

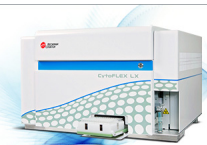


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Expression of CD1d Molecules by Human Schwann Cells and Potential Interactions with Immunoregulatory Invariant NK T Cells¹

Jin S. Im,* Nikos Tapinos,[‡] Gue-Tae Chae,[§] Petr A. Illarionov,[¶] Gurdyal S. Besra,[¶] George H. DeVries,^{||} Robert L. Modlin,^{**} Peter A. Sieling,[#] Anura Rambukkana,[‡] and Steven A. Porcelli^{2*†}

CD1d-restricted NKT cells expressing invariant TCR α -chains (iNKT cells) produce both proinflammatory and anti-inflammatory cytokines rapidly upon activation, and are believed to play an important role in both host defense and immunoregulation. To address the potential implications of iNKT cell responses for infectious or inflammatory diseases of the nervous system, we investigated the expression of CD1d in human peripheral nerve. We found that CD1d was expressed on the surface of Schwann cells in situ and on primary or immortalized Schwann cell lines in culture. Schwann cells activated iNKT cells in a CD1d-dependent manner in the presence of α -galactosylceramide. Surprisingly, the cytokine production of iNKT cells stimulated by α -galactosylceramide presented by CD1d⁺ Schwann cells showed a predominance of Th2-associated cytokines such as IL-5 and IL-13 with a marked deficiency of proinflammatory Th1 cytokines such as IFN- γ or TNF- α . Our findings suggest a mechanism by which iNKT cells may restrain inflammatory responses in peripheral nerves, and raise the possibility that the expression of CD1d by Schwann cells could be relevant in the pathogenesis of infectious and inflammatory diseases of the peripheral nervous system. *The Journal of Immunology*, 2006, 177: 5226–5235.

The CD1d-restricted invariant NKT (iNKT)³ cells express a unique invariant TCR- α rearrangement (V α 24-J α 18 in humans, V α 14-J α 18 in mice) paired with TCR β -chains showing markedly biased usage of one or a few V β gene segments (V β 11 in humans, and V β 2, V β 7, and V β 8 in mice) (1). Several naturally occurring and synthetic lipids have been reported to activate iNKT cells in association with CD1d proteins, the best studied of these being synthetic forms of α -galactosylceramide (α GalCer) (1). The iNKT cells participate in immunoregulation through their ability to rapidly produce large amounts of regulatory cytokines such as IFN- γ and IL-4, and also by influencing the recruitment and differentiation of dendritic cells (DCs) (2–4). Their immunoregulatory functions have been emphasized in studies of

multiple autoimmune diseases, in which defects of iNKT cell numbers or functions are associated with disease progression (5). In addition, there is abundant evidence implicating iNKT cells in protective immunity against microbial pathogens, and also in host responses to malignancies (6). Taken together, these findings suggest that iNKT cells contribute to normal homeostasis of the immune system, and also play an important role in modulating acute or chronic immune responses in a variety of diseases.

The pronounced ability of iNKT cells to inhibit autoimmune disease and promote tolerance to tissue allografts in murine models suggests that a central role for these cells in the immune system may be to maintain a delicate balance that is normally tipped in favor of immune tolerance (1). This role for iNKT cells may be most pronounced in tissues that are especially sensitive to the adverse effects of unrestrained inflammation, such as the bone marrow, the pancreatic islets, and the nervous system. These considerations led us to study the expression of CD1d molecules on Schwann cells, the myelin forming glial cells of the peripheral nervous system. Schwann cells not only form the myelin sheath that insulates the enclosed nerve axons, but also provide trophic and survival factors for neurons and facilitate regeneration following nerve injury (7). The degeneration of Schwann cells is involved in pathogenesis of peripheral neuropathies observed in autoimmune, inflammatory, and infectious diseases (8–11). In particular, Schwann cell invasion by *Mycobacterium leprae*, the causative organism of leprosy and the only known bacterial pathogen that infects Schwann cells, is the essential key step in immune-mediated nerve damage in leprosy (12). Moreover, Schwann cells are known to express MHC class I and class II molecules, particularly in the setting of inflammatory cytokines such as IFN- γ , and studies of *M. leprae*-specific T cells indicate that Schwann cells can present peptide Ags (13, 14). However, the potential for lipid Ag presentation and subsequent T cell activation by Schwann cells is unknown.

*Department of Microbiology and Immunology, and [†]Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461; [‡]Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, NY 10021; [§]Institute of Hansen's Disease, Catholic University, Seoul, South Korea; [¶]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom; ^{||}Edward Hines, Jr. Veterans Affairs Hospital, Hines, IL 60141; and [#]Division of Dermatology, ^{**}Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

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² Address correspondence and reprint requests to Dr. Steven A. Porcelli, Department of Microbiology and Immunology, Forchheimer Building, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

³ Abbreviations used in this paper: iNKT, invariant NKT; α GalCer, α -galactosylceramide; DC, dendritic cell; MPNST, malignant peripheral nerve sheath tumor.

In this study, we have investigated the expression of the lipid Ag-presenting molecule CD1d on human Schwann cells and their ability to present synthetic lipid Ags to activate CD1d-restricted iNKT cells. Our studies showed that Schwann cells express CD1d at detectable levels in both human peripheral nerve tissues and in culture as isolated primary Schwann cells or established Schwann cell lines. The CD1d proteins detected on Schwann cells were expressed on the cell surface in a mature glycosylated form, and were capable of binding and presenting α GalCer to iNKT cells. Most notably, activation of cultured human iNKT cells by Schwann cells led to a program of cytokine expression that was dominated by Th2-associated anti-inflammatory cytokines such as IL-13 and IL-5. These results suggest a potential role for CD1d expression by Schwann cells in orchestrating host immune responses in peripheral nerve tissue.

Materials and Methods

Cell lines and culture

Transformed human Schwann cell lines, ST88-14, T265-2C, and STS-26T and HeLa/CD1d transfectants were grown in DMEM supplemented with 10% FBS (HyClone Laboratories) (15–17). Human iNKT cell clones, DN2.D5, DN2.D6, and DN2.B10, and control CD4⁺ T cell clone T1.5B2 have been previously described (18), and murine iNKT hybridoma DN3A4.1-2 was a generous gift from Dr. M. Kronenberg, La Jolla Institute for Allergy and Immunology (San Diego, CA). All T cells were grown in RPMI 1640 medium supplemented with 10% FBS and additional additives (T Cell Media) (18). Short-term CD1d/Schwann cell transfectants were generated as previously described (17). All media and additives were purchased from Invitrogen Life Technologies.

Abs analysis

Mouse or hamster mAbs were used as either mouse ascite fluid or as IgG purified from hybridoma culture supernatants using protein G affinity chromatography (Amersham Biosciences). Anti-human CD1d mAbs used were CD1d27 (IgG1), CD1d42 (IgG1), CD1d51 (IgG2b), CD1d55 (IgG1), CD1d59 (IgM), CD1d75 (IgG1), C3D5 (IgG1), and NOR3.2 (IgG1; Serotec) (19, 20). The anti-p75 mAb (anti-neurotrophin receptor, IgG1) was a gift of Dr. P. Wood (University of Miami, Miami, FL). Other mAbs used (all obtained from American Type Culture Collection) were W6/32 (anti-HLA-A, anti-HLA-B, anti-HLA-C mAb, IgG2a), LB3.1 (anti-HLA-DR mAb, IgG1), OKT3 (anti-human CD3, IgG1), H57-597 (hamster anti-mouse TCR- β , IgG2), P3 \times 63Ag8 (isotype control, IgG1), MPC-11 (isotype control, IgG2b), and N-S.4.1 (isotype control, IgM). Biotinylated goat anti-mouse IgG/M (H+L) and streptavidin-PE were obtained from BioSource International and Phycollink (ProZyme), respectively.

Human peripheral nerve tissue and primary human Schwann cells

Peripheral nerve tissue from normal cauda equina from cadaveric organ donors was obtained from Dr. P. Wood through the University of Miami Organ Procurement Organization (Miami, FL). Isolation, purification, and characterization of primary Schwann cells were previously described (21, 22).

Immunostaining and immunohistochemistry

For immunostaining, cells were stained with a variety of mAbs against CD1d or other surface proteins, followed by biotinylated goat anti-mouse IgG/M (H+L) and streptavidin-PE, and then analyzed using a FACSCalibur apparatus (BD Biosciences). Acetone-fixed sections of human nerve biopsy specimens were used for immunohistochemistry as previously described, and slides were counterstained with hematoxylin (23). Images were acquired and processed by immunofluorescence microscopy (Olympus).

Immunoprecipitation and Western blotting of CD1d

CD1d proteins from soluble cell lysates were immunoprecipitated using mAb CD1d51 and deglycosylated by PNGase F treatment (New England Biolabs) (23). Western blotting was performed with a mixture of mAbs CD1d75, C3D5, and NOR3.2, followed by biotinylated goat anti-mouse κ -chain (Southern Biotechnology Associates) and streptavidin-poly-HRP (Bio-Rad). SuperSignal West Pico Chemiluminescent (Pierce) was used as substrate for imaging by ECL.

T cell stimulation assays

T cell stimulation and cytotoxic assays were done according to published protocols (24). Briefly, human or mouse T cells were stimulated by α GalCer pulsed, irradiated (5000 rad) APCs or by plate-bound anti-CD3 mAb. The APCs used in the assays were human primary Schwann cells, Schwann cell lines, monocyte-derived immature DCs, or HeLa/CD1d transfectants. Supernatants were analyzed for various cytokines after 24–48 h of stimulation using capture ELISA (BD Pharmingen), and remaining culture was used to determine T cell proliferation by measuring the incorporation of [³H]thymidine (ICN Pharmaceuticals). For cytotoxic assays, various numbers of human iNKT cells were incubated with α GalCer prepulsed ⁵¹Cr-labeled (New England Nuclear) target cells in the presence of anti-CD1d mAb CD1d59 or matching isotype control. Target cells were also incubated with medium alone or with 0.5% Triton X-100 to determine the spontaneous and maximum ⁵¹Cr release, respectively. The supernatants were analyzed for the release of ⁵¹Cr using a Microbeta Trilux liquid scintillation counter (Wallac). Assays were performed in quadruplicate, and results were expressed as the percentage of specific lysis defined as (experimental ⁵¹Cr release – spontaneous ⁵¹Cr release)/(maximum ⁵¹Cr release – spontaneous ⁵¹Cr release) \times 100.

Cytokine Ab arrays

Supernatants from human iNKT cells stimulated by α GalCer prepulsed APCs were analyzed for the presence of various cytokines and chemokines using Human Cytokine Ab Array 1.1 (RayBiotech). The individual spots corresponding to immunodetection of specific cytokines were quantized using ChemiImager 4000 (Alpha Innotech), normalized to internal positive and negative control spots provided on the array, and expressed as relative values in arbitrary units calculated as relative expression with the formula (cytokine spot intensity – negative control spot intensity)/(positive control spot intensity – negative control spot intensity).

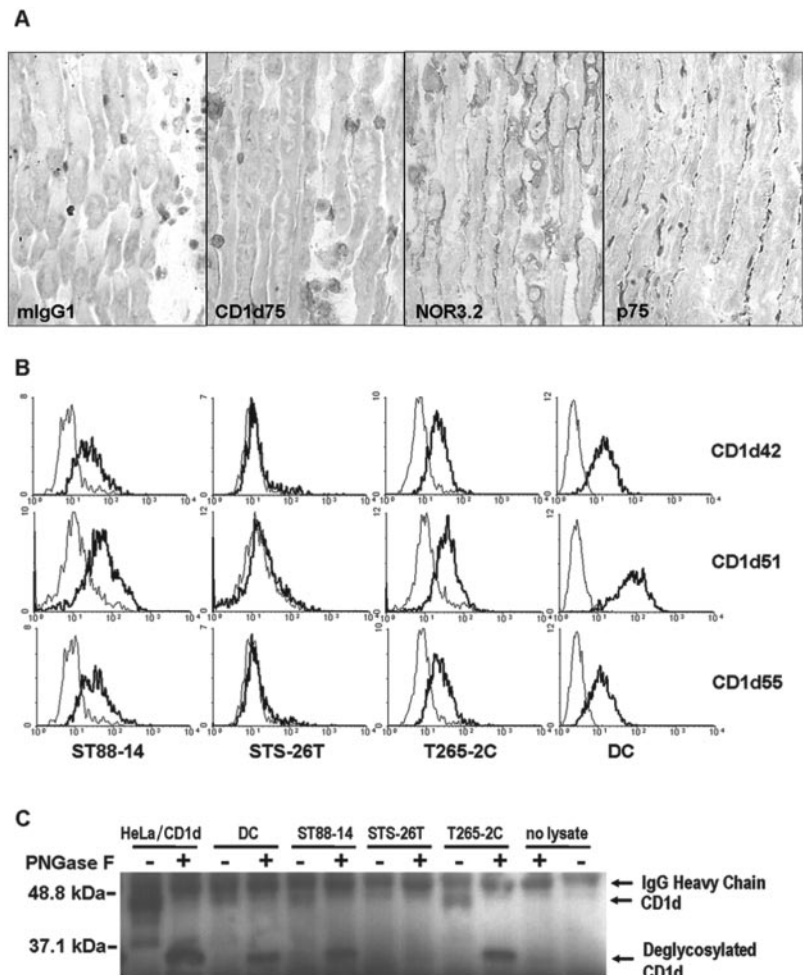
Results

CD1d expression by human Schwann cells

To investigate CD1d expression on Schwann cells in situ, immunohistochemistry was performed on cryopreserved human nerve tissue from normal cauda equina using mAbs specific for CD1d or for Schwann cell-specific markers (Fig. 1A). Staining with CD1d-specific mAb NOR3.2 strongly outlined the sheath of all axons in the nerve tissues, consistent with CD1d expression in situ on Schwann cell-axon units. Staining with another CD1d-specific mAb, CD1d75, revealed a similar pattern of staining as NOR3.2, although with lower intensity. This response may reflect the different sensitivity of the epitopes recognized by the two Abs to the tissue preparation and fixation methods. Staining with a mAb specific for the low affinity neurotrophin receptor (p75), a marker predominantly expressed on Schwann cells, revealed a similar linear staining pattern of axon sheaths. These results were consistent with expression of CD1d in the normal peripheral nerve in a pattern consistent with expression on Schwann cells.

To develop a model system to study the function of CD1d on Schwann cells, we characterized the CD1d expression by three different previously established human transformed Schwann cell lines, ST88-14, T265-2C, and STS-26T. The ST88-14 and T265-2C were established from a malignant peripheral nerve sheath tumor (MPNST), and STS-26T was derived from a benign schwannoma (15, 16). As previously described and confirmed in our hands, all three of these cell lines expressed the Schwann cell marker S100 β confirming their lineage of origin (data not shown). To directly assess CD1d protein expression in Schwann cell lines, the surface expression of CD1d was first examined by FACS analysis using a panel of mAbs specific for the native cell surface form of CD1d (Fig. 1B). All CD1d-specific Abs stained both MPNST-derived Schwann cell lines (ST88-14 and T265-2C), although they failed to stain the benign schwannoma-derived line STS-26T. The levels of anti-CD1d binding to the CD1d⁺ Schwann cell lines was similar to monocyte-derived DCs, which are known to be competent APCs for stimulation of CD1d-restricted iNKT cells.

FIGURE 1. CD1d expression on human Schwann cells. *A*, Immunohistochemical analysis of CD1d proteins on peripheral nerve tissue. Both CD1d-specific mAbs NOR3.2 and CD1d75 and Schwann cell-specific anti-p75 mAb generated similar staining patterns outlining the periphery of axon-Schwann cell units in normal peripheral nerve tissue. Control was a nonbinding mouse IgG1. *B*, FACS analysis of surface-expressed CD1d proteins from Schwann and DCs. Representative stainings with anti-CD1d mAbs CD1d42, CD1d51, and CD1d55 (thick line histogram) are shown overlaid on stainings with matching isotype controls (thin line histogram). *C*, Immunoprecipitation and Western blot analysis of total CD1d proteins from Schwann cells and DC and HeLa/CD1d transfectants. Immunoprecipitated CD1d proteins were deglycosylated where indicated using PNGase F.



Immunoprecipitation followed by Western blot analysis of lysates of the Schwann cell lines demonstrated that the m.w. of CD1d proteins expressed on Schwann cells was similar to that observed for DC and HeLa cell transfectants expressing CD1d (HeLa/CD1d), which express *N*-glycosylated CD1d. This observation suggested that CD1d proteins expressed by Schwann cells underwent extensive glycosylation as observed in HeLa/CD1d or DCs. This suggestion was confirmed by enzymatic deglycosylation of the immunoprecipitated CD1d using PNGase F, which showed that the core polypeptide of CD1d proteins from the Schwann cell lines was indistinguishable in size from those expressed by DC and HeLa/CD1d transfectants (Fig. 1C). Levels of immunoprecipitated CD1d from Schwann cell lines were roughly similar to the amount immunoprecipitated from an equivalent number of DCs, confirming the impression from the FACS staining that Schwann cells express levels of CD1d that are in a similar range as levels found on professional APCs.

Up-regulation of Schwann cell CD1d by IFN- γ treatment

It has been previously shown that *in vitro* treatment with IFN- γ can induce CD1d on keratinocytes, and enhanced expression of CD1d proteins was observed in psoriatic skin lesions, indicating that CD1d could be up-regulated on epithelial cells in the skin during the inflammation (23). To investigate the possibility that CD1d expression by Schwann cells can be similarly up-regulated, we cultured Schwann cell lines in medium containing a saturating amount of recombinant IFN- γ for 48 h and examined the change in the level of surface CD1d expression by FACS. This experiment

demonstrated that IFN- γ treatment significantly up-regulated the expression of membrane-bound CD1d proteins from MPNST-derived lines ST88-14 and T265-2C but not from schwannoma-derived line STS-26T (Fig. 2A). As previously shown for Schwann cells from different sources, all three Schwann cell lines highly up-regulated the expression of MHC class II and to a lesser extent MHC class I upon IFN- γ treatment (13, 25). Although Schwann cells expressed CD1d proteins to a lesser extent compared with MHC class I, they up-regulated the expression of CD1d proteins up to 5-fold upon IFN- γ treatment, as compared with an increase in MHC class I proteins of only about 2-fold (Fig. 2B). However, the highest level of CD1d proteins observed on IFN- γ -treated Schwann cells did not exceed that on DCs, suggesting that biogenesis of CD1d proteins might be tightly regulated so that it does not rise above a stringently set level (data not shown).

CD1d-dependent activation of iNKT cells by Schwann cells

Schwann cells are known to possess the ability to process and present *M. leprae* protein Ags to MHC class I and class II restricted T cells (13, 14). We thus investigated whether Schwann cells were able to present the CD1d-restricted synthetic glycolipid Ag α GalCer to iNKT cells. As iNKT cells are known to proliferate and produce IFN- γ and IL-4 upon activation, we examined these parameters as indicators of activation (24). We used a CD4⁻ T cell clone DN2.D5 that was previously shown to have the capacity to produce both IFN- γ and IL-4. We note that although some studies of freshly isolated circulating iNKT cells have found Th2-type cytokine production to be predominant in the CD4⁺ iNKT cell

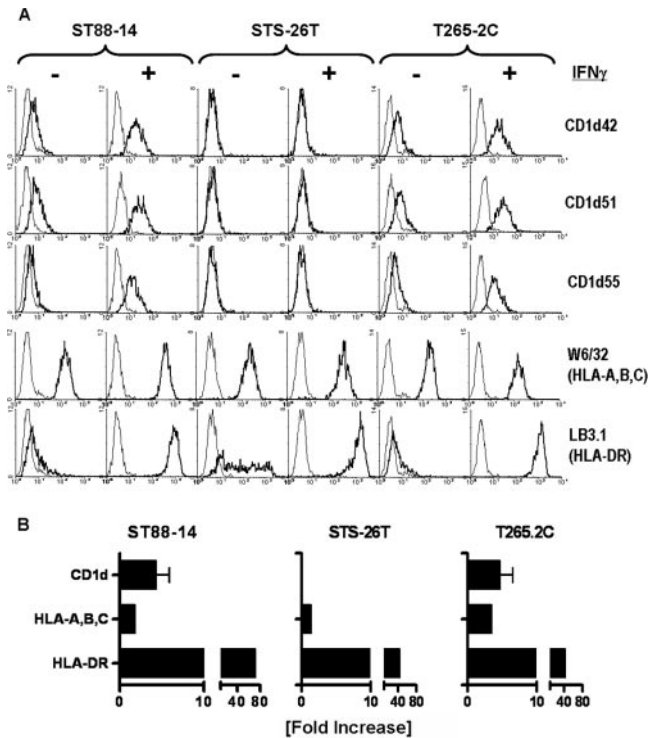


FIGURE 2. Up-regulation of CD1d expression on Schwann cells by IFN- γ treatment. *A*, The expression of CD1d, MHC class I and MHC class II proteins on Schwann cell treated with 1000 U/ml IFN- γ for 24 h were assessed by FACS analysis using various mAbs specific for CD1d, MHC class I or class II. Specific mAb staining (thick line histogram) are overlaid on isotype control mAb staining (thin line histogram). *B*, The mean fluorescence intensity (MFI) fold increase in expression of each protein was calculated as $(MFI_a - MFI_b)_{treated} / (MFI_a - MFI_b)_{untreated}$, where MFI_a is MFI of CD1d, MHC class I or class II, and MFI_b is MFI for matching isotype control. For CD1d, the mean fluorescence intensity used for the calculation was the average value of three stainings with CD1d42, CD1d51, and CD1d55.

subset, other studies indicate that nearly all cultured iNKT cell lines can produce detectable levels of Th2-type cytokines (26, 27). This result may in part reflect an increased state of activation of cultured iNKT cells lines resulting from in vitro stimulation and expansion. CD1d-expressing Schwann cell lines ST88-14 and T265-2C stimulated proliferation and IFN- γ and IL-4 production by human iNKT cell clone DN2.D5 in the presence of α GalCer, but not in the presence of the inactive β GalCer variant or with no glycolipid Ag present (Fig. 3A). Schwann cells cultured alone did not proliferate or produce detectable IFN- γ or IL-4 in response to α GalCer (data not shown).

We also observed that CD1d-expressing human Schwann cell lines could activate murine iNKT hybridoma DN3A4.1-2, consistent with the interspecies cross-reactivity that has been typically observed for this response (Fig. 3B). Four of six anti-CD1d mAbs tested gave nearly complete inhibition of both human and mouse iNKT cell responses to α GalCer presented by the Schwann cell lines (Fig. 3C), confirming the CD1d dependence of the responses. Of the two anti-CD1d mAbs that inhibited these responses weakly, one (CD1d27) is believed to have relatively low avidity for CD1d and the other (CD1d75) is reactive with an epitope that is poorly accessible on the cell surface. Overall, our results provided strong evidence that Schwann cells expressed fully functional CD1d proteins that were capable of activating iNKT cells by presenting the strong glycolipid agonist, α GalCer.

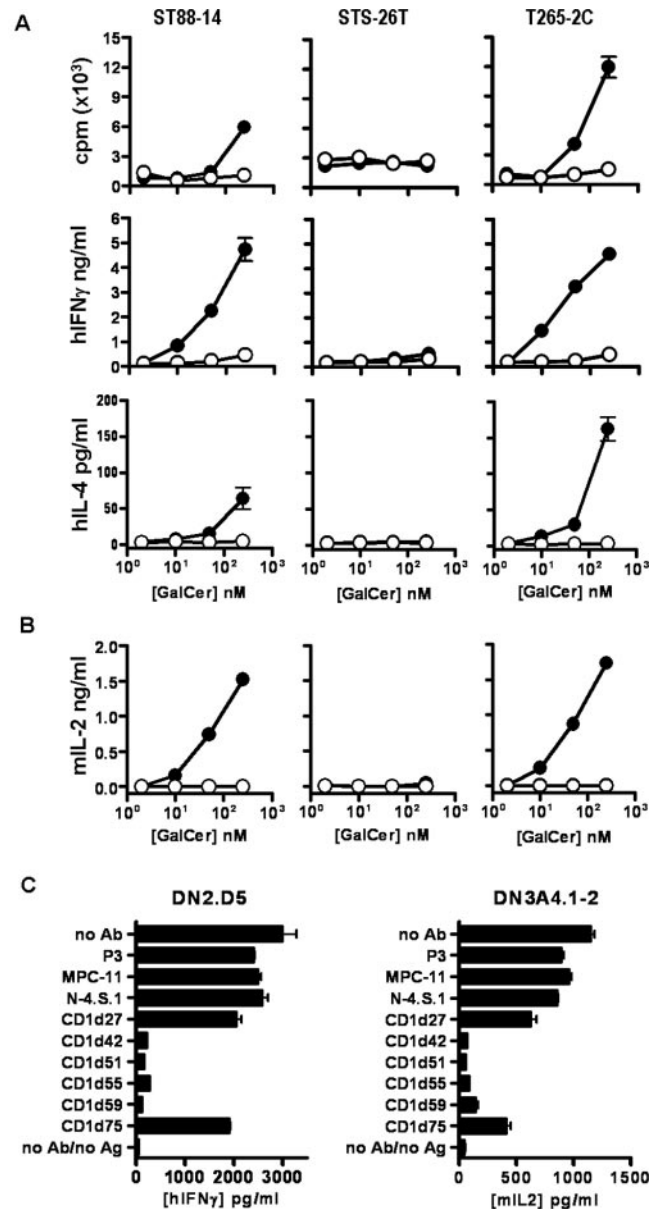


FIGURE 3. CD1d-dependent activation of iNKT cells by Schwann cells. Human iNKT cell clone DN2.D5 (*A*) or murine iNKT cell hybridoma DN3A4.1-2 (*B*) were cocultured with Schwann cell lines ST88-14 (*left*), STS-26T (*center*), or T265-2C (*right*) that were preloaded with the indicated concentrations of either α GalCer (●) or β GalCer (○). *A*, iNKT cell proliferation (*upper*), IFN- γ secretion (*middle*), or IL-4 secretion (*lower*) were measured. *B*, IL-2 secretion was measured. Similar results were obtained using additional iNKT cell clones and lines. *C*, Activation of iNKT cells by α GalCer presented by ST88-14 was blocked by addition of anti-CD1d mAbs. Stimulation of human iNKT cell clone DN2.D5 (*left*) and murine iNKT cell hybridoma DN3A4.1-2 (*right*) were assessed by IFN- γ and IL-2 secretion, respectively. Data show responses to 100 nM α GalCer in the presence of the indicated Ab. Results are representative of three independent experiments.

Lysis of Schwann cells by activated iNKT cells

Previously, it was reported that Schwann cells are susceptible to Ag-specific lysis by cytolytic CD4⁺ or CD8⁺ T cells, and that this process may contribute to the pathogenesis of peripheral neuropathy in leprosy patients (13, 14). As activated iNKT cells can also display potent killing of Ag-presenting target cells (28), we investigated whether iNKT cells activated by α GalCer-pulsed Schwann

cells could in turn lyse the Ag-presenting Schwann cells. HeLa/CD1d cells were used as positive control targets for cytotoxic activity of iNKT cells. As expected, iNKT cells lysed HeLa/CD1d cells very effectively in the presence of α GalCer, resulting in complete lysis within 2 h of incubation (Fig. 4B). The iNKT cells lysed HeLa/CD1d cells even in the absence of α GalCer to an appreciable level, which reflects the known autoreactivity directed against CD1d proteins expressed on certain cell types. Activated iNKT cells also lysed Schwann cells in the presence of α GalCer, although not as efficiently as compared with HeLa/CD1d target cells because longer incubation (4 h) was required for complete lysis of Ag-presenting Schwann cells. Additionally, iNKT cells did not lyse Schwann cells in the absence of α GalCer even with a prolonged incubation time, again confirming that iNKT cells do not exhibit easily detectable autoreactivity toward CD1d⁺ Schwann cells (Fig. 4A). The addition of anti-CD1d mAbs significantly blocked the lysis of α GalCer presenting Schwann cells by iNKT cells, confirming the requirement for direct recognition of CD1d in the cytolytic reaction.

Activation of iNKT T cells by human primary Schwann cells

To confirm the findings from experiments using CD1d⁺ transformed human Schwann cell lines, we conducted similar experiments using previously characterized human primary Schwann cells, which had been isolated from human adult peripheral nerve tissue and expanded up to 3–4 passages (22). Using fluorescence microscopy, we confirmed that IFN- γ -treated primary Schwann cells expressed CD1d in a diffuse and mostly homogenous distribution similar to that observed for MHC class I molecules (Fig. 5A). This pattern of staining was consistent with expression of CD1d predominantly on the plasma membrane of Schwann cells, in contrast to MHC class II molecules, which showed a more punctuate staining pattern indicative of substantial endosomal localization.

In vitro coculture of primary human Schwann cells with iNKT cells was conducted to assess the ability of these cells to stimulate CD1d-dependent T cell responses, as measured by proliferation and IFN- γ production. As shown in Fig. 5B, human iNKT cell clone DN2.B10, but not the control T cell clone T1.5B2, proliferated and secreted IFN- γ in response to α GalCer presented by human primary Schwann cells, although the magnitude of stimulation was significantly less than the response obtained using an identical number of monocyte-derived immature DCs.

We also assessed the recognition of CD1d on primary human Schwann cells by a murine iNKT cell hybridoma. DN3A4.1-2 produced substantial amounts of IL-2 in response to presentation of α GalCer by primary human Schwann cells (Fig. 5B). The magnitude of the response generated by primary human Schwann cells in

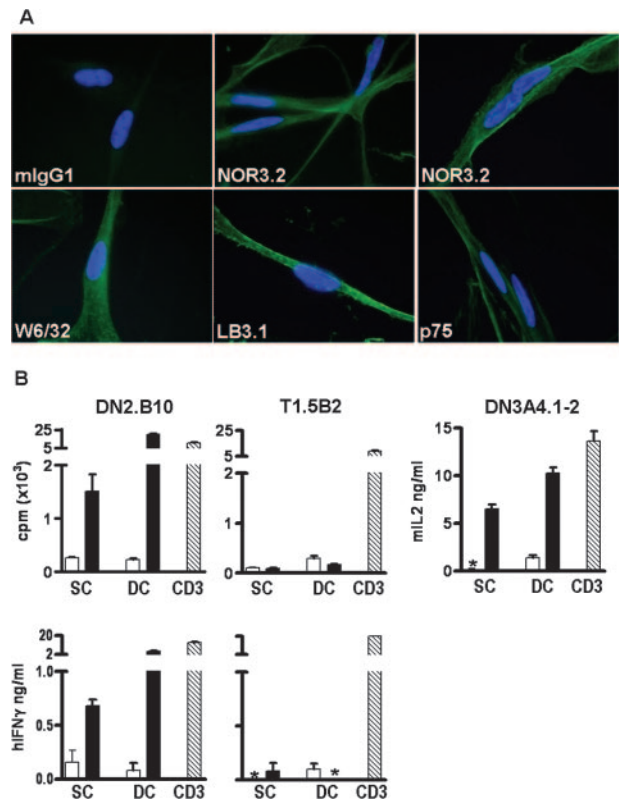


FIGURE 5. Activation of iNKT cells by primary human Schwann cells. **A**, The expression of CD1d, MHC class I (HLA-A, HLA-B, HLA-C), MHC class II (HLA-DR), and Schwann cell-specific marker p75 on primary Schwann cells were assessed by immunostaining with mAbs NOR3.2, W6/32, LB3.1, and anti-p75, respectively. Green indicates specific mAb staining with the indicated mAbs, and blue is nuclear staining (DAPI). One or two representative images for each staining are shown. **B**, Human iNKT cell clone DN2.B10, control conventional CD4⁺ T cell clone T1.5B2, or mouse iNKT hybridoma DN3A4.1-2 were stimulated by DCs or primary human Schwann cells (SC) that had been preincubated with 200 nM α GalCer (■) or medium only (□). For human T cell clones, proliferation (top) and IFN- γ secretion (bottom) were measured. For murine iNKT cell hybridoma cells, IL-2 secretion was measured. Schwann cells or DCs alone did not proliferate or produce any detectable cytokines in these experiments (data not shown). Anti-CD3 stimulation was included as a positive control (▨). Where indicated (*), <5 pg/ml cytokine is detected. The data shown are representative of three independent experiments.

this experiment was more similar to the level stimulated by immature DCs. Because iNKT hybridomas, unlike human iNKT cell clones, are generally not dependent on costimulation by B7

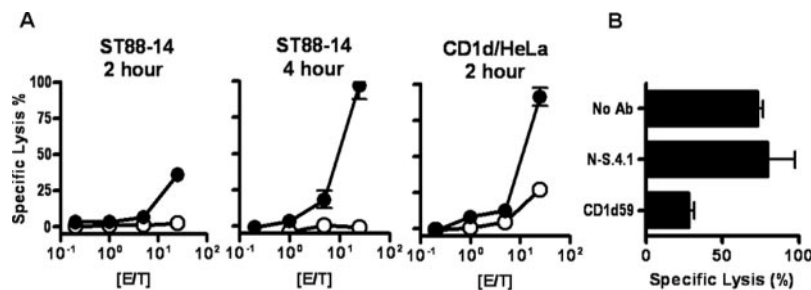


FIGURE 4. Lysis of Schwann cells by iNKT cells. **A**, Human iNKT cells (DN2.D6) were incubated for indicated time at various E:T ratios (E/T) with ⁵¹Cr-labeled target cells, which were either Schwann cell line ST88-14 or HeLa/CD1d transfectants. Target cells were either prepulsed (●) or not (○) with 200 nM α GalCer. **B**, Similar experiments using α GalCer-pulsed ST88-14 targets and 4 h of incubation were done with addition of anti-CD1d mAb CD1d59 or isotype-matched control mAb.

molecules or other accessory signals, these findings suggested that the relatively weak stimulation of human iNKT cell clone by primary Schwann cells may have reflected the lack of costimulation, rather than a lower level of Ag display compared with DCs.

Distinct cytokine profile following activation of iNKT cells by Schwann cells

Because CD1d-restricted iNKT cells are capable of secreting a variety of regulatory cytokines and chemokines upon TCR-mediated activation, we tested the pattern of secretion of these molecules by iNKT cells following CD1d-dependent activation by Schwann cells. This pattern was assessed by simultaneous measurement of 42 cytokines and chemokines in supernatant samples using an ELISA-based human cytokine Ab array and chemiluminescence detection system (Fig. 6A). This method was used to gain a broader view of the various soluble mediators produced by iNKT cells stimulated by coculture with either Schwann cells or DCs, in the presence or absence of α GalCer.

When cultured separately without iNKT cells, both human Schwann cell line ST88-14 and DCs constitutively produced the chemokines MCP-1 (CCL2) and IL-8 (CXCL8). In addition, human Schwann cells also constitutively produced IL-6 and GRO (CXCL3), whereas DCs produced the macrophage-derived chemokine (MDC, CCL22) and thymus and activation-regulated chemokine (TARC, CCL17) (Fig. 6B). Treatment with α GalCer did not modulate the expression of cytokines and chemokines by Schwann

cells or DCs (data not shown). Coculture of iNKT cells (clone DN2.D5) together with Schwann cell line ST88-14 did not lead to any changes in the pattern of cytokine secretion detected. However, similar coculture of iNKT cells with DCs stimulated the production of IL-13, IL-5, and RANTES (CCL5) (Fig. 6, B and C). With addition of α GalCer to the culture, iNKT cells cocultured with Schwann cells also showed significant production of IL-13, IL-5, and RANTES, but not IFN- γ or IL-4. It should be noted that stimulation of iNKT cells by α GalCer presenting Schwann cells did lead to detectable levels of IFN- γ when analyzed by sensitive capture ELISA (Figs. 3 and 5). However, the level produced was markedly less than was observed when DCs were used as the APCs, consistent with the overall Th2-biased or anti-inflammatory cytokine profile induced when Schwann cells were used as the presenting cells. In contrast, inclusion of α GalCer in cocultures of iNKT cells and DCs gave a broad spectrum of cytokine production, including easily detectable levels of many proinflammatory cytokines such as TNF- α , lymphotoxin/TNF- β , IFN- γ , IL-1 β , and IL-2 (Fig. 6, B and D). Overall, the analysis of an extended panel of cytokine production using the Ab array system indicated that iNKT cell activation by Schwann cells in the presence of a strong agonist such as α GalCer generated a cytokine response that was dominated by the production of Th2-associated immunoregulatory cytokines such as IL-13 and IL-5, whereas the same iNKT cells activated by DCs with α GalCer were capable of producing both Th1 and Th2 associated cytokines. These findings were confirmed

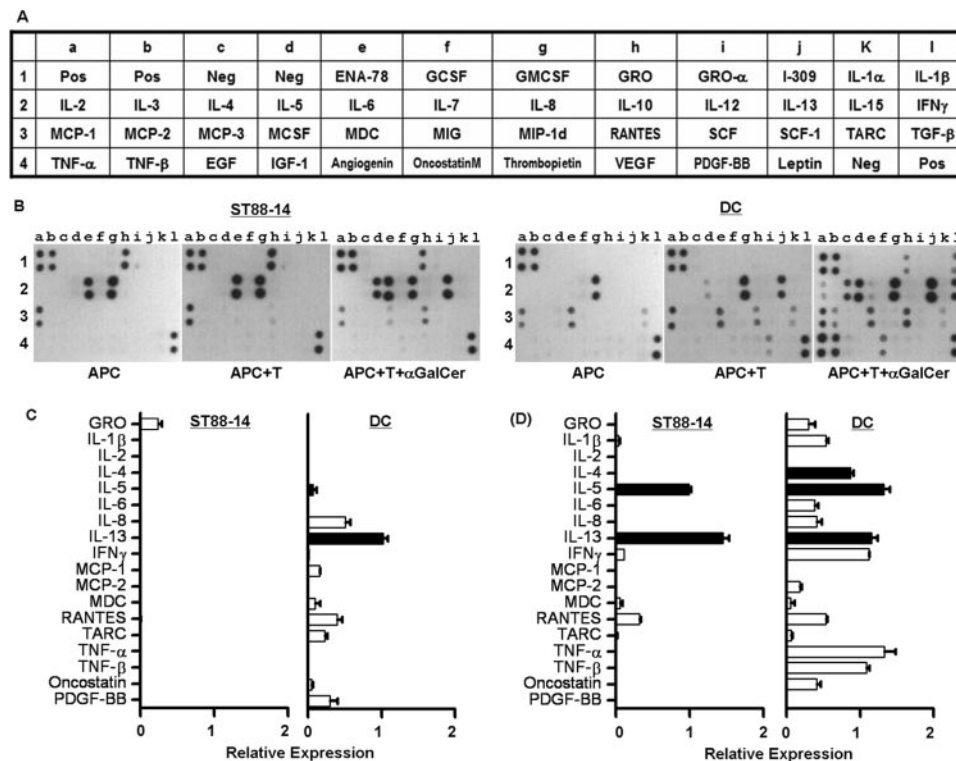


FIGURE 6. Cytokine production by iNKT cells activated by Schwann cells. Human iNKT cell clone DN2.D5 was stimulated by Schwann cell line ST88-14 or DCs in the presence or absence of α GalCer. A Human Cytokine Ab Array was used to analyze the presence of various cytokines and chemokines. A, A table of human cytokines and chemokines analyzed from Human Cytokine Ab Array is shown. B, Cytokine signals detected following development and imaging of cytokine arrays incubated with supernatant samples from cultures of APCs alone (APC), cocultures of iNKT cell clone DN2.D5 and APCs (APC+T), or similar cocultures containing 200 nM α GalCer (APC+T+ α GalCer). APCs were Schwann cell line ST88-14 for the group of three arrays shown on the left and monocyte-derived DCs for the group of three arrays shown on the right. No cytokines were detected by this method in supernatants of cultures of iNKT cells without APCs. C and D, Quantitation of cytokines and chemokines produced by iNKT cells activated by Schwann cells (left) or DCs (right) in the absence (C) or presence (D) of α GalCer. Similar results were obtained using another Schwann cell line (T265-2C) and additional CD4⁺ or CD4⁻ human iNKT cell clones and lines. Results shown are representative of three independent experiments. ■, Anti-inflammatory cytokines (IL-4, IL-5, and IL-13); □, remaining inflammatory cytokines or chemokines.

with similar results using additional CD4⁺ or CD4⁻ iNKT cell clones and lines using standard capture ELISA for selected cytokines, including IL4, IL-5, IL-13, IFN- γ , and TNF- α (Fig. 7 and data not shown).

Influence of IL-12 on iNKT cell activation by Schwann cells

Production of IFN- γ by autoreactive iNKT cells can be considerably augmented by the presence of exogenous IL-12, which is mainly produced by DCs and potentially other myeloid lineage cells upon exposure to products of microbial pathogens (29–33). The ability of IL-12 to synergize with signals generated by the recognition of CD1d-presented self Ags has been proposed as a central part of the mechanism by which iNKT cells are activated during infections (29). In a similar way, the presence of elevated levels of IL-12 produced by infiltrating macrophages and DCs in infected nerve tissue could also increase the production of IFN- γ from iNKT cells that would otherwise produce mainly immunosuppressive cytokines such as IL-5 and IL-13 upon activation by recognition of CD1d expressed by Schwann cells. To evaluate this model, we investigated whether exogenous IL-12 could increase the IFN- γ production from iNKT cells activated by recognition of α GalCer presented by Schwann cells.

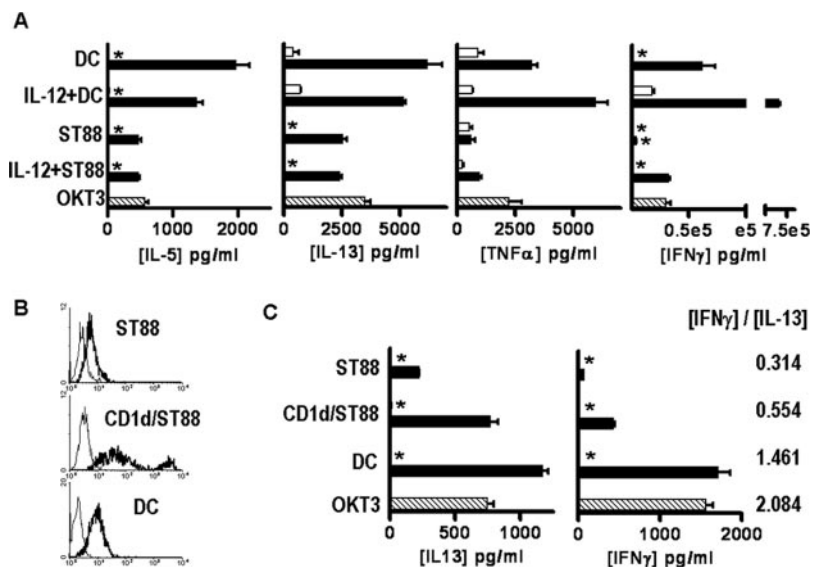
In general, exogenous IL-12 did not synergize in the production of anti-inflammatory cytokines such as IL-5 and IL-13, and only affected the production of Th1-associated inflammatory cytokines such as IFN- γ and TNF- α . In these experiments, the addition of IL-12 increased the level of IFN- γ from iNKT cells stimulated by DCs even in the absence of α GalCer, consistent with the mechanism of enhanced autoreactivity of iNKT cells that was previously proposed (29). In addition, exogenous IL-12 resulted in a significant increase in production of both IFN- γ and TNF- α from iNKT cells activated by presentation of α GalCer by DCs (Fig. 7A). In contrast, exogenous IL-12 did not induce any detectable level of IFN- γ production by iNKT cells cultured with Schwann cells, but did cause a modest up-regulation of IFN- γ production from iNKT cells in response to α GalCer presented by Schwann cells (Fig. 7A). However, this effect of IL-12 on IFN- γ was substantially less than the effect observed when DCs were used as APCs, and there was little or no effect on IL-5 and IL-13 production. These results suggested that a failure of Schwann cells to produce IL-12 may partially account for their preferential stimulation of anti-inflammatory Th2-type cytokines by iNKT cells.

Because CD1d levels on Schwann cell lines were observed to be slightly lower than those on DCs (Fig. 1), we considered whether this relatively low CD1d expression may have accounted for the observed cytokine polarization. To assess this consideration, we established short-term CD1d transfectants of Schwann cells with markedly increased levels of CD1d expression and compared their ability to stimulate cytokine production by iNKT cells (Fig. 7B). The overexpression of CD1d proteins on Schwann cells augmented the production of both IFN- γ and IL-13 by iNKT cells in response to α GalCer to a similar extent, and failed to significantly increase the ratio of Th1 (IFN- γ) to Th2 (IL-13) cytokine production (Fig. 7C). This indicated that the relatively low expression of CD1d by Schwann cells did not account for their unique property of directing activated iNKT cells to preferentially secrete Th2-associated cytokines.

Discussion

Unlike other members of the CD1 family, which are expressed mainly by myeloid lineage DCs and can be induced to high levels by various cytokines, CD1d proteins in professional APCs of monocytic lineage are constitutively expressed at a low but functionally significant level (20). In addition, nonhemopoietic cells such as keratinocytes, hepatocytes, intestinal epithelial cells, placental trophoblasts, and blood vessel endothelial cells also express CD1d proteins and are believed to have some capacity to activate iNKT cells (23, 34–37). Furthermore, the expression of CD1d has been observed to be up-regulated on nonhemopoietic cells in various inflammatory or infectious conditions, such as on keratinocytes in psoriatic lesions, on hepatic cells in hepatic C virus infection, and cardiac endothelial cells in Cocksackievirus infection (23, 34–36, 38, 39). These observations suggest a potential role for CD1d and CD1d-restricted iNKT cells in modulating and regulating adaptive immune responses at an early point in the development of inflammation in response to infection or autoimmunity. In the current study, we report that ectodermally derived Schwann cells of the peripheral nervous system express CD1d proteins on their plasma membrane and that they are capable of activating iNKT cells preferentially to produce anti-inflammatory cytokines. These findings extend the range of tissues in which iNKT cells may be activated to exert either their well-described proinflammatory or anti-inflammatory properties.

FIGURE 7. Effects of exogenous IL-12 and increased CD1d expression. **A**, Human iNKT cell clone DN2.D5 was stimulated with α GalCer-pulsed Schwann cells (ST88) or DCs in the presence or absence of IL-12, and production of various cytokines was analyzed by standard capture ELISA. **B**, FACS analysis showing levels of staining with anti-CD1d mAb CD1d55 (heavy line) or isotype control mAb (light line) on Schwann cell line ST88-14 that was either untransfected (upper), or transfected with an expression plasmid encoding CD1d (middle). CD1d55 staining of monocyte-derived DCs are shown for comparison (lower). **C**, Cytokine secretion (IL-13 and IFN- γ) following stimulation of human iNKT cell clone DN2.D5 cocultured with CD1d-transfected ST88-14 Schwann cell line expressing a high level of CD1d protein (CD1d/ST88), native ST88-14 cells, or DCs as indicated. Stimulation without α GalCer (\square), with 200 nM α GalCer (\blacksquare), or plate-bound anti-CD3 (hatched) stimulation is shown. Where indicated (*), <5 pg/ml cytokine is detected. Each ratio of IFN- γ to IL-13 secreted is shown to the right of the graph.



Our results give further evidence for the view that Schwann cells are not immunologically silent, but rather have the capacity to influence immune reactions that take place within the peripheral nervous system. A role for Schwann cells in Ag-specific T cell activation has been previously suggested by several studies demonstrating that proteins involved in this process, such as MHC class I and class II molecules, are expressed on isolated Schwann cells (9, 13, 14, 40–42). In addition, IFN- γ -treated Schwann cells have been shown using in vitro cell culture studies to process and present various endogenous or exogenous Ags in both an MHC class I- and class II-dependent manner, which can result in the killing of Schwann cells by Ag-specific CD4⁺ or CD8⁺ effector T cells (13, 14, 43). The expression of costimulatory proteins on Schwann cells is currently controversial. Whereas one group reported the presence of CD80 molecules on cultured primary Schwann cells (13), another study failed to detect CD80 and CD86 proteins in normal nerve tissue (9). Under the culture conditions used in the current study, we were unable to detect expression of CD80 on primary Schwann cells (data not shown). This discrepancy may be due to differences in culture conditions in our studies compared with those used in earlier work, which included crude supernatants from lymphokine-activated killer cells (13, 22). A variety of cytokines are known to be present in such supernatants, and one or more of those may have resulted in the induction of CD80. Whether or not such induction of costimulatory molecules occurs under any conditions on Schwann cells in vivo is an important point that will require further study.

In the current study, immunohistochemistry of normal peripheral nerve revealed staining with anti-CD1d mAbs in the periphery of most or all Schwann cell-axon units. CD1d proteins were also detected on primary Schwann cells isolated from healthy nerve biopsies (Figs. 1 and 5) (22). Furthermore, FACS and immunoprecipitation with Western blot analysis clearly demonstrated that CD1d proteins were present in Schwann cell lines that were derived from MPNST. In general, expression of CD1d proteins on Schwann cells was weak, and its clear demonstration required the use of staining procedures involving several steps of amplification. This observation may explain the failure to detect CD1d on nerve sections in a previous study (9). Nonetheless, the level of CD1d expression on Schwann cells was comparable to that expressed on monocyte-derived DCs, which are known to be potent iNKT activators in cell culture assays.

Despite the relatively low level of CD1d expressed on the surface of Schwann cells, our functional studies showed that Schwann cells could efficiently present the CD1d-restricted glycolipid Ag α GalCer to human and mouse iNKT cells (Fig. 3). In fact, functional studies using a murine iNKT cell hybridoma showed that presentation of α GalCer to these cells by Schwann cells gave responses that were of similar magnitude as those obtained using DCs as APCs (Fig. 5B). Because hybridoma cell lines are mainly sensitive to the level of Ag display and independent of accessory signals delivered by costimulatory molecules or cytokines, this finding indicated that the capacity for CD1d-restricted presentation of α GalCer by Schwann cells was comparable to that of specialized APCs such as myeloid DCs. In contrast, when α GalCer presentation was studied using human iNKT cell clones, which are more dependent on costimulation for their activation, DCs were obviously superior to Schwann cells in stimulating T cell proliferation or cytokine secretion (Figs. 5B and 6). The particular costimulatory molecules that could account for this difference and might be lacking from Schwann cells have not yet been determined. However, studies of murine iNKT cells have established that costimulation through CD28 and ICOS are required for maximal responses of these cells, suggesting that the ligands for these

costimulatory receptors may be lacking on the Schwann cells that we have studied (44, 45).

It is widely believed that iNKT cells express an inherent auto-reactivity toward one or more self-ligands that are presented by CD1d proteins (46–48). In addition, there is now strong evidence that certain bacteria such as *Sphingomonas* species express glycolipids that can serve as strong CD1d-presented agonists for activating proinflammatory functions of iNKT cells (49, 50). These findings raise the possibility that peripheral nerves infected with glycolipid-rich *M. leprae* may lead to local activation of iNKT cells. Indeed, we observed a weak enhancement of proliferation of iNKT cells activated by Schwann cells infected with *M. leprae* (data not shown). Even though this effect was relatively minor in our in vitro assays, we speculate that the chronicity of *M. leprae* infections in Schwann cells could eventually lead to the gradual accumulation of stimulatory iNKT cell agonists in sufficient amounts to influence the responses of these cells. The activation of iNKT cells in this setting could potentially lead to anti-inflammatory cytokine production, as we observed in vitro (Figs. 6 and 7). Alternatively, this activation could have deleterious effects from triggering cytotoxic activity of iNKT cells. Although our findings in the current study showed that iNKT cells could lyse Schwann cells in the presence of α GalCer, further studies will be needed to determine whether such lysis is observed against *M. leprae*-infected Schwann cells.

Rather than contributing to the stimulation of proinflammatory activities of iNKT cells, several findings in our studies point strongly to the alternate possibility that CD1d expression by Schwann cells may recruit the anti-inflammatory properties of iNKT cells. Using a cytokine array method to detect an extended panel of secreted products, we observed that activation of human iNKT cells by Schwann cells led to a markedly different cytokine profile compared with that obtained by activation with myeloid DCs. Thus, Schwann cells exhibited a tendency to stimulate the production of cytokines usually associated with Th2-type responses or inhibition of proinflammatory Th1-type responses (Figs. 6 and 7). In particular, the stimulation of iNKT cells by α GalCer presented by Schwann cells elicited strong production of IL-13, a cytokine that has been linked in previous studies to the immunosuppressive functions of CD1d-restricted T cells (51), and also to the ability of iNKT cells to enhance allergic airway reactivity (52). This bias toward stimulation of anti-inflammatory cytokines from iNKT cells activated by Schwann cells could be partially overcome by increasing CD1d expression or by the addition of exogenous IL-12 (Fig. 7B). In the setting of leprosy disease, it is likely that IFN- γ , which is a strong inducer of CD1d, and IL-12 can be produced by infiltrating intraneural macrophages infected with *M. leprae*. Thus, iNKT cells activated by Schwann cells in certain physiological settings could also produce proinflammatory mediators.

The migration and accumulation of iNKT cells toward inflammatory stimuli is well documented in various inflammatory diseases such as chronic viral hepatitis, bronchial asthma, leprosy lesions, periodontal disease tissue as well as chronic inflammatory peripheral neuropathy (38, 53–55), and is thought to be mediated through their constitutive expression of various chemokine receptors (56–59). It has been shown in mouse models that the initial recruitment of iNKT cells does not require the presence of CD1d proteins on cells within a focus of inflammation (60). However, the expression of CD1d proteins within an inflammatory site may be important for modulating the effector functions of iNKT cells arriving at the site. Accordingly, our findings on the expression of CD1d by Schwann cells and their capacity to polarize iNKT cell

responses may provide new insights into the pathogenesis of peripheral neuropathy in infectious diseases such as leprosy, chronic inflammatory peripheral neuropathy, or in various other inflammatory conditions affecting the nerves (53, 54). Taken together, the studies reported may also help to establish a complex model to explain the multitude of different and often contradictory activities that have been assigned to iNKT cells. Thus, activation of iNKT cells by DCs or other professional APCs, particularly in the presence of strong CD1d-presented glycolipid agonists or high levels of IL-12, can lead to a plethora of different cytokines and a predominance of the proinflammatory and adjuvant-like properties of iNKT cells (29, 49). In contrast, we propose that activation of these cells by weak or strong agonists presented by nonprofessional APCs such as Schwann cells can lead to the expression of a non-inflammatory or immunosuppressive program by iNKT cells. This latter situation may prevail throughout the body under conditions of normal homeostasis and may be strongly enforced under most conditions in tissues that are extremely sensitive to inflammatory damage, such as the peripheral nerve. Such responses in nerve tissue may thus be potentially exploited to halt nerve damage in infection or chronic inflammation, or to prevent demyelination and possibly also enhance regeneration of damaged nerves.

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Disclosures

S. A. Porcelli is a consultant for Vaccinex and is the inventor on a patent application for the development of iNKT cell activators. The patent is the property of the Albert Einstein College of Medicine. Certain aspects of the patent rights have been licensed by Albert Einstein College of Medicine to Vaccinex.

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