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# A Distinct IL-18-Induced Pathway to Fully Activate NK T Lymphocytes Independently from TCR Engagement<sup>1</sup>

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NK T lymphocytes are characterized by their ability to promptly generate IL-4 and IFN- $\gamma$  upon TCR engagement. Here, we demonstrate that these cells can also be fully activated in the absence of TCR cross-linking in response to the proinflammatory cytokine IL-18 associated with IL-12. NK T cells stimulated with IL-18 plus IL-12 proliferated, killed Fas<sup>+</sup> target cells, and produced high levels of IFN- $\gamma$  without IL-4. In these conditions, IFN- $\gamma$  production was at least 10-fold higher than that upon TCR cross-linking. Interestingly, a 2-h pretreatment with IL-12 plus IL-18 sufficed to maintain the high IFN- $\gamma$ -producing potential during subsequent stimulation with anti-TCR mAbs or with the specific Ag  $\alpha$ -galactosylceramide. Similar effects were observed in vivo, because splenic CD4<sup>+</sup> NK T cells from MHC class II-deficient mice secreted IFN- $\gamma$  without further stimulation when removed 2 h after a single injection of IL-12 plus IL-18. In conclusion, our evidence for activation of NK T lymphocytes in response to IL-18 plus IL-12 in the absence of TCR engagement together with the maintenance of preferential IFN- $\gamma$  vs IL-4 production upon subsequent exposure to specific Ags is consistent with the active participation of this cell population in innate as well as acquired cellular immune responses. *The Journal of Immunology*, 1999, 163: 5871–5876.

**T**he unusual NK T cell subset comprises both CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>+</sup> lymphocytes and is characterized by the expression of the NK1.1 surface molecule and the usage of an invariant V $\alpha$ 14-J $\beta$ 281 chain preferentially associated with a V $\beta$ 8.2 chain or, to a lesser extent, V $\beta$ 7 and V $\beta$ 2 chains (1, 2). NK T cells are positively selected by the nonpolymorphic MHC class I-like molecule CD1d and recognize CD1d-bound lipid ligands, such as ceramides or glycosylphosphatidyl inositols (1–4).

The physiological role of NK T lymphocytes is still unclear. Because of their capacity to promptly generate large amounts of IL-4 in response to TCR cross-linking, it has been proposed that they participate in the differentiation of naive T lymphocytes into Th2 cells (5–7). Yet, it has been shown that their absence in CD1 knockout mice does not prevent the development of typical Th2 responses (8, 9). Furthermore, recent evidence for the involvement of NK T cells in IL-12-induced tumor rejection and in the generation of CD8 effector functions against intracellular infections supports their implication in certain Th1 responses (10, 11).

In accordance with this dual regulatory potential, we recently demonstrated that the capacity of NK T cells to produce IFN- $\gamma$  or IL-4 depends on the cytokines present in their microenvironment (12–16). Thus, the secretion of high levels of IL-4 in response to TCR cross-linking required the presence of IL-7 (12–15), while

IFN- $\gamma$  was preferentially produced when IL-12 was present (16). This evidence together with the restricted Ag recognition by NK T cells due to the presentation by nonpolymorphic CD1 molecules and a biased TCR repertoire (1, 2) prompted us to evaluate their direct response to cytokines involved in early defense mechanisms against pathogens or other injuries, independently from TCR recognition. IL-18 or IFN- $\gamma$ -inducing factor, a proinflammatory protein produced by activated monocytes and dendritic cells which enhances NK cell activity, induces IFN- $\gamma$  production, and exerts a significant anti-tumor effect (17, 18), seemed a particularly good candidate for this purpose.

In the course of this study we found that IL-18 alone had only a slight stimulatory effect on NK T cells. Considering that in some models its activity can be enhanced by IL-12 through up-regulation of IL-18R expression (19), we examined whether the addition of this factor might unmask a more pronounced effect of IL-18 on NK T cells. In the absence of TCR stimulation, we found that 1) the cytotoxicity of NK T cells against Fas<sup>+</sup> targets was strikingly enhanced after exposure to IL-18 plus IL-12; 2) IFN- $\gamma$  production increased in these conditions both in vitro and in vivo, while IL-4 was no longer detected; and 3) the IFN- $\gamma$ -producing capacity of NK T cells in response to anti-TCR mAb or the specific Ag  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer)<sup>3</sup> was greatly enhanced by prior exposure to IL-18 plus IL-12.

## Materials and Methods

### Animals and reagents

Six- to eight-week-old wild-type and mutant ( $\beta_2\text{m}^{-/-}$  or A $\beta^0$ ) C57BL/6 mice were bred in our own facilities. RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (TechGen, Les Ulis, France), 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, 10 mM HEPES buffer (all from Life Technologies), and  $5 \times 10^{-5}$  M 2-ME was used as culture medium. Human rIL-7 (sp. act.,  $8.8 \times 10^6$  U/mg) was provided by Sanofi (Labege, France). Murine IL-4, IL-12, IL-18, and IFN- $\gamma$  were purchased from R & D Systems (Abingdon, U.K.). Anti-IL-4 mAbs (11B11 and BVD6-24G2.3 clones), anti-IFN- $\gamma$  mAbs (AN18 and

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<sup>3</sup> Abbreviations used in this paper:  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; A $\beta^0$ , MHC class II-deficient;  $\beta_2\text{m}^{-/-}$ ,  $\beta_2$ -microglobulin-deficient.

R46A2 clones), anti-CD3 mAb (clone 145-2C11), and anti-TCR $\alpha\beta$  mAb (clone H57-597) were purified in our laboratory. The BVD6-24G2.3 clone was obtained from DNAX (Palo Alto, CA). The following mAbs used for cell depletion were purified in our laboratory: CD4 (clone GK1.5), CD8 (clone 53.67), Gr-1 (clone RB6-8C5), and Mac1 (clone M1/70). CD4-PE (clone YTS 191.1), PE- or FITC-conjugated CD8 (clone YTS 169.4), CD3-FITC (500-A2), TCR $\alpha\beta$ -FITC (clone H57-597), anti-IFN- $\gamma$  (clone XMG1.2), rat IgG1-PE (isotype control), and streptavidin-PE were purchased from Caltag (Le Perray en Yvelines, France). Biotinylated anti-NK1.1 (clone PK136) and streptavidin-Cy-chrome were obtained from PharMingen (San Diego, CA). Anti-rat and anti-mouse Ig-coated magnetic beads were obtained from Dynal (Compiègne, France).

#### Expansion and stimulation of CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> lymphocytes

Enriched CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> thymocytes were obtained after expansion with IL-7 (40 ng/ml), as previously described (6, 20). In some experiments enriched CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> cells were stained with anti-TCR $\alpha\beta$  and/or anti-NK1.1. CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> thymocytes were then sorted using a FACS Vantage sorter (Becton Dickinson, Mountain View, CA). Purity was >99% after reanalysis.

In some experiments, freshly isolated splenocytes were incubated for 45 min with anti-CD4-coated magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) and positively sorted on a MACS positive selection column. In another series of experiments, TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> cells were sorted from freshly isolated thymocytes or splenocytes.

Enriched or sorted cells were then stimulated at a concentration of  $5 \times 10^5$  cells/ml with IL-18 (100 ng/ml) in the presence or the absence of IL-12 (10 ng/ml). The doses of cytokines used here have been previously assayed for optimal stimulation of NK T cells (data not shown). In some experiments, lymphocytes were stimulated with coated anti-TCR $\alpha\beta$  mAb (10  $\mu$ g/ml) or  $\alpha$ -GalCer (100 ng/ml; Kirin Brewery Co., Gunma, Japan) (21) with  $5 \times 10^5$  irradiated (20 Gy) autologous splenocytes/ml. Forty-eight hours later, supernatants were harvested and stored at  $-70^\circ\text{C}$  until IFN- $\gamma$  and IL-4 assays. The remaining cells were resuspended in 200  $\mu$ l of culture medium and pulsed for 18 h with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine. Cells were then harvested, and thymidine uptake was assessed using a beta counter (LKB Wallac, St. Quentin-en-Yvelines, France).

#### In vivo treatment

Mice received a single i.v. injection of 1  $\mu$ g of IL-18 plus 0.2  $\mu$ g of IL-12 diluted in a pyrogen-free solution containing BSA (1 mg/ml; Life Technologies/BRL, Gaithersburg, MD). Control mice were injected with an identical volume of the BSA solution alone. Mice were sacrificed 2 h after injection.

#### Flow cytometric analysis

Cells were stained in PBS containing 2% FCS and 0.01 M sodium azide and incubated for 30 min with appropriate dilutions of various mAbs coupled to biotin, PE, or fluorescein. When mAbs were biotinylated, streptavidin-PE or streptavidin-Cy-Chrome was used as a second step reagent.

Intracellular IFN- $\gamma$  was analyzed in TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> splenocytes freshly isolated 2 h after a single injection of IL-18 plus IL-12 as described above. CD8<sup>+</sup>, Gr-1<sup>+</sup>, and Mac1<sup>+</sup> and B splenocytes were depleted using magnetic beads as previously described (6). Enriched splenocytes were resuspended at  $10^6$ /ml and stained with anti-TCR $\alpha\beta$  and anti-NK1.1 mAb. After fixing with 4% formaldehyde for 5 min at room temperature, cells were washed with PBS containing 1% BSA and 0.5% saponin (Sigma, St. Louis, MO), incubated with anti-IFN- $\gamma$ -PE or isotype control for 30 min, washed again with PBS/BSA/saponin, and finally with PBS/BSA without saponin to allow membrane closure.

Flow cytometry was performed on a FACSscan (Becton Dickinson). Dead cells were excluded on the basis of forward and side scatter characteristics. At least  $10^4$  live lymphoid cells were acquired in each run.

#### IL-4 and IFN- $\gamma$ assays

IL-4 and IFN- $\gamma$  production was measured by ELISA, as previously described (6, 16). Samples were tested in duplicate, and the sensitivity of the ELISA was 40 pg/ml.

#### Cytotoxic NK T lymphocyte assay

Target cell lysis by NK T cells was measured by JAM (22) assay using the L1210 cell line transfected with Fas (L1210.Fas) and the control nontransfected (L1210) as target cells (23). Briefly, NK T cells were incubated with

Table I. NK T cells proliferate in response to IL-18 in combination with IL-12<sup>a</sup>

Stimulation	Cultured Lymphocytes (cpm)	
	CD4 <sup>+</sup> CD8 <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup>	CD4 <sup>+</sup> CD8 <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> NK1.1 <sup>+</sup>
IL-18	3,214 $\pm$ 490	279 $\pm$ 148
IL-12	2,504 $\pm$ 514	527 $\pm$ 236
IL-18 + IL-12	11,055 $\pm$ 722	9,209 $\pm$ 1,072
Medium	767 $\pm$ 254	109 $\pm$ 16

<sup>a</sup> Thymocytes sorted after expansion with IL-7 were stimulated at  $5 \times 10^5$  cells/ml for 72 h. [<sup>3</sup>H]-Thymidine was added during the last 18 h, and radioactivity was measured. Results represent means  $\pm$  SEM from at least three different experiments.

[<sup>3</sup>H]thymidine (Amersham, Les Ulis, France)-labeled L1210 or L1210.Fas cells ( $2.5 \times 10^4$ ) at various E:T cell ratios for 18 h. Assays were performed in 96-well U-bottom plates in a total volume of 200  $\mu$ l/well. After incubation, cells were harvested, and radioactivity was determined using a beta counter (LKB Wallac). The percent specific lysis was calculated as follows: [(spontaneous release – experimental release)/spontaneous release]  $\times$  100.

## Results and Discussion

### NK T lymphocytes proliferate in response to IL-18

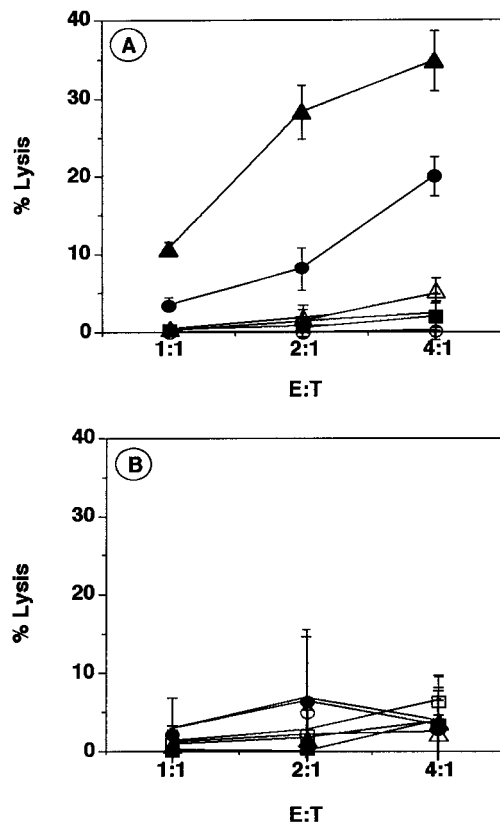
To date, most studies addressing the role of NK T lymphocytes in various experimental models of immune response have privileged their capacity to produce IL-4, which requires TCR engagement. However, only a restricted set of Ags has been found to stimulate NK T cells via the TCR in a CD1-dependent manner (1–4). Recently, we have reported that NK T lymphocytes produce detectable amounts of IFN- $\gamma$  in response to IL-12 plus IL-2 without prior TCR ligation (16). These data were in accordance with a possible involvement in early immune responses occurring independently from TCR engagement. To test this hypothesis, we evaluated the effect of IL-18, a proinflammatory cytokine that is secreted by the same cells as IL-12 and synergizes with the latter in several biological activities (18, 19), on the functional capacities of NK T cells.

We took advantage of the comparatively high incidence of CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> NK T cells in the thymus and their preferential expansion during a 4-day culture with IL-7 (6, 20) to assay IL-18 on the population obtained by this procedure. A slight proliferative response (Table I) occurred in these conditions. Since it has been documented that IL-12 can up-regulate IL-18 action (18, 19), we investigated its effect on the responsiveness of NK T cells to IL-18. As shown in Table I, the addition of IL-12 does effectively enhance the proliferative response promoted by IL-18, while it has little effect on its own.

Starting from this observation, we further purified NK T lymphocytes from the CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> thymocyte population derived from culture with IL-7, using the NK1.1<sup>+</sup> marker for positive selection. The results presented in Table I clearly show that sorted CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> thymocytes do not need TCR signaling to proliferate in response to IL-18 plus IL-12. A possible explanation for this effect is that, like IL-1, which according to our previous report induces NK T cell expansion (14), IL-18 signals via IL-1R-associated kinase (IRAK) and activates NF- $\kappa$ B (18) which is implicated in cell survival and thymocyte proliferation (24).

### IL-18 renders NK T cells cytotoxic against Fas<sup>+</sup> target cells

As illustrated in Fig. 1A, CD4<sup>+</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> thymocytes expanded in the presence of IL-7 and further stimulated for 24 h with IL-18 in combination with IL-12 became capable of killing target cells via the Fas pathway, as assessed on Fas-transfected L1210



**FIGURE 1.** Induction of NK T cell cytotoxicity by IL-18 in combination with IL-12. Total CD4<sup>-</sup>CD8<sup>-</sup>TCRαβ<sup>+</sup> (A) or CD4<sup>-</sup>CD8<sup>-</sup>TCRαβ<sup>+</sup>NK1.1<sup>-</sup> (B) thymocytes obtained after expansion with IL-7 were stimulated ( $5 \times 10^5$  cells/ml) with IL-18 (100 ng/ml; squares), IL-12 (10 ng/ml; circles), or IL-18 plus IL-12 (triangles) for 24 h. Cells were recovered, washed, and incubated with L1210Fas (filled symbols) or L1210 (open symbols) cells at different E:T cell ratios for 24 h. The cytotoxicity was determined using the JAM assay. Data represent the mean  $\pm$  SEM of at least three independent experiments.

cells (L1210.Fas) (23). Fig. 1A shows a significant cytotoxicity at an E:T cell ratio as low as 1:1, which did not affect control L1210 cells, thus proving the involvement of the Fas pathway. CD4<sup>-</sup>CD8<sup>-</sup>TCRαβ<sup>+</sup> thymocytes stimulated with IL-12 alone were already slightly cytotoxic against L1210.Fas cells, although much less than those treated together with IL-18 (Fig. 1A).

The failure of sorted CD4<sup>-</sup>CD8<sup>-</sup>TCRαβ<sup>+</sup>NK1.1<sup>-</sup> thymocytes stimulated with IL-18, IL-12, or both to kill L1210.Fas or L1210 cells (Fig. 1B) confirmed that NK T lymphocytes were responsible for the cytotoxicity. These results agree with previous evidence for up-regulation of Fas-mediated apoptosis by IL-18 and IL-12 (25, 26), but contrast with a recent report showing that IL-18 augments perforin- but not FasL-dependent cytotoxicity of liver NK T cells (27). A possible explanation for this discrepancy might be the requirement for anti-CD3 mAb costimulation in the latter situation, which could activate pathways amplifying perforin- rather than Fas-dependent cytotoxicity, as described in other models (28). In addition, in this particular study (27) NK T cells were not costimulated with IL-12, which, in our model, could up-regulate Fas-dependent cytotoxicity.

Several studies have provided evidence for inhibition of tumor development after injection of IL-18 and IL-12 (10, 18, 29). It has been reported that NK T cells are essential for IL-12-induced tumor rejection, which required neither TCR engagement nor Fas/FasL interactions (10). These results agree with the TCR-indepen-

**Table II.** IL-18 in combination with IL-12 induces IFN- $\gamma$  production by NK T cells<sup>a</sup>

Stimulation	IFN- $\gamma$ (ng/ml):	
	CD4 <sup>-</sup> CD8 <sup>-</sup> TCRαβ <sup>+</sup> NK1.1 <sup>+</sup>	
IL-18	4.4 $\pm$ 0.2	
IL-12	15.8 $\pm$ 1.8	
IL-18 + IL-12	3499 $\pm$ 967	
Medium	<0.04	

<sup>a</sup> Thymocytes sorted after expansion with IL-7 were stimulated at  $5 \times 10^5$  cells/ml. Forty-eight hours later IFN- $\gamma$  was measured in the supernatants. Results represent means  $\pm$  SEM from at least three different experiments.

dent stimulation of NK T cells in our model and imply that additional death-inducing ligands must be involved in the capacity of NK T cells to induce apoptosis.

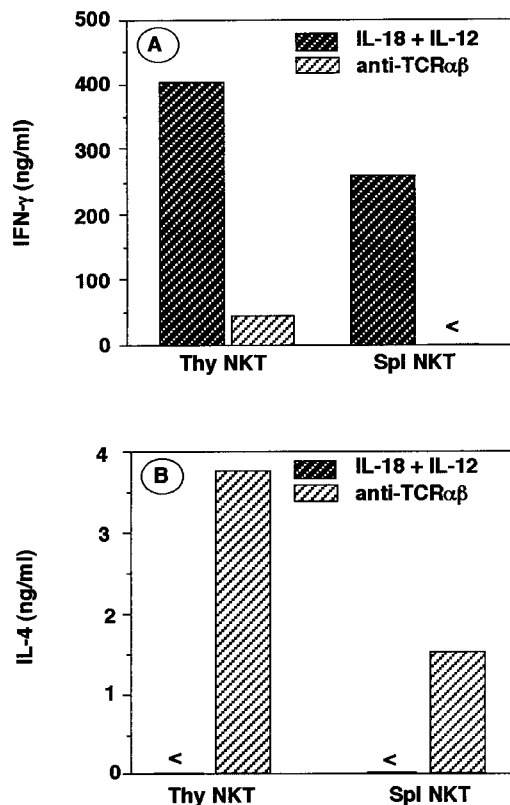
#### NK T lymphocytes produce large amounts of IFN- $\gamma$ upon in vitro stimulation with IL-18

A major feature of NK T cells is their capacity to promptly produce IL-4 and IFN- $\gamma$  in response to TCR cross-linking (5, 6). For this reason we measured these cytokines in supernatants from CD4<sup>-</sup>CD8<sup>-</sup>TCRαβ<sup>+</sup>NK1.1<sup>+</sup> thymocytes sorted after expansion with IL-7 and incubated for 48 h with IL-18 in the presence or the absence of IL-12. IFN- $\gamma$  production was strikingly increased when CD4<sup>-</sup>CD8<sup>-</sup>TCRαβ<sup>+</sup>NK1.1<sup>+</sup> cells were exposed to both IL-18 and IL-12 (Table II), while IL-4 could not be detected in these conditions (<0.04 ng/ml). Only low levels of IFN- $\gamma$  were generated in response to either cytokine alone in terms of both protein secretion (Table II) and mRNA expression (data not shown).

Costimulation with IL-18 plus IL-12 also induced marked IFN- $\gamma$  production by freshly isolated TCRαβ<sup>+</sup>NK1.1<sup>+</sup> thymocytes and splenocytes as shown in Fig. 2A. Interestingly, these cells produced at least 10 times more IFN- $\gamma$  in these conditions than in response to TCR cross-linking (Fig. 2A). As shown in Fig. 2B, thymic and splenic NK T cells secreted IL-4 upon TCR cross-linking, but not in response to IL-18 plus IL-12 (Fig. 2B). These results clearly establish the preferential effect of IL-18 plus IL-12 on the IFN- $\gamma$ -producing capacity of NK T cells.

NK T cells also produce IFN- $\gamma$  in response to specific Ags, such as  $\alpha$ -GalCer (30, 31). In our hands, this production was higher than that obtained in response to anti-TCR mAb. A possible explanation for this difference might be the addition of APC, which are potential IL-18 and IL-12 producers, during the incubation with  $\alpha$ -GalCer, while TCR ligation was performed with coated Abs (Fig. 3A). We have previously reported that the presence of IL-12 during anti-CD3 stimulation enhances IFN- $\gamma$  production by NK T cells (16). Furthermore, according to a recent study (32), the production of IL-12 by dendritic cells as well as their direct contact with NK T cells through CD40/CD40 ligand interactions are requisite for IFN- $\gamma$  production upon stimulation with  $\alpha$ -GalCer. It is plausible that IL-18, which is also generated by APC, participates in this biological activity. Indeed, the neutralization of IFN- $\gamma$  production by anti-IL-12 mAb (32) does not exclude such a possibility, because this treatment would also abolish the responsiveness of NK T cells to IL-18.

We further addressed the question of whether once activated by IL-18 plus IL-12, NK T cells continued to produce IFN- $\gamma$  in preference to IL-4 even when they are thereafter stimulated with anti-TCR mAb or specific Ag. For this purpose, CD4<sup>+</sup> splenocytes from class II-deficient (A $\beta$ <sup>o</sup>) mice were cultured for 2 h with IL-18 plus IL-12, washed, and further incubated with TCR- $\alpha\beta$  mAb or  $\alpha$ -GalCer. Fig. 3A shows that the amounts of IFN- $\gamma$  thus generated were strongly augmented after a preincubation with IL-18 plus

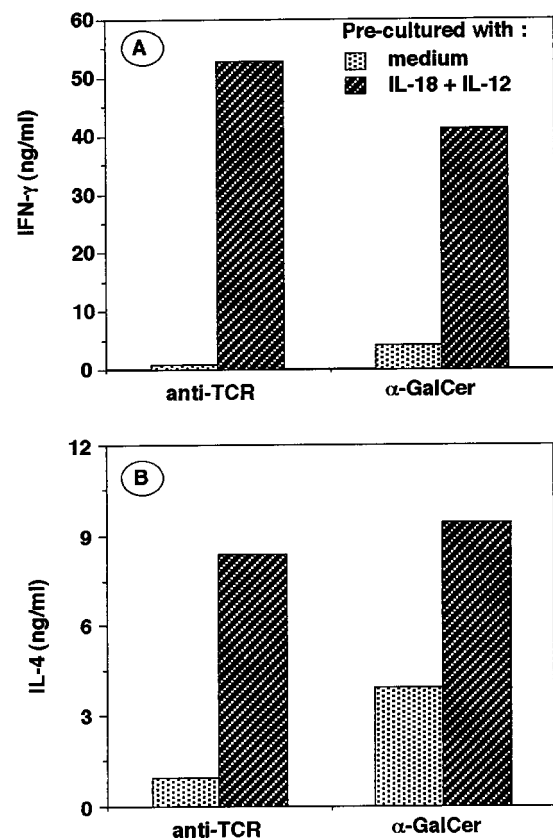


**FIGURE 2.** Freshly isolated NK T lymphocytes produce high levels of IFN- $\gamma$  in response to IL-18 plus IL-12. Sorted thymic (Thy NK T) or splenic (Spl NK T) TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> lymphocytes ( $5 \times 10^5$  cells/ml) from C57BL/6 mice were stimulated with IL-18 (100 ng/ml) plus IL-12 (10 ng/ml) or with coated anti-TCR $\alpha\beta$  mAb (10  $\mu$ g/ml). Culture supernatants were harvested after 48 h, and IFN- $\gamma$  (A) and IL-4 (B) levels were determined by ELISA. Data represent a typical experiment of three performed. <, <0.04 ng/ml.

IL-12. Because IL-4 was only slightly increased in these conditions (Fig. 3B), the IFN- $\gamma$ /IL-4 ratio was multiplied more than 5-fold, proving that the effect of IL-18 plus IL-12 persisted even after specific Ag stimulation. IFN- $\gamma$  levels induced by  $\alpha$ -GalCer would probably be higher than those given in Fig. 3A if purified dendritic cells had been used instead of total irradiated spleen cells. Indeed, NK T cells produced 40-fold more IFN- $\gamma$  when IL-18 and IL-12 were present both during pretreatment and during TCR stimulation, while IL-4 production was only slightly enhanced (data not shown).

#### NK T lymphocytes produce IFN- $\gamma$ after *in vivo* IL-18 plus IL-12 treatment

We verified whether the production of IFN- $\gamma$  by NK T cells stimulated with IL-12 plus IL-18 also occurred *in vivo*. To this end, C57BL/6 mice received a single injection of the two cytokines and were killed 2 h later. Purified CD4<sup>+</sup> splenocytes were then cultured for 24 h without further stimulation, and IFN- $\gamma$  was measured in the supernatants. CD4<sup>+</sup> cells from treated, but not from control, mice secreted IFN- $\gamma$ . However, this production was low, probably because the large majority of CD4<sup>+</sup> splenocytes in C57BL/6 mice is composed of conventional MHC class II-restricted T cells. Indeed, when class II-deficient ( $A\beta^0$ ) mice, which are devoid of this conventional population and hence enriched for NK T cells (33), received a single injection of IL-18 plus IL-12, the CD4<sup>+</sup> population generated high IFN- $\gamma$  levels without further stimulation (Table III) or in the presence of  $\alpha$ -GalCer (data not



**FIGURE 3.** Preculture of CD4<sup>+</sup> NK T splenocytes with IL-18 plus IL-12 greatly enhances their IFN- $\gamma$  production in response to anti-TCR $\alpha\beta$  mAb or  $\alpha$ -GalCer. CD4<sup>+</sup> splenocytes ( $5 \times 10^5$  cells/ml) from  $A\beta^0$  mice were cultured for 2 h with IL-18 (100 ng/ml) plus IL-12 (10 ng/ml). Cells were then washed and further stimulated with coated anti-TCR $\alpha\beta$  mAb (10  $\mu$ g/ml) or  $\alpha$ -GalCer (100 ng/ml) in the presence of  $5 \times 10^5$  irradiated autologous splenocytes/ml. Culture supernatants were harvested after 48 h, and IFN- $\gamma$  (A) and IL-4 (B) levels were determined by ELISA. Data represent a typical experiment of two performed.

shown). In contrast, as shown in the same table, splenocytes from  $\beta_2m^{-/-}$  mice, which comprise a high proportion of conventional CD4<sup>+</sup> and few NK T cells (33, 34) produced no detectable IFN- $\gamma$  (<40 pg/ml).

We further sorted CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> and CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>-</sup> NK T cell subsets from spleens of IL-18- plus IL-12-treated mice to analyze whether the two subpopulations were equally responsive after *in vivo* treatment with IL-18 plus IL-12. After a 24-h incubation without further stimulation, CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> secreted 2700 pg/ml, and CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>-</sup> secreted 2100 pg/ml of IFN- $\gamma$ /10<sup>6</sup> cells, showing that the two subsets share a similar potential for IFN- $\gamma$  production in response to stimulation with IL-12 plus IL-18 *in vivo*. IL-4 was detected in neither the CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>+</sup> nor the CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup>NK1.1<sup>-</sup> population (data not shown), and NK T cells from control mice secreted no detectable levels of IFN- $\gamma$  or IL-4 (data not shown).

Sorted NK T cells could eventually be costimulated by Abs used to perform the positive cell sorting. To avoid this possible costimulation and to confirm that IL-18 plus IL-12 induce IFN- $\gamma$  production independently of TCR or NK1.1 engagement, we used intracellular IFN- $\gamma$  staining to directly detect IFN- $\gamma$ -producing cells. To this end, C57BL/6 mice received a single injection of IL-18 plus IL-12 or vehicle. Two hours later, mice were sacrificed, and

Table III. Splenic CD4<sup>+</sup> NK T cells produce IFN-γ in response to in vivo treatment with IL-18 plus IL-12<sup>a</sup>

Expt.	IFN-γ (pg/ml)					
	Wild type		β <sub>2</sub> m <sup>-/-</sup>		Aβ <sup>o</sup>	
	Excipient	IL-18 + IL-12	Excipient	IL-18 + IL-12	Excipient	IL-18 + IL-12
1	<40	120	<40	<40	<40	1850
2	<40	90	<40	<40	<40	2160

<sup>a</sup> Wild-type and mutant (β<sub>2</sub>m<sup>-/-</sup> or Aβ<sup>o</sup>) C57BL/6 mice were treated with a single injection of IL-12 plus IL-18 or excipient. Two hours later mice were sacrificed, and purified CD4<sup>+</sup> splenocytes were cultured without further stimulation at 1 × 10<sup>6</sup> cells/ml. Twenty-four hours later IFN-γ was measured in the supernatants.

splenocytes were enriched for NK T cells by depletion of granulocytes, monocytes, CD8, and B lymphocytes. Enriched splenocytes were then stained with anti-TCRαβ, anti-NK1.1, and anti-IFN-γ mAb or isotype control and immediately analyzed. Fig. 4A indicates the windows used to further analyze IFN-γ staining among gated TCRαβ<sup>+</sup>NK1.1<sup>+</sup> (1) or TCRαβ<sup>+</sup>NK1.1<sup>-</sup> (2) splenocytes. TCRαβ<sup>+</sup>NK1.1<sup>+</sup> cells from IL-18- plus IL-12-treated, but not from vehicle-treated, mice produce IFN-γ (Fig. 4B, left panel). In contrast, we observed no significant frequency of IFN-γ<sup>+</sup> cells among TCRαβ<sup>+</sup>NK1.1<sup>-</sup> splenocytes from IL-18-plus IL-12-treated or control mice (Fig. 4B, right panel). The percentage of IFN-γ<sup>+</sup> cells among TCRαβ<sup>+</sup>NK1.1<sup>+</sup> and TCRαβ<sup>+</sup>NK1.1<sup>-</sup> splenocytes from IL-18- plus IL-12-treated mice attained 17 and 0.5%, respectively. The percentage of positive cells in the isotype control was always <1%. These results confirm that NK T cells produce IFN-γ in response to IL-18 plus IL-12 in a TCR- and NK1.1-independent manner.

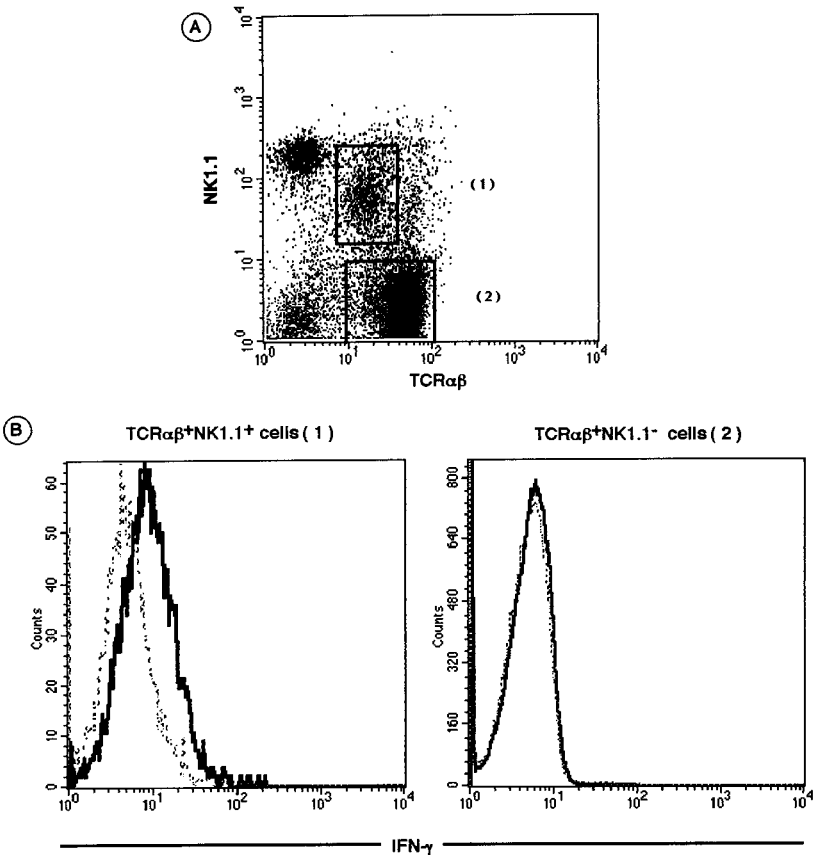
In conclusion, our findings clearly demonstrate that NK T cells do not need TCR cross-linking to acquire the capacity to kill target

cells in a Fas-dependent manner, to proliferate, or to secrete high levels of IFN-γ without IL-4 in response to IL-18 plus IL-12. In addition, this IFN-γ-producing potential conferred by the two proinflammatory cytokines was maintained during further stimulation with specific Ag. Knowing that monocytic and dendritic cells produce IL-18 and IL-12 after activation (18, 35–37), it might be speculated that this is the early direct stimulus NK T cells encounter in situ. Once activated, they conserve their functional capacities upon subsequent exposure to specific Ags. In conclusion, our data support the ideas that NK T cells are involved in both innate and acquired immune responses and that the presence of stimulatory factors in their environment will determine their final influence on immune responses.

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**FIGURE 4.** NK T lymphocytes produce IFN-γ in response to a single injection of IL-18 plus IL-12. Splenocytes from mice injected with IL-18 (1 μg/mouse) plus IL-12 (0.2 μg/mouse) or vehicle were depleted of monocytes, granulocytes, CD8, and B lymphocytes. Enriched cells were then stained with anti-TCRαβ-FITC, anti-NK1.1-biotin and streptavidin-Cy-chrome, and anti-IFN-γ-PE mAb. *A*, TCRαβ<sup>+</sup>NK1.1<sup>+</sup> (1) and TCRαβ<sup>+</sup>NK1.1<sup>-</sup> (2) splenocytes were gated. *B*, The intensities of IFN-γ staining among TCRαβ<sup>+</sup>NK1.1<sup>+</sup> (1, left panel) and TCRαβ<sup>+</sup>NK1.1<sup>-</sup> (2, right panel) cells from IL-18- plus IL-12-treated (solid line) or vehicle-treated (dotted line) mice was compared. Data represent a typical experiment of two performed.



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