Neonatal invariant Vα24$^+$ NKT lymphocytes are activated memory cells

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NKT cells are a small subset of T lymphocytes which express an invariant Vα24JαQ TCR and recognize glycolipids presented by CD1d. In adults, NKT cells have a memory phenotype, frequently associated with oligoclonal expansion, express NK cell markers, and produce T0 cytokines upon primary stimulation. Because of these features, NKT cells are regarded as lymphocytes of innate immunity. We investigated NKT cells from cord blood to see how these cells appear in the absence of exogenous stimuli. We found that NKT cells are present at comparable frequencies in cord blood and adult peripheral blood mononuclear cells and in both cases display a memory (CD45RO$^+$CD62L$^-$) phenotype. However, neonatal NKT cells differ from their adult counterparts by the following characteristics: (1) they express markers of activation, such as CD25; (2) they are polyclonal; (3) they do not produce cytokines in response to primary stimulation. Together, our data show that human NKT cells arise in the newborn with an activated memory phenotype, probably due to recognition of an endogenous ligand(s). The absence of oligoclonal expansion and primary effector functions also suggest that neonatal NKT cells, despite their activated memory phenotype, require a further priming/differentiation event to behave as fully functional cells of innate immunity.

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1 Introduction

Natural killer T (NKT) cells are a unique subpopulation of T lymphocytes which is highly conserved in both human and murine species [1]. NKT cells express some NK-specific surface markers, such as the C-type lectin NKRP-1A [2], thereby sharing some properties with classical NK cells. However, the most reliable marker to consistently identify this subset is their peculiar TCR. NKT cells are characterized by the usage of a highly homogenous TCR, consisting in humans of an invariant Vα24JαQ rearrangement paired preferentially with a variable Vβ11 chain [3]. In the mouse, NKT cells express an invariant Vα14Jα281 rearrangement paired with variable Vβ8, Vβ7 or Vβ2 [1]. In terms of coreceptor expression, NKT cells belong either to the single-positive CD4$^+$ or the double-negative CD4$^-$CD8$^-$TCRα$^+$/β$^+$ subsets of lymphocytes.

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Abbreviations: CBL: Cord blood lymphocytes **α-GalCer:** α-galactosylceramide

Although natural ligands of NKT cells have not yet been identified, these cells are activated when their TCR recognizes glycosylceramides derived from marine sponges, presented by CD1d [4–6]. This class of α-glycosylated ceramides is not readily detectable in mammals; however, they should share critical structural features with natural CD1d ligands, suggesting that NKT cells recognize antigens containing a hydrophobic (lipid) and a hydrophilic moiety. NKT cells have characteristic effector functions and secrete large amounts of both IFN-γ and IL-4 upon activation [7, 8]. Moreover, the phenotypic analysis of human NKT cells from peripheral blood showed constitutive expression of IL-2Rβ chain and of the memory marker (CD45RO), but low or not expression of activation markers such as CD69 or HLA-DR [8].

As little is known about the natural antigen recognized by these cells, we assessed phenotype and function of NKT cells in cord blood, assuming that neonatal NKT cells should be naive in terms of exogenous antigen stimulation. In the mouse, NKT cells are virtually absent in neonates and accumulate after birth, reaching their full

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development at 6–8 weeks of life [9, 10], whereas the only study on neonatal human NKT cells has found that these cells are already present at birth and that they can be expanded by α-galactosylceramide (α-GalCer) [11].

In the present study we found that human neonatal NKT cells display a memory phenotype similarly to their counterpart in the adult peripheral blood. At variance with adult NKT cells, neonatal NKT cells express CD25, a marker of recent activation, but they are incapable of secreting IFN-γ or IL-4 upon stimulation *ex vivo*. This uncoupling between phenotype and function in neonatal NKT cells suggests that at birth human NKT cells are not yet fully mature although present at frequencies comparable to those found in adults.

2 Results

2.1 Frequencies of NKT cells in cord blood and adult PBL

We first compared PBL from adults and umbilical cord blood lymphocytes (CBL) for their naive/memory phenotype (CD45RA and CD45RO) and expression of activation markers such as CD25. As expected, the PBL were a mixture of $CD45RA^+$ and $CD45RO^+$ cells whereas the CBL were mostly $CD45RA^+CD45RO^-$ (Fig. 1 A). Signs of recent activation, as indicated by expression of CD25, were poor on both PBL and CBL (Fig. 1 B). We then compared frequencies of $V\alpha24^+V\beta11^+$ T lymphocytes (NKT cells) present in the $CD3^+$ population from PBL or CBL. The representative experiment in Fig. 1 C shows that there was a similar frequency of NKT cells in adult and neonatal circulating lymphocytes. Fig. 1 D also shows that the majority of $V\alpha24^+V\beta11^+$ T lymphocytes expressed the NK marker NKRP-1A both in PBL and CBL. From this analysis we conclude that NKT cells are present at comparable frequencies in cord blood and adult PBL.

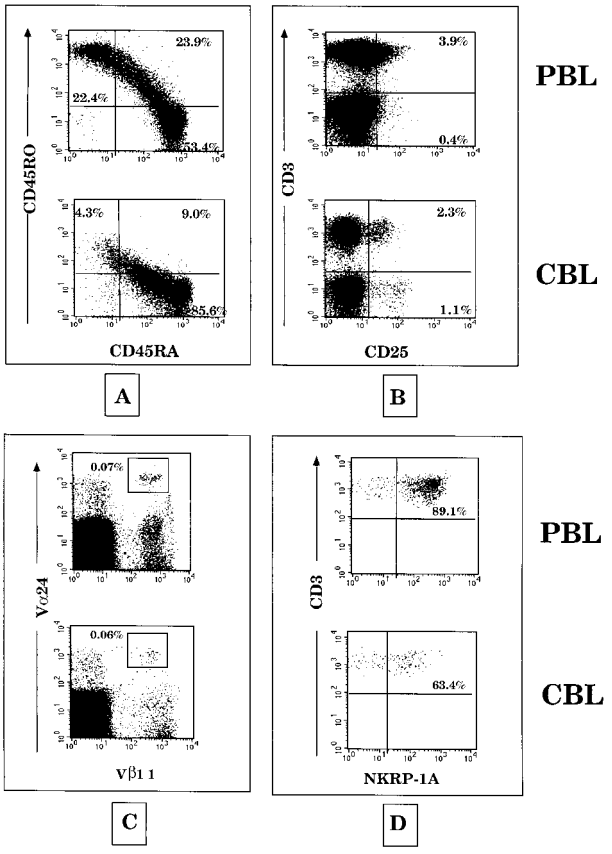

Fig. 1. Frequencies of NKT cells in cord blood and adult blood. PBL and CBL were examined for reactivity with mAb to (A) CD45RA, CD45RO and CD3; (B) CD3 and CD25; (C) Vα24, Vβ11, NKRP-1A and CD3. After gating on $V\alpha24^+V\beta11^+$ cells, representative dot plots for $CD3^+$ and $NKRP\text{-}1A^+$ cells were generated (D).

2.2 Neonatal NKT cells display an activated memory phenotype

To characterize the functional and activation state of neonatal NKT cells, we compared by four-color immunofluorescence a variety of surface markers in $V\alpha24^+V\beta11^+$ T cells present in CBL and PBL. The representative experiment in Fig. 2 A shows that, as previously reported, adult NKT cells were almost exclusively $CD45RO^+CD45RA^-$ [8]. Similarly, the vast majority (79.7 %) as median value of seven different CBL samples) of $V\alpha24^+V\beta11^+$ T cells from CBL were $CD45RO^+CD45RA^-$ (Fig. 2 B), while the $V\alpha24^+V\beta11^-$ as well as $V\alpha24^-V\beta11^+$ were virtually all $CD45RA^+CD45RO^-$ (data not shown). We next looked at the expression of the adhesion molecule CD62L (L-selectin), which is expressed by naive T cells and down-modulated on memory/effector cells which are ready to migrate into peripheral tissues [12]. NKT cells from PBL (Fig. 2 C) and CBL (Fig. 2 D) were both completely negative for CD62L expression, whereas the great majority of total CBL expressed high levels of CD62L (data not shown).

Further analysis of activation marker expression showed that, similar to what has been reported for NKT cells from adult PBL [8], neonatal $V\alpha24^+V\beta11^+$ T cells were negative for HLA-DR (Fig. 3 D) and CD69 (Fig. 3 B). However, we observed that a large percentage (67.1 % as median value of eight CBL samples) of neonatal NKT cells (Fig. 3 F) expressed the IL-2Rα chain (CD25), while their adult counterpart was completely $CD25^-$ (Fig. 3 E). We

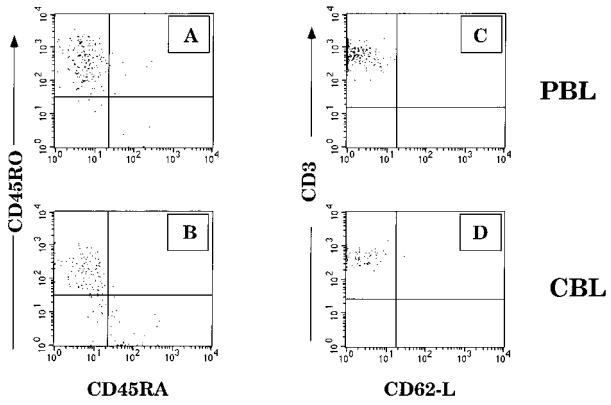

Fig. 2. Phenotype of Vα24$^+$Vβ11$^+$ NKT cells from adults and neonates. PBL (A, C) and CBL (B, D) were gated on Vα24$^+$Vβ11$^+$ cells and then analyzed for expression of CD45RA and CD45RO (A, B), or CD3 and CD62L (C, D).

therefore conclude that the majority of neonatal NKT cells have been activated recently.

2.3 Effector functions of cord blood NKT cells

A major difference between "naive" and "memory" or "effector" Th cells is in their effector functions. After stimulation, naive cells produce only IL-2, whereas the effector subset of memory cells [12] produces several cytokines. Depending on the type of cytokine produced, effector cells can be functionally distinguished into T1 (mainly making IFN-γ), T2 (mainly IL-4 producer) and T0 which can make both IFN-γ and IL-4 [13, 14]. We therefore assessed the ability of NKT cells to produce cytokines upon activation. We performed intracellular staining for IFN-γ and IL-4 in freshly isolated CBL and PBL stimulated for 4 h with PMA and ionomycin. As expected, NKT cells from PBL were T0 lymphocytes that could produce both cytokines (Fig. 4 A and B). By contrast, the Vα24$^+$Vβ11$^+$ cells from the cord blood did not produce either cytokine (Fig. 4 A and B). In the same experiment, we found that NK cells (CD3$^-$, CD16$^+$) from CBL cells produced significant amounts of IFN-γ (data not shown), indicating that the inability of neonatal NKT cells to produce cytokines is not solely due to a general impairment of effector function in CBL.

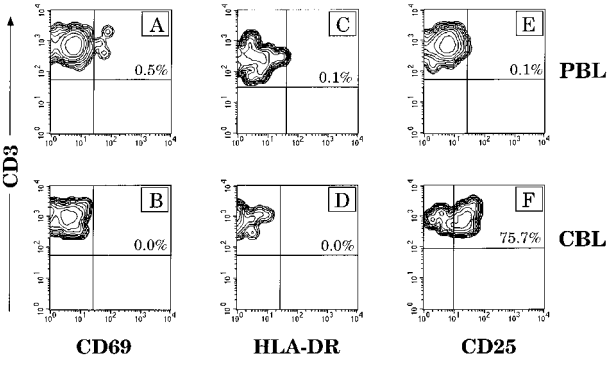

Fig. 3. The majority of cord blood NKT cells are CD25$^+$. PBL (A, C, E) and CBL (B, D. F) were gated on Vα24$^+$Vβ11$^+$ cells and then analyzed for expression of CD3 and CD69 (A, B), CD3 and HLA-DR (C, D), CD3 and CD25 (E, F).

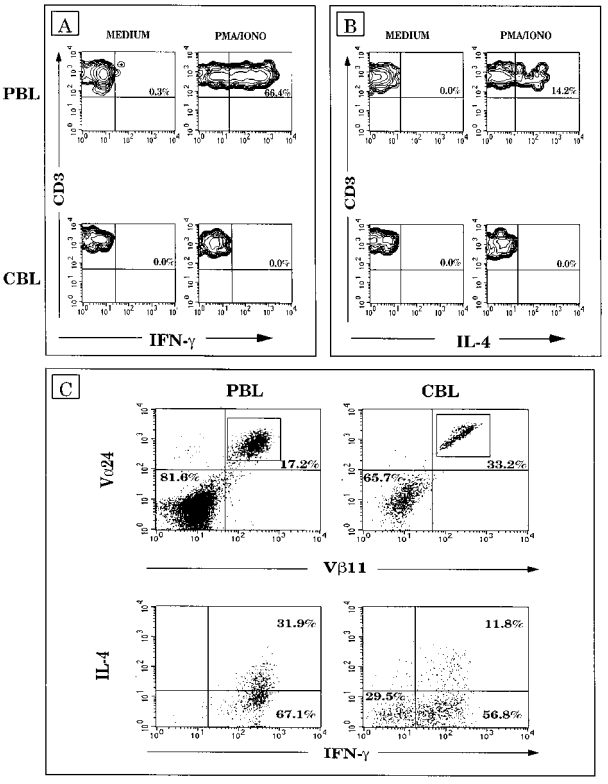

Fig. 4. IFN-γ and IL-4 production by Vα24$^+$Vβ11$^+$ NKT cells. (A, B) PBL and CBL were stimulated with PMA and ionomycin and stained with mAb to Vα24, Vβ11 and CD3. After fixation and permeabilization, the cells were stained again with mAb to either IL-4 or IFN-γ. Cytokine production by the NKT cells was analyzed by gating on CD3$^+$, Vα24$^+$ Vβ11$^+$ cells. The data shown are from a representative experiment out of five performed. (C) After 15 days of stimulation with α-GalCer, CBL and PBL were stained with mAb to Vα24, Vβ11 and CD3. All the Vα24$^+$ cells present in the culture were also Vβ11$^+$. These same cells were stimulated for 4 h with PMA/ionomycin and then stained with anti-CD3 and -Vα24 mAb. After fixation and permeabilization the cells were stained again for IFN-γ and IL-4 reactivity. The dot plots show the results obtained after gating on CD3$^+$ and Vα24$^+$ cells.

We then asked whether neonatal NKT cells can acquire the ability to secrete cytokines after secondary stimulation *in vitro*. CBL and PBL were expanded *in vitro* with the CD1d ligand α-GalCer and IL-2 [5, 6, 15]. After 2 weeks of culture, we obtained T cell lines highly enriched in Vα24$^+$Vβ11$^+$ cells from both PBL and CBL (Fig. 4 C), and then tested these cells for their ability to produce cytokines in response to PMA/ionomycin stimulation for 4 h. The representative experiment in Fig. 4 C shows that a sizeable fraction of neonatal NKT cells, upon secondary stimulation *in vitro*, was capable of producing IFN-γ only or both IFN-γ and IL-4. It is worth noting, however, that about a third of the CBL-derived NKT cells did not make either cytokine, whereas virtually all the NKT cells expanded from adult PBL made cytokines, suggesting that neonatal NKT cells are not functionally homogeneous even when expanded in culture.

2.4 Clonal analysis of Vα24$^+$/Vβ11$^+$ NKT cells from PBL and CBL

Despite their activated effector phenotype (CD45RO$^+$, CD25$^+$, CD62L$^-$), neonatal NKT cells do not display effector function upon primary stimulation *in vitro*, thus showing features of both naive and primed T cells. To obtain more insights into their activation experience, we assessed the clonal composition of neonatal invariant Vα24$^+$ NKT cells, and compared it to that of adult NKT cells. To enrich for a sizeable number of NKT cells, total mononuclear cells from either adult PBL or CBL were activated *in vitro* with α-GalCer for 14 days. The responding CD3$^+$Vα24$^+$Vβ11$^+$ T cells (Fig. 4C, top panels) were then purified to homogeneity by cell sorting, lysed, and subjected to PCR-heteroduplex analysis to determine either the Vα24 or Vβ11 clonal heterogeneity. The sorted samples contained just the canonical Vα24-JαQ rearrangement, confirming that the activation and sorting protocols had effectively enriched for NKT cells expressing the homogeneous Vα24 chain (data not shown). The analysis of the clonal heterogeneity of the TCR Vβ11 chains in each invariant Vα24$^+$ NKT cell population showed that seven out of nine (77 %) adult-derived invariant Vα24$^+$Vβ11$^+$ NKT cell populations displayed an oligoclonal pattern, characterized by the presence of discrete bands migrating in the heteroduplex gel (Fig. 5). Only a minority of adult donors (two out of nine, samples A5-1 or A5-2 and A7) had peripheral blood-derived Vα24/Vβ11 NKT cells bearing a polyclonal TCR Vβ11 repertoire, as documented by the presence of a continuos smear in the heteroduplex gel. Contrasting with the adult samples, all three neonatal invariant Vα24$^+$ NKT cell samples displayed a highly polyclonal TCR Vβ11 pattern. Interestingly, the neonatal sample N3 was obtained by plating 4000 mononuclear cells per well in the presence

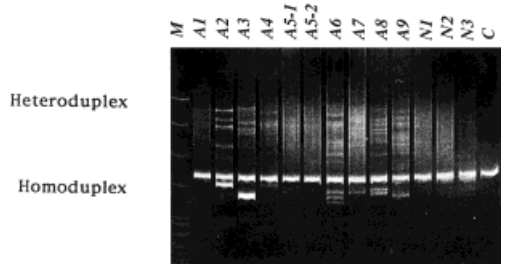

Fig. 5. Adult- and neonatal-derived invariant Vα24$^+$Vβ11$^+$ NKT cells display different clonal heterogeneity. To obtain adult peripheral blood (A1–9)- and neonatal cord blood-derived NKT cells (N1–3), invariant Vα24$^+$β11$^+$ NKT cells were expanded *in vitro* for 12 days with 100 ng/ml α-GalCer in the presence of 50 U/ml human recombinant IL-2, sorted by flow cytometer using anti-Vα24 and Vβ11 mAb, and immediately lysed for RNA extraction. Shown are the heteroduplex patterns of the TCR Vβ11 cDNA contained in each NKT cell population, performed on the Vβ11-Cβ PCR products obtained from the sorted samples according to Wack et al. [21]. M is the marker, A5-1 and 5-2 indicate the Vβ11 heteroduplex pattern of NKT cells obtained from peripheral blood of adult donor 5 at 1 month interval; C is the homoduplex produced by the Vβ11-Cβ carrier DNA alone.

of α-GalCer, thus generating microcultures containing a limited number of Vα24$^+$ NKT precursors. Cells from wells displaying growth were pooled before sorting, to re-create a heterogeneous Vβ11 repertoire. Indeed, even by using this protocol, prone to bias the number of responding T cell clones due the low frequency of Vα24$^+$Vβ11$^+$ cells in the cultures, the pattern of TCR Vβ11 rearrangements was still highly diverse, suggesting that this neonatal NKT cell sample was already quite heterogeneous before starting the cultures.

3 Discussion

We chose to investigate cord blood-derived NKT cells, reasoning that neonatal NKT cells should be naive with regard to exogenous antigen exposure. Previous studies in the mouse have shown that NKT cells are absent at birth, begin to appear 2–3 weeks later, and reach complete maturation at 6–7 weeks of life [9, 10], indicating that this population differentiates and/or expands after birth. Instead, in humans, it has been reported that NKT cells are already present in cord blood with frequencies similar to those found in adult PBL [11]. Our findings that neonatal NKT cells already exhibit an effector memory phenotype (CD45RO$^+$, CD45RA$^-$, CD62L$^-$) provide evidence that these cells develop and expand in the absence of exogenous stimuli. Furthermore, activation of neonatal NKT cells must take place around birth, given

the selective expression of the marker of recent activation CD25 on these cells. Together, these data suggest that neonatal NKT cells are recently activated, presumably by a self ligand.

It is intriguing that these cells do not respond to a stimulus with production of cytokines. This implies that from a functional perspective they are not yet fully mature, suggesting that additional steps for maturation are required. Two different mechanisms can be envisaged: NKT cells may arise as a consequence of a simple developmental program; alternatively, their maturation could be driven specifically by their encounter with an antigen. Our data showing that neonatal NKT cells produce cytokines in a secondary stimulation, after α-GalCer expansion, support the second hypothesis. This differs from their development in the mouse, where NKT cells take longer to appear, but once generated, appear fully functional, and suggests a fundamental difference between the predefined developmental programs of NKT cells in mice and humans.

A second indication that specific antigen encounter takes place in the developing fetus comes from the comparison of the TCR clonal composition of neonate *vs.* adult NKT cells. Thus, while neonatal NKT cells are polyclonal in their Vβ chain usage, the adult NKT cells exhibit more restricted Vβ usage. This could be explained by the existence *in utero* of a ligand which does not select for any particular clone, and results in a starting pool of $CD25^+$ NKT cells with a diverse Vβ repertoire. The same antigen may become less abundant or accessible after birth, thereby selecting only certain NKT cell subsets and resulting in the oligoclonality of adult NKT cells. Alternatively, a different antigen may be responsible for selecting a more homogeneous population of NKT cells from the starting pool.

The expression of a memory phenotype ($CD45RO^+$ $CD45RA^-$) and concomitant absence of effector function, which we describe for neonatal NKT cells, has been found recently among a subset of human memory T cells. It has been shown that within the $CD45RO^+$ cells, only the so-called peripheral memory subset, which does not express CCR7 and is very low for CD62L, can produce cytokines *in vitro* upon activation [12]. The central memory subset, which expresses both CCR7 and CD62L, does not produce cytokines upon primary activation, but can do so after secondary stimulation. The peripheral memory cells should be able to mediate effector function in tissues, whereas the central memory cells should divide and maintain the clonal size of the memory populations in blood and lymph. Interestingly, neonatal NKT cells do not fit in either subset, since they appear phenotypically as peripheral memory cells ($CD45RO^+$, $CD45RA^-$, $CD62L^-$), but behave functionally as central memory cells, as they do not produce cytokines upon primary stimulation, Therefore, neonatal NKT cells may serve a similar function as central memory cells by establishing a pool of cells that can be selected to become effector cells.

Previous analysis of NKT cells has revealed several general features which differ from those identified for conventional α/β T cells but resemble more those of innate immune responses. Our results provide more evidence that this lymphocyte subset straddles the adaptive and innate immune system. Similar to NK cells, NKT cells are already expanded in fetal life, but while NK cells are fully mature and well functioning [16], NKT cells, similar to T cells, need a second step to reach full maturation.

Using a mouse transgenic model, Alferink et al. [17] have shown trafficking of virgin T cells through peripheral tissues in the neonate. In the non-lymphoid tissues the cells encounter an antigen starting an activation process which results in induction of tolerance. The tolerized naive T cells show a memory-like phenotype but are effector function negative, thus resembling the neonatal NKT cells described here. Furthermore, these tolerized cells are diluted by the new (not tolerized) cells which have lost the ability to migrate to non-lymphoid organs. It is tempting to speculate that a fraction of neonatal NKT cells may be "naive", and indeed some $CD45RA^+CD45RO^-$ NKT cells could be found in the CBL (see Fig. 2 B). Similar to the naive T cells described by Alferink et al. [17], neonatal "naive" NKT cells might be destined to undergo a tolerizing process during which they acquire an activated memory phenotype ($CD25^+CD45RO^+$) but remain effector function negative. This interpretation is in agreement with the proposed regulatory role of NKT cells which has emerged from previous studies on systemic sclerosis patients [18], non-obese diabetic (NOD) mice and SJL mice [19, 20].

In conclusion, the present study demonstrates that neonate and adult humans bear invariant $V\alpha24^+V\beta11^+$ NKT cells at the same frequency and with the same memory phenotype ($CD45RO^+CD45RA^-$) but they are different in terms of activation markers, clonal diversity and effector functions.

4 Materials and methods

4.1 Preparation of PBL and CBL

Human umbilical cord blood samples were collected during labor after obtaining informed consent. Heparinized umbilical cord blood and peripheral blood obtained from healthy

donors were diluted vol/vol in PBS and separated on Ficoll-Paque density gradient (Pharmacia, Uppsala, Sweden). The cells obtained were used immediately or after thawing of aliquots kept in 90 %FCS/10 %DMSO in liquid N_3 until the day of the experiment.

4.2 Stimulation and staining for intracellular cytokines

CBL or PBL (10^5–10^6) were incubated with RPMI-10 %FCS with or without 1 μg/ml ionomycin (Sigma) and 50 ng/ml PMA (Sigma Chemical Co.) for 4 h. Brefeldin A (Sigma) was added at 10 μg/ml to the cells during the last 2 h of culture. Cells were then washed twice with sterile PBS and stained for surface markers. For the intracytoplasmic staining of cytokines, cells were fixed in 2 % paraformaldehyde (Sigma) for 15 min at 4 °C, washed again in PBS and permeabilized with PBS/1 % BSA/0.5 % saponin (S-7900; Sigma) for 15 min at room temperature. Cells were then stained with anti-IFN-γ and anti-IL-4 mAb (3 μl/test, either FITC- or PE-labeled) for 20 min at 4 °C, washed twice in PBS/1 %BSA, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Lymphocytes were gated according to the forward and side scatter parameters and results were analyzed using Cellquest software (Becton Dickinson).

4.3 *In vitro* expansion of NKT cells

α-GalCer (100 μg/ml in 100 %DMSO) was placed for 5 min at 65 °C, vortexed for 1 min and placed at 37 °C for at least 30 min. To activate the cells, the solution was used at 30 or 100 ng/ml. At days 2, 4 and 9, 50 U/ml IL-2 were added to the culture. Expansion of $V\alpha24^+V\beta11^+$ NKT cells was determined upon staining with a combination of anti-Vα24, anti-Vβ11, and anti-CD3 mAb.

4.4 Antibodies and flow cytometry

The following purified, FITC-, PE- or PerCP-conjugated mAb were used in various combinations for triple or quadruple staining: anti-CD3 (SK7, mouse IgG1), anti-Vα24 (C15, mouse IgG1; Coulter-Immunotech), anti-Vβ11 (C21, mouse IgG2a; Coulter-Immunotech), anti-NKRP-1A (DX12, mouse IgG1), anti-CD45RA (L48, mouse IgG1), anti-CD45RO (UCHL-1, mouse IgG2a), anti-CD25 (2A3, mouse IgG1), anti-CD69 (L78, mouse IgG1), anti-CD62L (Dreg-56, mouse IgG1) and anti-HLA-DR (L243, mouse IgG2a). Cells were washed twice with PBS and incubated with the required mAb for 20 min at 4 °C. After staining, the cells were washed, resuspended in PBS/2 % FCS and analyzed with a FACScalibur flow cytometer. Except were indicated all mAb were from Becton Dickinson.

In the case of multiple stainings including the biotinylated anti-Vα24, this antibody was first added to the cells for an incubation at 4 °C for 20 min. Cells were washed and incubated with streptavidin-PerCP as a second staining step together with the FITC- and PE-labeled mAb.

Lymphocytes were gated according to the forward and side scatter parameters of the FACS. Typically 100 000 to 500 000 events were acquired to ensure that a significant number of Vα24 T cells could be studied. Results were analyzed using Mac Cellquest software.

4.5 Heteroduplex analysis

Total RNA and cDNA was prepared as previously described [3]. To perform PCR and heteroduplex analysis, 1/20 of the cDNA from each sample was amplified by PCR using the following primers: Vβ11:5'TCAACAGTCTCCAGAATAAGGACG3'; Cβ:5'CACCCACGAGCTCAGCTCCACGTGGTC3'.

PCR conditions were: 94 °C for 5 min, followed by 35 cycles at 94 °C, 61 °C, 72 °C for 30 s each, and a final extension time of 10 min at 72 °C. A third of the PCR volume from each samples was mixed with 200 ng of a Vβ11-Cβ DNA carrier as described [21], and subjected to heteroduplex formation for 10 min at 94 °C, followed by 60 min at 50 °C. Heteroduplexes were separated on a native acrylamide gel as described in Wack et al. [21].

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