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Defective NK Cell Activity and Th1 Response in IL-18–Deficient Mice

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Summary

IL-18 is a cytokine that is secreted from activated macrophages and induces IFNγ production. To investigate the in vivo role of IL-18, we generated IL-18–deficient mice. In *Propionibacterium acnes (P. acnes)*–primed IL-18–deficient mice, LPS-induced IFNγ production was markedly reduced, despite normal IL-12 induction. Natural killer cell activity was significantly impaired. Th1 cell response after injection of *P. acnes* or *Mycobacterium bovis* (bacillus Calmette-Guérin [BCG]) was significantly reduced. Similar results were observed in IL-12–deficient mice. Interestingly, Th1 response was induced after BCG infection in IL-12–deficient mice. We therefore generated mice lacking both IL-18 and IL-12. In these mice, NK activity and Th1 response were further impaired. This demonstrates the important role of both IL-18 and IL-12 in NK activity, as well as in in vivo Th1 response.

Introduction

Interleukin-18 (IL-18) is a cytokine that was identified in the liver of mice sequentially treated with *Propionibacterium acnes (P. acnes)* and lipopolysaccharide (LPS) (Okamura et al., 1995a). This molecule was originally identified as interferonγ inducing factor (IGIF), a factor that induces interferonγ (IFNγ) production from splenocytes, liver lymphocytes, and type 1 T helper (Th1) cell clones (Okamura et al., 1995a, 1995b; Kohno et al., 1997; Matsui et al., 1997). In addition, IL-18 enhances natural killer (NK) cell activity and proliferation of activated T cells (Okamura et al., 1995a, 1995b; Ushio et al., 1996). Thus, IL-18 apparently shares its biological functions with IL-12, which is known to have immunoregulatory activities, including the induction of IFNγ production, the enhancement of NK cell cytotoxicity, and the enhancement of activated T cell proliferation (Trinchieri, 1994, 1995). IL-18 was purified from liver extracts and molecularly cloned, and this molecule, like IL-12, is shown to be secreted from activated macrophages (Okamura et al., 1995b; Ushio et al., 1996). In spite of the biological functions it shares with IL-12, the amino acid sequence of IL-18 has no similarity with that of IL-12. Interestingly, a three-dimensional homology search revealed that IL-18 has a striking similarity to the IL-1 family of cytokines (Bazan et al., 1996). IL-18 is synthesized as a precursor and becomes an active form by cleavage at the site of the aspartate residue (Okamura et al., 1995b; Ushio et al., 1996). Interleukin-1β converting enzyme (ICE) is a protease that has proteolytic activity for converting the precursor of IL-1β into the mature IL-1β (Cerretti et al., 1992; Thornberry et al., 1992). Recently, it has been demonstrated that ICE also cleaves the precursor of IL-18 into the mature form (Ghayur et al., 1997; Gu et al., 1997). Furthermore, the receptor for IL-18 was recently identified (Torigoe et al., 1997). This molecule is identical to the molecule designated as the IL-1 receptor–related protein (IL-1Rrp), which has a resemblance to the type 1 IL-1 receptor and can transduce IL-1 signaling (Parnet et al., 1996). In fact, IL-18 induces activation of IL-1 receptor–associated kinase and nuclear translocation of nuclear factor κB (Matsumoto et al., 1997; Robinson et al., 1997). Thus, IL-18 is similar to the IL-1 family of cytokines in the molecular mechanisms of its signaling.

IL-18 has other functions in addition to those it shares with IL-12. Treatment of *P. acnes*–sensitized athymic nude mice with anti–IL-18 antibody can prevent LPS-induced liver injury, indicating that IL-18 is involved in the pathogenesis of endotoxin-induced liver injury (Okamura et al., 1995b; Tsutsui et al., 1997). IL-18 up-regulates Fas ligand expression on NK cells (Tsutsui et al., 1996). Similarly, IL-18 enhances Fas ligand–mediated cytotoxicity of Th1 cells (Dao et al., 1996). IL-18 induces IFNγ production from anti-CD40 plus IL-4–stimulated B cells and inhibits Immunoglobulin E production in an autocrine manner (Yoshimoto et al., 1997). Additionally, IL-18 is produced from osteoblasts and inhibits osteoclast formation and is induced in the adrenal cortex under stressful conditions (Conti et al., 1997; Udagawa et al., 1997).

To determine the in vivo functions of IL-18, we generated IL-18–deficient mice by gene targeting. IL-18–deficient mice displayed reduced production of IFNγ, impaired NK cell activity, and defective Th1 cell response. Since these defects were not complete, we generated mice lacking both IL-18 and IL-12. In mice lacking both cyokines, NK cell activity and Th1 cell differentiation were further disturbed. These results clearly demonstrate the importance of in vivo cooperative action of IL-18 and IL-12 in NK lytic activity and the Th1 response.

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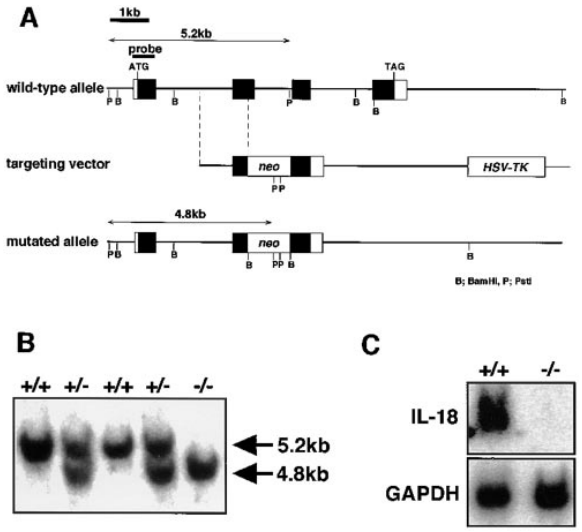

Figure 1. Targeted Disruption of the IL-18 Gene

(A) Maps of the IL-18 wild-type genome, the targeting construct, and the predicted disrupted gene. Open and closed boxes denote the non-coding and coding exons, respectively. The *neo* box represents the MC-1-*neo poly(A)*$^+$ gene. The HSV-tk box represents the herpes simplex virus–thymidine kinase gene. The 0.5 kb fragment corresponding to exon 2 was used as a probe in Southern blot hybridization. Restriction enzymes: B, BamHI; P, PstI.

(B) Southern blot analysis of tail DNA of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with PstI, electrophoresed, and hybridized with a probe indicated in (A). This hybridization resulted in the detection of a 5.2 kb band in the wild-type alleles, and a 4.8 kb band in the correctly targeted alleles.

(C) Northern blot analysis of macrophages activated by *P. acnes*. Mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes*. Four days after injection, peritoneal exudate cells were elicited, and cultured for 2 hr. Total RNA was extracted from the adherent macrophage monolayers, electrophoresed, transferred to nylon membrane, and hybridized with the mouse IL-18 cDNA probe. The same membrane was rehybridized with a GAPDH probe.

Results

Generation of IL-18–Deficient Mice

We disrupted the IL-18 gene by homologous recombination in embryonic day 14.1 embryonic stem cells. The mouse IL-18 genomic DNA consists of five exons and four introns and is about 20 kb in length. Exon 1 is >10 kb away from exon 2. A targeting vector was constructed to replace a 3.0 kb fragment of genomic DNA containing exons 3, 4, and 5 with the neomycin-resistance *(neo)* gene (Figure 1A). Embryonic stem cells were transfected with the linearized targeting vector and selected in the presence of G418 and gancyclovir. Homologous recombinant clones were screened by polymerase chain reaction. Thirteen correctly targeted clones out of 138 doubly resistant clones were verified by Southern blot analysis. Three of these targeted clones were microinjected into C57BL/6 blastocysts to generate chimeric mice. One line of these chimeric mice successfully contributed to the germline. Heterozygous mice were intercrossed to obtain mice homozygous for the IL-18 gene mutation. IL-18–deficient (IL-18$^{-/-}$) mice were born at the expected Mendelian ratios and were phenotypically normal and fertile (Figure 1B). To confirm that the IL-18 mutation inactivates the IL-18 gene, IL-18$^{-/-}$ and wild-type mice were intraperitoneally injected with 500 μg of *P. acnes*. At 4 days after injection, peritoneal macrophages were analyzed for IL-18 expression by Northern blot analysis. IL-18 mRNA could not be detected in macrophages of IL-18$^{-/-}$ mice (Figure 1C). Serum IL-18 concentration 1.5 hr after intravenous injection with LPS was analyzed by enzyme-linked immunosorbent assay (ELISA). In wild-type mice, increased production of IL-18 was observed. However, in IL-18$^{-/-}$ mice, the serum concentration of IL-18 was below the detectable level, indicating that the IL-18 gene mutation leads to no production of IL-18 (Figure 2A).

Flow cytometric analysis of lymphoid organs showed that lymphocyte composition was not altered in IL-18$^{-/-}$ mice. However, the intensity of CD3 expression in T cells from IL-18$^{-/-}$ mice was slightly lower than that in wild-type mice (data not shown). The dull CD3 expression was not enhanced by in vitro culture with IL-18, indicating that CD3 expression was affected during differentiation and maturation of IL-18$^{-/-}$ T cells (data not shown). Although CD3 expression was slightly dull in IL-18$^{-/-}$ T cells, these cells showed almost the same level of proliferative response to anti-CD3 antibody as did wild-type T cells. Moreover, IL-18$^{-/-}$ T cells proliferated to an almost equal degree in response to IL-2 plus anti-CD3 antibody as did wild-type T cells (data not shown). These results indicate that the activation of T cells through CD3 stimulation was not affected in IL-18$^{-/-}$ mice, in spite of the slight decrease in expression of CD3.

Reduced Production of IFNγ in IL-18$^{-/-}$ Mice

We analyzed IFNγ production after treatment with LPS in *P. acnes*–sensitized mice. Heat-killed *P. acnes* was injected intraperitoneally, and 7 days later LPS was injected intravenously. Serum IFNγ level was measured 6 hr after LPS challenge. The serum IFNγ level in IL-18$^{-/-}$ mice was about one fifth of that in wild-type mice, demonstrating the important role of IL-18 in in vivo IFNγ production (Figure 2B). Although IL-12 is known to induce IFNγ, the serum IL-12 level after LPS challenge in IL-18$^{-/-}$ mice was almost the same as that in wild-type mice (Figure 2C). This result suggests that the reduced IFNγ production was not secondary to low induction of IL-12.

Impaired NK Cell Activity in IL-18$^{-/-}$ Mice

IL-18 has been shown to augment NK cell activity (Okamura et al., 1995a, 1995b; Ushio et al., 1996). We analyzed the function of NK cells by YAC-1 cell killing activity. Spleen cells were incubated with ^{51}Cr-labeled YAC-1 NK target cells at the indicated effector/target ratios, and ^{51}Cr release was measured. The killing activity of NK cells from IL-18$^{-/-}$ mice was about one third of that from wild-type mice (Figure 3A). Intraperitoneal administration of exogenous IL-18 daily for 2 days dramatically restored the killing activity of IL-18$^{-/-}$ NK cells. (Figure 3B). More enhanced NK lytic activity was observed when treated with both IL-18 and IL-12.

Since NK activity in IL-18$^{-/-}$ mice after IL-18 treatment

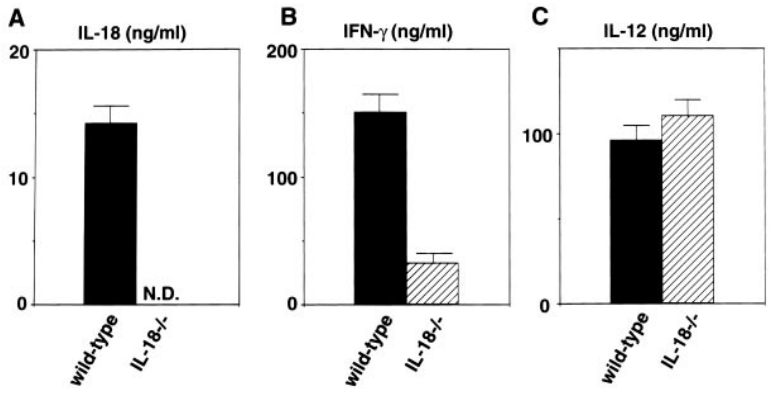

Figure 2. Serum Concentration of IL-18, IFNγ, and IL-12 after LPS Challenge in *P. acnes*–Sensitized Mice

Mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes*. LPS challenge was 7 days after injection.

(A) Sera were taken 1.5 hr after LPS challenge and analyzed for the production of IL-18 by ELISA.

(B and C) Sera were taken 6 hr after LPS challenge and analyzed for the production of IFNγ (B) or IL-12 (C) by ELISA. These data are the average of three or four mice analyzed.

was higher than that seen in wild-type mice, we examined whether expression of the IL-18 receptor IL-1Rrp was increased in IL-18$^{-/-}$ mice. We performed Northern blot analysis of spleen cells, using cDNA of the IL-18 receptor as a probe (Figure 3D). Expression of IL-1Rrp was below the detectable level and slightly induced after *P. acnes* injection in the splenocytes of wild-type mice. In contrast, abundant expression of IL-1Rrp was observed, and its expression was dramatically enhanced after *P. acnes* injection in splenocytes from IL-18$^{-/-}$ mice. Thus, it is possible that NK cells of IL-18$^{-/-}$ mice are highly responsive to IL-18 because of the increased expression of the IL-18 receptor.

IL-12 has been shown to enhance NK cell activity (Gately et al., 1994; Trinchieri, 1994, 1995). Therefore, we next analyzed the effect of IL-12 on NK activity of IL-18$^{-/-}$ mice. Intraperitoneal administration of 5 ng of IL-12 enhanced NK activity in wild-type mice (Figure 3C). Almost no enhancement of NK lytic activity was observed in IL-18$^{-/-}$ mice after administration of 5 ng of IL-12. When the dose of IL-12 was increased to 10 ng, the increase in NK activity was still severely reduced in IL-18$^{-/-}$ mice as compared with wild-type mice (Figure 3C). However, when the dose of IL-12 was increased to 100 ng, NK lytic activity in the IL-18$^{-/-}$ mice was enhanced to an almost equal degree with that in the wild-type. Taken together, these results demonstrate that the combined action of IL-18 and IL-12 plays an important role in the activation of NK cells, although administration of a high amount of IL-12 could enhance NK activity to the normal level in the absence of IL-18.

Defective Th1 Response in IL-18$^{-/-}$ Mice

Like IL-12, IL-18 is shown to activate Th1 cells and induce IFNγ production from these cells (Okamura et al., 1995a, 1995b; Kohno et al., 1997; Matsui et al., 1997). Recent studies demonstrate that IL-18 does not in itself drive Th1 development but synergizes with IL-12 for production of IFNγ from developing Th1 cells, arguing for the potential role of IL-18 in IL-12–primed Th1 cell development (Matsui et al., 1997; Robinson et al., 1997). Therefore, we examined Th1 cell development in vivo using IL-18$^{-/-}$ mice. Mice were intraperitoneally injected with heat-killed *P. acnes*, which was previously shown

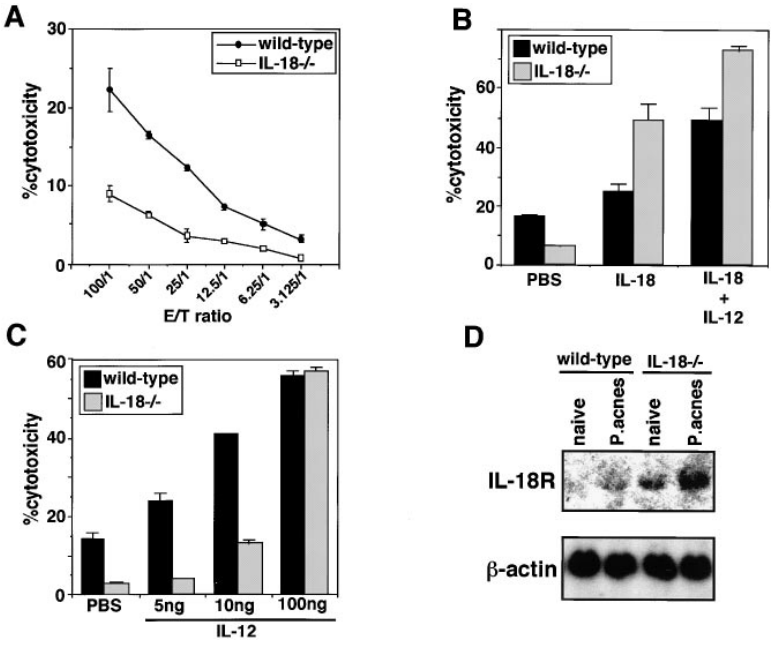

Figure 3. NK Cell Activity in IL-18$^{-/-}$ Mice

(A) Naive spleen cells were analyzed for NK lytic activity against ^{51}Cr–labeled YAC-1 target cells. ^{51}Cr–releases in supernatants of cultures at the indicated effector/target (E/T) ratios were measured.

(B) Mice were intraperitoneally injected with PBS, 1000 ng of IL-18, or 100 ng of IL-12 and IL-18 daily for 2 days. On the next to last day of injection, spleen cells were analyzed for NK lytic activity. The data are the activity at a 50/1 E/T ratio.

(C) Mice were intraperitoneally injected with PBS or 5, 10, or 100 ng of IL-12 daily for 2 days. Spleen cells were analyzed for NK activity. The data are the activity at a 50/1 E/T ratio.

(D) Expression of the IL-18 receptor in spleen. Total RNA was extracted from spleens of noninfected mice (naive) or heat-killed *P. acnes* injected mice (*P. acnes*), electrophoresed, transferred to nylon membrane, and hybridized with the mouse IL-18 receptor cDNA probe.

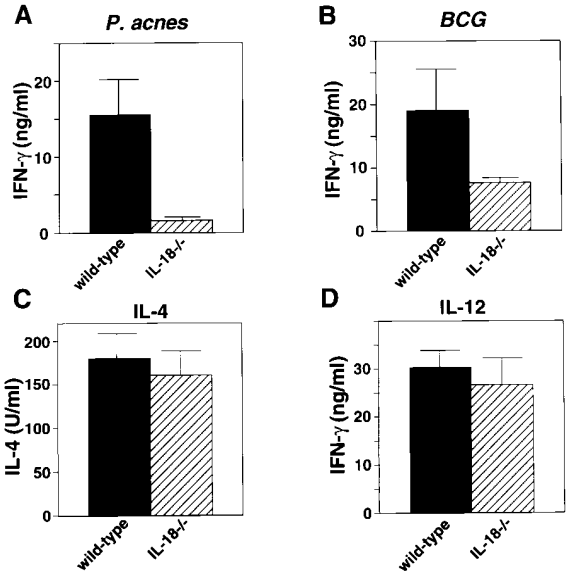

Figure 4. Th1 Cell Development In Vivo and In Vitro in IL-18$^{-/-}$ Mice

(A) Mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes*. Seven days after injection, splenic T cells were purified and stimulated by immobilized anti-CD3 antibody for 24 hr. The concentrations of IFNγ in culture supernatants were measured using ELISA.

(B) Mice were intravenously injected with 2 mg of BCG. Fourteen days after injection, production of IFNγ from splenic T cells was analyzed using ELISA.

(C) Naive splenic T cells were cultured for 4 days in the presence of 1000 U/ml of IL-4 in anti-CD3 antibody-coated plates. After 4 days of culture, T cells were washed and stimulated by immobilized anti-CD3 antibody for 24 hr. The concentrations of IL-4 in culture supernatants were measured using ELISA.

(D) Naive splenic T cells were cultured for 4 days in the presence of 2 ng/ml IL-12 in anti-CD3 antibody-coated plates. The production of IFNγ from the stimulated T cells was analyzed using ELISA.

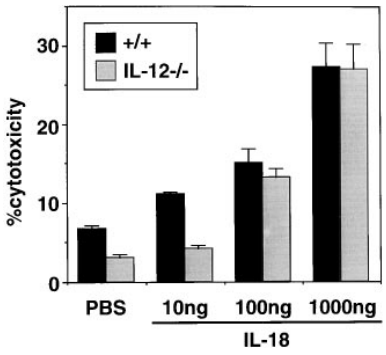

Figure 5. NK Cell Activity in IL-12$^{-/-}$ Mice

Wild-type or IL-12$^{-/-}$ mice were intraperitoneally injected with PBS or 10, 100, or 1000 ng of IL-18 daily for 2 days. On the next to last day of injection, spleen cells were analyzed for NK lytic activity against YAC-1 target cells. The data are the activity at 50/1 effector/target ratio.

to induce Th1 response in vivo (Okamura et al., 1982; Matsui et al., 1997). Seven days after injection, splenic T cells were purified and stimulated with anti-CD3 antibody. At the 24 hr culture period, culture supernatants were analyzed for IFNγ production by ELISA. Elevated levels of IFNγ production were observed in wild-type mice, while IFNγ production from IL-18$^{-/-}$ T cells was markedly reduced (Figure 4A). We next infected the mice with *Mycobacterium bovis* (bacillus Calmette-Guérin [BCG]), which is also known to induce IFNγ production in vivo (Kawamura et al., 1992), and assayed for IFNγ production. The IFNγ production from IL-18$^{-/-}$ T cells was about one third of that from wild-type T cells (Figure 4B). These results demonstrate that the in vivo Th1 cell response was impaired in IL-18$^{-/-}$ mice.

We studied in vitro differentiation of naive Th cells into Th1 or Th2 cells. Naive splenic T cells were cultured for 4 days in the presence of IL-12, which induces Th1 cell differentiation, or in the presence of IL-4, which induces Th2 cell differentiation. They were then restimulated with anti-CD3 antibody for 24 hr (Paul, 1991; Trinchieri, 1994, 1995). When T cells were cultured with IL-4, production of IL-4 from both wild-type and IL-18$^{-/-}$ T cells was at almost the same level, indicating that Th2 cell differentiation was not impaired in IL-18$^{-/-}$ mice (Figure 4C). Similarly, T cells from *Nippostrongylus brasiliensis*–infected IL-18$^{-/-}$ mice produced almost the same level of IL-4 as did wild-type T cells, further supporting the normal Th2 response in IL-18$^{-/-}$ mice (data not shown). When stimulated with IL-12 in vitro, IL-18$^{-/-}$ T cells produced almost the same level of IFNγ as compared with wild-type T cells (Figure 4D). These results indicate that high amounts of IL-12 can induce Th1 cell development in vitro in the absence of IL-18, although *P. acnes*– or BCG-infected IL-18$^{-/-}$ mice displayed impaired in vivo Th1 response.

IL-18 Enhanced NK Cell Activity in IL-12$^{-/-}$ Mice

Although IL-18$^{-/-}$ mice displayed decreased IFNγ production, impaired NK activity, and defective Th1 cell response, these functions were not completely disturbed. In addition, administration of high doses of IL-12 restored the impaired NK cell activity, and in vitro stimulation with IL-12 induced almost normal Th1 cell development in IL-18$^{-/-}$ mice. Recently, IL-12 p40-deficient (IL-12$^{-/-}$) mice also showed partial impairment in these functions (Magram et al., 1996a). To investigate the role of IL-18 more precisely, we analyzed the effect of exogenous IL-18 on NK cell activity seen in IL-12$^{-/-}$ mice. As previously reported, NK cell activity in IL-12$^{-/-}$ mice was about two-thirds of that in wild-type mice (Magram et al, 1996a). We intraperitoneally injected 10, 100, or 1000 ng of IL-18 daily for 2 days, and measured NK lytic activity against YAC-1 target cells. In wild-type mice, administration of IL-18 enhanced NK activity in a dose-dependent manner (Figure 5). In contrast, 10 ng of IL-18 did not activate NK cells in IL-12$^{-/-}$ mice. However, administration of 100 ng or 1000 ng of IL-18 to IL-12$^{-/-}$ mice resulted in almost the same enhancement of NK activity as in the wild-type mice (Figure 5). These results indicate that IL-18 acts on NK cells in the absence of IL-12.

We next examined in vitro activation of NK cells in response to IL-12 or IL-18, using IL-12$^{-/-}$ or IL-18$^{-/-}$ mice. Spleen cells isolated from wild-type, IL-12$^{-/-}$, or IL-18$^{-/-}$ mice were cultured with IL-12 or IL-18 for 24 hr, and measured for cytolytic activity against YAC-1

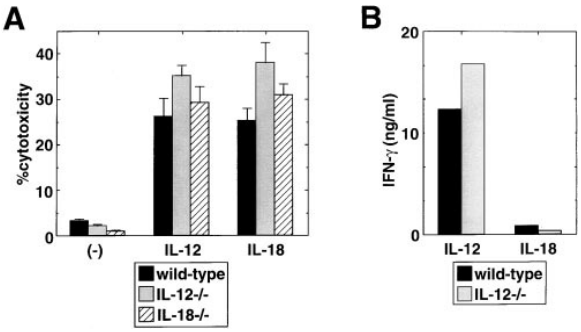

Figure 6. In Vitro Stimulation of NK Cells or Induction of Th1 Cell Development by IL-18 in IL-12$^{-/-}$ Mice

(A) Splenocytes from wild-type, IL-12$^{-/-}$, or IL-18$^{-/-}$ mice were incubated with IL-18 or IL-12 for 24 hr. Their NK activities against YAC-1 target cells were measured. The data are the activity at a 50/1 E/T ratio.

(B) Splenic T cells from wild-type or IL-12$^{-/-}$ mice were cultured for 4 days in the presence of 2 ng/ml of IL-12 or 20 ng/ml of IL-18 in anti-CD3 antibody-coated plates. After 4 days of culture, T cells were washed and stimulated by immobilized anti-CD3 antibody for 24 hr. The concentrations of IFNγ in culture supernatants were measured by ELISA.

cells (Figure 6A). NK activity of IL-18$^{-/-}$ cells, when incubated with IL-12 in vitro, was enhanced almost equally compared with that of wild-type mice. Reciprocally, spleen cells from IL-12$^{-/-}$ mice showed almost the same enhancement of NK lytic activity as those from wild-type mice did after in vitro stimulation with IL-18. Thus, in vitro study also demonstrates that IL-12 activates NK cells in the absence of IL-18, and vice versa.

In contrast, splenic T cells from wild-type or IL-12$^{-/-}$ mice did not differentiate into IFNγ-producing Th1 cells by in vitro stimulation with IL-18, although in vitro stimulation with IL-12 induced almost normal Th1 cell development in IL-18$^{-/-}$ mice (Figures 4D and 6B). This is consistent with the previous report stating that IL-18 in itself does not induce Th1 cell development (Matsui et al., 1997; Robinson et al., 1997).

NK Cell Activity and Th1 Response in Mice Deficient in Both IL-12 and IL-18

Analysis of NK cell activity in IL-18$^{-/-}$ or IL-12$^{-/-}$ mice revealed that high amounts of IL-12 or IL-18 compensates for the deficiency of each cytokine. In addition, the impairments observed in IL-18$^{-/-}$ or IL-12$^{-/-}$ mice were not complete. These findings prompted us to propose that the synergistic action of IL-12 and IL-18 leads to activation of NK cells and Th1 cell development in vivo. Therefore, we next generated and characterized mice deficient in both the IL-12 and IL-18 genes (IL-12$^{-/-}$IL-18$^{-/-}$ mice) to examine these functions more precisely.

IL-12$^{-/-}$IL-18$^{-/-}$ mice were viable and showed no overt developmental abnormality. We first measured NK lytic activity of spleen cells from IL-12$^{-/-}$ and/or IL-18$^{-/-}$ mice. Although about one third and two thirds levels of NK activities was observed in IL-18$^{-/-}$ and IL-12$^{-/-}$ mice, respectively, further decreased lytic activity was observed in IL-12$^{-/-}$IL-18$^{-/-}$ mice, indicating that the cooperative action of IL-12 and IL-18 is indispensable for the in vivo activation of NK cells (Figure 7A).

We finally analyzed Th1 cell development in BCG-infected mice. As mentioned above, IL-18$^{-/-}$ showed decreased Th1 response, the level of which was about 3-fold lower than that of wild-type mice. Th1 response in BCG-infected IL-12$^{-/-}$ mice was reduced to about one fourth of that in wild-type mice (Figure 7B). In the case of IL-12$^{-/-}$IL-18$^{-/-}$ mice, IFNγ production from BCG-infected T cells was severely diminished, to a level about 12-fold lower than that of wild-type mice (Figure 7B).

Thus, IL-12$^{-/-}$IL-18$^{-/-}$ mice displayed marked reduction in NK activity and severely impaired Th1 cell development. These results clearly demonstrate that the cooperative action of both IL-12 and IL-18 is essential for activation of NK cells and induction of Th1 cell response in vivo.

Discussion

In this study, we describe the generation and characterization of IL-18$^{-/-}$ mice. These mice developed normally, were fertile, and showed no obvious signs of disease until 28 weeks of age, indicating that IL-18 is not involved in normal mouse development. However, several immunological defects were identified in IL-18$^{-/-}$ mice. Furthermore, generation of double-knockout mice lacking IL-18 and IL-12 have revealed that the combined action of these cytokines plays a key role in NK activity as well as in in vivo Th1 development.

Defective IFNγ Production in IL-18$^{-/-}$ Mice

Several in vitro studies have shown that IL-18 shares biological properties with IL-12. These properties include the induction of IFNγ production and the enhancement of NK cell activity. However, the contribution of IL-18 to these functions in vivo has not been studied. IL-12 has been described as the factor most responsible for IFNγ production and Th1 cell development. IL-12 exerts these functions together with several costimulants (Trinchieri, 1994, 1995). IFNγ has been shown to be one of the potent costimulants for IL-12 in the induction of the Th1 response (Seder et al., 1993; Schmitt et al., 1994). Recently, IL-12–deficient mice have been generated in order to examine the in vivo role of IL-12. In IL-12–deficient mice, defects in IFNγ production and the Th1 response were observed, but these defects were not complete (Magram et al., 1996a, 1996b). It was therefore expected that several other cytokines, including IL-18, could contribute to the low level of IFNγ production and the remaining Th1 response observed in the absence of IL-12. Like IL-12$^{-/-}$ mice, IL-18$^{-/-}$ mice displayed reduced production of IFNγ in vivo, although IL-12 was secreted almost normally after LPS challenge. This finding demonstrates that IL-18 is also an important factor for IFNγ production in vivo, and that IL-18 deficiency cannot be compensated for by IL-12 and other cytokines.

Role of IL-18 in Th1 Cell Development

Although a severely impaired Th1 response was shown in *P. acnes*–injected IL-18$^{-/-}$ mice, a weak Th1 response was still observed in BCG-infected IL-18$^{-/-}$ mice. In

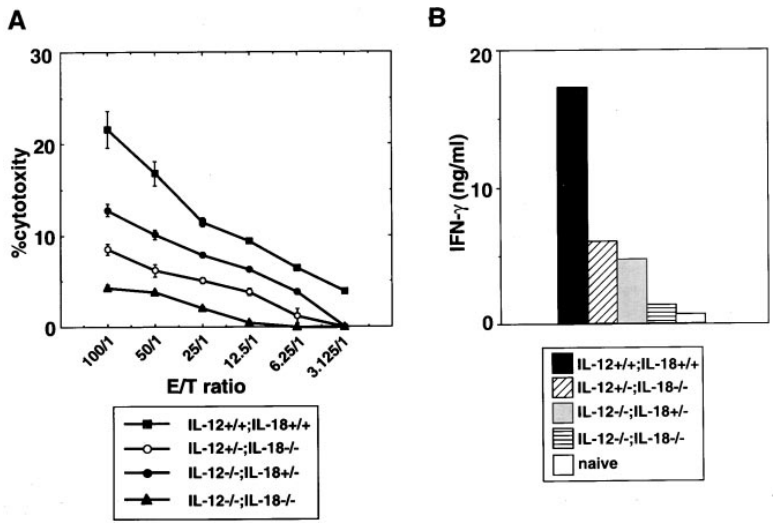

Figure 7. NK Activity and Th1 Response in IL-12$^{-/-}$IL-18$^{-/-}$ Mice

(A) Naive spleen cells from wild-type, IL-18$^{-/-}$, IL-12$^{-/-}$, or IL-12$^{-/-}$IL-18$^{-/-}$ mice were analyzed for NK lytic activity against ^{51}Cr-labeled YAC-1 cells. ^{51}Cr-releases in supernatants of cultures at the indicated effector/target (E/T) ratios were measured.

(B) Mice were intravenously injected with 2 mg of BCG. Fourteen days after injection, splenic T cells were purified and stimulated by immobilized anti-CD3 antibody for 24 hr. The concentrations of IFNγ in culture supernatants were measured using ELISA.

addition, in vitro stimulation with IL-12 induced almost normal Th1 cell development in IL-18$^{-/-}$ mice, indicating that the presence of high concentrations of IL-12 is sufficient for induction of the Th1 response in the absence of IL-18. These findings appear to be consistent with previous reports showing that IL-12 is a key factor in Th1 cell development and that IL-18 in itself does not induce the differentiation of naive Th0 cells into Th1 cells and acts only on IL-12–primed Th1 developing cells for IFNγ production (Matsui et al., 1997; Robinson et al., 1997). In addition, it is shown that IL-12–stimulated Th1 cell lines exhibit enhanced binding capacity to IL-18 (Ahn et al, 1997). Our data demonstrate that naive Th cells display increased mRNA expression of the IL-18 receptor when stimulated with IL-12 (T. Y., unpublished data).

From these findings, the following scenario may be established: naive Th cells, when primed with IL-12, express the IL-18 receptor and then develop into fully differentiated Th1 cells in response to both IL-12 and IL-18. However, BCG-infected IL-12$^{-/-}$ mice displayed an impaired but significant level of Th1 response. This demonstrates that naive Th cells actually develop into Th1 cells in the absence of IL-12. Furthermore, IL-12$^{-/-}$ IL-18$^{-/-}$ mice displayed more profound impairment in the Th1 response after BCG infection as compared with IL-12$^{-/-}$ or IL-18$^{-/-}$ mice. Thus, IL-18 deficiency affects Th1 cell development in IL-12$^{-/-}$ mice, indicating that IL-18 accounts for Th1 cell development in vivo even in the absence of IL-12. This result led us to the speculation that IL-12$^{-/-}$ naive Th cells might acquire responsiveness to IL-18 by expressing the IL-18 receptor independently of IL-12 after BCG infection, and differentiate into Th1 cells in response to IL-18. Nevertheless, fully differentiated wild-type Th1 cells after BCG infection did not produce augmented levels of IFNγ by in vitro stimulation with IL-18 alone. On the contrary, these T cells produced a significant level of IFNγ in response to IL-12 alone as well as a high level of IFNγ by stimulation with the combination of IL-12 and IL-18 (data not shown). These results indicate that IL-12–independent Th1 cell development could be induced by the cooperative action of IL-18 and other factors. These factors for Th1 response may include IFNγ itself (Szabo et al., 1997), IFNα (Manetti et al., 1995; Rogge et al., 1997), IL-1α/β (Robinson et al., 1997), or an as yet unidentified factor. Indeed, IL-18 acted on NK cells to produce IFNγ that may induce Th1 cell development in the absence of IL-12 (H. T., unpublished data). These factors might also contribute to the remaining Th1 response observed in BCG-infected IL-12$^{-/-}$IL-18$^{-/-}$ mice. The present study clearly demonstrates that the Th1 cell response can be induced by IL-18 independently of IL-12, although IL-12 is a central factor in Th1 cell development.

Both IL-12 and IL-18 Contribute to Functional Development of NK Cells as well as to Their Activation

The total cell number of splenic NK cells from IL-18$^{-/-}$, IL-12$^{-/-}$, or IL-12$^{-/-}$IL-18$^{-/-}$ mice, as determined by the CD3^{-}, IL-2Rβ^{+} population, was almost the same as that from wild-type mice, indicating that either IL-12 or IL-18 is not involved in the early development of NK cells (data not shown). However, NK activity of naive spleen cells from either IL-12$^{-/-}$, IL-18$^{-/-}$, or IL-12$^{-/-}$IL-18$^{-/-}$ mice was attenuated in this order, strongly suggesting that both IL-12 and IL-18 are involved in the functional maturation of NK cells.

Naive splenic T cells have no mRNA expression of the IL-18 receptor, whereas they come to express the IL-18 receptor during Th1 cell development. In contrast, splenic NK cells from wild-type mice are able to respond to IL-18 and exert their facilitated killing activity against YAC-1 cells. Additionally, splenic NK cells freshly isolated from wild-type mice showed enhanced killing activity in response to in vitro stimulation with IL-18 without any help from IL-12. These findings prompted us to investigate whether endogenous IL-12 is mandatory for the up-regulating action of IL-18 on NK activity. As expected, IL-18 augmented the NK activity of spleen cells from IL-12$^{-/-}$ mice, indicating that NK cells do not require any stimulation with IL-12 for the response to IL-18. Recently, we observed that splenic nonadherent cells from SCID mice, lacking T cells or B cells but having intact NK cells, constitutively express the IL-18 receptor

mRNA and produce IFNγ in response to stimulation with IL-18 (H. T., unpublished data). IL-12 also activates cytolytic activity of spleen cells against YAC-1 cells independently of IL-18, because spleen cells from IL-18$^{-/-}$ mice, when stimulated in vitro with IL-12, had almost the same cytotoxic activity against YAC-1 cells as those from wild-type mice. Thus, the action of IL-18 on NK cells does not need intrinsic IL-12, and vice versa.

Experimental Procedures

Generation of IL-18–Deficient Mice

IL-18 genomic DNA was screened from an 129/SvJ mouse genomic library (Stratagene), subcloned into the pBluescript vector, and characterized by restriction enzyme mapping and DNA sequencing. A targeting vector was designed to replace a 3.0 kb genomic fragment containing exons 3, 4, and 5 with pMC1-*neo* (Stratagene). The targeting vector was flanked by the 5.2 kb fragment at the 3′ end and the 1.0 kb fragment at the 5′ end and contains a HSV-tk cassette at the 3′ end of the vector. The targeting vector was linearized with SalI and electroporated into embryonic day 14.1 embryonic stem cells. The resistant clones to G418 and gancyclovir were screened for homologous recombination by polymerase chain reaction and confirmed by Southern blot analysis with the probe shown in Figure 1A. Generation of chimeric mice and mutant mice was essentially as described previously (Takeda et al., 1997).

Mice and Reagents

IL-12 p40-deficient mice were generated and maintained as described previously (Magram et al., 1996a). IL-12$^{-/-}$ mice were crossed with IL-18$^{-/-}$ mice to generate mice heterozygous for both the IL-12 and IL-18 genes. These mice were intercrossed to create IL-12$^{-/-}$IL-18$^{-/-}$ mice. Offspring from these intercrosses were used for the experiments.

Recombinant mouse IL-12 and IL-18 were supplied by Hayashibara Biochemical Laboratories, Inc. LPS (*Escherichia coli* strain 055:B5) was purchased from DIFCO.

Northern Blot Analysis

Mice were injected intraperitoneally with 500 μg of heat-killed *P. acnes.* Four days after injection, peritoneal exudate cells were harvested by washing the peritoneal cavity with 5 ml of PBS. The cells were cultured for 2 hr. Nonadherent cells were removed. Total RNA was extracted from the remaining macrophage monolayers with TRIzol reagent (GIBCO), electrophoresed, transferred to nylon membrane, and hybridized with the ^{32}P-labeled mouse IL-18 cDNA.

Serum Concentration of IL-18, IFNγ, and IL-12

Mice were injected intraperitoneally with 500 μg of heat-killed *P. acnes*. Seven days after injection, mice were injected intravenously with 1 μg of LPS. Mice were bled 1.5 or 6 hr after LPS injection. Serum concentration of IL-18 after 1.5 hr of LPS challenge was determined by ELISA as described previously (Gu et al., 1997). Levels of IFNγ and IL-12 after 6 hr of LPS challenge were determined using ELISA kits (Genzyme).

In Vitro Induction of Th1 or Th2 Cell Differentiation

Splenic T cells were purified essentially as described previously (Takeda et al., 1996). Splenic T cells were enriched by two rounds of nylon wool column passage. Enriched T cells were incubated with anti-I-A^b antibody and anti-IgM antibody for 30 min on ice, then washed, and incubated with rabbit complement (Low-Tox-M, Cedar Lane) for 30 min at 37°C. Cell purity was analyzed by flow cytometry using fluorescein isothiocyanate–conjugated anti-CD3 antibody (PharMingen).

T cells purified to more than 95% (2×10^6 cells) were incubated on anti-CD3 antibody (20 mg/ml)–coated plates in the presence of 2 ng/ml IL-12 to induce a Th1 response or 1000 U/ml IL-4 to induce a Th2 response. After 4 days of culture, cells were harvested and washed with Hanks' buffered salt solution. 2×10^5 cells were stimulated on anti-CD3 antibody (20 μg/ml)–coated 96-well plates for 24 hr. Concentration of IFNγ or IL-4 in culture supernatants was determined by ELISA.

In Vivo Induction of Th1 or Th2 Cell Differentiation

Mice were injected intraperitoneally with 500 μg of heat-killed *P. acnes* to induce a Th1 response in vivo. Seven days after injection, splenic T cells were purified and stimulated on anti-CD3 antibody-coated 96-well plates for 24 hr as described above. Level of IFNγ in culture supernatants was determined by ELISA. In the case of BCG infection, mice were injected intravenously with 2 mg of BCG (Kyowa, Japan). Fourteen days after injection, splenic T cells were purified and assayed for IFNγ production, as described above.

Analysis of NK Cell Activity

Naive spleen cells were incubated with ^{51}Cr-labeled YAC-1 target cells at the indicated effector/target ratios. After 4 hr of culture, supernatants were counted for ^{51}Cr release by γ counter. For administration of IL-18 or IL-12, mice were intraperitoneally injected with PBS or the indicated doses of IL-18 or IL-12 daily for 2 days. Spleen cells of these mice at day 3 were assayed for NK lytic activity against ^{51}Cr-labeled YAC-1 target cells.

In the case of in vitro activation of NK cells, spleen cells were incubated with or without 2 ng/ml IL-12 or 20 ng/ml IL-18 for 24 hr. Their cytolytic activity against YAC-1 cells was measured.

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