Inhibition of T Cell Differentiation into Effectors by NKT Cells Requires Cell Contacts[1]

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NKT cells are potent regulatory T cells that prevent the development of several autoimmune diseases. Analysis of NKT cell regulatory function in the NOD mouse has revealed that NKT cells inhibit the development of type 1 diabetes by impairing the differentiation of anti-islet T cells into Th1 effector cells. In the present study, we have performed in vitro and in vivo experiments to determine the respective role of cytokines and cell contacts in the blockade of T cell differentiation by NKT cells. These experiments reveal that cytokines such as IL-4, IL-10, IL-13, and TGF-β, that have been involved in other functions of NKT cells, play only a minor role if any in the blockade of T cell differentiation by NKT cells. Diabetes is still prevented by NKT cells in the absence of functional IL-4, IL-10, IL-13, and TGF-β. In contrast, we show for the first time that cell contacts are crucial for the immunoregulatory function of NKT cells. ***The Journal of Immunology,* 2005, 174: 1954–1961.**

Natural killer T cells are unconventional T cells that have been conserved through mammalian evolution. NKT cells express several surface markers of the NK lineage and are restricted by a MHC class I-like molecule, CD1d, that presents self and exogenous glycolipid Ags (1–3). Most NKT cells express a highly biased $\alpha\beta$TCR using a canonical TCRα chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) and a restricted set of Vβ genes (4). Although the natural ligand of NKT cells is not known, α-galactosylceramide (α-GalCer)[3] isolated from marine sponges is specifically recognized by both human and mouse NKT cells (5, 6). Upon activation via their TCR with anti-CD3 mAb or α-GalCer, NKT cells release massive amounts of cytokines, including IFN-γ and IL-4 (7).

Many studies have shown a link between NKT cells and autoimmunity (8). Quantitative and qualitative defects of the NKT cell population have been reported in patients with type 1 diabetes (9, 10), lupus, and multiple sclerosis (11–13). Similarly, NOD mice, that spontaneously develop type 1 diabetes and are highly susceptible to experimental autoimmune encephalomyelitis, have subnormal numbers of NKT cells, which show subnormal cytokine production on activation (14–16). Mice can be protected against these autoimmune diseases either by increasing the number of NKT cells by transgenesis (Vα14-Jα281 transgenic mice) or transfer, or by activating NKT cells with their ligand α-GalCer (17–23). Other studies have shown an immunoregulatory role of NKT cells. For example, NKT cells have been implicated in tolerance induction in anterior chamber-associated immune deviation (24) and in the inhibition of antitumoral immune responses (25).

Previous studies have shown that cytokines play an important role in the immunoregulatory functions of NKT cells. Protection against type 1 diabetes induced by diabetogenic T cell transfer or cyclophosphamide treatment requires the presence of IL-4 and/or IL-10 (19, 26). Also, NKT cell activation by specific ligands such as α-GalCer can prevent experimental autoimmune encephalomyelitis in an IL-4-dependent fashion. NKT cell-mediated protection was associated with Th2 responses in these studies (22, 23, 27). Both autoimmune diseases are associated with Th1-autoreactive T cells, and IL-4, which induces a shift toward a Th2 profile, is protective (28, 29). Tolerance induction after soluble Ag injection in the anterior chamber of the eye is also associated with a shift toward a Th2 state and is dependent on IL-10 production by NKT cells (30). NKT cell blockade of CD8 responses to tumor cells is dependent on IL-13 secreted by NKT cells and on the subsequent production of TGF-β by granulocytes (31).

We recently showed that NKT cells could prevent type1 diabetes without inducing a Th2 shift of autoreactive T cells. When naive autoreactive anti-islet CD4$^+$ T cells were transferred into recipient mice containing a large number of NKT cells, these autoreactive T cells were primed in pancreatic lymph nodes but did not differentiate into Th1 effector cells. Instead, they become anergic and were unable to induce diabetes (32). This suggests that IL-4 release might not be the only mechanism by which NKT cells induce tolerance. We therefore examined the role of IL-4 and other cytokines known to play a role in the immunoregulatory functions of NKT cells in the blockade of T cell differentiation. We also analyzed the role of cell contacts, since other regulatory T cells (CD4$^+$ CD25$^+$ T cells) require such contacts to exert their immunosuppressive effect in vitro (33–35). We took advantage of our previously described in vitro and in vivo systems in which NKT cells inhibit the differentiation of naive autoreactive T cells into Th1 effector cells (32). We found that cell contacts were required and that cytokines, including IL-4, appear to play little, if any, role.

Materials and Methods

Mice

The Vα14-Jα281 (Vα14) C$\alpha^{-/-}$ and Vα8-Jα37 (Vα8) C$\alpha^{-/-}$ transgenic NOD lines and Thy1.1$^+$BDC2.5 C$\alpha^{-/-}$ NOD mice have been described elsewhere (17, 26, 32, 36, 37). Vα14 IL-4$^{-/-}$C$\alpha^{-/-}$ NOD mice were generated by backcross onto IL-4$^{-/-}$ NOD (38) (kindly donated by D. Serreze, The Jackson Laboratory, Bar Harbor, ME). β2m$^{-/-}$ NOD mice were kindly provided by C. Benoist and D. Mathis (Harvard Medical School,

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[3] Abbreviations used in this paper: α-GalCer,α-galactosylceramide; β-GluCer, β-glucosylceramide.

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Boston, MA) (39). All of the mice used in this study were raised and housed in strictly controlled specific pathogen-free conditions.

BDC2.5 cell preparation and sorting

BDC2.5 T cells were obtained from the spleens of Thy1.1$^+$BDC2.5 C$\alpha^{-/-}$ NOD mice at 5–7 wk of age. Splenocytes were pooled from at least two mice. RBC were lysed with NH_4Cl and B lymphocytes were removed by using sheep anti-mouse IgG-coated beads (Dynal). CD62L$^+$ splenocytes were positively selected with biotinylated anti-CD62L mAb (Mel-14) and streptavidin microbeads (Miltenyi Biotec). Cells were then stained with anti-CD5 mAb (53-7.3; BD Pharmingen) and anti-CD62L (Mel-14; BD Pharmingen) and sorted with a FACSVantage cell sorter (BD Biosciences). The purity of sorted cells was between 97 and 99%.

Preparation and sorting of NKT and control T cell populations

NKT cells were obtained from Vα14C$\alpha^{-/-}$ NOD mice or Vα14IL-4$^{-/-}$C$\alpha^{-/-}$ NOD mice at 6–8 wk of age. Cells from spleens and pancreatic and mesenteric lymph nodes were pooled from at least two mice. After removal of RBC and B lymphocytes, cells were stained with anti-CD5 mAb and CD5$^+$ cells were sorted with a FACSVantage sorter. Control T cell populations used in several experiments were obtained from Vα8C$\alpha^{-/-}$ NOD mice, NOD mice, and β2m$^{-/-}$ NOD mice and were prepared and sorted as described for NKT cells. The purity of sorted CD5$^+$ T cells was between 97 and 99%. The NKT cell population corresponding to CD5$^+$ cells from Vα14C$\alpha^{-/-}$ transgenic mice contained ~70% of CD1-α-GalCer tetramer-positive cells.

In vitro culture

Sorted CD62L$^+$ BDC2.5 T cells (5×10^4/well) were incubated with 5×10^4 APC/well (obtained from the peritoneal cavity of C$\alpha^{-/-}$ NOD mice and irradiated with 3000 rad) in complete IMDM for 72–120 h at 37°C. In some experiments, APC were obtained from IL-4$^{-/-}$C$\alpha^{-/-}$ NOD mice. Cells were incubated with 10 U/ml recombinant mouse IL-2 and with or without 10 ng/ml peptide 1040–51 (40). NKT cells or control T cell populations (2×10^5/well) were added to some wells at the beginning of the culture period. Transwells with 0.2-μm Anopore membranes in 96-well plates (NuncBrand Products) were used in several experiments. In these experiments, BDC2.5 T cells (2.5×10^4/well) and APC (2.5×10^4/well) were incubated in the lower chamber and NKT cells (10^5/well) and APC (2.5×10^4/well) were incubated either in the lower chamber or in the upper chamber. The upper chamber was removed after 96–120 h of culture and BDC2.5 T cells were analyzed.

Abs and reagents

In some experiments, cytokine- and cytokine receptor-blocking mAbs were added to the cultures at a concentration of 100 μg/ml, or were injected i.v. into recipient mice (0.5 mg/mouse) on days 0, 3, 6, and 10. Blocking anti-IL-4 mAb (11B11) (41), blocking anti-IL-10R mAb (1B1) (42), and blocking anti-TGF-β mAb (2G.7) (43) were purified from ascites. IL-13E13K was added to cultured cells at 1 μg/ml or injected i.p. (3 μg/mouse) twice daily for 7 days. IL13E13K, a powerful IL-13 antagonist, in which the glutamic acid at position 13 is replaced by a lysine, was expressed and purified as previously described (44). The efficiency of each cytokine-blocking reagent was confirmed by in vitro or in vivo assays. Anti-IL-4 mAb was tested by ELISA. IL-13K13K was tested using the IL-13-dependent cell line TF-1 (44). The efficiency of anti-IL-10 receptor and anti-TGF-β mAbs was confirmed by their ability to induced colitis after in vivo treatment. Synthetic α-GalCer, β-glucosylceramide (β-GluCer), either purified from human sources (Matreya 1057), or synthetic PBS35 were used at 50 ng/ml in some in vitro experiments.

Flow cytometry

Cells were stained at 4°C in PBS containing 5% FCS and 0.1% azide after FcγR blockade with the anti-FcγR mAb 2.4G2. Surface staining was performed with biotinylated anti-Thy-1.1 mAb (HO22.1) plus streptavidin-allophycocyanin, PerCP-conjugated anti-CD4 mAb (RM4-5), and PE-conjugated anti-CD69 mAb, anti-CD25 mAb, or anti-CD44mAb. All reagents were obtained from BD Pharmingen. To analyze in vitro proliferation, sorted BDC2.5 T cells were incubated with 5 μM CFSE (Molecular Probes) diluted in PBS-0.5% BSA before culture. To analyze cytokine production, BDC2.5 T cells were incubated for 4 h with PMA (100 ng/ml) plus ionomycin (500 ng/ml) and brefeldin A (10 μg/ml). Intracytoplasmic staining was performed as previously described (32, 36) using the following PE-conjugated mAbs: anti-mouse IL-2 (JES6-5H4; BD Pharmingen) anti-mouse IFN-γ (XMG1.2; BD Pharmingen), anti-mouse IL-4 (BVD4-1D11; BD Pharmingen), and anti-mouse IL-10 (JES5-16E3; BD Pharmingen). Cells were analyzed with a FACSCalibur device (BD Biosciences) and CellQuest software.

In vivo transfer, diagnosis of diabetes, and pancreatic histology

In vivo transfer of purified CD62L$^+$ BDC2.5 T cells was performed as previously described (32). Purified CD62L$^+$ BDC2.5 T cells (2.5×10^5) were injected i.v. into 6- to 7-wk-old C$\alpha^{-/-}$ NOD or Vα14C$\alpha^{-/-}$ NOD recipient mice. In some experiments, the recipient mice were IL-4$^{-/-}$. The mice were tested daily (after day 5) for diabetes onset using Glukotest and Hemoglukotest kits (Boehringer, Mannheim). Insulitis was evaluated on 4-μm-thick pancreas sections. At least 40 islets per mouse were scored. Peri-insulitis was recorded when islets were surrounded by inflammatory cells, and insulitis was recorded when islets were invaded by inflammatory cells.

Statistical analysis

Statistical analysis was based on Student's unpaired two-tailed test, with Prism software.

Results

NKT cells inhibit BDC2.5 T cell proliferation and differentiation

BDC2.5 T cells specifically recognize pancreatic β cell peptide presented by IA^{g7}, and these CD4$^+$ T cells are able to induce diabetes in NOD-background recipient mice (37, 45). We have recently shown that the presence of NKT cells inhibits diabetes induction by BDC2.5 T cells due to blockade of BDC2.5 differentiation into pathogenic Th1 cells (32). To determine the respective roles of cytokines and cell contacts in NKT cell-mediated immunoregulation, we set up an in vitro model using BDC2.5 T cells. CD62L$^+$ BDC2.5 T cells were stimulated in vitro with a specific peptide (1040–51) in the presence of APC and rIL- 2. Phenotypic and functional analysis showed that BDC2.5 T cells were strongly activated in these conditions (Fig. 1 and Table I). On day 3, up to 60% of BDC2.5 T cells had acquired activation markers such as CD69, CD25, and CD44^{high} and had up-regulated Thy1.1 and CD4 (Fig. 1*A*). On day 5, most BDC2.5 T cells had divided, and ~80 and 50% of them produced IL-2 and IFN-γ, respectively (Fig. 1*B*). The addition of NKT cells to the culture inhibited BDC2.5 T cell activation and differentiation. On day 3, acquisition of activation markers by BDC2.5 T cells and CD4 and Thy1.1 up-regulation were less pronounced in the presence of NKT cells. This inhibition ranged from 20 to 40% according to the marker. NKT cells inhibited 40 and 70%, respectively, of IL-2 and IFN-γ production by BDC2.5 T cells. However, BDC2.5 T cell proliferation was not affected by the presence of NKT cells. Similarly, IL-4 and IL-10 production, that is low in BDC2.5 T cells, was not modified by NKT cells. The in vitro inhibition of BDC2.5 T cell differentiation by NKT cells was reminiscent of our previous observations in vivo (32). To further analyze the effect of NKT cells on BDC2.5 T cell activation, α-GalCer, the specific ligand of NKT cells, was added to the culture. NKT cells thus activated were even more potent in blocking BDC2.5 T cell activation. CD4 and Thy1.1 up-regulation was totally blocked, and acquisition of activation markers (except for CD69) was strongly inhibited. BDC2.5 T cell proliferation was totally blocked and their cytokine production remained at background levels, as observed in the absence of peptide 1040–51. In contrast, addition of β-GluCer did not influence the effect of NKT cells on BDC2.5 T cell responses (Fig. 1*C*). These results confirm the immunoregulatory role of NKT cells and show that activation by α-GalCer increases their immunosuppressive activity.

Specificity and efficacy of NKT cell inhibitory activity

To confirm the specificity of the observed suppressive activity of nonstimulated NKT cells, BDC2.5 T cells were stimulated with peptide 1040–51 in the presence of other T cell populations (Table

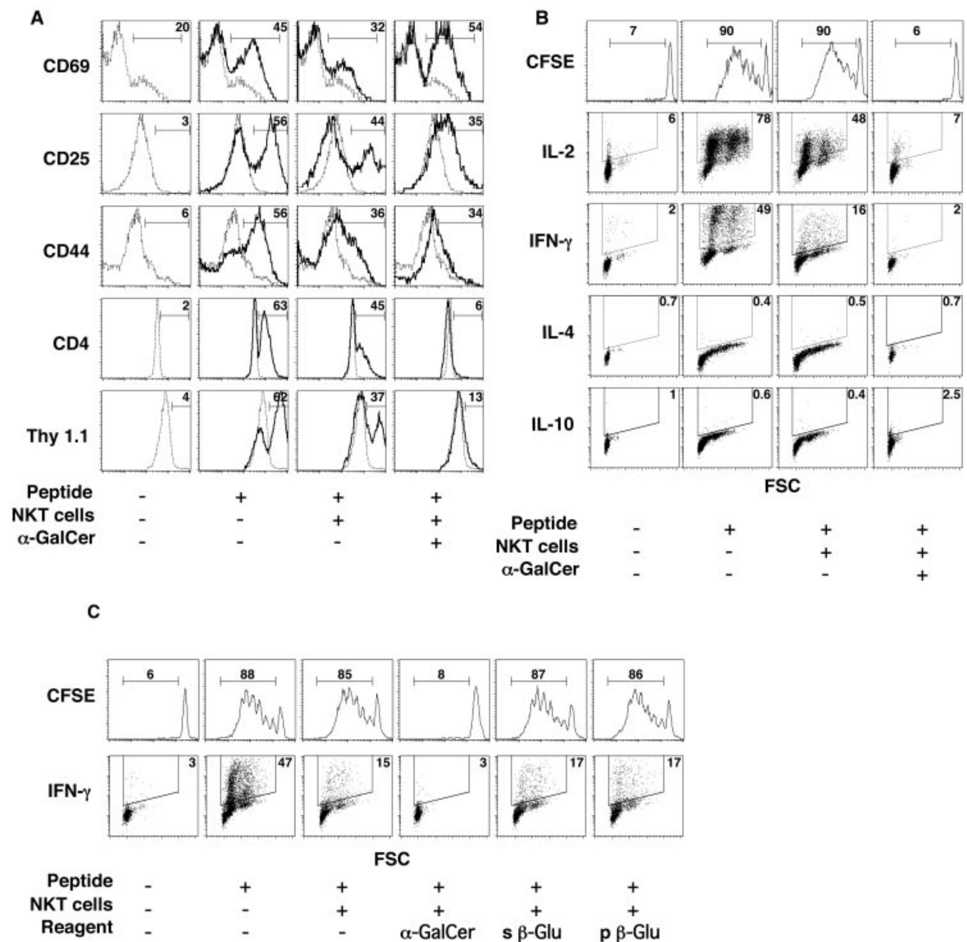

FIGURE 1. NKT cells inhibit BDC2.5 T cell activation, proliferation, and cytokine production. Sorted $CD62L^+$ BDC2.5 T cells were incubated in 96-well plates in the presence of their specific peptide (peptide 1040–51), APC, and rIL-2. NKT cells were added to some wells, with or without α-GalCer. In some experiments, β-GluCer, either purified or synthetic, was used as negative controls (purified (p) and synthetic (s) β-Glu). Histograms and dot plots correspond to BDC2.5 T cells ($CD4^+Thy1.1^+$). *A,* The phenotype was analyzed on day 3. Values shown are percentages of positive BDC2.5 T cells. *B* and *C,* Proliferation was measured by CFSE dilution on day 5. Values are the percentages of recovered BDC2.5 T cells that had divided. IL-2, IFN-γ, IL-4, and IL-10 production was detected by intracytoplasmic staining on day 5. Values are the percentages of BDC2.5 T cells producing cytokines. Similar data were repeatedly obtained.

I). NKT cells were obtained from $V\alpha14C\alpha^{-/-}$ NOD mice. A control T cell population was obtained from $V\alpha8C\alpha^{-/-}$ NOD mice, another α-chain-transgenic line that is not associated with NKT cells and that we have previously characterized (32). Control T cells were also obtained from wild-type NOD mice and from $\beta2m^{-/-}$ NOD mice. IL-2 and IFN-γ production by BDC2.5 T cells was similar in the presence and absence of control T cell populations. In striking contrast, NKT cells inhibited both IL-2 and IFN-γ production by BDC2.5 T cells. Thus, the suppression of BDC2.5 T cell differentiation was specifically mediated by NKT cells.

Having established that NKT cells inhibit BDC2.5 T cell differentiation in vitro, we examined their inhibitory activity on BDC2.5 T cells at various NKT cell ratios. Constant numbers of BDC2.5 T cells were incubated with various numbers of NKT cells, and IFN-γ production by BDC2.5 T cells was measured on day 5 (Fig. 2). IFN-γ production was inhibited by a NKT cell to BDC2.5 T cell ratio as low as one. Because NKT cells activated by α-GalCer also blocked the proliferation of BDC2.5 T cells, we then tested the effect of activated NKT cells on both BDC2.5 T cell proliferation and IFN-γ production. Activated NKT cells still efficiently inhibited BDC2.5 T cell division and IFN-γ production at the ratio of one NKT cell to eight BDC2.5 T cells. These ratios are similar to those reported with other regulatory T cells (33–35).

Role of cytokines in NKT cell-mediated immunosuppression

Because NKT cells can produce large amounts of cytokines with immunosuppressive properties, such as IL-4, IL-10, IL-13, and TGF-β, we examined the possible role of these cytokines in the inhibition of BDC2.5 T cell differentiation by NKT cells (Fig. 3). Anti-IL-4, anti-TGF-β, and anti-IL-10 receptor mAbs and an IL-13 antagonist were added to BDC2.5 T cell cultures containing NKT cells. When NKT cells were stimulated with α-GalCer, none of the cytokine-blocking reagents abolished NKT cell immunosuppression of BDC2.5 T cells, even when all four cytokine-blocking reagents were added together; CD25 expression, cell division, and IFN-γ production remained at background levels (Fig. 3, *A–C*). In the absence of α-GalCer, CD25 expression on BDC2.5 T cells was still inhibited by NKT cells when cytokine-blocking reagents were added (Fig. 3*D*). Interestingly, anti-IL-4 mAb attenuated the inhibitory effect of nonstimulated NKT cells on the production of IFN-γ by BDC2.5 T cells. The other blocking reagents

Table I. *Inhibitory activity is specific of NKT cells*[a]

Added T cells	1040–51 Peptide	% of BDC2.5 T Cells Producing	
		IL-2	IFN-γ
−	−	7.1 ± 1.01	3.2 ± 0.29
−	+	80.9 ± 1.13	38.3 ± 1.80
Vα14	+	41.9 ± 2.05	12.8 ± 1.03
Vα8	+	67.7 ± 2.10	41.1 ± 1.45
NOD	+	73.8 ± 1.00	37.1 ± 2.25
$\beta2M^{-/-}$	+	74.2 ± 4.55	38.3 ± 2.60

[a] $CD62L^+$ BDC2.5 T cells were incubated with 1040–51 peptide, APC, and rIL-2. T cells from various mouse strains were added to the culture and IL-2 and IFN-γ production by BDC2.5 T cells was detected by intracytoplasmic staining on day 5. Values are the average of triplicates. Similar data were obtained in three independent experiments.

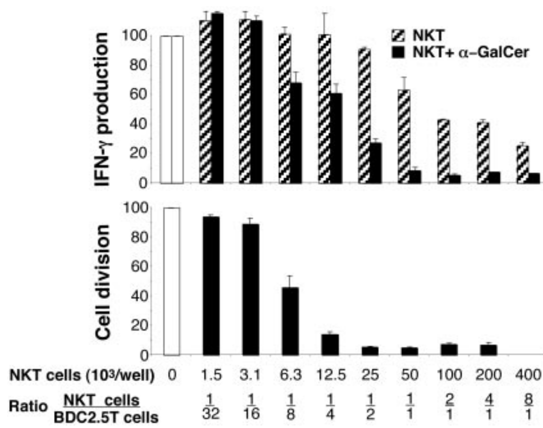

FIGURE 2. Inhibition of BDC2.5 cell differentiation by various NKT cell ratios. Sorted CD62L$^+$ BDC2.5 T cells were incubated in 96-well plates for 5 days in the presence of their specific peptide (peptide 1040–51), APC, and rIL-2. Variable numbers of NKT cells (from 1.5×10^3 to 4×10^5) were added to the wells. α-GalCer was added (■) or not added (▨). IFN-γ production by BDC2.5 T cells was detected on day 5 by intracytoplasmic staining. Proliferation of BDC2.5 T cells was measured by CFSE dilution. Values are percentages of IFN-γ-producing cells or divided cells, respectively, 100% corresponding to the data obtained with BDC2.5 T cells cultured without NKT cells. The ratio of NKT cell numbers to BDC2.5 T cell numbers is indicated. Values correspond to the average of triplicates, and similar data were obtained in two independent experiments.

had no effect in similar cultures (Fig. 3*E*). The role of IL-4 was confirmed by using NKT cells and APC from IL-4$^{-/-}$ mice (Fig. 3*F*). IL-4 produced by both NKT cells and APC was involved in the inhibition of BDC2.5 T cell differentiation by NKT cells. Together, these results suggested that only IL-4 had a significant role in the immunosuppression mediated by nonactivated NKT cells and that none of the cytokines tested was necessary for the immunosuppressive activity of α-GalCer-activated NKT cells.

Cell contacts are required for NKT cell-mediated immunosuppression

We performed Transwell experiments to examine the role of cell contacts in the immunosuppression mediated by NKT cells (Fig. 4). We first analyzed the inhibitory activity of NKT cells in the absence of α-GalCer (Fig. 4*A*). In the presence of peptide 1040–51 and APC, 45% of BDC2.5 T cells produced IFN-γ; the addition of NKT cells to the lower chamber, along with BDC2.5 T cells, reduced IFN-γ production to 16%. When NKT cells and BDC2.5 T cells were separated by a membrane, in experiments in which APC were present in both the upper and lower chambers, 33% of BDC2.5 T cells produced IFN-γ. The histograms shown in Fig. 4*A*, summarizing the results several experiments, show that cell contacts were indeed required for optimal NKT cell inhibitory activity. In other experiments, NKT cells were stimulated with α-GalCer (Fig. 4, *B* and *C*). These activated NKT cells totally blocked BDC2.5 T cell proliferation and IFN-γ production in experiments in which BDC2.5 T cells, NKT cells, and APC were all present in the lower chamber. When activated NKT cells and APC were placed in the upper chamber and BDC2.5 T cells and APC were placed in the lower chamber, NKT cells had no effect on BDC2.5 T cell proliferation (89% of dividing BDC2.5 T cells in the absence of NKT cells and 85% when NKT cells were in the upper chamber). The presence of NKT cells in the lower chamber, along with BDC2.5 T cells and APC, was also required to suppress IFN-γ production by BDC2.5 T cells. The residual inhibitory effect

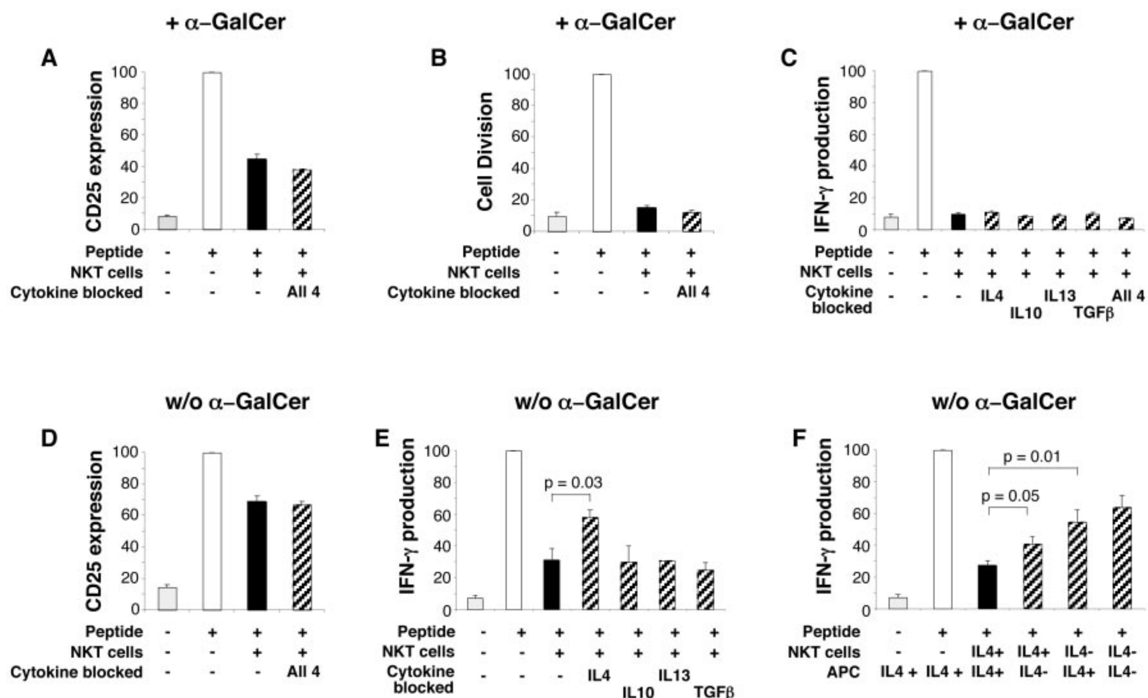

FIGURE 3. Role of cytokines in NKT cell-mediated inhibition. CD62L$^+$ BDC2.5 T cells were incubated with 1040–51 peptide, APC, and rIL-2. In some wells, NKT cells and α-GalCer were added as indicated. *A* and *D,* CD25 expression by BDC2.5 T cells was analyzed on day 3. *B,* Proliferation of BDC2.5 T cells was measured by CFSE dilution on day 5. *C, E,* and *F,* IFN-γ production by BDC2.5 T cells was detected by intracytoplasmic staining on day 5. *A–E,* NKT cells and various cytokine-blocking reagents were added to the culture. IL-4, IL-10, IL-13, and TGF-β activities were either inhibited separately or all together (all four). *F,* NKT cells and APC obtained from IL-4$^{+/+}$ or IL-4$^{-/-}$ mice were added to the culture. The data are the average of two to four independent experiments. Values are percentages of BDC2.5 T cell responses, 100% corresponding to BDC2.5 T cell results when they were cultured with peptide in the absence of NKT cells.

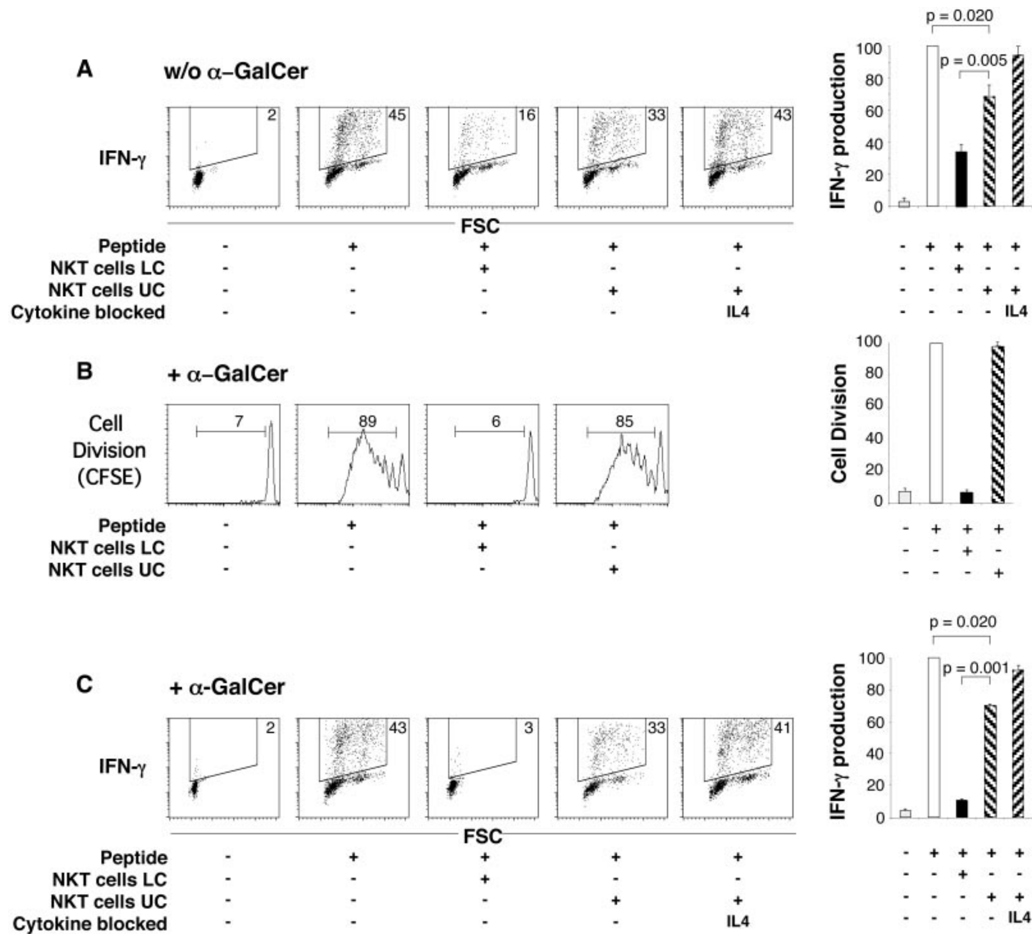

FIGURE 4. Cell contacts are required for NKT cell-mediated inhibition. Sorted CD62L$^+$ BDC2.5 T cells were incubated in the lower chamber (LC) of Transwells in the presence of peptide 1040–51, APC, and rIL-2. NKT cells were added to the lower chamber or to the upper chamber (UC). On day 5, IFN-γ production was detected by intracytoplasmic staining and cell division was measured by CFSE dilution. Dot plots and histograms correspond to BDC2.5 T cells (CD4$^+$Thy1.1$^+$). *A* and *C,* Values in the dot plots are the percentages of BDC2.5 T cells producing IFN-γ. *B,* Values are the percentages of recovered BDC2.5 T cells that had divided. Each graph on the *right* corresponds to the average of three independent experiments. *B* and *C,* α-GalCer was added to the culture.

of NKT cells, observed when cell contacts were prevented in Transwell culture, was due to IL-4, as it was blocked by anti-IL-4 mAb. Together, these data clearly showed that contacts between NKT cells and BDC2.5 T cells and/or their stimulating APC were crucial for the immunosuppressive effect of NKT cells.

IL-4, IL-10, IL-13, and TGF-β are not required for NKT cell inhibition of BDC2.5 T cell differentiation in vivo

In vitro experiments suggested that IL-10, IL-13, and TGF-β were not required for the immunosuppressive activity of NKT cells activated by α-GalCer and that only IL-4 was involved when NKT cells were not activated by α-GalCer and when cell contacts were prevented. We then examined the role of these cytokines in vivo. We have previously reported that NKT cells prevent diabetes induced by BDC2.5 T cell transfer (32). Indeed, Vα14C$\alpha^{-/-}$ recipient mice, which contain abundant NKT cells, were protected against diabetes onset, contrary to recipient mice lacking NKT cells (Table II and Ref. 32). In this study, we found that BDC2.5 T cells still protected Vα14 $\alpha^{-/-}$ recipient mice from diabetes when IL-4, IL-10, IL-13, and TGF-β were absent or inhibited (Table II). We also graded insulitis and examined BDC2.5 T cell expansion and differentiation in this model, since we had previously observed that NKT cells inhibited the expansion and differentiation of diabetogenic BDC2.5 T cells and attenuated insulitis (32). As shown in Fig. 5, the absence of functional IL-4, IL-10, IL-13, and TGF-β had little influence on the inhibitory effect of NKT cells on BDC2.5 T cells. The frequency and absolute number of BDC2.5 T cells remained low in all recipient mice containing NKT cells. Analysis of IFN-γ production by BDC2.5 T cells confirmed that these cytokines were not required for the inhibitory effect of NKT cells. Similarly, severe insulitis was still prevented in all recipient mice containing

Table II. *Incidence of diabetes in recipient mice treated with cytokine-blocking agents*[a]

Recipients	Cytokine Blocked				
	None	IL-10	TGF-β	IL-10, TGF-β	IL-13
C$\alpha^{-/-}$	7/7	4/4	—	—	—
C$\alpha^{-/-}$IL4$^{-/-}$	7/7	4/4	4/4	4/4	—
Vα14C$\alpha^{-/-}$	1/7	1/8	—	—	0/6
Vα14C$\alpha^{-/-}$ IL4$^{-/-}$	0/9	1/9	0/6	0/6	—

[a] CD62L$^+$ BDC2.5 T cells were injected into recipient mice containing (Vα14C$\alpha^{-/-}$) or not containing (C$\alpha^{-/-}$) NKT cells and producing or not producing IL-4. Recipient mice were treated with various cytokine inhibitors and monitored daily for signs of diabetes. Values are the incidence of diabetes 20 days after BDC2.5 T cell transfer.

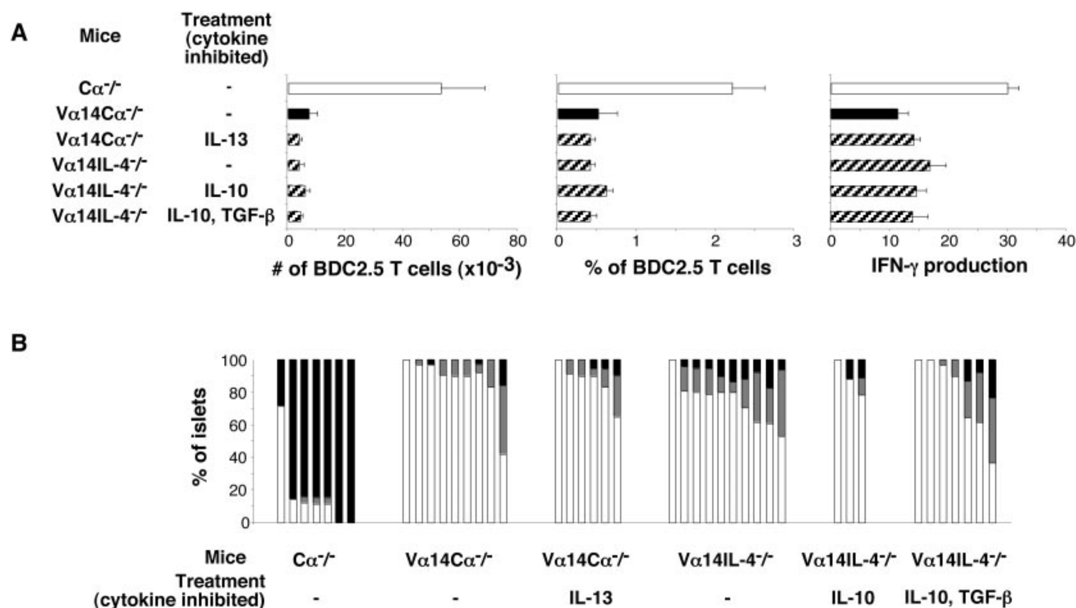

FIGURE 5. Role of cytokines in BDC2.5 T cell inhibition by NKT cells in vivo. $CD62L^+$ BDC2.5 T cells were injected into recipient mice containing ($V\alpha14C\alpha^{-/-}$) or not containing ($C\alpha^{-/-}$) NKT cells. Recipient mice were treated with various cytokine-blocking reagents. On day 15 after BDC2.5 T cell transfer, mice were killed and lymphoid organs were analyzed. *A,* Absolute numbers and percentages of BDC2.5 T cells and their IFN-γ production were determined by immunofluorescence staining. *B,* Pancreatic histology was performed on days 15–20 for the six groups of mice. Insulitis was scored as described in *Materials and Methods*. Each recipient mouse corresponds to a bar.

NKT cells when IL-4, IL-10, IL-13, and TGF-β were absent or inhibited. These experiments demonstrated that none of the four cytokines previously suggested to mediate NKT cell suppressive activity was necessary for the inhibitory effect on BDC2.5 T cells in vivo.

Discussion

This study shows that cell contacts are required for the inhibitory effects of NKT cells and suggests that cytokines are not always the principal mediators of these actions. The key role of cell contacts was observed in CD4 BDC2.5 T cell cultures with peptide 1040–51 and NKT cells, in which NKT cells strongly inhibited the differentiation of naive BDC2.5 T cells into Th1 effector cells. This inhibitory effect observed in vitro is very similar to what is observed in vivo when naive BDC2.5 T cells are primed in the pancreatic lymph nodes of $V\alpha14C\alpha^{-/-}$ NOD mice containing large numbers of NKT cells (32, 36). Both in vitro and in vivo, the presence of NKT cells during naive BDC2.5 T cell priming inhibits its BDC2.5 T cell IL-2 and IFN-γ production and does not induce them to shift toward a Th2 profile. Indeed, IL-4 and IL-10 production by BDC2.5 T cells remained very low (Ref. 32 and Fig. 1). In vivo and in vitro, in the absence of α-GalCer, NKT cells do not influence the early proliferative response of BDC2.5 T cells. The immunoregulatory effect of NKT cells does not seem to involve apoptosis of BDC2.5 T cells, since the percentage of 7-aminoactinomycin D^-annexin V^+ BDC2.5 T cells was not affected by the presence of NKT cells (data not shown). Importantly, the observed inhibition of BDC2.5 T cell differentiation in vitro was specific to NKT cells, as it was not observed with several other T cell populations. All of these observations suggest that NKT cells exhibit similar inhibitory effects in vitro and in vivo. Moreover, NKT cell suppressive effect was observed when they were added at the initiation of the culture but not when added at day 3 (data not shown). This is reminiscent of our previous observation in vivo that NKT were not able to prevent diabetes induced by already differentiated BDC2.5 T cells (32). Interestingly, α-GalCer addition to cultures markedly up-regulated the inhibitory effect of NKT cells, as BDC2.5 T cell proliferation and cytokine production remained at the background level observed in the absence of peptide 1040–51. However, BDC2.5 T cell expression of activation markers such as CD69 and CD25 was still up-regulated in these conditions.

In vitro systems can unambiguously demonstrate the role of cell contacts. When NKT cells were separated from BDC2.5 T cells in Transwell culture, the inhibitory effect of NKT cells was largely or totally abolished. With regard to IFN-γ production, cell contacts could account for 65–80% of the inhibitory effect of NKT cells in the absence or presence of α-GalCer, respectively. Interestingly, in the absence of both cell contact and IL-4, the inhibitory effect of NKT cells on IFN-γ production by BDC2.5 T cells was fully abolished. The inhibition of BDC2.5 T cell proliferation by α-GalCer-activated NKT cells was completely dependent on cell contact. APC were present in both the upper wells containing NKT cells and the lower wells containing BDC2.5 T cells and their specific peptide. It remains to be determined what cell types are involved in these cell contacts, namely, NKT cells with BDC2.5 T cells or NKT cells with APC? Interestingly, cell contacts are also required for immunoregulation by $CD4^+CD25^+$ regulatory T cells (33–35). The molecules involved in these cell contacts have not yet been identified. Several molecules could interfere with the induction of T cell tolerance, such as CTLA-4 (46), OX-40 (47), and membrane-bound TGF-β (48). As previously reported for $CD4^+CD25^+$ cells (33, 49), none of these molecules seems to be involved in immunoregulation by NKT cells in vitro (data not shown). Addition of a blocking anti-CTLA-4 mAb, an activating anti-OX40 mAb, or a blocking anti-TGF-β mAb had no influence on the inhibition of BDC2.5 T cell differentiation by NKT cells. The possible involvement of CD1d in contacts between NKT cells and APC or BDC2.5 T cells is currently under investigation.

The second finding of this study is that IL-4, IL-10, IL-13, and TGF-β are not required for the inhibition of BDC2.5 T cell differentiation by NKT cells in vivo, contrary to previous suggestions. IL-4, IL-10, and IL-13 can be produced by NKT cells (7, 23, 25, 30), whereas TGF-β production is indirectly induced by NKT

cells (31). These four cytokines can also inhibit the differentiation of Th1 cells. However, in vivo blockade of BDC2.5 T cell expansion and differentiation did not require any of these cytokines. Only in two in vitro experimental settings was IL-4 required to obtain full NKT cell inhibitory activity, namely, in the absence of α-GalCer or in the absence of cell contacts. These observations suggest that IL-4 might not be required in vivo, where cell contacts can occur and NKT cells are activated during the immune response induced by BDC2.5 T cells. Indeed, after BDC2.5 T cell transfer to Vα14C$\alpha^{-/-}$ NOD mouse recipients, NKT cells up-regulate CD69 and down-regulate their TCR, reflecting their activation (L. Beaudoin, J. Novak, and A. Lehuen, manuscript in preparation). It is also interesting to note that, in the in vitro experiment showing a role of IL-4, exogenous IL-4 did not mimic the effect of NKT cells. When rIL-4 was added into the culture instead of NKT cells, the concentration of rIL-4 required to inhibit IFN-γ production by BDC2.5 T cells induced massive IL-4 production by BDC2.5 T cells; this was never observed when NKT cells were present in the culture (data not shown). Together, our data show that IL-4 is not a key player in the inhibition of BDC2.5 T cell differentiation by NKT cells. The difference in IL-4 requirement for diabetes prevention by NKT cells between ours and previous studies could relate to the different models of type 1 diabetes induction, either by naive transgenic BDC2.5 T cells or by cyclophosphamide treatment of NOD mice and by transfer of primed diabetogenic T cells from NOD mice. Interestingly, this differential involvement of IL-4 in the inhibitory effect of NKT cells could be related to the functional status of anti-islet T cells. In this study, we found that NKT cells inhibited the differentiation of naive T cells; in contrast, in both previous studies showing a role of IL-4, NKT cells prevented the onset of diabetes induced by preprimed T cells in 10-wk-old NOD mice treated with cyclophosphamide (26), and in immunoincompetent recipient mice transferred with diabetogenic T cells (19).

Our findings thus suggest that NKT cells can prevent diabetes by hindering the differentiation of naive anti-islet T cells into Th1 effectors. Previous studies on the immunoregulatory functions of NKT cells have emphasized a key role of cytokines. Depending on the experimental model, IL-4, IL-10, IL-13, and TGF-β appeared to mediate the immunoregulatory activity of NKT cells. Our findings clearly show that none of these four immunoregulatory cytokines is required for the inhibition of BDC2.5 T cell differentiation by NKT cells in vivo or for the prevention of diabetes induced by BDC2.5 T cells. In contrast, we show for the first time that cell contacts are crucial for the inhibitory function of NKT cells. The molecule(s) involved in these contacts could represent targets for treatments designed to control NKT cell functions.

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