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Phenotypic and Functional Characterization of Long-Term Cultured Rhesus Macaque Spleen-Derived NKT Cells

Balgansuren Gansuvd,* William J. Hubbard,* Anne Hutchings,* Francis T. Thomas,* Jeanine Goodwin,* S. Brian Wilson,† Mark A. Exley,‡ and Judith M. Thomas

Natural killer T cells are immunoregulatory cells, which have important roles in tolerance and autoimmunity, as demonstrated primarily in mice and humans. In this study, we define the phenotype and function of Vα24+ T cells derived from the spleens of rhesus macaques, a species increasingly used in models of immune tolerance. Vα24+ cells were isolated and expanded with monocyte-derived immature dendritic cells in the presence of α-galactosylceramide, IL-2, and IL-15. Rhesus NKT cells were stained with mAbs against both Vα24 and the invariant complementarity-determining region 3 epitope of the human Vα24/JoQ TCR. The cells were CD4+, CD8+ double negative (DN) and expressed CD56. Rhesus NKT cells also exhibited moderate to high expression of CD95, CD45RO, CD11a, and β2 integrin, but did not express CD45 RA, CD62L, CCR7, CD28, and other activation, costimulatory molecules (CD69 and CD40L). By intracellular staining, >90% of unstimulated rhesus NKT cells expressed IL-10, but not IFN-γ. However, the latter was strongly expressed after stimulation. Rhesus NKT secreted large amounts of TGF-β, IL-13, and IL-6, and modest levels of IFN-γ, whereas IL-10 secretion was negligible and no detectable IL-4 was observed either intracellularly or in culture supernatants. Functionally, the NKT cells and their supernatants suppressed T cell proliferation in allogeneic MLR. We conclude that long-term cultured rhesus macaque spleen-derived Vα24+ T cells are semi-invariant double-negative cells with effector memory phenotype. These cells are semianergic, polarized to a uniquely Th3 > T regulatory-1 regulatory cell phenotype, and have regulatory/suppressive function in vitro. The Journal of Immunology, 2003, 171: 2904–2911.
The mechanism of immune regulation by NKT cells remains poorly defined, possibly related to differences in cytokines produced by NKT subsets. Many invariant NKT react with CD1d tetramers, and CD4+ CD1d tetramer+ cells produce both Th1 and Th2 cytokines, but are relatively biased to Th2, whereas CD4- cells produce IFN-γ and TNF-α (16, 17). Human BM-derived CD1d-reactive NKT cells that suppress MLR also have a Th2 bias (13). Costimulatory pathways involving CD28/CD80/CD86 and CD40-CD154 may contribute to the regulation of Th1 and Th2 cytokine functions of NKT cells (31). For example, type 2 dendritic cells (DC2) can enhance the development of neonatal NKT cells into NKT2 cells, which reorganize toward NK1T in the presence of DC1 cells (32). Thus, NKT cytokine polarization probably depends on coreceptor expression as well as the type of APC exposure.

To date, detailed studies of NKT cells have not been reported in nonhuman primates, the penultimate preclinical model for interventional research in AIDS and organ transplantation tolerance. In this study, we provide the first description of rhesus macaque spleen-derived NKT cells and characterize their suppressive effect in vitro.

Materials and Methods

Animals

Tissues from three normal, male rhesus macaques (Macaca mulatta), who were selected for islet transplantation, were studied. All were free of known pathogens. Surgical procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Primates under supervision of the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Preparation of mononuclear cells (MNCs)

Spleen cells were prepared from tissue specimens of normal donor rhesus macaques. Spleens were cut into small pieces and minced gently on metal mesh filter (75 μm diameter). The filtered cells were washed twice and suspended into RPMI 1640 (Life Technologies, Gaithersburg, MD) with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies) containing 10% heat-inactivated FCS. The MNCs were isolated by Ficoll-Hypaque density (G = 1.077 g/ml) gradient centrifugation at 1500 rpm for 30 min.

Monocyte-derived DCs

CD14+ cells were isolated from MNCs using MACS column (Miltenyi Biotec, Auburn, CA) after staining with Microbeads conjugated with mouse anti-human CD14 mAb (Miltenyi Biotec). Monocytes were cultured in RPMI 1640 supplemented with 10% FCS in the presence of 800 U/ml human rGM-CSF (rGM-CSF; R&D Systems, Minneapolis, MN) and 10 ng/ml rhL-4 (R&D Systems) for 4 days at 37°C in a humidified atmosphere with 5% CO2. Cells were washed with PBS containing 0.05% BSA and 2% EDTA before use.

Enrichment and expansion of Va24+ NKT cells

MNCs (1 x 10⁶) isolated from rhesus macaques, as described above, were incubated with FITC-conjugated mouse anti-human Va24 mAb (Beckman Coulter, Kansas, MO), followed by anti-FITC MACS beads (Miltenyi Biotec), and applied onto a MACS column. The positively selected cells (3–5 x 10⁶) were stimulated by irradiated (2300 rad) autologous CD14+ cells pulsed with α-GalCer (100 ng/ml; Kirin Brewery, Maebashi, Gunma, Japan) in the presence of rhL-2 (100 U/ml; R&D Systems) and rhL-15 (10 ng/ml; BioSource International, Camarillo, CA). From the third week, the NKT cells were stimulated by allogeneic monocyte-derived immature DCs (iDC), pulsed with α-GalCer in the same cytokines, as mentioned above. The cytokines and α-GalCer were added every 2–3 days.

Antibodies

Anti-human mAbs known to cross-react with rhesus macaques were selected for this study. These included mAbs Va24 FITC (C15; Immunotech, Marseille Cedex, France); Vβ11 PE (C21; Immunotech); CD8αa PC5 (B9.11; Immunotech); CD8β PE2ST8 (5H7; Immunotech); mAb clone 6B11-biotin, recognizing complementarity-determining region 3 (CDR3) of Va24+ NKT cells (33); CD3e FITC (SP34; BD PharMingen, San Diego, CA); CD4 FITC (M-T477; BD PharMingen); CD161 FITC (DX12; BD PharMingen); CD56 PE (MY31; BD PharMingen); CD45RA FITC (5H9; BD PharMingen);CCR7 unconjugated (2H4; BD PharMingen); CD28 PE (CD28.2; BD PharMingen); CD69 FITC (FN50; BD PharMingen); CD69 FITC/PE (TRAP; BD PharMingen); CD122 PE (Mik52, PharMingen); CD11a FITC (G25.2; BD Biosciences, San Jose, CA); β2 integrin PE (FB504; BD Biosciences); CD26L2 PE (SK11; BD Biosciences); CD45RO biotin R-PE (UCHL1; Ancell, Bayport, MN); CD1d unconjugated (CD1d55; kindly provided by S. Porcelli, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY). For intracellular staining, anti-IFN-γ FITC (MD-1; Biosource); anti-IL-4 FITC, PE (860A-4B3; Biosource); and anti-IL-10 PE (JE53-9D7; Caltag Laboratories, Burlingame, CA) mAbs were used. FITC-conjugated goat anti-mouse IgG (BD Biosciences) was used as a secondary Ab for the anti-human CD1d mAb.

Cell surface phenotyping by flow cytometry

Before staining, the cells were washed and resuspended in PBS containing 1% BSA and 0.01% NaN3, and subsequently incubated with the mAbs for 30 min on ice. After washing twice, 2 x 10⁵ labeled cells were subjected to two- or three-color FACScan flow cytometry using an Epics Elite Analyzer (Beckman Coulter).

Intracellular staining

For intracellular IFN-γ, IL-10, and IL-4 staining, 2–5 x 10⁶ NKT cells were stimulated for 4 h at 37°C with 20 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml ionomycin (Sigma-Aldrich) in 1 ml of complete medium. For intracellular staining the intracellular transport inhibitor brefeldin A (10 μg/ml; Sigma-Aldrich). The cells were then washed with staining buffer (0.05% BSA/EDTA in PBS) and permeabilized with FACS permeabilizing solution (BD Biosciences) for 10 min at room temperature. These cells were resuspended in staining buffer and incubated for 30 min on ice in the dark with FITC-conjugated anti-IFN-γ, PE-conjugated anti-human IL-10, or FITC/PE-conjugated anti-human IL-4 mAbs. Unbound Abs were removed by two washes with staining buffer. Finally, the cells were resuspended in PBS and analyzed by flow cytometry.

Measurement of cytokines in culture supernatants

NKT cells (10⁶/well) were cultured in a 24-well plate (Corning, Corning, NY) with α-GalCer-pulsed iDCs (10⁷/well) in the presence of rhL-2 and rhL-15 for 24, 48, 72, or 96 h. Cell-free supernatants were collected from each well and stored at −80°C before analysis. IFN-γ, IL-10, and IL-4 concentrations in culture supernatants were determined by ELISA kit (BioSource), according to the manufacturer’s recommended procedure.

Proliferation assay

MNCs (6 x 10⁴/well) were cultured in a 96-well round-bottom plate with irradiated mature DCs (10⁷/well) and PMA-stimulated (20 ng/ml, for 4 h) or unstimulated NKT cells (3 x 10⁴/well, 1.2 x 10⁷/well, or 6 x 10⁷/well). After 6 days, the cells were labeled with 1 μc/well of [³⁵S]thymidine (Life Science Products, Boston, MA) for 16 h and harvested with an automated cell harvester (Tomtec, Orange, CT). Incorporation of the radioisotope was measured by a liquid scintillation counter (Pharmacia Wallac, Turku, Finland).

Results

Va24+ rhesus macaque NKT cells use the same CDR3 region, but different Vβ TCR than human invariant NKT cells

We first asked whether rhesus macaque NKT cells express the same Vα and Vβ invariant TCR and CDR3 regions that are used by human invariant NKT cells. Using anti-human Va24 and anti-human Vβ11 mAbs that are cross-reactive with rhesus monkeys, we examined expression in rhesus peripheral blood, spleen, and culture-expanded cells. In fresh normal peripheral blood MNCs, and in freshly obtained normal spleen cells, only a small percentage of cells exhibited either Vβ11 (MNC mean 0.3 ± 0.14 SD, n = 7; spleen mean 0.25 ± 0.1 SD, n = 4) or Va24 expression (PBL mean 0.27 ± 0.12 SD; spleen mean 0.15 ± 0.05 SD). The percentage of Vβ11/Va24 double-positive T cells among the Va24+ fresh spleen cells was also low (mean 1.0 ± 0.7 SD, n = 3). After 3-wk expansion in culture, purified Va24+ spleen cells
expressed a high (65%) percentage of Vα24+ cells, but relatively few (4%) Vβ11+ cells. In longer term cultures (>2 mo), in which the cells were stimulated weekly with α-GalCer-pulsed iDC in the presence of rhIL-2 and rhIL-15, expression of Vα24 was nearly homogenous (>98%), while Vβ11 staining was less than 1% (Fig. 1a). Most (89%) of these Vα24+ cells also stained with mAb 6B11, which is specific for the CDR3 region of Vα24/JαQ TCR (Fig. 1b) and binds to human invariant NKT cells (33). Rhesus Vα24+ cells also specifically proliferated in response to α-GalCer-pulsed CD1d-transfected HeLa cells, but not to α-GalCer-pulsed, mock-transfected CD1d− control HeLa cells (data not shown). These data indicate that cultured rhesus NKT cells are semi-invariant, expressing Vα24 and invariant CDR3 epitopes, but not Vβ11.

The expression of T and NK cell markers by rhesus macaque NKT cells

It has been previously demonstrated that most Vα24+ invariant NKT cells display a DN or CD4+ phenotype, but rarely a CD8+ phenotype. We found that rhesus Vα24+ invariant TCR was coexpressed with CD3ε (Fig. 2a). CD8αβ and CD4 were essentially undetectable (Fig. 2, c and d). However, a high frequency of these cells expressed CD8αα− (Fig. 2b, mean 53 ± 10 SD, n = 4), which may have been up-regulated due to culture in the presence of IL-2 and IL-15 (18). Hereafter, we refer to these rhesus NKT cells as CD4/8DN because they were negative for CD4 and CD8β.

Earlier studies have reported that most invariant NKT cells in human and mouse coexpress NK cell markers, such as CD161 (7, 10, 34). CD161 is also expressed by many other human T cells (4). Before testing rhesus spleen cells with anti-human CD161 reagents, the available mAbs were validated for binding activity to freshly isolated human CD16+ NK cells (mean 19.9 ± 0.3 SD; n = 3), CD3+ T cells (mean 3.8 ± 1.9 SD; n = 6), and long-term (>1 mo) cultured CD3+ T cells (mean 37.5 ± 3.8 SD; n = 3). Normal rhesus PBL stained with anti-CD161 FITC (clone DX12) exhibited a small number (mean 0.4 ± 0.2 SD, n = 6) of positive cells, although both short- and long-term cultured NKT cells were consistently negative. In contrast, as shown in Fig. 3, >80% of rhesus Vα24+CD3+ cells were positive for CD56, a molecule commonly expressed on human NK cells (17). These results show that rhesus macaque spleen-derived NKT cells are mostly CD4/8DN and preferentially coexpress CD56.

Central and effector memory phenotype expression in NKT cells derived from rhesus macaque spleen

The tissue distribution of invariant NKT cells has been reported to affect the phenotype of these cells. Thymus- and liver-derived
NKT cells in mice have been described as memory phenotype-positive cells, whereas spleen- and bone marrow-derived murine NKT cells displayed a naive phenotype (20). Human PB and cord blood NKT cells express an activated memory phenotype (22). Freshly obtained rhesus NKT cells expressed a primed memory phenotype, but with variable levels of expression. The uncultured spleen cells, gated for the Vα24+ NKT cell population, strongly expressed CD45RO (mean 78.3 ± 11.4 SD, n = 3). Two of three rhesus were positive for CCR7 expression (mean 40.4 ± 17.6 SD), but negative for CD62L (mean 0.7 ± 0.5 SD), CD28 (mean 4.9 ± 2.1 SD), CD45RA (mean 0.2 ± 0.2 SD), β7 integrin (mean 1.9 ± 1.9 SD), and CD95 (mean 2.3 ± 2.3 SD). One of three displayed strong to moderate expression of CD62L, β7 integrin, CD45RA, CD95, and CD28. The expression of CD45RO was consistently characteristic of naive cells is consistent with an effector memory phenotype. The cells were stained with appropriate mAb or isotype control Ab, described in Materials and Methods, and analyzed by flow cytometry. Open histograms represent isotype control staining. Representative data from three independent experiments are shown.

**FIGURE 4.** Spleen-derived NKT cells show effector memory phenotype. a. Histogram profiles gated on lymphocyte forward/side scatter properties showed high to moderate expression of adhesion molecule CD11a, β7 integrin, and memory cell markers CD45RO and CD95. b. The absence of CD Ags characteristic of naive cells is consistent with an effector memory phenotype. The cells were stained with appropriate mAb or isotype control Ab, described in Materials and Methods, and analyzed by flow cytometry. Open histograms represent isotype control staining. Representative data from three independent experiments are shown.

For functional analysis, we harvested NKT cells 4 days after weekly stimulation with α-GalCer-pulsed DC, rhIL-2, and rhIL-15, typically a time of peak activation for NKT cells (36, 37). Although the NKT cells displayed a semianergic phenotype, >95% of unstimulated NKT cells expressed intracellular IL-10 (Fig. 6a), but no detectable IL-4 and IFN-γ (data not shown). After stimulation with PMA and ionomycin, >90% of the NKT cells coexpressed both IL-10 and IFN-γ (Fig. 6b). However, IL-4 expression was still negligible (not shown). The possibility of non-specific staining with anti-human IL-10 mAb was excluded by the staining of positive and negative control cells and inhibition of positive staining by rhIL-10 (Fig. 6c, d). In supernatants of NKT cells, large amounts of TGF-β, IL-13, and IL-6 were detected, but only low levels of IL-4 and IL-10 were observed, using ELISA reagents that efficiently detect rhesus IL-4 and IL-10 in our experience (38) (Fig. 7). Culturing the NKT cells with α-GalCer increased the production of IL-13 and IL-10 by 1.4- to 2.6-fold, compared with cultures without α-GalCer (data not shown). The cytokine production profile of anergic T cells following stimulation with high doses of cross-linked anti-CD3 mAb is distinct from Th1, Th2, or Th0 cells. Tr1/Th3 cells are typically anergic (39, 40), and their most striking feature is low proliferative capacity, unusually high levels of IL-10 and TGF-β, and significant amounts of IFN-γ, IL-5, but no IL-4 or IL-2 (41–43). In this context, we conditions, rhesus spleen-derived NKT cells, in contrast, did not express CD69, CD40L, but these markers were up-regulated after PMA and ionomycin stimulation (Fig. 5, a–d). Intracellular production of IL-2 (Fig. 5, e and f) and expression of CD25 (IL-2Rα) and CD122 (IL-2Rβ) failed to arise after stimulation with PMA/ionomycin (data not shown). Moreover, the NKT cells grew slowly, even though they were stimulated weekly with α-GalCer-pulsed DC in the presence of rhIL-2 and rhIL-15. This has previously been reported to be an optimal condition for expansion of human NKT cells (18). These results suggest that rhesus spleen-derived NKT cells are semianergic under regular culture conditions.

**Th3/T regulatory-1 (Tr1)-polarized, cytokine profile**

In the long-term cultures, there was relatively homogeneous expression of CD45RO, CD95, the adhesion molecule CD11a, and the mucosal homing receptor β7 integrin, indicating retention of a memory phenotype (Fig. 4a). In contrast, there was a consistent lack of expression of the secondary lymphoid tissue migration molecules, CD62L, CCR7, and also CD28, characteristic of the naive phenotype (35). These results are consistent with expression of the effector memory phenotype in long-term cultured spleen-derived NKT cells of rhesus macaques.

**Spleen-derived NKT cells exhibit semianergic phenotype in regular culture conditions**

CD69, an early activation marker of T and NK cells, is constantly expressed on the NKT cell surface in mice and humans (17, 19). In addition, a high expression of CD25 (IL-2Rα) has been observed in human cord blood NKT cells (22). Under regular culture conditions, rhesus spleen-derived NKT cells, in contrast, did not express CD69, CD40L, but these markers were up-regulated after PMA and ionomycin stimulation (Fig. 5, a–d). Intracellular production of IL-2 (Fig. 5, e and f) and expression of CD25 (IL-2Rα) and CD122 (IL-2Rβ) failed to arise after stimulation with PMA/ionomycin (data not shown). Moreover, the NKT cells grew slowly, even though they were stimulated weekly with α-GalCer-pulsed DC in the presence of rhIL-2 and rhIL-15. This has previously been reported to be an optimal condition for expansion of human NKT cells (18). These results suggest that rhesus spleen-derived NKT cells are semianergic under regular culture conditions.
Spleen-derived NKT cells suppress allogeneic T cell responses in vitro

Next, we investigated how the resting and activated NKT cells regulate T cell responses in allogeneic MLR. Both unstimulated/resting NKT cells and also PMA-stimulated NKT cells consistently suppressed allogeneic T cell responses in a dose-dependent manner. To elicit strong MLR responses, we used DCs from MHC class II DRB1-mismatched donors as a source of APC. As shown in Fig. 8, unstimulated NKT cells strongly inhibited (43%) T cell responses at a responder/NKT ratio of 2:1, while the inhibition was decreased from 21 to 13% at a responder/NKT ratio of 5:1 and 10:1, respectively. In contrast, using PMA-stimulated NKT cells, the strongest inhibition (47%) of T cell responses was found at a responder/NKT ratio of 10:1, and was dose dependent, decreasing from 36 to 28% at a responder/NKT ratio of 5:1 and 2:1, respectively. Supernatants from both unstimulated and stimulated NKT cells suppressed T cell proliferation to a lesser extent than the strongest inhibition of NKT cells. Taken together, these results indicate that rhesus macaque spleen-derived NKT cells can be induced as regulatory cells with suppressive function.

Discussion

NKT cells have been studied in humans, mice, and to a lesser extent rats, but, to our knowledge, NKT cells have not been defined in nonhuman primates. In this study, we obtained NKT cells from the spleens of rhesus macaques, expanded them in vitro, and characterized their phenotype and function. Due to the high homology between humans and rhesus macaques, many anti-human mAbs cross-react with rhesus CD Ags, including those reported in this work. The cross-reactivity of each mAb was confirmed on at least three healthy monkeys before using them in additional experiments. Our results indicate that rhesus spleen cells, positively selected for Vα24, consistently expressed the invariant CDR3 epitope, which is also expressed on human invariant NKT cells (33). However, unlike their human counterparts, rhesus Vα24+ NKT cells highly expressed CD56 and did not express Vβ11.

Discrimination of naive and memory phenotypic traits can provide insight into the biological function of NKT and conventional cells. It is known that naive T cells express high levels of L-selectin (CD62L) and CCR7 that promote their recruitment via high endothelial venules into lymph nodes. In contrast, memory T cells lose these two receptors, and instead express high levels of integrins (CD11a and β2) and chemokine receptors that promote recruitment into nonlymphoid tissues (44). The central memory cells recirculate through lymph nodes, where they encounter Ag. Primed central memory cells (CD45RO+CCR7+) are thought to represent a precursor population of further differentiated effector memory cells (CD45RO−, CCR7+) that have polarized cytokine production, reduced costimulatory requirements, and home to nonlymphoid tissues. Pitcher et al. (35) recently reported an increased ratio of memory (CD28−, CCR7+, CD62L−, CD45RO+) to naive (CD28+, CCR7+, CD62L+, CD45RO+) T cells in rhesus macaque spleen compared with blood, confirming that rhesus spleen is both a secondary lymphoid tissue (white pulp) and a potential effector site (red pulp) (45). In this context, we expected both naive and memory cells might coexist in our spleen NKT cells. However, we did not find evidence for naive cells in fresh rhesus spleen Vα24+ cell population. Most fresh spleen cells gated for the Vα24+ population expressed CCR7, and these cells consistently expressed CD45RO, showing a central memory phenotype. Consistent with this outcome, most Vα24+ cells were positive for CD11a, the α-chain of the adhesion molecule LFA-1. Vα24 positively enriched NKT cells lost their expression of CCR7, CD62L, and CD28, and developed elevated expression of CD45RO and CD95 after short-term (5 days) culture (data not shown). Likewise, in the long-term cultured NKT cells, the memory cell marker CD45RO was moderate to strongly expressed throughout the culture period. However, further studies of the long-term cultured NKT cells indicated a lack of expression of CD28, CD62L, and CCR7, along with high expression of CD11a and β2 integrin, and modest to high expression of CD95, confirming acquisition of an
effector memory phenotype in these spleen-derived rhesus macaque NKT cells. From these findings, we speculate that rhesus Vα24+ NKT cells home into the spleen as primed central memory cells, and following Ag challenge they differentiate into effector memory cells, able to home into nonlymphoid sites.

Although rhesus spleen NKT cells displayed the same effector memory phenotype as mouse and neonatal and adult human NKT cells (16, 17, 20, 21), they did not express activation markers in regular culture conditions. Following activation with PMA and ionomycin, CD69 and CD40L expression were up-regulated, but other activation and costimulatory molecules, particularly CD25 (IL-2Rα), CD122 (IL-2Rβ), and CD28, remained negative. Regardless of weekly stimulation with α-GalCer-pulsed DC in the presence of rhIL-2 and rhIL-15, the growth of rhesus NKT cells was slow, compared with that of human Vα24+ neonatal cord blood NKT cells (46). In addition, rhesus NKT cells consistently failed to produce intracellular IL-2 following stimulation with PMA and ionomycin. Several factors may have contributed to this outcome. For one, the lack of IL-2R on the rhesus NKT cells could explain why they failed to proliferate, despite the presence of high rhIL-2 concentrations. We speculate that the lack of costimulatory molecules on iDC, added weekly to the NKT cultures, may have also played a role. Except for conditions in which there is high membrane Ag density, stimulation of the TCR with Ag is generally insufficient to generate a productive conventional T cell response with cytokine secretion and clonal expansion (47). Instead, suboptimal cross-linking of the TCR by Ag tends to lead to anergy, which can be prevented by costimulation provided by accessory molecules or the IL-2R (48, 49). Fujii et al. (50) showed that α-GalCer stimulation alone causes NKT cell anergy. In our experiments, frequent administration of free α-GalCer every 2–3 days, between the weekly stimulation with DC, might have driven the rhesus NKT cells to become anergic. At the same time, the high concentrations of supplemental rhIL-2 and rhIL-15, acting via low density cytokine receptors, might have prevented them from becoming fully anergic, resulting in their long-term maintenance, albeit slow growth in vitro. Overall, we suggest that a semianergic state characterizes rhesus macaque spleen NKT cells in our regular culture conditions.

The semianergic NKT cells exhibited a regulatory cytokine profile, common to that of Th2, Tr1, and Th3 regulatory cells. As with conventional T cells, iDCs might induce NKT cells to differentiate into regulatory cells (51). Roncarolo et al. (52) recently demonstrated that CD4+ Tr1 cell clones produce high levels of IL-10 and TGF-β, moderate amounts of IFN-γ and IL-5, but little or no IL-2 or IL-4, when activated via the TCR. In addition, Tr1 cell clones rapidly produce IL-10 within 12–24 h after stimulation (53). In contrast, T cells that are stimulated by the oral route primarily secrete TGF-β, and are described as Th3 regulatory cells (54). However, T cell lines generated from tolerant animals, following oral administration of copolymer 1, produce high levels of IL-10.

**FIGURE 6.** Dot plot profiles of Vα24+ NKT cells stained for intracellular cytokines. a, Unstimulated NKT cells routinely produced intracellular IL-10, but minimal IFN-γ. b, PMA/ionomycin stimulation up-regulated intracellular IFN-γ production. The cells were harvested at day 4 after weekly stimulation and incubated with or without PMA/ionomycin for 4 h at 37°C in complete medium containing brefeldin A (10 μg/ml), then washed, fixed, permeabilized, and stained with anti-human IL-10 and anti-human IFN-γ or isotype control Abs. Intracellular anti-human IL-10 mAb was preincubated with rhIL-10 (0.25 μg/test) at room temperature for 30 min. Then HEK cells were separately stained with preblocked or unblocked anti-human IL-10 mAb. Negative cell surface IL-10 staining is shown on unpermeabilized HEK cells, and positive staining for intracellular IL-10 is shown on permeabilized cells using anti-human IL-10 mAb. d, Rhesus spleen-derived CD3+Vα24+ T cells were stained with anti-human IL-10 or isotype control mAbs after 4-h stimulation with PMA/ionomycin, following fixation and permeabilization. Representative data from three independent experiments are shown.

**FIGURE 7.** Cytokine secretion of NKT cells in ambient culture supernatants measured by ELISA. Culture supernatants were collected from the cells at day 4 after weekly stimulation with α-GalCer-pulsed DC, but without PMA/ionomycin stimulation. Concentrations were calculated from the mean of three wells for each cytokine. Representative data from three to five independent experiments are shown.
Additionally, neutralizing Ab against TGF-β release in vitro. They showed that TSP-1-induced activation of cells, including T cells (56), can inhibit IL-10 and enhance IL-6.

Strikingly, they found that rhesus NKT cells in resting conditions produced IL-10 at the single cell level and secreted a large amount of TGF-β and IL-13. However, the secretion of IL-10 was low at all time points tested (24–72 h; data not shown), and production of both IL-4 and IL-2 was negligible. In this context, the cytokine profile of rhesus NKT cells more closely resembles that of Th3 regulatory cells than Tr1 cells. However, the high intracellular expression of IL-10 in the rhesus cells leads us to speculate that they belong to a Tr1 lineage.

The discrepant pattern of positive intracellular IL-10 and negative extracellular IL-10 suggests that an inhibitory mechanism may be interfering with IL-10 secretion in vitro. Yamauchi et al. (57) recently demonstrated that thrombospondin-1 (TSP-1), an extracellular matrix glycoprotein synthesized by various types of cells, including T cells (56), can inhibit IL-10 and enhance IL-6 release in vitro. They showed that TSP-1-induced activation of TGF-β1, or exogenously added TGF-β1 inhibited IL-10 release. Additionally, neutralizing Ab against TGF-β1 reversed TSP-1-induced inhibition of IL-10 release. Collectively, these findings led us to speculate that latent TGF-β might be activated by TSP-1 in the rhesus NKT cell cultures, leading to inhibition of IL-10 release into the supernatants. This mechanism, currently under examination, might explain the paradoxical high intracellular IL-10 and low IL-10 secretion pattern that we have observed.

Both PMA-stimulated and resting rhesus NKT cells and their culture supernatants inhibited T cell responses in allogeneic MLR. This is analogous to and extends reports in human and mouse (13, 28). In view of their different cytokine and cell surface phenotypes, we expected that the suppressive effects of rhesus NKT on MLR might be different between PMA-stimulated and resting NKT cells. Indeed, PMA-stimulated NKT cells suppressed MLR responses more strongly than did resting NKT cells, possibly related to the augmented TGF-β produced by PMA-stimulated NKT cells. However, the suppression by stimulated NKT cells occurred in an inverse dose-response manner. The latter outcome might be explained by the heightened expression of CD40L and IFN-γ after PMA stimulation. Resting NKT cells expressed no detectable CD40L or IFN-γ, whereas >90% of the stimulated cells expressed both. In this context, it is conceivable that the PMA-stimulated NKT cells enhanced the Ag-presenting activity of allogeneic DC in MLR via CD40-CD40L interactions, promoting IL-12 and IFN-γ responses. In the MLR assays that contained the highest numbers of NKT cells, this mechanism, along with the increased expression of IFN-γ by PMA-stimulated cells, could have defeated the suppressive action of the PMA-stimulated NKT cells. Given the striking conservation of NKT cells, another possibility is that resting NKT cells responded to allogeneic CD1d+/CD161+ APC in MLR in a manner distinct from that of pharmacologically (PMA/ionomycin)-activated NKT cells. Taken together, our results suggest that NKT cells from rhesus macaques have a novel invariant TCR, but Vβ11+ CD56+ CD161+ and memory phenotype coupled to a distinct regulatory suppressive function.

In conclusion, we have characterized spleen-derived rhesus macaque NKT cells, which have been cultured and expanded in vitro. This expansion is necessary because the numbers of NKT cells present in normal rhesus tissues are too low to allow for extensive analysis. Our results allow several conclusions regarding cultured rhesus NKT cells. First, they are highly homologous with human NKT cells and less so with murine counterparts. Second, they display a semi-invariant TCR (Vα24Jα18Qβ1+) and are CD4+ CD8− DN, CD161−, and CD56+ cells with effector memory phenotype. Third, rhesus NKT cells and their culture supernatants suppress allogeneic MLR, and have a cytokine expression pattern most consistent with Th3 cells. Although these properties might be skewed by in vitro expansion, the studies provide original information that will be useful for future characterization of uncultured NKT cells.

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References

FIGURE 8. Suppressive effect of NKT cells on DC-stimulated allogeneic T cell proliferation. NKT cells and culture supernatants were harvested at day 4 after weekly stimulation. PMA/ionomycin stimulation was in the absence of brefeldin A; R, responder T cell; DC, allogeneic mature DC; sup., NKT culture supernatant; ratio of R:DC, 20:1. Ratio of R:NKT, 2:1, 5:1, and 10:1. Representative data of four independent, triplicate experiments are shown.


