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IL-21 Is Produced by NKT Cells and Modulates NKT Cell Activation and Cytokine Production[1]

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The common γ-chain cytokine, IL-21, is produced by CD4$^+$ T cells and mediates potent effects on a variety of immune cells including NK, T, and B cells. NKT cells express the receptor for IL-21; however, the effect of this cytokine on NKT cell function has not been studied. We show that IL-21 on its own enhances survival of NKT cells in vitro, and IL-21 increases the proliferation of NKT cells in combination with IL-2 or IL-15, and particularly with the CD1d-restricted glycosphingolipid Ag α-galactosylceramide. Similar to its effects on NK cells, IL-21 enhances NKT cell granular morphology, including granzyme B expression, and some inhibitory NK receptors, including Ly49C/I and CD94. IL-21 also enhanced NKT cell cytokine production in response to anti-CD3/CD28 in vitro. Furthermore, NKT cells may be subject to autocrine IL-21-mediated stimulation because they are potent producers of this cytokine following in vitro stimulation via CD3 and CD28, particularly in conjunction with IL-12 or following in vivo stimulation with α-galactosylceramide. Indeed, NKT cells produced much higher levels of IL-21 than conventional CD4 T cells in this assay. This study demonstrates that NKT cells are potentially a major source of IL-21, and that IL-21 may be an important factor in NKT cell-mediated immune regulation, both in its effects on NK, T, and B cells, as well as direct effects on NKT cells themselves. The influence of IL-21 in NKT cell-dependent models of tumor rejection, microbial clearance, autoimmunity, and allergy should be the subject of future investigations. *The Journal of Immunology,* **2007, 178: 2827–2834.**

Natural killer T cells are a unique population of T cells capable of regulating a broad range of immune responses, including autoimmunity, allergy, infection, and tumor rejection (1). In mice, NKT cells express a biased TCR repertoire characterized by an invariant Vα14-Jα18 chain coupled with Vβ8.2, Vβ7, or Vβ2, whereas human NKT cells express the homologous TCR α genes, Vα24-Jα18 in conjunction with Vβ11 (2). This biased TCR usage appears to confer on NKT cells the ability to recognize glycolipids presented in the context of the MHC class I-like molecule, CD1d (3).

The most commonly used glycolipid for the study of NKT cell activation is α-galactosylceramide (α-GalCer)[3] (4). α-GalCer is presented by CD1d-expressing APCs and potently activates NKT cells to rapidly produce both Th1 and Th2 cytokines such as IFN-γ and IL-4 (1). Importantly, activation of NKT cells with α-GalCer leads to potent downstream activation of CD8 T cells (5, 6), NK cells (7, 8), and APC such as dendritic cells (DC) (9, 10) and B cells (11). Bystander activation of these cells is crucial to the protective antitumor and microbial immunity mediated by α-GalCer (12–14). The potent immunoregulatory activity of NKT cells in mice and humans has led to α-GalCer being used in clinical trials as an antitumor agent (15–18).

Recently, we demonstrated that the stimulatory effects of α-GalCer could be dramatically enhanced when used in combination with the common γ-chain (γ_c) receptor cytokine IL-21, resulting in enhanced protection in a number of different tumor models (19). IL-21 enhanced the antitumor effects of α-GalCer by optimizing NK cell cytotoxicity through increased IFN-γ and perforin production, although the direct impact of IL-21 on NKT cells was not investigated (19). IL-21 shares sequence homology with IL-2 and IL-15, and acts through a unique IL-21R, coupled to the γ_c receptor (20, 21). IL-21R is expressed by multiple lymphoid cell types, including NKT cells (22). Since its discovery, the influence of IL-21 on a number of cell types has been investigated, including B, T, NK cells, and DC, but not NKT cells. The findings indicate that IL-21 may play a very significant role in immunity by triggering T and B cell proliferation (22–24), B cell Ig class switching to IgG production (25), dampening DC function (26, 27), and enhancing the differentiation of effector and central memory T cells (28, 29). IL-21 also exhibits potent antitumor function by virtue of enhancing NK and CD8 T cell cytotoxicity and IFN-γ production (30–33), and this has led to its use in the first phase of clinical trials in advanced cancer patients (34). Increased IL-21 responsiveness has also been associated with impaired T cell homeostasis and type 1 diabetes in NOD mice (35), and an absence of IL-21 may contribute to the B cell phenotype observed in X-linked SCID (25). Although α-GalCer is the most common reagent used for NKT cell stimulation, NKT cells are also susceptible to activation and regulation by cytokines, including γ_c receptor cytokines such as IL-2, IL-7, and IL-15 (36–40), as well as DC-derived cytokines such as IL-12 and IL-18 (41–45). These cytokines have been found to enhance NKT cell cytokine production and some are also known to

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[3] Abbreviations used in this paper: α-GalCer, α-galactosylceramide; 7-AAD, 7-aminoactinomycin D; DC, dendritic cell; γ_c, common γ-chain.

stimulate NKT cell cytotoxicity against tumors in vitro and in vivo (43, 46).

Given that NKT cells constitutively express the IL-21R, we hypothesized that IL-21 would influence NKT cell function. Furthermore, although the main source of IL-21 is considered to be activated conventional $CD4^+$ T cells (24), it is known that NKT cells are one of the most potent subsets of $CD4^+$ T cells, responsible for production of many different cytokines. The contribution of NKT cells to $CD4^+$ T cell-derived IL-21 production has not been tested. In this study, we examine the influence of IL-21 on NKT cell function, and also test NKT cells for their ability to produce this cytokine.

Materials and Methods

Mice

C57BL/6 wild-type mice were bred in-house at the Department of Microbiology and Immunology Animal House, Melbourne University or the Peter MacCallum Cancer Centre Animal Facility (Melbourne, Victoria, Australia). IL-12-deficient mice were bred at the Peter MacCallum Cancer Centre Animal Facility. Mice age 7–14 wk were used in all experiments. All experiments were conducted with the approval of appropriate institutional animal ethics committees.

Lymphocyte isolation

Lymphocytes were isolated from the liver by first perfusing the liver with 10 ml of PBS via the hepatic portal vein, and then gently passing it through 200-μm wire mesh sieves into PBS containing 2% FCS (JRH Biosciences). Lymphocytes were separated from hepatocytes and cellular debris by 33% isotonic Percoll density gradient (GE Healthcare) at room temperature. Lymphocytes were harvested from spleen and thymus by gently grinding them between two frosted glass slides into PBS containing 2% FCS. Splenocytes were depleted of RBC by RBC lysis buffer (Sigma-Aldrich).

NKT cell enrichment

For thymus-derived NKT cell enrichment, thymocytes were labeled with anti-CD8 (clone 3.155) and anti-CD24 (J11D), and tagged cells were depleted using rabbit complement (C-SIX Diagnostics) in the presence of DNase (Roche Diagnostics). Viable cells were collected using a Histopaque gradient (1.083 g/ml; Sigma-Aldrich) at room temperature. Cells were then washed before being cultured or before being labeled for flow cytometric purification. NKT cells typically represented >25% of enriched thymocytes.

CFSE labeling

NKT cell-enriched thymocytes or fresh ex vivo splenocytes were washed once in 0.1% BSA in PBS before being labeled in 1 ml of 0.1% BSA in PBS with 5 μl of 1 mM CFSE (Molecular Probes) for 10 min at 37°C in the dark. The reaction was stopped with PBS containing 20% FCS before cells were washed twice in complete tissue culture medium (see below on cell culture conditions).

List of Abs and flow cytometry

All Abs used are from BD Pharmingen unless otherwise specified. Abs used include $\alpha\beta$ TCR-allophycocyanin (clone H57-597), NK1.1-PE/CY7 (PK-136), CD4-FITC (RM4-5), Ly49D-FITC (4E5), Ly49F-PE (HBF-719), NKG2A/C/E-FITC (20d5), NKG2D-PE (CX5), Ly49C/I-FITC (5E6), Ly49G2-allophycocyanin (LGL-1), CD94-FITC (MP-3D9), KLRG1-biotin (2F1), and mouse anti-human granzyme B-allophycocyanin (GB11; Caltag Laboratories). 7-Aminoactinomycin D (7-AAD) (Bioscientific) was added to stained samples where appropriate at a final concentration of 1 μg/ml before cells were analyzed by flow cytometry. Mouse CD1d tetramer loaded with α-GalCer was produced in-house, as previously described (47), using recombinant baculovirus encoding *his*-tagged mouse CD1d and mouse β_2-microglobulin, originally provided by Prof. M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA). Flow cytometry was performed using a FACSCalibur or LSR-II (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

In vivo mouse stimulation

Mice were administered with 2 μg of α-GalCer (provided by Kirin Brewery) prepared in 200 μl of PBS and i.p. injected as previously described (48).

Cell culture conditions

Cells were cultured in medium containing RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 15 mM HEPES buffer (Invitrogen Life Technologies), 10% FCS, and 50 μM 2-ME (Sigma-Aldrich), either in 96-well or 48-well plates (Nunc). For in vitro stimulation assays, no azide low endotoxin anti-CD3 (145-2C11; BD Pharmingen) and anti-CD28 (37.51; BD Pharmingen) were used at a concentration of 10 μg/ml to coat plates. α-GalCer was used at a concentration of 100 ng/ml. Recombinant murine IL-2 (PeproTech) was used at 50 IU/ml, IL-15 (Bioscientific) at 50 ng/ml, and IL-21 (a gift from Zymogenetics) at between 1 and 300 ng/ml and usually at 100 ng/ml.

Flow cytometric purification

Following staining with α-GalCer/CD1d tetramer and $\alpha\beta$ TCR, NKT cell populations from enriched thymus, liver, or spleen preparations were sorted using either a FACSAria (BD Biosciences) or MoFlo (DakoCytomation). A sample of sorted cells was always analyzed to assess the purity of these populations. Purified NKT cell populations were always >95% pure.

Quantitative RT-PCR

Following electronic sorting of NKT cells from livers and spleens of mice, RNA was isolated using the RNeasy kit (Qiagen), and cDNA was synthesized by using a Sensiscript kit (Qiagen) according to the manufacturer's instructions with random hexamers (Invitrogen Life Technologies). Primers for quantitative analysis of IL-21 were designed using Primer Express software (Applied Biosystems). Quantitative RT-PCR was performed using SYBR green (Applied Biosystems) with 18 S rRNA as the normalizer. The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 20 s, ending with a melting curve to validate product specificity. Samples were run on an Applied Biosystems ABI7700 Real-Time PCR machine. Results were analyzed using Sequence Detector software.

ELISA analysis

For IL-4 and IFN-γ ELISA, capture anti-IL-4 (11B11) and anti-IFN-γ (AN18) were grown in-house, and biotinylated detection Ab for IFN-γ (XMG1.2) and IL-4 (BVD6-24G2) were purchased from BD Pharmingen. IL-21 ELISA and IL-13 ELISA were performed using the DuoSet ELISA kits from R&D Systems. The Ab reagents (polyclonal) were titrated and used as follows: IL-21 kit (catalog no. DY594) capture Ab used to coat plate at 0.8 μg/ml in PBS; biotinylated detection Ab used at 0.8 μg/ml; IL-13 kit (catalog no. DY413) capture Ab used to coat plate at 8 μg/ml; and biotinylated detection Ab used at 0.2 μg/ml. Bound detection Ab was detected using streptavidin HRP with tetramethylbenzidine (Sigma-Aldrich) as the substrate and the plates were read using a LabSystems Multiskan multisoft machine.

Results

IL-21 enhances survival and proliferation of NKT cells in vitro

IL-21 is known to have a broad range of effects on different cell types. For T and B cells, IL-21 increases their proliferative potential in vitro following stimulation via CD3 or CD40, respectively (22–24). However, cultures of NK cells expanded in IL-15 indicate that although addition of IL-21 promotes progression to a more mature NK cell phenotype, it decreases NK cell survival and proliferation (49, 50). To investigate a potential role for IL-21 in NKT cell activity, NKT cell-enriched thymocytes were first cultured over a period of 5 days in the presence of varying doses of IL-21 (1–300 ng/ml), and the viability of NKT cells in culture was monitored by flow cytometry using 7-AAD uptake to indicate dead cells (Fig. 1*A*). IL-21 enhanced NKT cell survival in culture over 5 days in a dose-dependent manner, with 100 ng/ml representing the maximal effective dose for NKT cell survival (Fig. 1*A*). However, in comparison to other γ_c cytokines such as IL-2 and IL-15, IL-21 appeared to be less effective at promoting NKT cell survival (Fig. 1*B*). To determine whether IL-21 may affect the proliferation of NKT cells, NKT cell-enriched thymocytes were labeled with CFSE before culture. The presence of IL-21 alone in culture did not lead to any significant proliferation of NKT cells, in stark

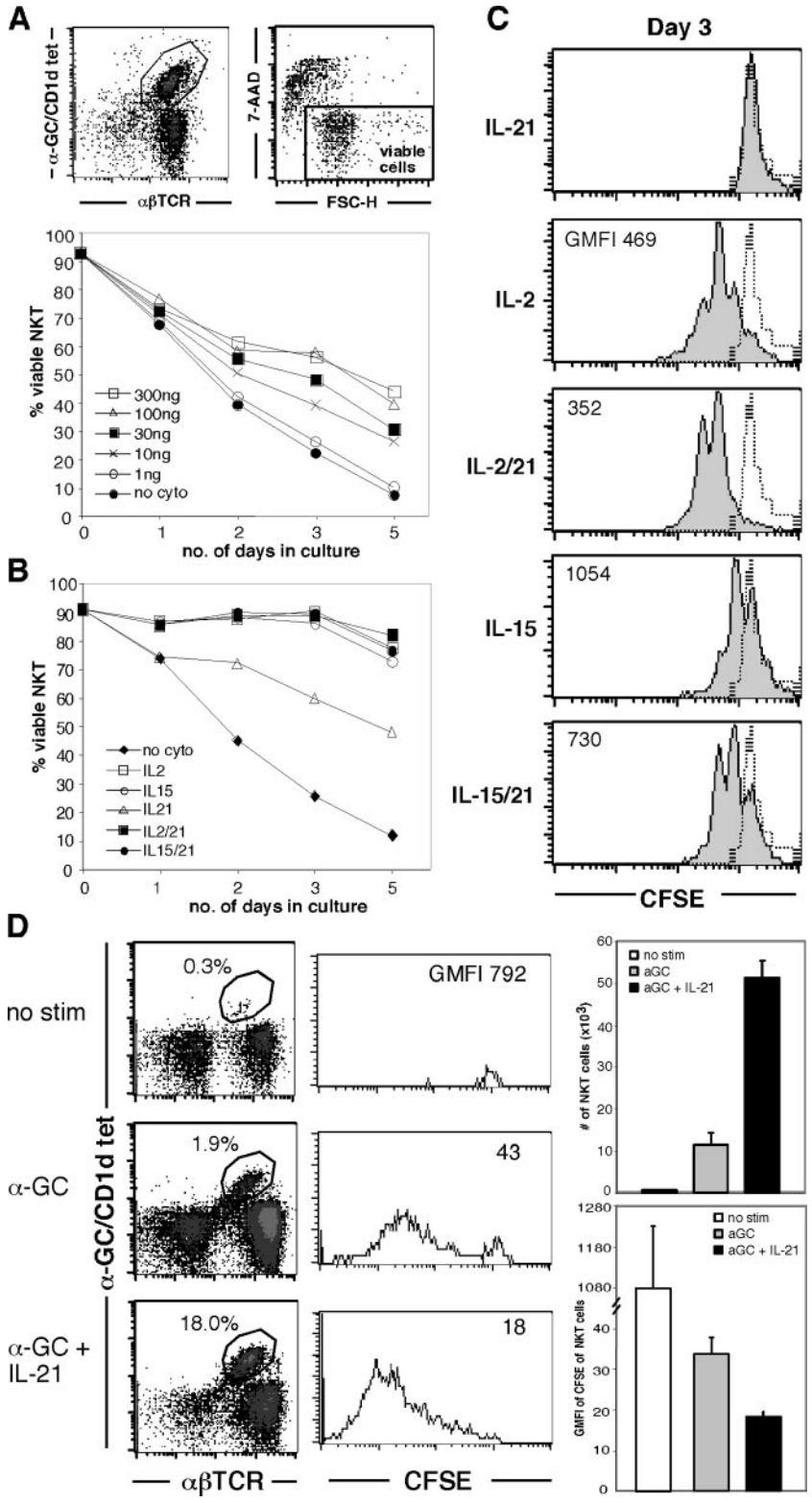

FIGURE 1. IL-21 has unique effects on NKT cell survival and proliferation. NKT cell-enriched thymocyte preparations were cultured in the presence or absence of cytokines for 5 days. *A*, Cells were cultured in the presence of various doses of IL-21 (1–300 ng/ml). An aliquot was taken at the time points indicated. NKT cells were identified as α-GalCer/CD1d tetramer$^+$/$\alpha\beta$ TCR$^+$ (α-GC/CD1d tet) as shown in the density plot, and the percentage of viable NKT cells was determined based on 7-AAD staining also shown. Data represent the mean of two independent experiments each consisting of duplicate wells. *B*, Cells were cultured in the presence of 50 IU/ml IL-2, 50 ng/ml IL-15, 100 ng/ml IL-21, or a combination of these cytokines and viability was measured as described. Data shown for cytokine treated groups are the mean from four independent experiments in which the result from each individual experiment was the mean of two to three separate wells. The "no cytokine" group was included in two of the four experiments. *C*, Before culture, cells were stained with CFSE. Histograms represent dilution of labeling of CFSE on gated NKT cells at the day 3 time point, and the geometric mean fluorescence intensity (GMFI) for NKT cells is shown. The shaded histogram represents NKT cells cultured with the cytokine indicated, whereas the open histogram represents NKT cells cultured in the absence of cytokines. Data are representative of three experiments. *D*, Splenocytes were labeled with CFSE and cultured for 3 days (1.25×10^6 cells per well in a 48-well plate). The 100 ng/ml α-GalCer (α-GC) $\pm$ 100 ng/ml IL-21 was added to cultures as indicated and after culture, cells were stained for α-GalCer/CD1d tetramer and $\alpha\beta$ TCR. The gate used for NKT cells and the corresponding percentage is shown in each density plot. The histograms indicate the CFSE profile of the gated NKT cells. The number of viable NKT cells after 3 days of culture was determined based upon total cell counts multiplied by percentage of viable NKT cells as determined by flow cytometry. The geometric mean fluorescence intensity for CFSE intensity of NKT cells is also graphed. Data are the mean $\pm$ SEM of individual samples from eight separate mice analyzed over two independent experiments ($n = 4$ mice per experiment).

contrast to the effects of IL-2 or IL-15 alone (Fig. 1*C*). However, IL-21 enhanced NKT cell proliferation induced by IL-2 or IL-15 as illustrated by a reduction in CFSE labeling intensity at this time point (Fig. 1*C*). To test whether IL-21 could affect NKT cell proliferation and expansion following antigenic stimulation, IL-21 was added to CFSE-labeled whole splenocyte cultures in the presence of α-GalCer. The presence of IL-21 led to increased proliferation of NKT cells, reflected by a dilution in the CFSE intensity and by the dramatically increased proportion and number of NKT cells at the end of the culture period (Fig. 1*D*). Similar results were observed for liver lymphocyte cultures (data not shown).

IL-21 affects NKT cell granularity and NK receptor expression

IL-21 has been shown to increase NK cell granularity in culture (50). To test whether IL-21 also influenced NKT cell granularity, NKT cells cultured in the presence of IL-2, IL-15, or either of

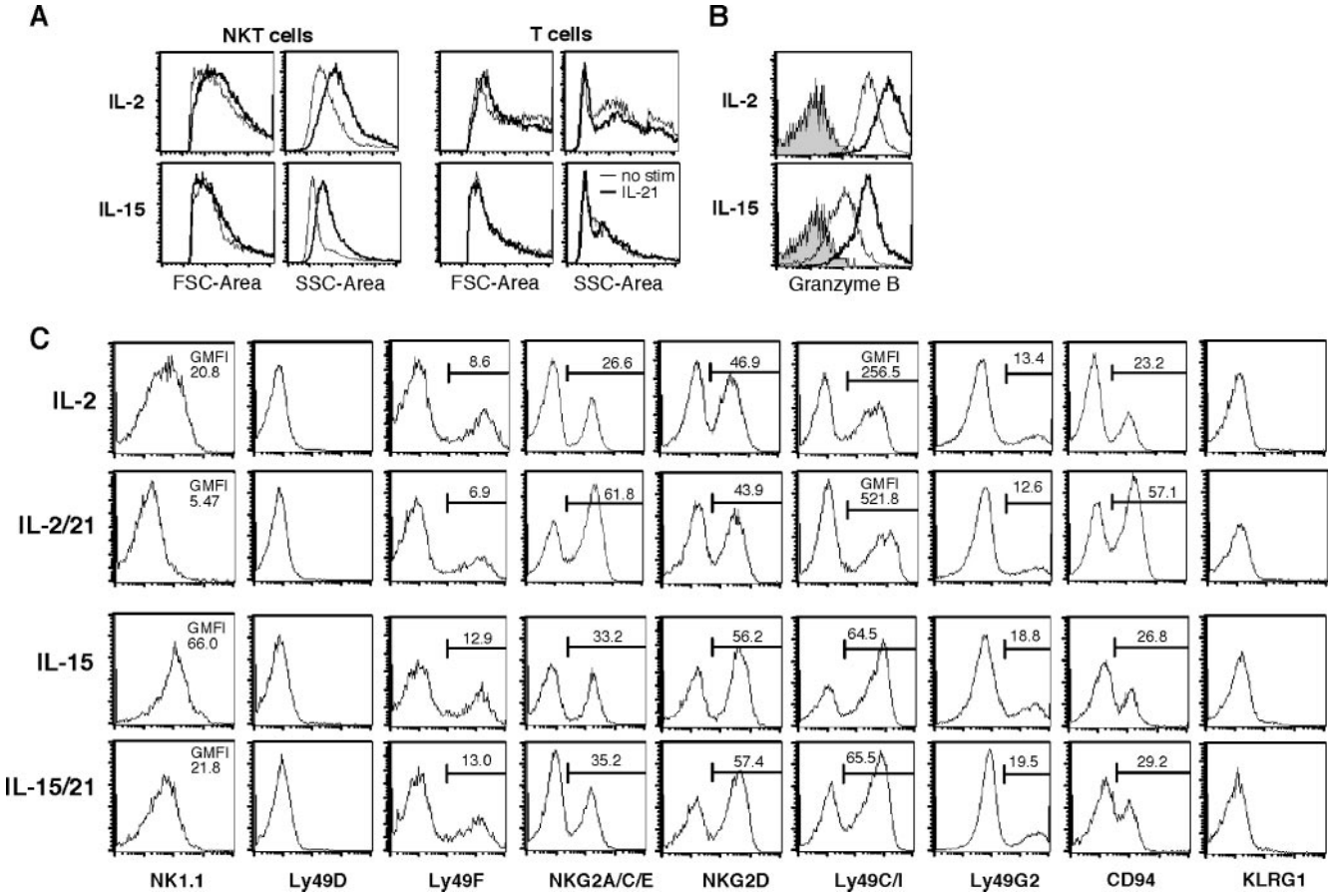

FIGURE 2. IL-21 affects NKT cell morphology and NK receptor expression. NKT cell-enriched thymocyte preparations were cultured in the presence of IL-2, IL-15, IL-21, or a combination of cytokines as indicated. *A*, Histograms depict forward and side scatter properties of NKT and conventional T (α-GalCer/CD1d tetramer$^-$/$\alpha\beta$ TCR$^+$) cells after 5–7 days of culture. NKT cells cultured in the presence of the cytokine indicated (50 IU/ml IL-2 or 50 ng/ml IL-15) (thin line histogram) and the NKT cells cultured in the presence of 100 ng/ml IL-21 as well as the cytokine indicated (bold line histogram) are shown. Data are representative of three to four independent experiments. *B*, Histograms depict intracellular granzyme B expression of NKT cells after 5 days of culture. Thin and bold line histograms are representative of NKT cells cultured as described in *A*. Intracellular granzyme B expression of fresh ex vivo thymus-derived NKT cells (shaded histogram) is shown and is the same in both the IL-2 and IL-15 histograms. Data are representative of two independent experiments. *C*, The expression of NK receptors on the NKT cell surface following culture is depicted. The percentage of NKT cells positive for that marker in the set region is shown, unless geometric mean fluorescence intensity (GMFI) is shown. Data are representative of three to four independent experiments that ranged between 5 and 7 days of culture.

these cytokines in combination with IL-21 were analyzed by flow cytometry after 5–7 days of culture. NKT cells, but not conventional T cells, cultured in the presence of IL-21 were clearly more granular than NKT cells cultured in the absence of IL-21, as measured by the side scatter property of these cells (Fig. 2*A*). Furthermore, this increased granularity correlated with an increase in intracellular granzyme B protein (Fig. 2*B*). NK cells cultured in the presence of IL-21 also exhibit altered NK receptor expression, including decreased NK1.1, and increased CD94, CD154, and KLRG-1 (50). We tested whether IL-21 would have similar effects on NK receptor expression by NKT cells. After 5–7 days of culture in the presence of IL-2 and IL-21, there was a considerable increase in the proportion of NKT cells that were CD94$^+$, NKG2A/C/E$^+$, as well as an increase in Ly49C/I intensity but not percentage, and a decrease in the NK1.1 expression when compared with NKT cells cultured in IL-2 alone (Fig. 2*C*). Importantly, NKT cells cultured in IL-2 alone have a very similar NK receptor profile to fresh ex vivo NKT cells (data not shown and (51)). In contrast, when NKT cells were cultured in the presence of IL-15 and IL-21 together, NKG2A/C/E and Ly49C/I expression was no different from NKT cells cultured in IL-15 alone, although NK1.1 expression was decreased. The presence of IL-21 alone in culture did not appear to impact on NK receptor expression of NKT cells (data not shown). Although most of the experiments, including those depicted, were performed on enriched thymus-derived NKT cells that were then electronically gated using α-GalCer/CD1d tetramer, very similar effects were observed when purified NKT cells were used, in terms of survival, proliferation, granularity, and NK receptor expression (data not shown).

IL-21 enhances NKT cell cytokine production

IL-21 is known to increase the production of both Th1 and Th2 cytokines from NK cells and IFN-γ from CD8 T cells (49, 50). To investigate the influence of IL-21 on NKT cell cytokine production, NKT cells from the thymus and liver were purified and separately cultured in anti-CD3/CD28-coated plates for 24 h in the presence or absence of IL-21. Most strikingly, IL-21 enhanced the production of IL-13 by both liver- and thymus-derived NKT cells (Fig. 3). IL-4 production by NKT cells was also generally increased in cultures with IL-21. However, the impact of IL-21 on NKT cell IFN-γ production was less clear with liver-derived NKT cells appearing to produce more IFN-γ in the presence of IL-21 in most experiments, but thymic NKT cells showing variable responsiveness. These effects were not observed when IL-2 was used

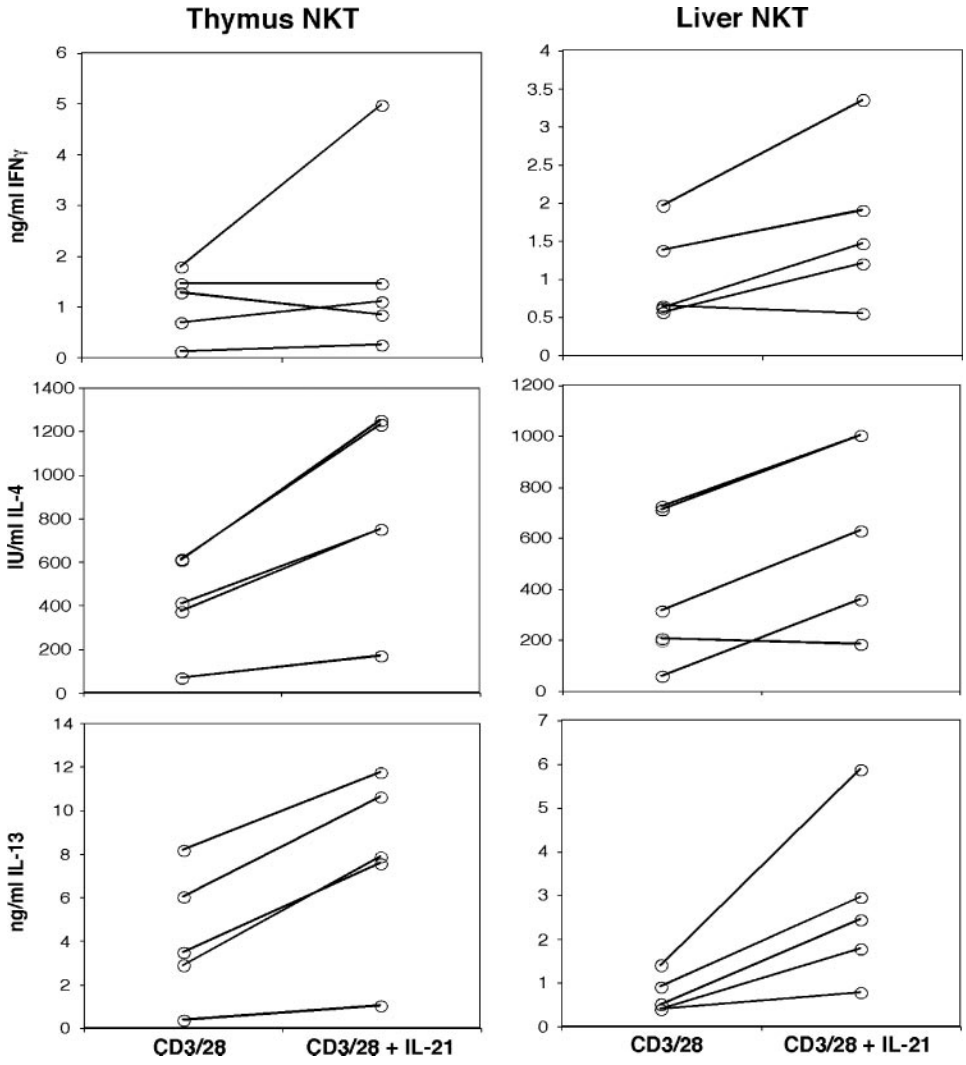

FIGURE 3. IL-21 induces NKT cell cytokine production and expansion. Thymus- and liver-derived NKT cells from C57BL/6 mice were purified by flow cytometry. From 4×10^4 to 1×10^5 NKT cells (depending on cell sorting yields) were cultured in wells previously coated with anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml). Then 100 ng/ml IL-21 was added to culture and 24 h later, supernatants were taken and analyzed for the presence of IL-4, IL-13, and IFN-γ by ELISA. Graphs depict the production of IFN-γ (*upper*), IL-4 (*middle*), IL-13 (*lower*) in the presence or absence of IL-21. Each symbol (○) represents the mean result from replicate wells (two or more) within one experiment, except for one liver experiment in which NKT cell purification yields were low and only one well was set up for each condition. The lines between symbols link samples from individual experiments.

instead of IL-21 (data not shown). Thus, IL-21 enhances NKT cell cytokine production and appears to bias thymic, but not liver NKT cell cytokine production toward a Th2 profile.

NKT cells are capable of potent IL-21 production

$CD4^+$ T cells produce IL-21 mRNA following PMA/ionomycin stimulation or CD3 and CD28 ligation (24). To determine whether NKT cells (many of which are $CD4^+$) also produce IL-21, purified NKT cells from liver, thymus, and spleen of C57BL/6 mice were stimulated in vitro in anti-CD3/CD28-coated plates for up to 3 days, and culture supernatants were tested by ELISA. Spleen- and thymus-derived NKT cells were both capable of making IL-21 protein under these conditions and this production was delayed, detectable only on days 2 and 3 of culture (Fig. 4*A*). Recent findings that IL-12 could stimulate IL-21 mRNA production from T cells (52) prompted us to test whether this cytokine also boosted NKT cell IL-21 production. The addition of IL-12 to cultures of liver-derived NKT cells resulted in a dramatic increase in IL-21 production. IL-21 production by spleen- and thymus-derived NKT cells was also increased by IL-12, although to a lesser extent. Conventional $CD4^+$ T cells purified from the spleen also produced IL-21 over the 3 days of culture, although at much lower levels than NKT cells. Importantly, the increased IL-21 production in the presence of IL-12 did not appear to be a result of increased cell survival or expansion in the presence of this cytokine over the 3 days of culture (data not shown).

The ability of NKT cells to produce IL-21 in vivo was also tested, using C57BL/6 wild-type and IL-12-deficient mice. Mice were i.p. injected with α-GalCer, and 2 or 48 h later NKT cells from the livers and spleens were purified and analyzed for IL-21 mRNA production by quantitative RT-PCR. NKT cells from the liver showed IL-21 mRNA production at both time points, whereas spleen NKT cells showed detectable IL-21 mRNA primarily at the later time point (Fig. 4*B*). Furthermore, the results suggest that the presence of endogenous IL-12 was necessary for optimal IL-21 mRNA production in vivo. IL-21 mRNA was not detected in NKT cells derived from unstimulated mice under the assay conditions used for these experiments. Although the presence of IL-21 mRNA after short-term in vivo stimulation seems inconsistent with the in vitro results in which IL-21 was not detected until 2–3 days, in vivo stimulation with α-GalCer is a very potent stimulus that also drives potent production of other cytokines such as IFN-γ and IL-4 within 1–2 h of stimulation at levels that we never see with in vitro CD3/CD28 stimulation based upon intracellular cytokine staining (our unpublished data).

$CD4^+$ as well as $CD4^-$ NKT cells produce IL-21 mRNA

Previous studies have shown that $CD4^+$ T cells but not $CD8^+$ T cells are the primary producers of IL-21 (24), which suggests that IL-21 production is limited to $CD4^+$ T cells. Thus, we tested whether IL-21 production within the NKT cell population was also limited to the $CD4^+$ subset. $CD4^+$ and $CD4^-$ NKT cells from the

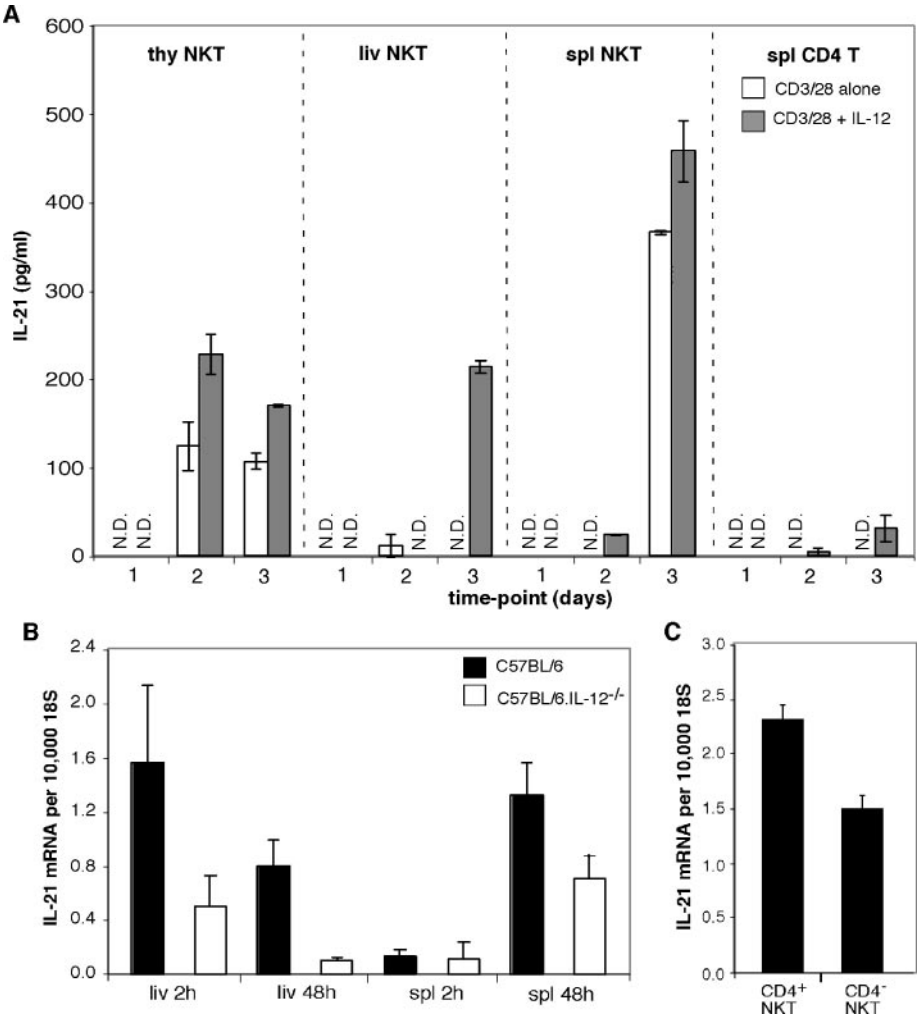

FIGURE 4. NKT cells produce IL-21. *A*, NKT cells were purified by flow cytometric sorting from thymus, liver, and spleen of C57BL/6 mice and added to wells coated with anti-CD3 and anti-CD28. IL-12 (100 ng/ml) was added to wells as indicated. Supernatants were harvested at days 1, 2, and 3 and screened for the presence of IL-21 by ELISA. Representative data from one of four to five experiments are shown and error bars represent the range of values for that culture condition. Each experiment tested in two to four replicate wells. N.D., Not detected. *B*, C57BL/6 or IL-12-deficient mice were i.p. injected with 2 μg of α-GalCer. The livers and spleens of mice were harvested 2 or 48 h later and NKT cells were purified by flow cytometry. RNA was isolated from these cells and quantitative RT-PCR performed to assess levels of IL-21 mRNA relative to 18S rRNA. Data depict the mean $\pm$ SEM of IL-21 mRNA relative to 18S rRNA of three to five separate samples, where each sample represents a separate mouse, except for the liver at 2 h in which two livers were sometimes pooled to acquire sufficient NKT cells, and this exception was counted as one sample. Data are from two to three independent experiments. *C*, The spleens of C57BL/6 mice were harvested 48 h after α-GalCer injection and $CD4^+$ and $CD4^-$ NKT cells were purified by flow cytometry. Data depict the mean $\pm$ SEM of IL-21 mRNA relative to 18S rRNA in separate samples from four mice.

spleens of B6 mice stimulated with α-GalCer for 48 h were purified separately by flow cytometry and analyzed for IL-21 mRNA production. Both $CD4^+$ and $CD4^-$ NKT cells were capable of IL-21 mRNA production, although $CD4^+$ NKT cells showed slightly higher IL-21 mRNA levels upon stimulation (Fig. 4*C*).

Discussion

IL-21 is known to influence T, B, NK cells and DC, and has potent anti-tumor effects, although the precise mechanisms by which this cytokine acts are unclear. In this study, we have demonstrated that IL-21 also has potent effects on NKT cells and furthermore, that NKT cells are also potent producers of IL-21. The latter finding is particularly significant because previous studies have suggested that IL-21 production is restricted to isolated conventional $CD4^+$ T cells (24). Importantly, our study demonstrated that NKT cells were clearly more capable of IL-21 production than conventional $CD4^+$ T cells in an in vitro assay and highlights the need to distinguish between conventional $CD4^+$ T cells and nonconventional $CD4^+$ cells, such as NKT cells, when investigating IL-21 production. Furthermore, we demonstrated that IL-21 production was not limited to $CD4^+$ cells because $CD4^-$ NKT cells also expressed mRNA for this cytokine.

This study is one of the first to measure IL-21 protein production by T cells, which represents a significant improvement in the field because the majority of studies thus far have been limited to quantifying IL-21 mRNA, including a very recent finding demonstrating IL-21 mRNA expression in a microarray comparison of immature and mature NKT cells in the thymus (53). We have also shown that IL-21 production is distinct between NKT cells from different organs. Intriguingly, IL-21 production by liver-derived NKT cells seemed to be dependent on the presence of IL-12, whereas thymus- and spleen-derived NKT cells were able to produce IL-21 in the absence of IL-12.

The importance of IL-21 production in NKT cell-dependent disease models remains to be tested. The ability of NKT cells to mediate potent antitumor responses is quite well characterized and relies on NKT cell IFN-γ production, as well as NK and CD8 T cell function (6, 12, 13, 48), although the precise mechanism by which these two cell types are induced remains unclear. The known ability of IL-21 to promote antitumor responses in a number of models by enhancing NK and CD8 T cell function (29, 30, 54, 55) suggests that NKT cells may regulate the response of these downstream effector cells at least in part by virtue of their IL-21 production.

Paradoxically, in addition to promoting tumor rejection, NKT cells are also able to mediate the suppression of autoimmune disease, allograft rejection and promotion of allergy via their production of IL-4 and IL-13 (1). In this study, we showed that IL-21 could enhance the production of these Th2 cytokines by NKT cells, suggesting a potential role for IL-21 and NKT cells in the regulation of these diseases. Supporting this theory are recent findings that NKT cells are pivotal in eliciting a potent Th2 response following helminth infection via their production of IL-13 and IL-4 (56), and also that IL-21 is crucial for optimal IL-13 and IL-4 production and eventual granuloma formation following infection

with this parasite (57). Taken together, these studies are consistent with a role for NKT cell-derived IL-21 in the regulation of Th2 immune responses. Conversely, studies investigating the role of IL-21 in the NOD mouse model (35) and experimental allergic encephalomyelitis (58) suggest that IL-21 exacerbates disease via the enhanced proliferation of autoreactive CD8 T cells and enhanced NK cell function. This probably reflects the influence of the cellular microenvironment in overall disease outcome and suggests that the function of NKT cell-derived IL-21 will likely depend upon the activation of downstream cells and other cytokines present.

Although the functional importance of NKT cell-derived IL-21 remains unclear, we may hypothesize that NKT cells play a role in bridging the innate and adaptive immune responses via their production of this cytokine. This role makes sense when considering findings showing that although IL-21 can enhance NK cell function, it also increases the death of NK cells (49, 50), increases inhibitory NK receptor expression on both NK (50) and NKT cells (our study), and down-regulates costimulatory molecule expression on DC (26, 27). Conversely, because IL-21 enhances Ag-specific Ab production (25, 59), CTL responses (49, 60), and the generation of central and effector memory T cells (28, 29), the delayed production of IL-21, which we observed in vitro and also in vivo for spleen-derived NKT cells, may be timed to optimally enhance these responses. Thus, NKT cell derived IL-21 may be an important factor underlying the well-established ability of NKT cells to enhance Ag-specific Ab production and CTL responses (1, 3, 61).

In summary, the results from this study provide the first evidence of IL-21 production by thymic and peripheral NKT cells, and furthermore, that this cytokine can potently regulate NKT cell function. We hypothesize that IL-21 may play a role in a number of NKT cell-mediated immune responses by affecting the activation of downstream effector cells and also the activation of NKT cells themselves. It will therefore be important to investigate NKT cell-mediated immune responses in IL-21- and IL-21R-deficient mice to determine whether this cytokine is a critical regulator of NKT cell function in vivo.

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References

1. Godfrey, D. I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J. Clin. Invest.* 114: 1379–1388.
2. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol.* 4: 231–237.
3. Brigl, M., and M. B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu. Rev. Immunol.* 22: 817–890.
4. Kawano, T., J. Q. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. Cd1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* 278: 1626–1629.
5. Nishimura, T., H. Kitamura, K. Iwakabe, T. Yahata, A. Ohta, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, et al. 2000. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int. Immunol.* 12: 987–994.
6. Nakagawa, R., T. Inui, I. Nagafune, Y. Tazunoki, K. Motoki, A. Yamauchi, M. Hirashima, Y. Habu, H. Nakashima, and S. Seki. 2004. Essential role of bystander cytotoxic CD122$^+$CD8$^+$ T cells for the antitumor immunity induced in the liver of mice by α-galactosylceramide. *J. Immunol.* 172: 6550–6557.
7. Eberl, G., and H. R. MacDonald. 2000. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur. J. Immunol.* 30: 985–992.
8. Carnaud, C., D. Lee, O. Donnars, S. H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 163: 4647–4650.
9. Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. van Kaer, et al. 1999. The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 189: 1121–1127.
10. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J. Exp. Med.* 198: 267–279.
11. Kitamura, H., A. Ohta, M. Sekimoto, M. Sato, K. Iwakabe, M. Nakui, T. Yahata, H. X. Meng, T. Koda, S. Nishimura, et al. 2000. α-Galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cell. Immunol.* 199: 37–42.
12. Crowe, N. Y., M. J. Smyth, and D. I. Godfrey. 2002. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* 196: 119–127.
13. Smyth, M. J., N. Y. Crowe, and D. I. Godfrey. 2001. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int. Immunol.* 13: 459–463.
14. van Dommelen, S. L., H. A. Tabarias, M. J. Smyth, and M. A. Degli-Esposti. 2003. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. *J. Virol.* 77: 1877–1884.
15. Nieda, M., M. Okai, A. Tazbirkova, H. Lin, A. Yamaura, K. Ide, R. Abraham, T. Juji, D. J. Macfarlane, and A. J. Nicol. 2004. Therapeutic activation of Vα24$^+$Vβ11$^+$ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* 103: 383–389.
16. Giaccone, G., C. J. A. Punt, Y. Ando, R. Ruijter, N. Nishi, M. Peters, B. M. E. von Blomberg, R. J. Scheper, H. J. J. van der Vliet, A. J. M. van den Eertwegh, et al. 2002. A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin. Cancer Res.* 8: 3702–3709.
17. Ishikawa, A., S. Motohashi, E. Ishikawa, H. Fuchida, K. Higashino, M. Otsuji, T. Iizasa, T. Nakayama, M. Taniguchi, and T. Fujisawa. 2005. A phase I study of α-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin. Cancer Res.* 11: 1910–1917.
18. Chang, D. H., K. Osman, J. Connolly, A. Kukreja, J. Krasovsky, M. Pack, A. Hutchinson, M. Geller, N. Liu, R. Annable, et al. 2005. Sustained expansion of NKT cells and antigen-specific T cells after injection of α-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J. Exp. Med.* 201: 1503–1517.
19. Smyth, M. J., M. E. Wallace, S. L. Nutt, H. Yagita, D. I. Godfrey, and Y. Hayakawa. 2005. Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. *J. Exp. Med.* 201: 1973–1985.
20. Asao, H., C. Okuyama, S. Kumaki, N. Ishii, S. Tsuchiya, D. Foster, and K. Sugamura. 2001. Cutting edge: the common γ-chain is an indispensable subunit of the IL-21 receptor complex. *J. Immunol.* 167: 1–5.
21. Habib, T., S. Senadheera, K. Weinberg, and K. Kaushansky. 2002. The common γ chain (γ_c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3. *Biochemistry* 41: 8725–8731.
22. Jin, H., R. Carrio, A. Yu, and T. R. Malek. 2004. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. *J. Immunol.* 173: 657–665.
23. Mehta, D. S., A. L. Wurster, M. J. Whitters, D. A. Young, M. Collins, and M. J. Grusby. 2003. IL-21 induces the apoptosis of resting and activated primary B cells. *J. Immunol.* 170: 4111–4118.
24. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408: 57–63.
25. Ozaki, K., R. Spolski, C. G. Feng, C. F. Qi, J. Cheng, A. Sher, H. C. Morse, III, C. Liu, P. L. Schwartzberg, and W. J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science* 298: 1630–1634.
26. Brandt, K., S. Bulfone-Paus, D. C. Foster, and R. Ruckert. 2003. Interleukin-21 inhibits dendritic cell activation and maturation. *Blood* 102: 4090–4098.
27. Brandt, K., S. Bulfone-Paus, A. Jenckel, D. C. Foster, R. Paus, and R. Ruckert. 2003. Interleukin-21 inhibits dendritic cell-mediated T cell activation and induction of contact hypersensitivity in vivo. *J. Invest. Dermatol.* 121: 1379–1382.
28. Eberl, M., R. Engel, E. Beck, and H. Jomaa. 2002. Differentiation of human γ-δ T cells towards distinct memory phenotypes. *Cell. Immunol.* 218: 1–6.
29. Zeng, R., R. Spolski, S. E. Finkelstein, S. Oh, P. E. Kovanen, C. S. Hinrichs, C. A. Pise-Masison, M. F. Radonovich, J. N. Brady, N. P. Restifo, et al. 2005. Synergy of IL-21 and IL-15 in regulating CD8$^+$ T cell expansion and function. *J. Exp. Med.* 201: 139–148.
30. Ma, H. L., M. J. Whitters, R. F. Konz, M. Senices, D. A. Young, M. J. Grusby, M. Collins, and K. Dunussi-Joannopoulos. 2003. IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN-γ. *J. Immunol.* 171: 608–615.
31. Kishida, T., H. Asada, Y. Itokawa, F. D. Cui, M. Shin-Ya, S. Gojo, K. Yasutomi, Y. Ueda, H. Yamagishi, J. Imanishi, and O. Mazda. 2003. Interleukin (IL)-21 and IL-15 genetic transfer synergistically augments therapeutic antitumor immunity and promotes regression of metastatic lymphoma. *Mol. Ther.* 8: 552–558.

32. Wang, G., M. Tschoi, R. Spolski, Y. Lou, K. Ozaki, C. Feng, G. Kim, W. J. Leonard, and P. Hwu. 2003. In vivo antitumor activity of interleukin 21 mediated by natural killer cells. *Cancer Res.* 63: 9016–9022.
33. Leonard, W. J., and R. Spolski. 2005. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat. Rev. Immunol.* 5: 688–698.
34. Curti, B. D. 2006. Immunomodulatory and antitumor effects of interleukin-21 in patients with renal cell carcinoma. *Expert Rev. Anticancer Ther.* 6: 905–909.
35. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117: 265–277.
36. Hameg, A., C. Gouarin, J. M. Gombert, S. M. Hong, L. van Kaer, J. F. Bach, and A. Herbelin. 1999. IL-7 up-regulates IL-4 production by splenic NK1.1$^+$ and NK1.1$^-$ MHC class I-like/CD1-dependent CD4$^+$ T cells. *J. Immunol.* 162: 7067–7074.
37. Kadowaki, N., S. Antonenko, S. Ho, M. C. Rissoan, V. Soumelis, S. A. Porcelli, L. L. Lanier, and Y. J. Liu. 2001. Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. *J. Exp. Med.* 193: 1221–1226.
38. Lin, H., M. Nieda, and A. J. Nicol. 2004. Differential proliferative response of NKT cell subpopulations to in vitro stimulation in presence of different cytokines. *Eur. J. Immunol.* 34: 2664–2671.
39. Ohteki, T., S. Ho, H. Suzuki, T. W. Mak, and P. S. Ohashi. 1997. Role for IL-15/IL-15 receptor β-chain in natural killer 1.1$^+$ T cell receptor-$\alpha\beta^+$ cell development. *J. Immunol.* 159: 5931–5935.
40. Matsuda, J. L., L. Gapin, S. Sidobre, W. C. Kieper, J. Y. T. Tan, R. Ceredig, C. D. Surh, and M. Kronenberg. 2002. Homeostasis of Vα14i NKT cells. *Nat. Immunol.* 3: 966–974.
41. Baxevanis, C. N., A. D. Gritzapis, and M. Papamichail. 2003. In vivo antitumor activity of NKT cells activated by the combination of IL-12 and IL-18. *J. Immunol.* 171: 2953–2959.
42. Smyth, M. J., M. Taniguchi, and S. E. A. Street. 2000. The anti-tumor activity of IL-12: mechanisms of innate immunity that are model and dose dependent. *J. Immunol.* 165: 2665–2670.
43. Cui, J. Q., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623–1626.
44. Leite-De-Moraes, M. C., A. Hameg, A. Arnould, F. Machavoine, Y. Koezuka, E. Schneider, A. Herbelin, and M. Dy. 1999. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J. Immunol.* 163: 5871–5876.
45. Leite-De-Moraes, M. C., A. Hameg, M. Pacilio, Y. Koezuka, M. Taniguchi, L. Van Kaer, E. Schneider, M. Dy, and A. Herbelin. 2001. IL-18 enhances IL-4 production by ligand-activated NKT lymphocytes: a pro-Th2 effect of IL-18 exerted through NKT cells. *J. Immunol.* 166: 945–951.
46. Smyth, M. J., K. Y. T. Thia, S. E. A. Street, E. Cretney, J. A. Trapani, M. Taniguchi, T. Kawano, S. B. Pelikan, N. Y. Crowe, and D. I. Godfrey. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* 191: 661–668.
47. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192: 741–753.
48. Smyth, M. J., N. Y. Crowe, D. G. Pellicci, K. Kyparissoudis, J. M. Kelly, K. Takeda, H. Yagita, and D. I. Godfrey. 2002. Sequential production of interferon-γ by NK1.1$^+$ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood* 99: 1259–1266.
49. Kasaian, M. T., M. J. Whitters, L. L. Carter, L. D. Lowe, J. M. Jussif, B. Deng, K. A. Johnson, J. S. Witek, M. Senices, R. F. Konz, et al. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity* 16: 559–569.
50. Brady, J., Y. Hayakawa, M. J. Smyth, and S. L. Nutt. 2004. IL-21 induces the functional maturation of murine NK cells. *J. Immunol.* 172: 2048–2058.
51. Uldrich, A. P., N. Y. Crowe, K. Kyparissoudis, D. G. Pellicci, Y. Zhan, A. M. Lew, P. Bouillet, A. Strasser, M. J. Smyth, and D. I. Godfrey. 2005. NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J. Immunol.* 175: 3092–3101.
52. Monteleone, G., I. Monteleone, D. Fina, P. Vavassori, G. Del Vecchio Blanco, R. Caruso, R. Tersigni, L. Alessandroni, L. Biancone, G. C. Naccari, et al. 2005. Interleukin-21 enhances T-helper cell type I signaling and interferon-γ production in Crohn's disease. *Gastroenterology* 128: 687–694.
53. Matsuda, J. L., Q. Zhang, R. Ndonye, S. K. Richardson, A. R. Howell, and L. Gapin. 2006. T-bet concomitantly controls migration, survival and effector functions during the development of Vα14i NKT cells. *Blood* 107: 2797–2805.
54. Dou, J., G. Chen, J. Wang, F. Zhao, J. Chen, X. Fang, Q. Tang, and L. Chu. 2004. Preliminary study on mouse interleukin-21 application in tumor gene therapy. *Cell. Mol. Immunol.* 1: 461–466.
55. Di Carlo, E., A. Comes, A. M. Orengo, O. Rosso, R. Meazza, P. Musiani, M. P. Colombo, and S. Ferrini. 2004. IL-21 induces tumor rejection by specific CTL and IFN-γ-dependent CXC chemokines in syngeneic mice. *J. Immunol.* 172: 1540–1547.
56. Mallevaey, T., J. P. Zanetta, C. Faveeuw, J. Fontaine, E. Maes, F. Platt, M. Capron, M. L. de-Moraes, and F. Trottein. 2006. Activation of invariant NKT cells by the helminth parasite *Schistosoma mansoni*. *J. Immunol.* 176: 2476–2485.
57. Pesce, J., M. Kaviratne, T. R. Ramalingam, R. W. Thompson, J. F. Urban, A. W. Cheever, D. A. Young, M. Collins, M. J. Grusby, and T. A. Wynn. 2006. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J. Clin. Invest.* 116: 2044–2055.
58. Vollmer, T. L., R. Liu, M. Price, S. Rhodes, A. La Cava, and F. D. Shi. 2005. Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. *J. Immunol.* 174: 2696–2701.
59. Nakano, H., T. Kishida, H. Asada, M. Shin-Ya, T. Shinomiya, J. Imanishi, T. Shimada, S. Nakai, M. Takeuchi, Y. Hisa, and O. Mazda. 2006. Interleukin-21 triggers both cellular and humoral immune responses leading to therapeutic antitumor effects against head and neck squamous cell carcinoma. *J. Gene Med.* 8: 90–99.
60. Li, Y., M. Bleakley, and C. Yee. 2005. IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. *J. Immunol.* 175: 2261–2269.
61. Hansen, D. S., and L. Schofield. 2004. Regulation of immunity and pathogenesis in infectious diseases by CD1d-restricted NKT cells. *Int. J. Parasitol.* 34: 15–25.