the development of 1D. Reports of the beneficial effects of type II NKT cells in other autoimmune diseases suggest that PD-L1 and ICOS/ICOS-L pathways. Taken together with other reports of the beneficial effects of type II NKT cells in other autoimmune disease models, our findings encourage further exploration of the immunoregulatory potential of these cells in the development of tolerization strategies to prevent or reduce autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

References


