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Article *in* The Journal of Immunology · September 2000

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Cytokine Production and Killer Activity of NK/T-NK Cells Derived with IL-2, IL-15, or the Combination of IL-12 and IL-18[1]

Bernard R. Lauwerys,* Nathalie Garot,* Jean-Christophe Renauld,†‡ and Frédéric A. Houssiau[2]*

NK cell populations were derived from murine splenocytes stimulated by IL-2, IL-15, or the combination of IL-12 and IL-18. Whereas NK cells derived with the latter cytokines consisted of an homogeneous population of NK cells ($DX5^+CD3^-$), those derived with IL-2 or IL-15 belonged to two different populations, namely NK cells ($DX5^+CD3^-$) and T-NK cells ($DX5^+CD3^+$). Among NK cells, only those derived with IL-12/IL-18 produced detectable levels of cytokines, namely IFN-γ, IL-10, and IL-13 (with the exception of IL-13 production by NK cells derived with IL-2). As for T-NK cells, IL-2-stimulated cells produced a wide range of cytokines, including IL-4, IL-5, IL-9, IL-10, and IL-13, but no IFN-γ, whereas IL-15-derived T-NK cells failed to produce any cytokine. Switch-culture experiments indicated that T-NK cells derived in IL-2 and further stimulated with IL-12/IL-18 produced IFN-γ and higher IL-13 levels. Next, we observed that NK/T-NK cell populations exerted distinct effects on Ig production by autologous splenocytes according to the cytokines with which they were derived. Thus, addition of NK cells derived in IL-12/IL-18 inhibited Ig production and induced strong cytotoxicity against splenocytes, whereas addition of NK or T-NK cells grown in IL-2 or IL-15 did not. Experiments performed in IFN-γR knockout mice demonstrated that IFN-γ was not involved in the killer activity of IL-12/IL-18-derived NK cells. The hypothesis that their cytotoxic activity was related to the induction of target apoptosis was confirmed on murine A20 lymphoma cells. Experiments performed in MRL/*lpr* mice indicated that IL-12/IL-18-derived NK cells displayed their distinct killer activity through a Fas-independent pathway. Finally, perforin was much more expressed in IL-12/IL-18-derived NK cells as compared with IL-2- or IL-15-derived NK cells, an observation that might explain their unique cytotoxicity. *The Journal of Immunology,* **2000, 165: 1847–1853.**

Natural killer cells are thought to play a critical role in the defense mechanisms against pathogens and transformed cells. They display MHC-unrestricted cytotoxicity and produce various cytokines, such as IFN-γ and TNF-α, that further enhance their cytotoxic effects and contribute to modulate both innate and acquired immune responses. Thus, IFN-γ production by NK cells may skew immune responses toward a Th1 pattern, in particular during infection with *Listeria monocytogenes* (1). Similarly, IL-5 production by NK cells was shown to contribute to the eosinophilic reaction in a mouse model of allergic inflammation (2).

The proliferation and activation of NK cells are controlled by cytokines such as IL-2 (3, 4), IL-12 (5, 6), IL-15 (7–9), and IL-18 (10–15). Interestingly, observations made in knockout (KO)[3] mice have indicated that IL-15, but not IL-2, is required for NK cell ontogeny. Thus, mice genetically deficient for the IL-2R γ-chain gene, involved in IL-2 and IL-15 signaling pathways, lack NK cells (16), whereas IL-2-KO mice have NK cells, although their function is impaired (17, 18). The important role of IL-12 and IL-18 as NK cell-activating factors was confirmed by the observation that IL-18- and IL-12-deficient mice displayed impaired NK cell activity (19).

We and others recently unmasked a striking synergy between IL-12 and IL-18 for the proliferation and activation of murine NK cells (20, 21). NK cells derived with the latter cytokines consisted of a homogeneous population, whereas those derived with IL-2 or IL-15 belonged to two different populations, namely NK and T-NK cells, the latter expressing both NK and T cell surface markers. Interestingly, NK cells stimulated with IL-12/IL-18 secreted large amounts of IFN-γ, whereas NK cells grown in IL-2 or IL-15 did not. This observation prompted us to further compare the functional characteristics of NK/T-NK cells derived in IL-2, IL-15, or IL-12/IL-18.

Here, we describe 1) that murine NK and T-NK cell populations display distinct cytokine production profiles according to the growth factors they are derived and stimulated with and 2) that IL-12/IL-18-derived NK cells exert a unique killer activity compared with NK cells derived in IL-2 or IL-15.

Materials and Methods

Cytokines and Abs

Murine rIL-18 was purchased from Peprotech EC (London, U.K.). Murine rIL-12 and human rIL-15 (which is active on murine cells) were obtained from R&D Systems Europe (Abingdon, U.K.). Human rIL-2 was purchased from Eurocetus (Chiron, Amsterdam, The Netherlands), and murine

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Received for publication November 12, 1999. Accepted for publication May 31, 2000.

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[1] This work was supported in part by grants from the Centre de Recherche Interuniversitaire en Vaccinologie; the Fonds National de la Recherche Scientifique et Médicale; the Belgian Federal Service for Scientific, Technical, and Cultural Affairs; the Opération Télévie; and the Actions de Recherche Concertées (Communauté Française de Belgique, Direction de la Recherche Scientifique). J.-C.R. is a Research Associate with the Fonds National de la Recherche Scientifique, Belgium.

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[3] Abbreviations used in this paper: KO, knockout; FasL, Fas ligand.

0022-1767/00/$02.00

rIFN-γ was a kind gift from Dr. W. Fiers (University of Ghent, Ghent, Belgium). The following cytokine concentrations were used: 1 ng/ml for IL-12; 100 ng/ml for IL-18; 50 ng/ml for IL-15 (except if otherwise indicated); 100 U/ml for IL-2; and 100 ng/ml for IFN-γ. Anti-Fas ligand (FasL) mAb was purchased from PharMingen (San Diego, CA) and used at a concentration of 20 μg/ml. An Armenian hamster IgG1 purchased from PharMingen (20 μg/ml) was used as isotype-matched control.

Animals and cellular preparations

(NZB $\times$ NZW)F_1 hybrids (BWF_1), C57BL/6, 129, and IFN-γR-KO 129 mice were bred in our animal facility. The latter animals, initially derived by Dr. S. Huang and Dr. M. Aguet (22), were obtained through the courtesy of Dr. F. Brombacher (Max Planck Institute for Immunobiology, Freiburg, Germany). MRL/*lpr* mice were purchased from Harlan (Oxon, U.K.). For most experiments, we used BWF_1 spleen cells that spontaneously produce huge amounts of Ig.

Splenocytes from 8-wk-old female mice were prepared by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. Adherent cells were removed by nylon wool filtration, and the remaining cells were cultured at a density of 3×10^6 cells/well in 24-well plates in DMEM supplemented with 10% FCS, 50 μM 2-ME, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine, in the presence of IL-2, IL-15, or a combination of IL-12 and IL-18. After 3 days, cells were harvested and centrifuged on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient. Magnetic cell sorting was performed on the low density fraction using a mixture of anti-CD4- and anti-CD8-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), to eliminate T cells. For cells derived with IL-12/IL-18, the negatively selected $CD4^-CD8^-$ cell fraction consisted of an homogeneous population of $DX5^+CD3^-$ NK cells (Fig. 1*A*), referred to as NK IL-12/IL-18 (between 95 and 98% $DX5^+$ and $<1\%$ $CD3^+$). By contrast, for cells derived with IL-2 or IL-15, the negatively selected fraction contained two distinct populations, namely $DX5^+CD3^-$ NK cells and $DX5^+CD3^+$ T-NK cells (Fig. 1, *B* and *C*), that were further separated by flow cytometry (FACSvantage SE, Becton Dickinson, Mountain View, CA) using a FITC-labeled anti-CD3 mAb into NK or T-NK cells, referred to as NK IL-2/T-NK IL-2 or NK IL-15/T-NK IL-15. After sorting, NK IL-2 and NK IL-15 cells were $>98\%$ $DX5^+$ and $< 1\%$ $CD3^+$, whereas T-NK IL-2 and T-NK IL-15 cells were $>98\%$ $DX5^+$ and $>98\%$ $CD3^+$. The anti-CD3 and anti-DX5 mAbs used to determine the purity of T and T-NK cell populations were purchased from PharMingen. Labeling experiments were performed in the presence of an anti-CD16/CD32 (FcγRIII/II) mAb (10 μg/ml) purchased from PharMingen. Because BWF_1 mice lack NK1.1 expression, we did not use this NK cell marker.

Cytokine and Ig production assays

Purified NK and T-NK cells were seeded for 24 h in triplicate or quadruplicate cultures at a density of 3×10^5/well in microtiter plates in the presence of the indicated cytokine(s). IL-4 and IFN-γ titers were determined in the supernatants using ELISA kits purchased from BioSource Europe (Nivelles, Belgium). IL-5 and IL-13 were measured using ELISA kits supplied by Amersham Life Science (Little Chalfont, U.K.) and R&D Systems (Minneapolis, MN), respectively. Total Igκ titers were determined by a standard ELISA technique using LO-MK-2 as coating Ab and peroxidase-labeled LO-MK-1 as a detecting Ab (both are anti-κ light chain Abs purchased from the Unit of Experimental Immunology, Université catholique de Louvain, Brussels, Belgium).

Coculture experiments

Purified NK and T-NK cells (5×10^4/well) were cocultured with syngeneic splenocytes (5×10^5/well) in quadruplicate microtiter wells. In other experiments, purified NK cells (1×10^4/well) were added to murine A20 lymphoma cells (ATCC TIB-208; 5×10^4/well). Thymidine incorporation studies (overnight pulse with 0.5 μCi [^{3}H]thymidine/well) and cell countings after trypan blue exclusion (Seromed, Berlin, Germany) were performed after 2 days of coculture. Supernatants were harvested at day 7 for measurement of total Igκ titers. For double chamber experiments, 5×10^5 purified NK cells were cocultured with 3×10^6 syngeneic splenocytes in 24-well plates, in either single or double chambers, using cell culture inserts (Nalge Nunc, Naperville, IL). LPS was purchased from Sigma (St. Louis, MO).

Cytotoxicity assays

Yac-1 cells (1×10^3 cells/well), a murine NK cell target, were ^{51}Cr labeled and incubated for 4 h in U-shaped microtiter wells with purified NK cells at various E:T ratios in quadruplicate cultures. ^{51}Cr release was determined in supernatants, and specific lysis was calculated as the ratio: ([measured ^{51}Cr release $-$ minimal ^{51}Cr release (targets alone)]/[maximal ^{51}Cr release (targets in 1% Triton) $-$ minimal ^{51}Cr release]) $\times$ 100.

Annexin V labeling assays

Purified NK cells were seeded at 1×10^4/well in U-shaped microtiter plates with 1×10^5 A20 cells. After 24 h, cells were tested for apoptosis by a 15-min incubation with FITC-conjugated annexin V (Genzyme Diagnostics, Cambridge, MA; 0.5 μg/ml) and propidium iodide (Sigma, 1 μg/ml) in HEPES buffer (10 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) before being analyzed by flow cytometry.

RNA extraction and RT-PCR analyses

Purified NK and T-NK cells were lyzed in TriPur (Boehringer Mannheim, Mannheim, Germany), and total RNA was purified by chloroform extraction. cDNA was synthesized by using oligo(dT) primers (Boehringer Mannheim) and murine Moloney leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). Diluted cDNA was amplified by PCR using recombinant *Taq* DNA polymerase (Takara, Shiga, Japan) and specific primers for murine β-actin (5′-ATGGATGACATATCGCTGC-3′; 3′-GTCTCCGTCTCCGTCCAC-5′), IL-4 (5′-ATGGGTCTCAACCCCCAGCTA-3′; 3′-GCATGGTGGCTCAG TACTACG-5′), IL-5 (5′-AAGGATGCTTCTGCACTTGA-3′; 3′-ACAC CAAGGAACTCTTGCA-5′), IL-9 (5′-GATGATTGTACCACACCGTG-3′; 3′-CCTTTGCATCTCTGTCTTCTGG-5′), IL-10 (5′-GAGACTTGCTCTT GCACTAC-3′; 3′-CCTGGAGTCCAGCAGACTCA-5′), IL-13 (5′-ATG GCGCTCTGGGTGACTGCAG-3′; 3′-GAAGGGGCCGTGGCGAAA CAGTTG-5′), IFN-γ (5′-GACAATCAGGCCATCAGCAAC-3′; 3′-CGCAATCACAGTCTTGGCTAA-5′), and perforin (5′-TGCTAC ACTGCCACTCGGTCA-3′; 3′-TTGGCTACCTTGGAGTGGGAG-5′). PCR was performed as follows: 1 min at 94°C, followed by 1 min at 63°C for IL-13, 60°C for β-actin, 57°C for perforin, 55°C for IL-4 and IFN-γ, 52°C for IL-9 and IL-10, 49°C for IL-5, and by 2 min at 72°C for 20 (β-actin and IFN-γ), 30 (IL-4 IL-5, IL-10, and perforin), 33 (IL-13), and 35 (IL-9) cycles. The RT-PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis. For semiquantitative perforin gene expression studies by RT-PCR analyses, serially diluted cDNA was amplified, and net fluorescence intensity of the PCR products was measured on a Kodak ImageStation 440CF (Eastman Kodak, Rochester, NY). Ratios between perforin and β-actin counts were calculated for dilutions where amplification was linear. PCR products were sequenced with an automated fluorescence base system (Applied Biosystems 377) using cycle sequencing with standard FS dye Deoxy Terminator chemistry (Perkin-Elmer Applied Biosystems, Foster City, CA) and their homology with the *Mus musculus* cDNA perforin sequence (accession no. M23182) was confirmed.

Results

Distinct cytokine production by NK/T-NK cells derived with IL-2, IL-15, or IL-12/IL-18

NK cell populations can be readily derived from murine splenocytes by addition of IL-2, IL-15, or the combination of IL-12 and IL-18. Whereas NK cells derived in response to the latter cytokines consist of a homogeneous population of $DX5^+CD3^-$ NK cells (Fig. 1*A*), those derived with IL-2 or IL-15 belong to two different populations, namely $DX5^+CD3^-$ NK cells and $DX5^+CD3^+$ T-NK cells (Fig. 1, *B* and *C*). In a first set of experiments, we investigated whether these different T and T-NK cell populations displayed distinct patterns of cytokine production. NK and T-NK cells were purified from spleen cells stimulated with IL-2, IL-15, or IL-12/IL-18 as described in *Materials and Methods* and further cultured with the same cytokine(s) for 24 h. Cytokine gene expression analyses were performed by RT-PCR, and cytokine concentrations were measured by ELISA in NK or T-NK cell culture supernatants. As shown in Fig. 2, only NK cells derived in IL-12/IL-18 and T-NK cells derived in IL-2 expressed detectable amounts of cytokine messages, with the exception of IL-13 production by IL-2-stimulated NK cells. Interestingly, T-NK cells stimulated with IL-2 produced IL-4, IL-5, and IL-9 but no IFN-γ, whereas NK cells derived in IL-12/IL-18 produced IFN-γ but no IL-4, IL-5, or IL-9. Both T-NK cells cultured in IL-2 and NK cells derived in IL-12/IL-18 produced IL-10 and IL-13. NK and T-NK

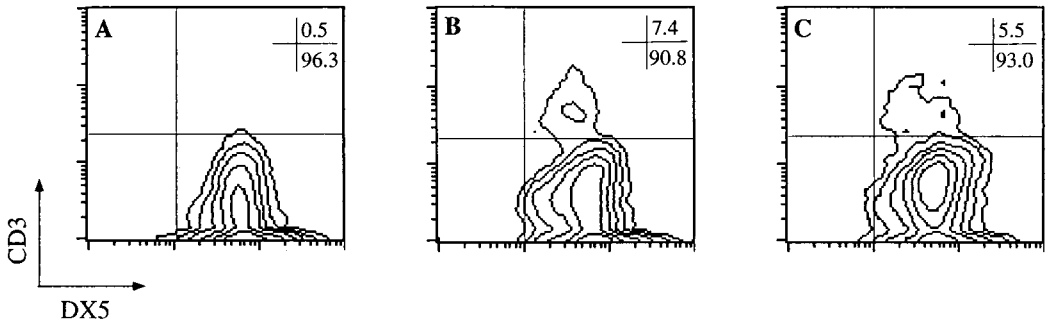

FIGURE 1. NK/T-NK cell populations derived from splenocyte cultures in response to IL-12 and IL-18 (*A*), IL-2 (*B*), or IL-15 (*C*). Nylon wool-filtered BWF_1 splenocytes were supplemented with IL-2, IL-15, or IL-12 and IL-18. After a 3-day culture, cells were centrifuged on a discontinuous Percoll gradient. The low density cells were further depleted from T cells by cell sorting using a mixture of anti-CD4- and anti-CD8-coated magnetic beads. The resulting $CD4^-CD8^-$ cells were double-labeled with an anti-DX5 and an anti-CD3 mAb. The upper right $DX5^+CD3^+$ population and the lower right $DX5^+CD3^-$ population correspond to T-NK and NK cells, respectively.

cells derived in IL-15 produced none of the aforementioned cytokines, even when the cytokine was added at a concentration of 500 ng/ml.

Next, we investigated whether the pattern of cytokine production by T-NK cells derived in IL-2 could be reversed by a switch-culture in IL-12/IL-18 and whether cytokine expression of NK cells derived in IL-12/IL-18 was influenced by a further pulse in IL-2. As indicated in Fig. 3*A*, T-NK cell populations derived in IL-2 and further stimulated with IL-12/IL-18 produced IFN-γ. Moreover, their IL-13 production was up-regulated, and their IL-4 and IL-5 productions were down-regulated. Conversely, NK cells derived with IL-12/IL-18 down-regulated their IFN-γ and IL-13 productions, when further cultured with IL-2 (Fig. 3*B*).

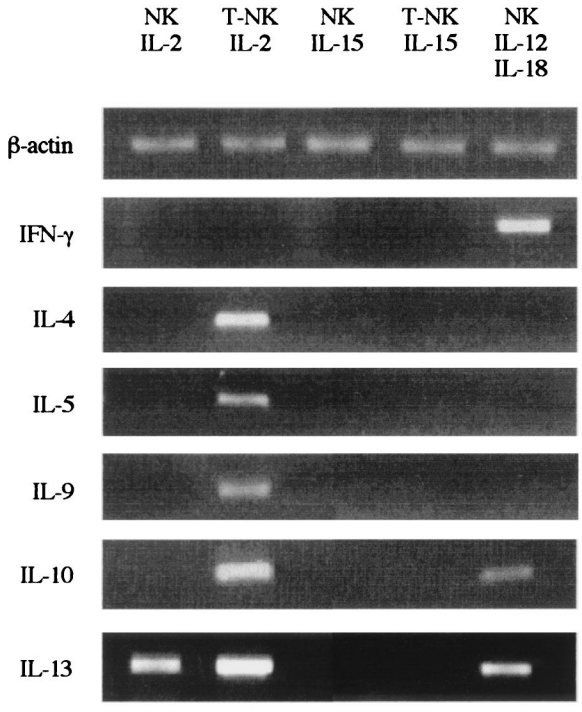

FIGURE 2. Cytokine gene expression by NK/T-NK cell populations. RT-PCR analyses were performed with primers specific for murine β-actin, IL-4, IL-5, IL-9, IL-10, IL-13, and IFN-γ on cDNA synthesized from RNA extracted from purified BWF_1 NK/T-NK cells derived and restimulated for 24 h with the indicated cytokines. RT-PCR products were analyzed by agarose gel electrophoresis.

Distinct effects of NK/T-NK cells on Ig production by syngeneic splenocytes

The observation that IL-2-stimulated T-NK cells produced type 2 cytokines such as IL-4, IL-5, and IL-9, whereas IL-12/IL-18-stimulated NK cells did not, prompted us to evaluate whether NK cell populations exerted distinct regulatory effects, in particular on spontaneous Ig production by autologous splenocytes. BWF_1 mice were used for these experiments because their splenocytes spontaneously produce large amounts of Ig. NK cell populations were purified from spleen cells stimulated with IL-2 or IL-12/IL-18, washed, and cocultured with freshly isolated syngeneic splenocytes. Supernatants were tested for Igκ titers after a 7-day culture. As shown in Fig. 4, addition of NK cells derived in IL-12/IL-18 inhibited Ig production, whereas NK cells or T-NK cells derived in IL-2 did not. Rather, addition of T-NK cells grown in IL-2 up-regulated Ig production by autologous splenocytes.

Given the striking synergy between IL-12 and IL-18 for IFN-γ induction (23) and given the inhibitory effects of IFN-γ on Ig production in certain experimental systems (24, 25), we evaluated whether the inhibitory effects exerted by IL-12/IL-18-derived NK cells on Ig production and cell survival were mediated by IFN-γ. As indicated in Fig. 5, LPS-induced Ig production by IFN-γR-KO 129 splenocytes was inhibited by addition of syngeneic IL-12/IL-18-derived NK cells as potently as Ig production by wild-type 129 splenocytes, thereby demonstrating that IFN-γ did not mediate the inhibitory effects of IL-12/IL-18-derived NK cells.

Induction of apoptosis by NK cells derived with IL-12/IL-18

We hypothesized that the inhibitory effect of IL-12/IL-18-derived NK cells on Ig production was due to their cytotoxic activity against splenocytes. As indicated in Table I, addition of IL-12/IL-18 NK cells to autologous BWF_1 splenocytes increased cell mortality, as assessed by cell counting after trypan blue exclusion. By contrast, NK or T-NK cells derived in response to IL-2 or IL-15 displayed no cytotoxicity at the same E:T ratio. Similar experiments performed on Con A- and PMA-stimulated T cell blasts yielded similar results (data not shown). The distinct cytotoxic activity of IL-12/IL-18-derived NK cells, as compared with NK cells grown in IL-2 or IL-15, was further evidenced by a ^{51}Cr release assay using Yac-1 cells as targets (Fig. 6).

We evaluated whether the cytotoxic activity of IL-12/IL-18-derived NK cells on syngeneic splenocytes required a cell-to-cell

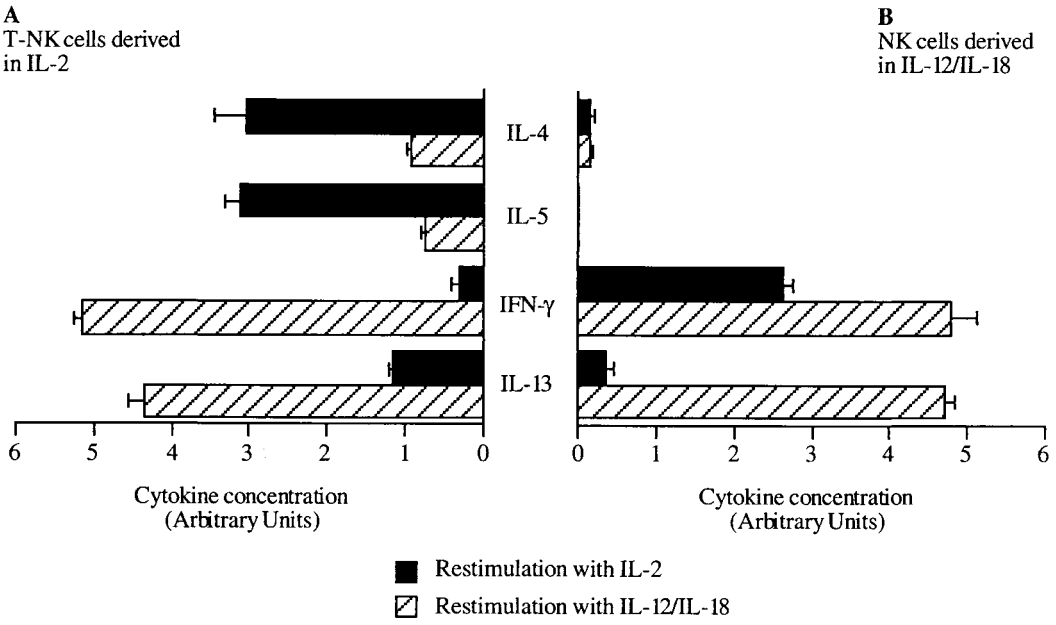

FIGURE 3. Cytokine production by NK/T-NK cell populations. BWF_1 IL-12/IL-18 NK cells (*right*), and IL-2 T-NK cells (*left*) were purified as described in *Materials and Methods* and restimulated with IL-2 (■) or with IL-12/IL-18 (▨). Cytokine concentrations (mean ± SEM) were measured by ELISA in supernatants from duplicate cultures. One arbitrary unit corresponds to 100 pg/ml for IL-4, IL-5, and IL-13 and 2000 pg/ml for IFN-γ. Data are representative of two distinct experiments. Similar results were observed with C57BL/6 splenocytes (data not shown). In two additional experiments, no IL-4 production was detected in supernatants from T-NK cells stimulated with as high an IL-15 concentration as 500 ng/ml (data not shown).

interaction or was mediated by (a) soluble factor(s). Double-chamber culture experiments indicated that spontaneous Ig production was down-regulated only when effector (NK) and target (splenocytes) cells were cocultured in the same chamber (Fig. 7), thereby demonstrating that the cytotoxic effect of NK cells required a cell-to-cell contact.

The possibility that IL-12/IL-18 NK cells killed their targets *via* induction of apoptosis was investigated on murine A20 lymphoma cells. As shown in Fig. 8*A*, addition of NK cells derived with IL-12/IL-18 strongly inhibited the proliferation of A20 cells at an E:T ratio (1:5) with which no similar effect could be observed with NK cells derived in IL-2 or IL-15. Induction of A20 cells apoptosis by NK cell populations was evaluated by annexin V labeling assays performed in the presence of propidium iodide to discriminate apoptotic from necrotic cells. As illustrated in Fig. 8*B*, IL-12/IL-18-derived NK cells strongly induced apoptosis of A20 cells.

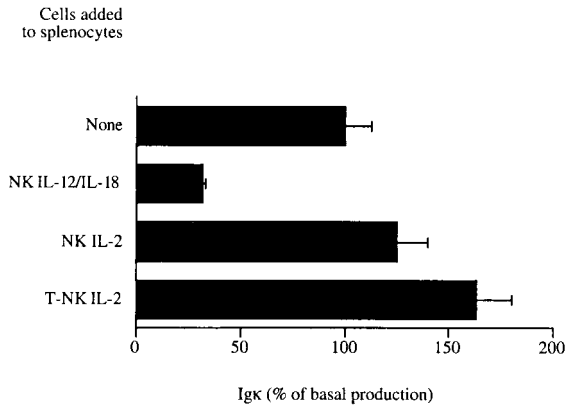

FIGURE 4. Effects of NK cells on spontaneous Ig production by autologous splenocytes. Total Igκ titers were measured in the supernatants of BWF_1 splenocyte cultures in the presence or in the absence of autologous NK/T-NK cells derived with the indicated cytokines. Results are expressed as mean (±SEM) percent of basal Ig production measured in quadruplicate cultures. Data are representative of two distinct experiments.

Next, we wondered whether the proapoptotic activity of IL-12/IL-18-derived NK cells was dependent on Fas/FasL interaction. Two lines of evidence argued, however, against this possibility. First, addition of a neutralizing anti-FasL mAb did not reverse the proapoptotic activity of IL-12/IL-18-derived NK cells on A20 cells, as assessed by annexin V labeling assays (data not shown). Moreover, and more convincingly, NK cells purified from Fas-mutated MRL/*lpr* splenocytes stimulated with IL-12/IL-18 also inhibited the spontaneous Igκ production by autologous splenocytes (Fig. 9), thereby demonstrating that IL-12/IL-18-derived NK cells exerted their unique killer activity through a Fas-independent pathway. Finally, using semiquantitative RT-PCR analyses, we investigated whether perforin gene expression was up-regulated in IL-12/IL-18-derived NK cells. As shown in Fig. 10, expression of the perforin gene was strongly enhanced in NK cells derived in IL-12/IL-18 compared with NK cells grown in IL-2 or IL-15, an observation that might explain their distinct cytotoxicity.

Discussion

The data presented here demonstrate 1) that murine NK and T-NK cell populations display distinct cytokine production profiles according to the growth factors they are derived with and 2) that IL-12/IL-18-derived NK cells exert a unique killer activity compared with NK cells derived in IL-2 or IL-15.

Although numerous recent studies, including our own, have identified the critical role of IL-18 (alone or in synergy with IL-12 or IL-10) as a potent IFN-γ inducer in NK cells (10, 12, 15, 20), the data available regarding other cytokines produced by NK cells are more difficult to interpret. The cytokine expression profile of NK cell populations is indeed influenced not only by the growth factor(s) used to expand and to stimulate them but also by the level of purity of the NK preparations. The latter issue is of the utmost

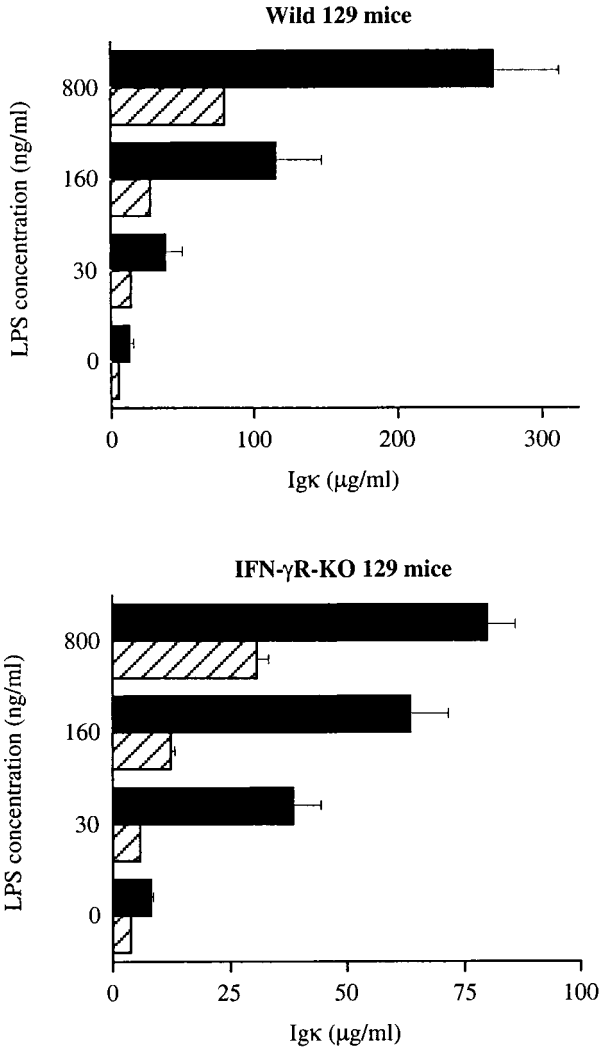

FIGURE 5. Effects of IL-12/IL-18-derived NK cells on Ig production by IFN-γR-KO mice. Splenocytes from wild-type (*top*) or IFN-γR-KO (*bottom*) 129 mice were cultured with the indicated doses of LPS in the presence (▨) or in the absence (■) of autologous NK cells derived in response to IL-12/IL-18. Igκ titers were measured in the supernatants. Results are expressed as mean (±SEM) concentrations measured in triplicate cultures. Data are representative of two distinct experiments.

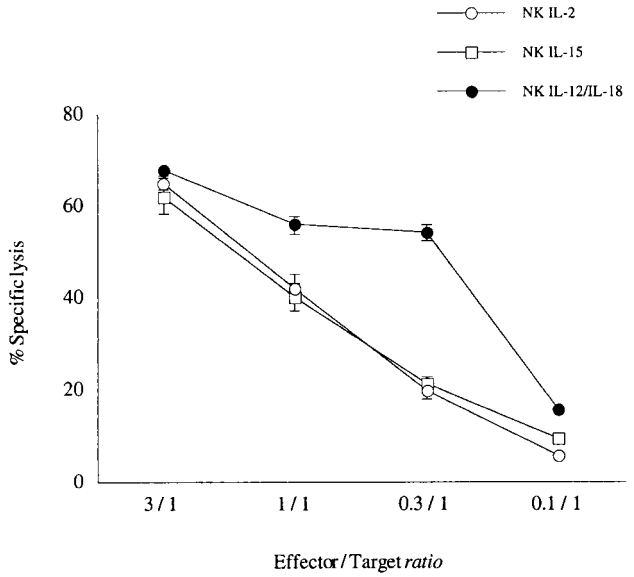

FIGURE 6. Cytotoxic activity of NK cells against Yac-1 cells. ^{51}Cr-labeled Yac-1 cells were incubated in quadruplicate with NK cells derived with the indicated cytokines. Results are expressed as mean (±SEM) percent of specific lysis at various E:T ratios.

importance for NK blasts derived in response to IL-2 or IL-15, because stimulation by either of these two cytokines generates two different cellular populations bearing NK markers, namely $DX5^+CD3^-$ NK cells and $DX5^+CD3^+$ T-NK cells (21), which may both contribute to cytokine production. In this respect, our observation that T-NK cells derived in response to IL-2 (and not NK cells derived and tested under the same experimental conditions) produce type 2 cytokines, such as IL-4, IL-5, and IL-9, suggests that T-NK cells can provide some help to humoral immune responses. Accordingly, we noted that addition of T-NK cells derived in IL-2 to autologous spleen cells augmented their Ig production (Fig. 4), likely via induction of type 2 cytokines. By contrast, and well in line with previous findings, we confirmed that NK cells derived in IL-12/IL-18 produce IFN-γ but no IL-4, IL-5

Table I. *Effects of NK/T-NK cells on splenocyte survival*[a]

Cells Added to Splenocyte Cultures	% of Viable Cells	
	Expt. 1	Expt. 2
None	83.5	72.4
NK IL-2	90.0	84.7
T-NK IL-2	91.9	88.2
NK IL-15	83.7	81.9
T-NK IL-15	86.8	88.2
NK IL-12/IL-18	34.8	17.7

[a] BWF_1 splenocytes were cultured in the presence or in the absence of autologous NK/T-NK cells derived with the indicated cytokines. Cell survival was evaluated by counting after trypan blue exclusion after a 3-day culture.

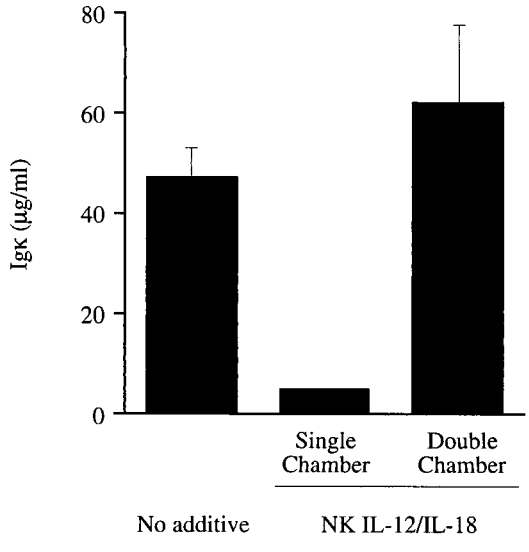

FIGURE 7. Effects of IL-12/IL-18-derived NK cells on spontaneous Ig production in double-chamber cultures. BWF_1 splenocytes were cultured in the absence or in the presence of autologous NK cells derived in IL-12/IL-18 under either single-chamber or double-chamber culture conditions. Igκ titers were measured in the supernatants. Results are expressed as mean (±SEM) concentrations measured in duplicate cultures. Data are representative of two distinct experiments.

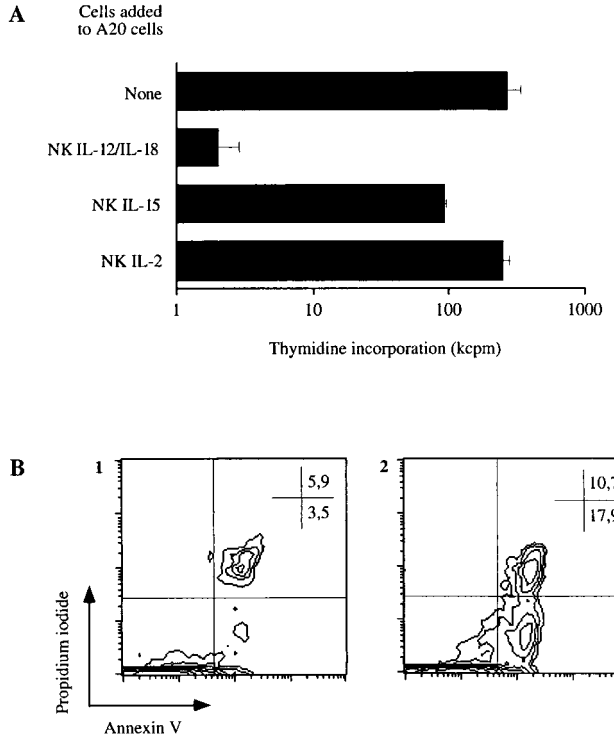

FIGURE 8. Effects of NK cells on A20 lymphoma cells. A20 cells were cultured for 3 days in the presence or in the absence of BWF_1 NK cells derived with the indicated cytokines, at an E:T ratio of 1:5 for the proliferation assays (*A*) and 1:10 for the annexin V labeling assays (*B*). Proliferations (mean kcpm ± SEM) were measured by [^{3}H]thymidine incorporation studies. Apoptosis was evaluated by labeling with FITC-conjugated annexin V in the presence of propidium iodide on A20 cells cultured alone (*B1*) or in the presence of BWF_1 NK cells derived in IL-12/IL-18 (*B2*). Data are representative of two distinct experiments for the proliferation assays and five distinct experiments for the annexin V labeling assays.

or IL-9. It should be stressed, however, that we did not observe a strict type 1/type 2 cytokine dichotomy between T-NK cells derived in IL-2 on the one hand and NK cells grown in IL-12/IL-18 on the other hand, because both subsets produced IL-10 and IL-13, two other purported type 2 cytokines.

Although IL-15 strongly stimulated the growth of NK and T-NK cells from bulk spleen cell cultures, we could not detect any cytokine production in NK/T-NK cells derived in IL-15, even added at a concentration of 500 ng/ml. The striking difference between IL-2 and IL-15 with respect to cytokine induction in T-NK cells is puzzling because IL-2 and IL-15 are purported to share the same signaling pathway through the β- and γ-chains of the IL-2R (26).

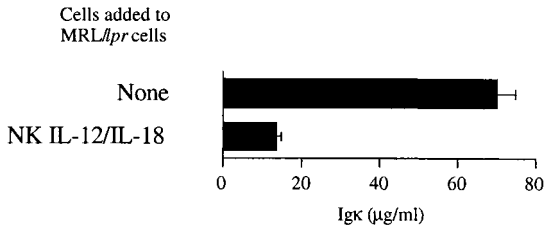

FIGURE 9. Effects of IL-12/IL-18-derived NK cells on Ig production by MRL/*lpr* splenocytes. Igκ production (mean ± SEM) was determined in supernatants from MRL/*lpr* splenocytes cultured alone or in the presence of IL-12/IL-18-derived syngeneic NK cells. Data are representative of two experiments.

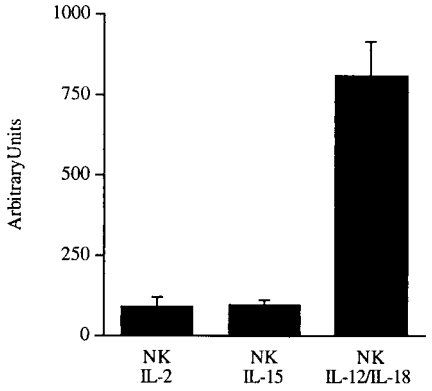

FIGURE 10. Perforin gene expression in NK cells. RNA was extracted from purified NK cells derived with the indicated cytokines and cDNA was synthesized as indicated in *Materials and Methods*. Serially diluted cDNA was amplified using specific primers for β-actin and perforin. Results are expressed as mean (±SEM) arbitrary units calculated as the ratio between luminescence intensity of perforin and β-actin PCR products where amplification was linear. The data of two experiments were pooled.

Our observation that IL-2 and IL-15 have distinct effects on cytokine expression by T-NK cells suggests that their signaling pathways might differ at least in certain cell types. On stimulation with an anti-CD3 mAb, T-NK cells produced large amounts of IL-4 and IFN-γ, irrespective of the cytokines (IL-2 or IL-15) they were derived with. A functional difference between IL-15 and IL-2 was also recently reported for cytokine production by TCR$\alpha\beta$-positive intestinal intraepithelial lymphocytes which were shown to produce IFN-γ after IL-2, but not IL-15, stimulation (27).

Switch-culture experiments indicated that cytokine production by NK/T-NK cells was not strictly committed by their initial culture condition. Thus, IFN-γ- and IL-13-producing NK cells derived in IL-12/IL-18 down-regulated their IFN-γ and IL-13 production when pulsed in IL-2. Conversely, type 2 cytokine-producing T/NK cells grown in IL-2 and further cultured in IL-12/IL-18 expressed IFN-γ, augmented their IL-13 production and down-regulated IL-4 and IL-5 expression. These results confirm that cytokine production by NK/T-NK cells is a reversible process depending on the cytokine(s) with which they are stimulated and is not associated with a fixed phenotype acquired during ontogeny.

Our results demonstrate for the first time that murine NK cells derived in response to IL-12 and IL-18 are much more cytotoxic effectors against syngeneic and tumoral targets than NK cells grown in IL-2 or IL-15. Interestingly, the cytotoxicity of NK cells derived in IL-2 or IL-15 was not enhanced to the level observed with IL-12/IL-18 NK cells by a 72-h pulse with IL-12/IL-18 (data not shown), while their IFN-γ and IL-13 productions were up-regulated as indicated above. Given the potent synergy between IL-12 and IL-18 for IFN-γ production by different lymphocyte subsets, including NK cells (21, 28–30), we first investigated whether the latter cytokine was responsible for the potent killer activity of NK cells derived with IL-12 and IL-18. Experiments performed on IFN-γR-KO-splenocytes convincingly ruled out this hypothesis. Double-chamber experiments, by indicating that cell-to-cell contact was required for the cytotoxicity of NK cells to occur, suggested that the effect of NK cells derived in IL-12 and IL-18 was to induce target apoptosis, a hypothesis that was confimed by annexin V labeling assays performed on murine A20 lymphoma cells.

The mechanisms explaining the different level of proapoptotic activity between distinct NK cell populations according to the cytokine(s) they were derived with, remain unclear. Thus, Tsutsui et

al. (11) observed that IL-18 up-regulated FasL expression on liver-derived murine NK cell clones and increased their Fas/FasL-mediated cytotoxicity. Our observations that addition of anti-FasL Ab did not inhibit the cytotoxicity of IL-12/IL-18 NK cells and that MRL/*lpr* NK cells derived in response to IL-12 and IL-18 inhibited Ig production by autologous spleen cells indicated that Fas/FasL interaction was not required for the cytotoxicity of IL-12/IL-18 NK cells to occur.

The effects of IL-12 and IL-18 on NK cell expression of other molecules involved in cytotoxicity have recently been studied by Hyodo et al. (31). They found that IL-12, but not IL-18, enhances mRNA expression of perforin and granzyme B in murine NK cells. Moreover, splenocytes from perforin-deficient mice incubated for 24 h with IL-12 and IL-18 failed to display NK activity, thereby demonstrating that perforin is required for the cytotoxicity of IL-12/IL-18-stimulated spleen cells. Using semiquantitative RT-PCR analyses, we observed an increased expression of the perforin gene in NK cells derived in response to IL-12/IL-18, compared with NK cells grown in IL-2 or IL15. These results, although they do not address, strictly speaking, the mechanisms underlying the unique lytic activity of IL-12/IL-18-derived NK cells, lend support to the possibility that induction of the perforin lytic pathway accounts for their preferential cytotoxicity.

Taken together, our results indicate 1) that very potent cytotoxic NK cells can be derived in response to IL-12 and IL-18, compared with IL-2 or IL-15, and 2) that type 2 cytokines are produced by IL-2-derived T-NK cells, which thereby might contribute to modulate humoral immune responses.

Acknowledgments

We thank A. Tonon for running the cell sorting experiments.

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