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IL-18 Up-Regulates Perforin-Mediated NK Activity Without Increasing Perforin Messenger RNA Expression by Binding to Constitutively Expressed IL-18 Receptor[1]

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IL-18 is a powerful inducer of IFN-γ production, particularly in collaboration with IL-12. IL-18, like IL-12, also augments NK activity. Here we investigated the molecular mechanism underlying the up-regulation of killing activity of NK cells by IL-18. IL-18, like IL-12, dose dependently enhanced NK activity of splenocytes. This action was further enhanced by costimulation with IL-12. Treatment with anti-IL-2R Ab did not affect IL-18- and/or IL-12-augmented NK activity, and splenocytes from IFN-γ-deficient mice showed enhanced NK activity following stimulation with IL-12 and/or IL-18. Splenocytes from the mice deficient in both IL-12 and IL-18 normally responded to IL-18 and/or IL-12 with facilitated NK activity, suggesting that functional NK cells develop in the absence of IL-12 and IL-18. IL-18R, as well as IL-12R mRNA, was constitutively expressed in splenocytes from SCID mice, which lack T cells and B cells but have intact NK cells, and in those from IL-12 and IL-18 double knockout mice. NK cells isolated from SCID splenocytes expressed IL-18R on their surface. IL-18, in contrast to IL-12, did not enhance mRNA expression of perforin, a key molecule for exocytosis-mediated cytotoxicity. However, pretreatment with concanamycin A completely inhibited this IL-18- and/or IL-12-augmented NK activity. Furthermore, IL-18, like IL-12, failed to enhance NK activity of splenocytes from perforin-deficient mice. These data suggested that NK cells develop and express IL-12R and IL-18R in the absence of IL-12 or IL-18, and that both IL-18 and IL-12 directly and independently augment perforin-mediated cytotoxic activity of NK cells. ***The Journal of Immunology,*** **1999, 162: 1662–1668.**

IL-18 is a multifunctional cytokine produced by activated macrophages (1–3). IL-18 induces IFN-γ production by lymphocytes including T cells, B cells, and NK cells (1–5). IL-18 up-regulates Fas ligand (FasL)[3] expression on cloned NK cells, resulting in their facilitated killing activity against Fas-expressing cells in a DNA-fragmenting manner (6). IL-18 also up-regulates NK activity in a Fas/FasL-independent manner against FasL-resistant target cells (1, 2). In vivo administration of IL-18 augments NK activity in mice (7), and in vitro treatment with IL-18 elevates NK activity of human PBMC and murine splenocytes (2, 7). Although IL-18 is a potent factor to induce IFN-γ production by T cells and B cells, IL-12 is prerequisite to exert the action of IL-18 on these cells (1–3, 8, 9). This is because IL-18R, which has recently been proved to be identical to IL-1R-related protein (IL-1RrP) (10), is not constitutively expressed but rather induced by the stimulation with IL-12 on T and B cells (3, 9, 11). Thus, the function of IL-18 on T cells and B cells completely depends on the coexistence of IL-12. However, NK cells have the capacity to respond to IL-18 in a distinct fashion. IL-18 induces IFN-γ production by cloned NK cells without any help by IL-12 (5). Recently, we have demonstrated that up-regulating action of IL-18 on NK activity, in contrast to its capacity to induce IFN-γ production by T cells, does not require endogenous IL-12, using IL-12 null mutant mice (7, 12, 13).

In this study, we investigated the precise mechanism of how IL-18 augments NK activity, particularly comparing with IL-12, a second potent up-regulating factor for NK activity (14), because both cytokines are occasionally secreted by activated macrophages at the same time (1, 5). We confirmed that YAC-1 cells, murine target cells of NK activity, were not susceptible to the FasL-mediated killing. Thus, we addressed here the effect of IL-18 and/or IL-12 on Fas/FasL-independent killing activity of NK cells. We found that IL-18 as well as IL-12 up-regulated NK activity in an IL-2- or IFN-γ-independent manner and that NK cells constitutively expressed IL-12R and IL-18R, even in the absence of endogenous IL-12 or IL-18. Furthermore, we investigated the molecular mechanism underlying the up-regulation of NK activity by IL-18, and found that IL-18 did not increase the expression of

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[3] Abbreviations used in this paper: FasL, Fas ligand; IL-1RrP, IL-1R-related protein; SCID, Fox Chase SCID C.B-17/ICR-*scid* Jcl; IL-2Rα, IL-2R α-chain; IL-2Rβ, IL-2R β-chain; IL-12Rβ1, IL-12Rβ1 chain; IL-12Rβ2, IL-12Rβ2 chain; PE, phycoerythrin.

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perforin, an essential molecule for exocytosis-mediated killing action by NK cells and CTL, whereas IL-12 enhanced it. Surprisingly, up-regulating the effect of IL-18 on NK cells nevertheless depended totally on perforin, because splenocytes from perforin-deficient mice did not show NK activity even after stimulation with IL-18.

Materials and Methods

Mice

Female C57BL/6 mice (5–8 wk old) were purchased from Japan SLC (Sizuoka, Japan), and female Fox Chase SCID C.B-17/ICR-*scid* Jcl (SCID) mice 5–8 wk old (15, 16) were from Clea Japan (Tokyo, Japan). IFN-γ-deficient mice (17) were backcrossed to C57BL/6 mice for F8 generations, and females 5–8 wk old were used in this study. Mice deficient in both IL-12 and IL-18 and their littermates with wild genotype were also used (7, 12, 13). Perforin-deficient mice with C57BL/6 backgrounds were purchased from Taconic (Germantown, PA). Sprague Dawley rats were from Charles River Japan (Yokohama, Japan). The animals were kept under specific pathogen-free conditions.

Abs and reagents

Murine rIL-18 and rIL-12 were produced and purified as described previously (1). Murine rIL-2 was purchased from Genzyme (Boston, MA). mAbs against murine IL-2R α-chain (IL-2Rα) (7D4, rat IgG2b) and mAb against IL-2R β-chain (IL-2Rβ) (TM-β1, rat IgG2b) were purchased from PharMingen (San Diego, CA). PE-conjugated anti-IL-2Rβ mAb and FITC-conjugated anti-rat IgG1 mAb were also from PharMingen. Purified rat IgG was from Cappel (West Chester, PA). Concanamycin A, a potent inhibitor for perforin-mediated cytotoxicity (18), was purchased from Wako Pure Chemical Industries (Osaka, Japan). YAC-1, a Moloney virus-induced lymphoma of A/Sn origin (NK-sensitive tumor cell line), was obtained from American Type Culture Collection (ATCC, Manassas, VA). The murine T cell lymphoma cell line L5178Y, which does not express Fas, and its subline transfected with a full-length cDNA encoding murine Fas (A-1) (6) were kind gifts from Dr. S. Yonehara of Kyoto University (Kyoto, Japan). mFasL/L5178Y was a subline of L5178Y transfected with a full-length cDNA encoding FasL from BALB/c mice (19). The culture medium generally used in this study was RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM of 2-ME, and 2 mM L-glutamine.

mAb against murine IL-18R

Soluble murine IL-18R was purified from the supernatant of COS-1 cells transfected with cDNA encoding murine IL-1RrP (11). We confirmed that soluble murine IL-18R used in this study could inhibit binding of [^{125}I]IL-18 to murine IL-18R-transfected COS-1 cells. Rats were i.p. immunized with 50 μg of soluble murine IL-18R, followed by booster injections three times. Spleen cells were prepared from the rats i.v. injected with soluble IL-18R 4 days before and fused with Y3Ag1.2.3 cells (Lou rat myeloma cell line) (10). Hybridoma supernatant reactivities were assayed by the system of inhibition of binding of [^{125}I]-labeled murine IL-18 to the COS-1 cells transfected with murine IL-18R. After a limiting dilution method, we obtained cloned hybridoma-producing mAb against murine IL-18R, mAb-MuIL-18R-Y38 (IgG1κ). mAb-MuIL-18R-Y38 inhibited IFN-γ production by IL-18-responsive cloned NK cells (5) in response to IL-18 but not IL-12 and had no action like IL-18. mAb was purified from hamster ascites fluid by protein G column (20).

Assay for killing activity

Spleen cells were prepared from C57BL/6, both IL-12- and IL-18-disrupted mice, their wild-type littermates, IFN-γ-deficient mice, or perforin-deficient mice. Spleen cells were incubated with or without various doses of IL-12 and/or IL-18 for 24 h. For some experiments, spleen cells from C57BL/6 mice were incubated with various combinations of IL-12 and IL-18, or 100 U/ml of IL-2 in the presence or absence of 10 μg/ml of 7D4 plus 10 μg/ml of TMβ-1 or 20 μg/ml of rat IgG for 24 h. Cytotoxic activity of variously treated spleen cells against YAC-1 cells was determined by 4-h ^{51}Cr-release assay, and the percentage of cytotoxicity was calculated (6). Briefly, YAC-1 cells were labeled with [^{51}Cr]sodium chromate and incubated with the variously treated spleen cells at various E:T ratios for 4 h. The radioactivity in the supernatant was measured by a gamma counter. The killing activity of FasL transfectant against YAC-1 and A-1 was also determined by the same method described above, using ^{51}Cr-labeled YAC-1 cells and [^{51}Cr]A-1 cells, respectively (6).

Cell preparation from SCID mice

Spleen cells (5×10^6/ml) from 20 SCID mice were pooled and incubated in plastic dishes for 1 h at 37°C. Plastic nonadherent cells (5×10^6/ml) were additionally incubated in fresh plastic dishes for another 1 h, and nonadherent cells were collected and used as splenic lymphocytes. The cells obtained had almost no contamination with macrophages (data not shown) and contained no surface IgM$^+$ cells and CD3$^+$ cells. The splenic lymphocytes (2×10^6/ml) from SCID mice were incubated with various combinations of IL-12 and IL-18 for 24 h, and their total RNA was extracted. IL-2Rβ$^+$ cells were isolated by cell sorting and used as NK cells for the detection of their surface expression of IL-18R by immunohistochemistry.

FACS analysis and sorting

For determination of IL-18R expression on NK cells, after FcR blocking using anti-FcγR Ab (6), splenic lymphocytes from SCID mice were incubated with anti-murine IL-18R mAb followed by FITC-conjugated anti-rat IgG1 mAb and PE-conjugated IL-2Rβ Ab. Stained cells were analyzed using a dual laser FACScalibur (Becton Dickinson, Mountain View, CA). Ten thousand cells were analyzed and data were processed with CellQuest (Becton Dickinson). For sorting experiments, an Epics XL Flow Cytometer (Coulter, Hialeah, FL) was used.

Immunohistochemistry

IL-2Rβ-positive cells were isolated from splenic lymphocytes from SCID mice by FACS sorting. Surface expression of IL-18R on the isolated NK cells were determined histochemically, according to the method described before except using 3,3′-diaminobenzidine (Sigma, St. Louis, MO) for coloring substrate (6). Isotype-matched control Ab instead of anti-IL-18R mAb was used for the control study.

RT-PCR

Splenocytes from SCID mice (2×10^6/ml) or IL-12 and IL-18 double-knockout mice were prepared. For some experiments, they were incubated with 2 ng/ml IL-12 and/or 20 ng/ml IL-18 for 24 h. Total RNA was isolated by the method described previously (6), reverse transcribed, and amplified. The sense primer for murine IL-18R was CGT GAC AAG CAG AGA TGT TG and the anti-sense primer was ATG TTG TCG TCT CCT TCC TG (11). Thermocycle conditions for IL-18R were 95°C for 30 s for denaturing, 58°C for 30 s for annealing, and 72°C for 30 s for extension. The sense primer for murine β1 chain of IL-12R (IL-12Rβ1) was GCA AAC ACA TCA CCT TCC TCC TGC, and the antisense primer was GTG TGT CAC CAT CTT GGC AGG ATC (21). The sense primer for murine β2 chain of IL-12R (IL-12Rβ2) was GGC ACA GAC TGT AGA ATG CTC, and the antisense primer was TGC AGA AGC GCC TTT TGA GTT GGT (22). The sense primer for perforin was TGC TAC ACT GCC ACT CGG TCA, and the antisense primer was TTG GCT ACC TTG GAG TGG GAG (23). The sense primer for murine granzyme B was CTC CAC GTG CTT TCA CCA AA, and the antisense primer was GGA AAA TAG TAC AGA GAG GCA (24). Thermocycle conditions were 95°C for 30 s for denaturing, 64°C for 30 s for annealing, and 72°C for 1 min for extension. Thirty-five cycles were performed for IL-18R, IL-12Rβ1, IL-12Rβ2, and perforin. The primers and amplifying conditions for β-actin were described previously (4). The PCR products were electrophoresed in 1.7% agarose gel and visualized by ethidium bromide fluorescence. For some experiments, densitometric analysis was performed with Densitograph (Atto, Tokyo, Japan). Relative expression of perforin mRNA or granzyme B mRNA was calculated as follows: Relative expression of molecule mRNA examined = ((densitometric units of IL-12- and/or IL-18-treated samples)/(densitometric units of their β actin mRNA))/((densitometric units of untreated samples)/(densitometric units of β-actin mRNA of untreated samples)).

Results

IL-18 up-regulates NK activity in collaboration with IL-12

IL-18 augmented NK activity of splenocytes. As shown in Fig. 1*A*, IL-18 elevated killing activity of spleen cells against YAC-1 cells in a dose-dependent manner, reaching a plateau at 20 ng/ml of IL-18. The killing activity of splenocytes was also enhanced when they were incubated with IL-12 (Fig. 1*B*). The maximal cytotoxicity of splenocytes after incubation with IL-12 was almost similar to that after incubation with IL-18. The minimum dose of IL-12 that was required to give maximal cytotoxic activity was one-tenth of that of IL-18. Next, we examined whether IL-18 collaborates

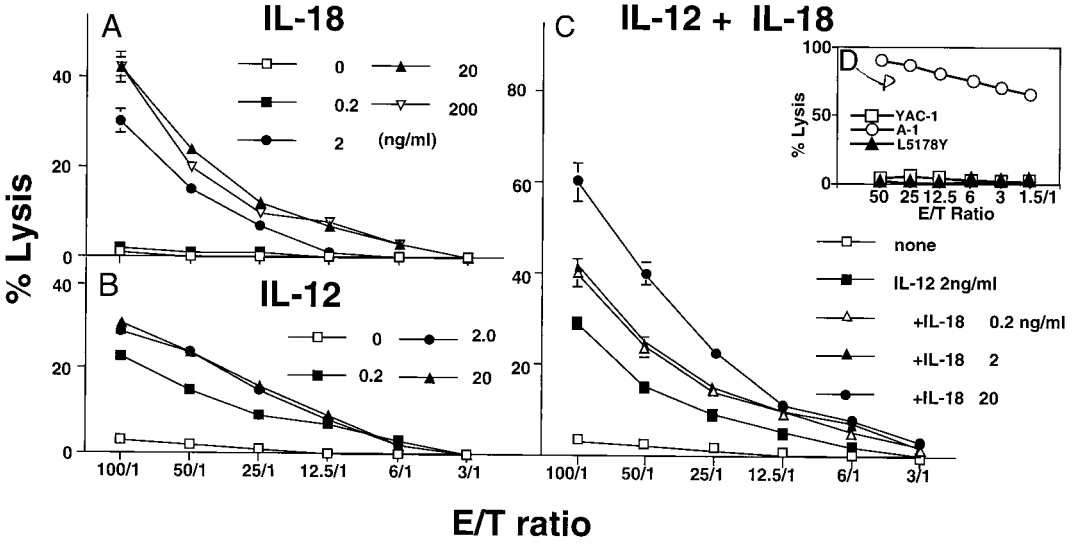

FIGURE 1. IL-18 and IL-12 independently upregulate NK activity. Spleen cells from C57BL/6 mice were incubated with various doses of IL-18 (*A*) and/or IL-12 (*B* and *C*) for 24 h, and their cytotoxic activity against YAC-1 was determined. The variously treated spleen cells were incubated with ^{51}Cr-labeled YAC-1 cells (1×10^5/ml) at the indicated E:T ratios for 4 h. mFasL/L5178Y cells were incubated with ^{51}Cr-labeled YAC-1 cells, ^{51}Cr-labeled L5178Y cells, or ^{51}Cr-labeled A-1 cells at the indicated E:T ratios for 4 h (*D*). The radioactivity in each supernatant was determined by a gamma counter, and the percentage of lysis was calculated. The data indicate the mean ± SD of triplicate samples of one experiment. The results shown are representative of three independent experiments.

with IL-12 to enhance NK activity, because both IL-12 and IL-18 are produced by activated macrophages sometimes simultaneously (1, 5). As shown in Fig. 1*C*, IL-18 dose dependently increased NK activity of splenocytes in the presence of optimal dose of IL-12 (2 ng/ml). Thus, IL-12 and IL-18 collaboratively augment NK activity of splenocytes. To investigate whether YAC-1 cells were killed by the Fas/FasL-mediated killing pathway, we incubated YAC-1 cells with FasL-transfected cells (19). As shown in Fig. 1*D*, YAC-1 cells were not killed by FasL-expressing effector cells that were able to kill Fas-expressing A-1 target cells, indicating that up-regulating action of IL-12, as well as IL-18, on YAC-1 killing by splenocytes was independent of the Fas/FasL-mediated killing pathway.

IFN-γ- or IL-2-independent augmentation of NK activity by IL-12 and/or IL-18

We examined whether IL-12 (2 ng/ml) and/or IL-18 (20 ng/ml), at their optimal concentration, augmented NK activity through the induction of IL-2, another potent NK cell activator (25). Splenocytes were cultured with IL-12 and/or IL-18 in the presence of both anti-IL-2Rα Ab plus anti-IL-2Rβ Ab. The combination of these Abs completely canceled the cytolytic activity augmented by 100 U/ml of IL-2, but not that by IL-12 and/or IL-18 (Fig. 2*A*). The amount of mAbs used completely inhibited the proliferative response of splenocytes to Con A (data not shown). Furthermore, IL-2 was not detected in the supernatant of the splenocytes incubated with IL-12 and/or IL-18, determined by CTLL-2 proliferation assay (data not shown). Control Ab did not affect the augmenting effect of IL-12 and/or IL-18 on NK activity (Fig. 2*A*). Next, we investigated whether IL-18 and/or IL-12 enhanced NK activity via the induction of IFN-γ, because both IL-18 and IL-12 are upstream cytokines of IFN-γ. We analyzed the effect of IL-12 and/or IL-18 on spleen cells from IFN-γ-deficient mice instead of using anti-IFN-γ Ab, because production of a tremendous amount of IFN-γ is induced by the combinational stimulation with IL-12 and IL-18 (1–5) and may be much higher than the maximal IFN-γ dose that can be neutralized by anti-IFN-γ Abs. As shown in Fig. 2*B*, IL-18, as well as IL-12, enhanced NK activity of spleen cells from IFN-γ-deficient mice, excluding the contribution of endogenous IFN-γ to the enhanced NK activity.

IL-12 and/or IL-18 enhanced NK activity of spleen cells from both IL-12- and IL-18-deficient mice

Next, we measured the net responsiveness of spleen cells to exogenous IL-18 and/or IL-12 in NK activity. Splenocytes from wild-type mice used so far might have been contaminated with the cells that had been previously stimulated with endogenous IL-12 and/or IL-18 in vivo. Furthermore, spleen cells used in these experiments contained macrophages that are able to produce IL-12 and IL-18 in vivo or in vitro upon stimulation. Thus, we used spleen cells from IL-12 and IL-18 null mice. The mice deficient in both IL-12 and IL-18 showed very weak NK activity (7, 12), suggesting that there are few in vivo activated NK cells in these mice. Using these unstimulated NK cells, we examined their capacity to gain NK activity following stimulation with IL-12 and/or IL-18. As shown in Fig. 3, compared with splenocytes from wild-type mice, those from IL-12$^{-/-}$IL-18$^{-/-}$ mice showed rather higher NK activity in response to stimulation with IL-12 and/or IL-18, suggesting that functional NK cells develop even in the absence of IL-12, IL-18, or IFN-γ (Figs 2*B* and 3).

Constitutive expression of IL-12R and IL-18R on NK cells

The responsiveness of NK cells to IL-12 and IL-18 observed above suggested that NK cells should express IL-18R and IL-12R constitutively. We then investigated the expression of IL-18R and IL-12R mRNA in NK cells by RT-PCR. To demonstrate this, we freshly isolated nonadherent spleen cells from SCID mice as NK cells, because SCID mice lack both T cells and B cells but have mature NK cells (26–28). IL-18R mRNA was constitutively expressed in splenic nonadherent cells from SCID mice (Fig. 4*A*). Surface IL-18R was also constitutively expressed on NK cells from SCID mice. As shown in Fig. 4*B*, highly enriched NK cell fraction contained the cells whose surface was stained by anti-IL-18R mAb. The cells were not stained with isotype-matched control

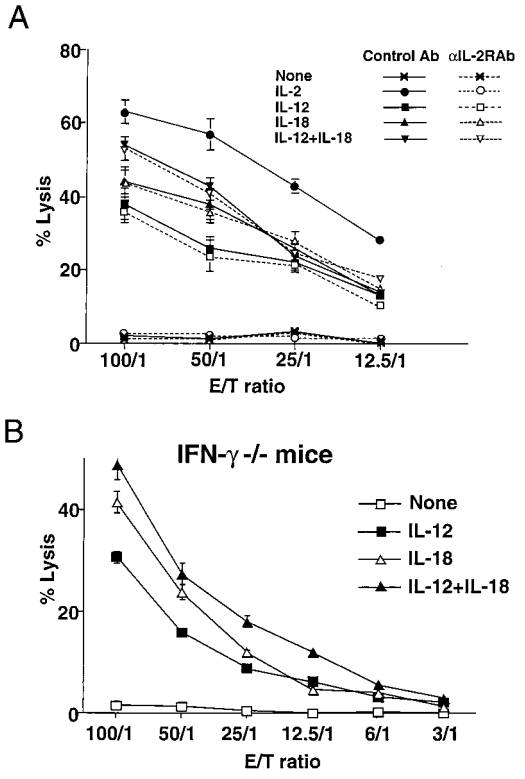

FIGURE 2. IL-2- or IFN-γ-independent action of IL-12 and/or IL-18 on NK activity. Spleen cells from C57BL/6 mice were cultured alone or incubated with IL-2 (100 U/ml), IL-12 (2 ng/ml), and/or IL-18 (20 ng/ml) in the presence or absence of 10 μg/ml of anti-IL-2Rα plus 10 μg/ml of anti-IL-2Rβ (αIL-2R Ab), or 20 μg/ml of rat IgG (control Ab) for 24 h (*A*). Spleen cells from IFN-γ-deficient mice were incubated with IL-12 (2 ng/ml) and/or IL-18 (20 ng/ml) for 24 h (*B*). Their cytotoxic activity was measured as described in the legend to Fig. 1. The data indicate the mean ± SD of triplicate samples of one experiment. The results shown are representative of three independent experiments.

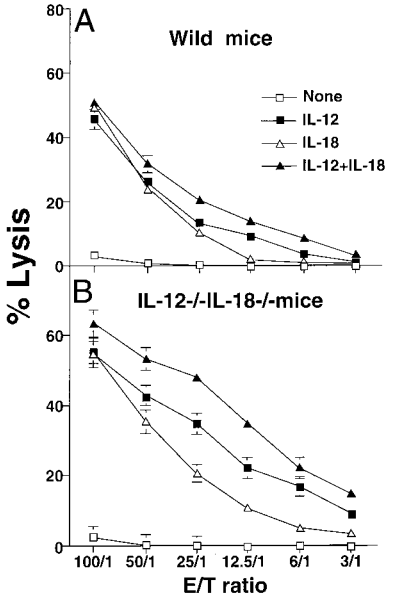

FIGURE 3. IL-18 and/or IL-12 augments NK activity of splenocytes from both IL-12- and IL-18-deficient mice. Spleen cells from wild-type (*A*) or from mutant littermates deficient in both IL-12 and IL-18 (*B*) were incubated with IL-12 (2 ng/ml) and/or IL-18 (20 ng/ml) for 24 h. Their cytotoxicity against YAC-1 was determined as described in the legend to Fig. 1. The data represent the mean ± SD of triplicate samples of one experiment. The results shown are representative of three independent experiments.

Ab (data not shown). To measure the proportions of IL-18R-expressing NK cells, we stained splenic nonadherent cells from SCID mice with both anti-IL-18R mAb and anti-IL-2Rβ mAb. As shown in Fig. 4*C*, calculated proportion of IL-18R-expressing IL-2Rβ$^+$ cells is about 35%. This was also the case for spleen cells from euthymic wild-type mice (data not shown). These cells also constitutively expressed mRNA for IL-12Rβ1 and IL-12Rβ2, which constitute functional IL-12R (Fig. 4).

Next, we investigated whether IL-18R or IL-12R expression required intrinsic IL-12 and/or IL-18. To examine this possibility, splenocytes from IL-18 and IL-12 double-knockout mice, which were able to respond to the stimulation with IL-18 as well as IL-12 (Fig. 3), were used. As shown in Fig. 4*A*, both splenocytes from the double-mutant mice and from wild-type mice constitutively expressed IL-18R as well as IL-12R. Since naive T cells and B cells have no responsiveness to IL-18 unless they have been stimulated (3, 4, 11), IL-18R expression in splenocytes from the double-knockout mice might be due to that in NK cell fraction. In a separate experiment, we examined the expression of IL-18R mRNA in spleen cells from IFN-γ-deficient mice. We found that they also constitutively expressed IL-18R mRNA (data not shown).

IL-18 enhances perforin-dependent cytotoxicity of splenocytes

Next, we examined the molecular mechanism by which IL-18 and/or IL-12 enhanced NK activity. To investigate whether IL-18 and/or IL-12 augment perforin-mediated killing activity, we incubated the IL-18- and/or IL-12-treated spleen cells with concanamycin A, a potent inhibitor for perforin-mediated cytotoxicity (18). As shown in Fig. 5*A*, pretreatment with concanamycin A completely abrogated the enhancing effects of IL-12 and/or IL-18 on NK activity.

We also examined whether IL-12 and/or IL-18 affect the NK activity of spleen cells from perforin-deficient mice. As shown in Fig. 5*B*, stimulation with either IL-12 and/or IL-18 did not induce NK activity in splenocytes from perforin-knockout mice.

Since it has been shown that IL-12 as well as IL-2 augments NK activity by up-regulating the expression of perforin (23, 29), we examined whether IL-18 also augments perforin mRNA expression in NK cells. To exclude the possible contamination of T cells, we used splenic nonadherent cells from SCID mice. IL-18 did not increase the expression of perforin mRNA, while IL-12 did increase it (Fig. 5*C*). This was also the case for the mRNA expression of granzyme B, another cytotoxic molecule responsible for exocytosis-dependent cytotoxicity of NK cells and CTL (29).

Discussion

Innate immunity is an essential system to eliminate microbes, particularly in the early infectious phase before the elicitation of specific immunity. NK cells, an important constituent of this system, are localized not only in the immune tissues but in any organs and in the circulation (30–34) to make a first line of host defense in cooperation with macrophages. NK cells play a critical role in activation or modulation of innate and acquired immunity through their production of proinflammatory cytokines such as TNF-α (30), a powerful effector cytokine, as well as adhesion molecule-inducing factor and IFN-γ (30), a potent macrophage activating factor and a modulating factor for acquired immunity. NK cells also participate in innate immunity as effector cells, which eliminate virus-infected cells in an MHC-unrestricted manner and also

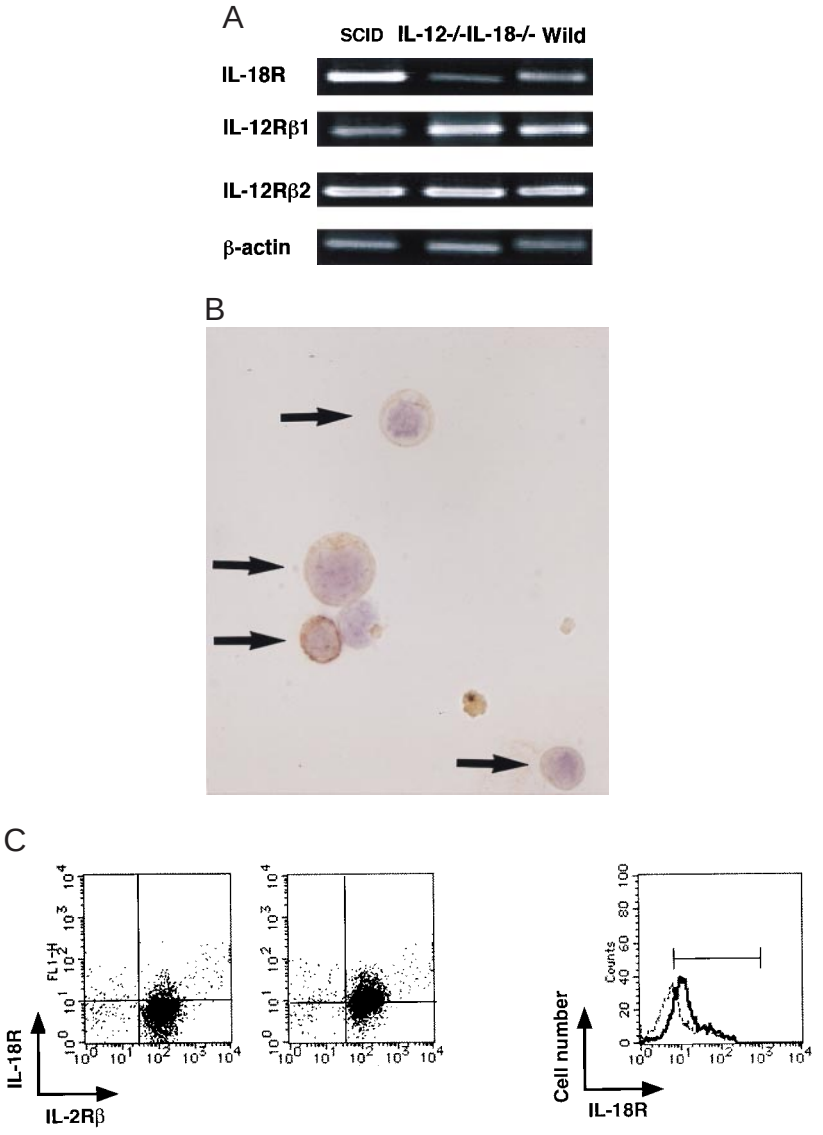

FIGURE 4. NK cells constitutively express both IL-18R and IL-12R. *A*, Splenic nonadherent lymphocytes from 20 SCID mice were pooled (*left lane*). Splenocytes were also prepared from IL-12, IL-18 double-knockout mice (*middle lane*) and wild-type mice (*right lane*). Total RNA was extracted from freshly isolated splenic lymphocytes. RT-PCR was performed using each specific primer. The results shown are representative of three independent experiments. The RT-PCR was done in triplicate for each experiment. *B*, FACS-sorted NK cells from splenic nonadherent cells of SCID mice were histochemically stained with anti-IL-18R mAb (×400). Arrows indicate positively stained cells with anti-IL-18R mAb. The result represents typical results of three independent experiments. *C*, Splenic nonadherent cells were stained with anti-IL-18R mAb followed by FITC-conjugated anti-rat IgG1 and PE-conjugated anti-IL-2Rβ mAb (*middle panel*). The *left panel* indicates background staining with PE-conjugated anti-IL-2Rβ mAb and FITC-conjugated anti-rat IgG1 alone. Histogram (*right panel*) shows proportion of IL-18R$^+$ cells gated in the IL-2Rβ^+ cell population. Dotted line indicates background staining from *left panel* and solid line from *middle panel*. The results shown are representative of three independent experiments.

play a role in tumor surveillance (35, 36). Indeed, severity of some bacterial and viral infections have been reported to be reversely related to NK cell functions (28, 30, 37–39). Since NK cells are an essential component of the innate immunity, regulation of NK cell activity via NK receptors is now under extensive studies (40, 41). Cytokine-induced activation of NK cells, particularly by IL-18 and IL-12, is also of great interest.

We here demonstrated that NK cells constitutively express functional IL-18R as well as IL-12R. Recently, Torigoe et al. reported that N-terminal and internal amino acid sequences of affinity-purified IL-18R were identical to those predicted from cDNA encoding the IL-1RrP (10). They also confirmed that COS-1 cells transfected with the cDNA were able to respond to human IL-18 with nuclear translocation of NF-κB (10), indicating that the cDNA they cloned encodes functional IL-18R. COS-1 cells transfected with cDNA encoding murine IL-1RrP (11) bind murine rIL-18, indicating that IL-1RrP is also the receptor for IL-18 in murine system (M. Kurimoto, unpublished observations). Recently, we have shown that splenic T cells isolated from NK cell-depleted mice did not respond to the stimulation with IL-18, whereas those cells exhibited proliferation and IFN-γ production in response to IL-18 once they were preincubated with IL-12, suggesting that T cells require the costimulation with IL-12 for their acquisition of the responsiveness to IL-18 (3, 4, 9, 11). Indeed, T cells incubated with IL-12 express IL-18R, but naive T cells do not (11). In contrast, splenic nonadherent cells freshly isolated from SCID mice express IL-18R without any stimulation (Fig. 4). The splenic nonadherent cells from SCID mice are predominantly constituted by NK cells (15, 42). Furthermore, NK cells isolated from splenocytes of SCID mice by FACS sorting were able to be stained with anti-murine IL-18R mAb (Fig. 4, *B* and *C*). Therefore, NK cells appear to constitutively express IL-18R, at least in SCID mice. Functional IL-12R is composed of two subunits, IL-12Rβ1 and IL-12Rβ2 (21, 22). IL-12Rβ1 is expressed in NK cells and T cells and is equally expressed in Th1 and Th2 subpopulations (43, 44). The former is defined by particularly skewed cytokine production profile of IL-2 and IFN-γ, and the latter of IL-4, IL-5, IL-10, and IL-13 (45). Recently, it has been reported that IL-12Rβ2 is expressed only in Th1 cells but not in Th2 cells (43, 44). In the case of splenic nonadherent cells freshly isolated from SCID mice, IL-12Rβ2 as well as IL-12Rβ1 was expressed under normal condition (Fig. 4*A*). In fact, splenic nonadherent cells from SCID mice showed enhanced NK activity by the stimulation with IL-18 and/or IL-12, but with a little weaker activity than did those from C57BL/6 (data not shown). To exclude the possibility that endogenous IFN-γ contributed to the constitutive expression of IL-12R

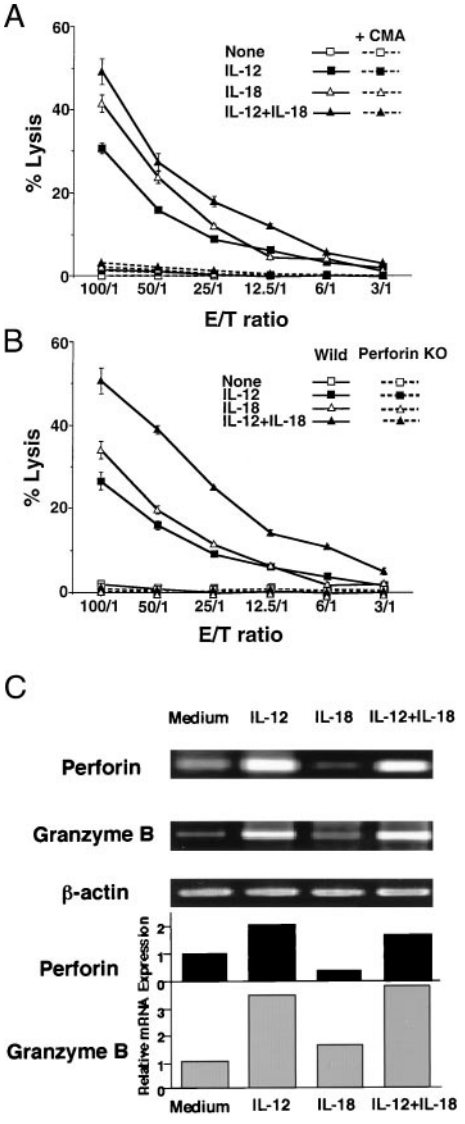

FIGURE 5. IL-18 enhances perforin-dependent NK activity without up-regulating their perforin expression. *A*, The spleen cells from C57BL/6 mice were incubated with IL-12 (2 ng/ml) and/or IL-18 (20 ng/ml) for 24 h, additionally incubated with or without 100 nM concanamycin A (CMA) for 2 h at 37°C, and their killing activity was determined. The data indicate the mean ± SD of triplicate samples of one experiment. The results shown are representative of three independent experiments. *B*, Splenocytes from perforin-deficient mice or from wild-type mice were incubated with IL-18 (20 ng/ml) and/or IL-12 (2 ng/ml) for 24 h, and their NK activity was determined. The data represent the mean ± SD of triplicate samples of one experiment. The results shown are representative of three independent experiments. *C*, Pooled splenic nonadherent cells from SCID mice were incubated with IL-18 and/or IL-12 for 24 h. Total RNA was extracted, and their perforin and granzyme B mRNA expressions were determined by RT-PCR (*upper*). The densitometric analysis was performed, and relative expression of perforin and granzyme B was calculated (*lower*). The results shown are representative of three independent experiments. The RT-PCR was done in triplicate for each experiment.

or IL-18R in NK cells, we examined the responsiveness of splenocytes from IFN-γ-deficient mice to the stimulation with IL-12 and/or IL-18, and we observed that IFN-γ null splenocytes also similarly respond to IL-12 and/or IL-18 (Fig. 2*B*), indicating that both IL-12R and IL-18R expressions in NK cells do not require the intrinsic IFN-γ. Constitutive expression of IL-18R also does not require endogenous IL-12 or IL-18, because lymphocytes from IL-18, IL-12 double-deficient mice expressed IL-18R under normal condition (Fig. 4*A*) and their NK activity was equally or rather more strongly augmented by IL-12 and/or IL-18 as compared with the lymphocytes from wild-type mice (Fig. 3). IL-18 and IL-12 do not appear to cooperatively activate killing of NK cells from wild-type mice with a background of both C57BL/6 and 129/SvJ (Fig. 3), whereas these stimuli cooperatively activated that of those from C57BL/6 mice (Figs. 1 and 5*B*). This may imply that sensitivity of NK cells to IL-12 and/or IL-18 differ between C57BL/6 mice and 129/SvJ mice. Constitutive expression of IL-12R on NK cells is also independent of endogenous IL-12 or IL-18 because of the same reasons (Figs. 3 and 4). These data strongly suggested that immune competent NK cells can develop without IL-12 or IL-18 and that peripheral NK cells are ready to respond immediately and independently to both IL-18 and IL-12. This may account for the fact that NK cells have the capacity to make a first line of host defenses (37, 38) together with macrophages that produce IL-12 and IL-18 in response to microbes and their products (1, 5).

Perforin and granzyme B have been shown to be key molecules for cytolytic action of NK cells (29). Perforin is localized in the cytoplasmic granules of NK cells and CTL and is accumulated at the site where target cells interact, followed by exocytosis of these molecules onto target cells leading to cell lysis (29). IL-12, like IL-2, has been reported to up-regulate production of perforin by cytotoxic lymphocytes (25). This was also the case for nonadherent splenocytes from SCID mice (Fig. 5*C*) or splenocytes from C57BL/6 mice (data not shown). In contrast, IL-18 did not up-regulate the perforin mRNA expression (Fig. 5*B*). We also measured mRNA expression of granzyme B, which facilitates target cell lysis in concert with perforin (25), in similarly treated nonadherent splenocytes from SCID mice and found that IL-18, unlike IL-12, did not enhance its expression (Fig. 5*B*). In separate experiments, we did time course study from 3 h to 48 h and found no obvious elevation of either perforin or granzyme B mRNA in IL-18-stimulated, nonadherent SCID splenocytes at any time point (data not shown). However, IL-18, like IL-12, did not enhance cytotoxic activity of splenocytes from perforin-deficient mice (Fig. 5*B*). Taken together, these data indicate that IL-18 enhances perforin-dependent cytotoxic activity of NK cells without up-regulating perforin or granzyme B expression. This is in clear contrast with IL-12 that enhances NK activity by up-regulating these molecules and may be responsible for the cooperative effect of IL-12 and IL-18 (Fig. 1). The unique effect of IL-18 on NK activity may be explained by several possibilities. First, IL-18 may up-regulate an undefined effector molecule other than granzyme B, which functions in perforin-dependent cytotoxicity. Second, IL-18 may activate some intracellular mechanism leading to exocytosis. Third, IL-18 may facilitate the processing of perforin from an inactive precursor to an active form (18) and the intracellular transport. Further studies are needed to address these possibilities.

In summary, we here demonstrated that NK cells constitutively express functional IL-18R as well as IL-12R and up-regulate perforin-dependent cytotoxic activity in response to IL-18 and IL-12 by apparently distinct mechanisms. We are now investigating the physiological roles of IL-18 in NK cell-dependent host defenses.

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