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IL-1 Receptor-Associated Kinase 4 Is Essential for IL-18-Mediated NK and Th1 Cell Responses[1]

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IL-18 is an important cytokine for both innate and adaptive immunity. NK T cells and Th1 cells depend on IL-18 for their divergent functions. The IL-18R, IL-1R, and mammalian Toll-like receptors (TLRs) share homologous intracellular domains known as the TLR/IL-1R/plant R domain. Previously, we reported that IL-1R-associated kinase (IRAK)-4 plays a critical role in IL-1R and TLR signaling cascades and is essential for the innate immune response. Because TLR/IL-1R/plant R-containing receptors mediate signal transduction in a similar fashion, we investigated the role of IRAK-4 in IL-18R signaling. In this study, we show that IL-18-induced responses such as NK cell activity, Th1 IFN-γ production, and Th1 cell proliferation are severely impaired in IRAK-4-deficient mice. IRAK-4$^{-/-}$ Th1 cells also do not exhibit NF-κB activation or IκB degradation in response to IL-18. Moreover, AP-1 activation which is triggered by c-Jun N-terminal kinase activation is also completely inhibited in IRAK-4$^{-/-}$ Th1 cells. These results suggest that IRAK-4 is an essential component of the IL-18 signaling cascade. ***The Journal of Immunology*****, 2003, 170: 4031–4035.**

Interleukin-18, a member of the IL-1 cytokine family, mediates both IFN-γ production by Th1 cells and NK cell activity and acts synergistically with IL-12 to potentiate these responses (1–3). IL-18 exerts its effects via the IL-18R, a heterodimeric complex consisting of a ligand-binding α-chain (IL-18Rα/IL-1R-related protein) and an associating β-chain (IL-18Rβ/accessory protein-like) (2, 4). IL-18Rα contains an evolutionarily conserved domain called the Toll-like receptor (TLR)[3]/IL-1R/plant R gene (TIR) domain, a cytoplasmic region homologous among the IL-1R and mammalian TLRs (4).

The signal transduction pathways mediated by IL-1R and TLRs are very similar (5). Upon specific ligand binding, these receptors associate with the adaptor protein myeloid differentiation factor 88 (MyD88) (6–8), which recruits IL-1R-associated kinases (IRAKs) (5, 9–11) to the receptor complex. IRAKs then associate with TNFR-associated factor 6 (12, 13) to trigger downstream signaling pathways, which ultimately lead to the activation of NF-κB and various stress kinases, including c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase.

Because of the TIR domain in the cytoplasmic tail, the IL-18R signaling pathway may resemble that of IL-1R or TLRs. Indeed, through gene targeting studies, MyD88 and IRAK-1 have been shown to play a role in IL-18 signaling (6, 9, 14). MyD88 has been shown to interact with the IL-18R complex and IRAK-1, and IL-18-induced IFN-γ production and NK cell activity are impaired in MyD88-deficient mice (6). Moreover, IL-18-activated NF-κB and JNK are completely blocked in MyD88-deficient cells. Interestingly, IRAK-1-deficient mice exhibit only a partial impairment of NF-κB activation and JNK induction, but decreased IFN-γ expression in IL-18-challenged Th1 cells as compared with wild-type controls (9). In vivo induction of NK cytotoxicity by IL-18 is also impaired in IRAK-1$^{-/-}$ mice (9).

Because IL-18 signaling is not completely abolished in cells lacking IRAK-1, some other molecules similar to IRAK-1 may also be involved in the signaling cascade. Recently, our group generated IRAK-4-deficient mice and observed that in vivo and in vitro responses to IL-1, LPS, and various TLR ligands are profoundly impaired in IRAK-4$^{-/-}$ mice and cells (5). These mice exhibit no residual cytokine production or NF-κB activation in response to IL-1, nor any residual cytokine production upon LPS or TLR ligand stimulation (5). Because these results suggest that IRAK-4 may occupy an important position in TIR-containing receptor signaling, we analyzed the role of IRAK-4 in IL-18 signaling using IRAK-4-deficient Th1 and NK cells in this study. Our results showed that IRAK-4 is the critical factor for IL-18-induced signaling pathways.

Materials and Methods

Phenotypic analysis of T cells

Thymocytes and splenocytes were stained with CD4- and CD8-specific Abs (BD PharMingen, San Diego, CA). Enriched CD4$^+$ T cells before and after 6 days of differentiation were stained with Abs specific for CD25 and CD44 (BD PharMingen). After Ab staining, cells were analyzed with a FACS (FACSCalibur; BD Biosciences, Mountain View, CA).

Preparation of Th1/Th2 cells

CD4$^+$ T cells were purified from lymph node cells by MACS CD4-positive selection kit (Miltenyi Biotec, Auburn, CA) following the manufacturer's

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; TIR, TLR/IL-1R/plant R gene; MyD88, myeloid differentiation factor 88; IRAK, IL-1R-associated kinase; JNK, c-Jun N-terminal kinase; IKK, IκB kinase.

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protocol. The purity of $CD4^+$ T cells in different preparations was ~95%. Enriched $CD4^+$ T cells were activated with immobilized anti-CD3 mAb (145-2C11; BD PharMingen), which was coated overnight onto 24-well plates at 3 μg/ml. Th1 differentiation was induced by addition of 10 ng/ml IL-2 (R&D Systems, Minneapolis, MN), 5 ng/ml IL-12 (R&D Systems), and 5 μg/ml anti-IL-4 Ab (BD PharMingen) in RPMI medium with 10% FCS. For Th2 cell differentiation, supplementing culture medium with 10 ng/ml IL-2, 5 ng/ml IL-4 (R&D Systems), and 5 μg/ml anti-IFN-γ Ab plus anti-IL-12 Ab (BD PharMingen) was added to $CD4^+$ T cells. After 6 days, the cytokine profile of Th1 or Th2 cells was determined by plating the cells at 2×10^5 cells/well in 96-well plates with 5 μg/ml PMA and ionomycin, and culture supernatants were collected after 24 h for cytokine detection by ELISA.

Cytokine detection

IFN-γ and IL-4 in serum samples and culture supernatants were determined by using commercially available ELISA kits (Genzyme, Cambridge, MA), and recombinant cytokines provided by the manufacturer were used as standards.

Cell proliferation assay

Th1 cells were plated in 96-well plates at 10^5 cells/well and treated with different cytokines for 24 h. [^{3}H]Thymidine (1 μCi/well) was then added for 8 h, and radioactivity incorporated in dividing cells was measured using a Topcount Microplate scintillation counter (Packard, Meriden, CT).

Generation of activated splenocytes and their in vitro response to IL-18

Spleens were harvested from IRAK-4-deficient mice and heterozygous littermates and transferred to medium (RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin). Spleen cells were dispersed by grinding the spleen between two frosted glass slides. After filtration of the ground spleen through a cell strainer, RBCs were removed by hypotonic lysis. Spleen cells (4×10^6 cells/ml) were washed with medium and stimulated with immobilized anti-CD3 mAb (1 μg/ml) for 3 days. Cells were washed with medium, transferred to a 24-well plate (1×10^6 cells/well), and incubated with varying concentrations of murine IL-18 in the absence or the presence of 10 ng/ml murine IL-12 for 24 h. Culture supernatants were collected and assayed for IFN-γ production by ELISA.

Western blot analysis

Western blots of cell extracts were prepared and incubated with anti-IκB Abs (New England Biolabs, Beverly, MA) or anti-β-actin Ab (Fisher Scientific, Pittsburgh, PA). Protein bands were visualized using secondary HRP-conjugated Abs and the ECL detection system (Amersham Pharmacia, Piscataway, NJ).

Gel mobility shift assay

Cell extracts were prepared from Th1 cells following treatment with either 10 ng/ml mouse IL-18 (R&D Systems) or 10 ng/ml mouse TNF-α (R&D Systems). Nuclear extracts were harvested from IL-18-treated and control Th1 cells according to previously described protocols (5). The Bio-Rad (Hercules, CA) protein assay was used to adjust for equal amounts of nuclear or cytoplasmic proteins in each sample. Nuclear extracts were assayed by gel mobility shift assay, as previously described (5). Nuclear extracts were incubated with an end-labeled, double-stranded NF-κB- and AP-1-specific oligonucleotide probe, and the reaction and gel fractionation were performed according to previously described protocols.

NK cytotoxic assay

Mice were injected i.p. daily with PBS alone as controls or PBS containing 1 μg of IL-18 for two consecutive days. Spleen cells prepared from these mice were incubated with ^{51}Cr-labeled YAC-1 target cells for 4 h at 37°C at different E:T ratios. After 4 h of incubation, ^{51}Cr released from target cells was counted using a gamma counter (Packard). Specific lysis was calculated as follows: ((measured ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)) × 100. Maximum release was determined based on acid-lysed target cells. Spontaneous release was determined by incubating target cells in the absence of effector cells.

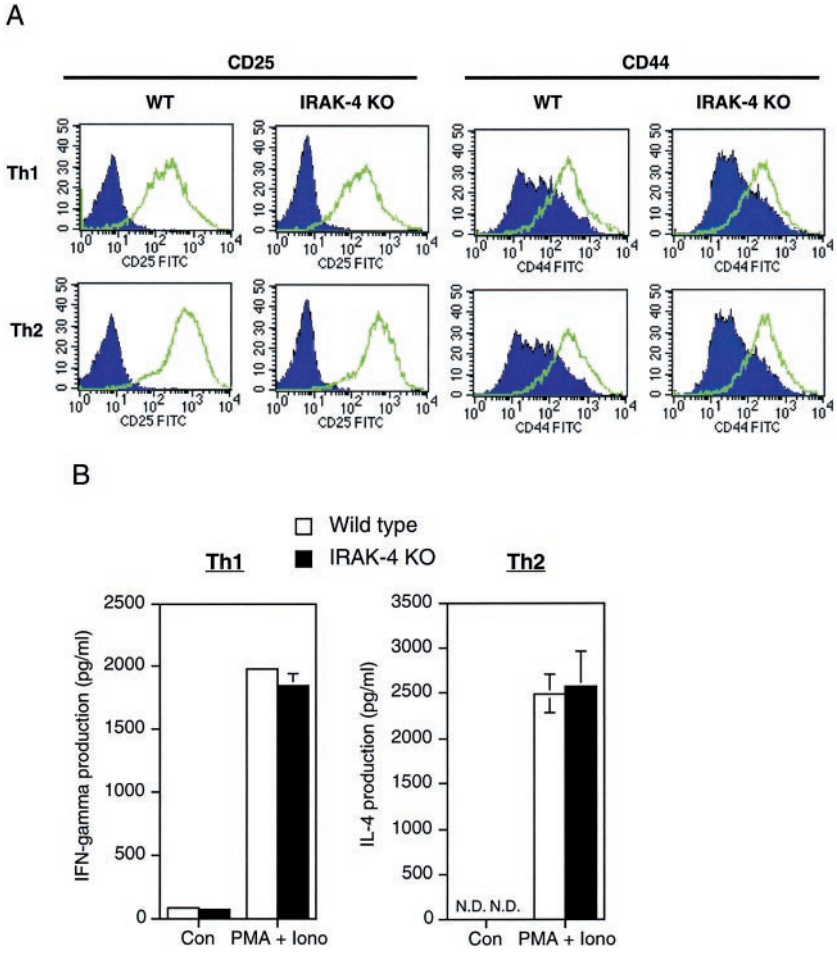

FIGURE 1. Th1/Th2 differentiation in IRAK-4-deficient T cells. *A*, Normal expressions of CD25 and CD44 in IRAK-4-deficient T cells. $CD4^+$ T cells purified from the spleens and lymph nodes of IRAK-4-deficient and wild-type mice were stimulated in anti-CD3-coated plates in the presence of IL-12 and anti-IL-4 for Th1 differentiation, or IL-4 and anti-IFN plus anti-IL-12 for Th2 differentiation. Enriched $CD4^+$ T cells before (filled histogram) and after (empty histogram) 6 days of stimulation were stained with Abs specific for CD25 and CD44 for flow cytometry analysis. *B*, Cytokine profile of Th1 and Th2 cells. After 5 days of Th1 or Th2 differentiation, cells were washed and restimulated with PMA plus ionomycin for 24 h. IFN-γ and IL-4 levels in culture supernatants were determined by ELISA.

FACS analysis for IL-18R expression

For determination of IL-18R expression on $CD4^+$ or $CD8^+$ T cells, after FcR blocking, splenocytes were incubated with anti-murine IL-18R mAb followed by FITC-conjugated anti-rat IgG1 mAb, together with PE-conjugated anti-CD4, or -CD8 (BD PharMingen). Stained cells were analyzed using a dual laser FACSCalibur (BD Biosciences). Ten thousand cells were analyzed, and data were processed with CellQuest (BD Biosciences).

Results

Normal differentiation of Th1 and Th2 cells in IRAK-4-deficient mice

First, we examined Th cell development in IRAK-4-deficient mice. The development of T cells in the thymus and distribution of mature $CD4^+$ and $CD8^+$ T cells in the primary and secondary lymphoid organs of IRAK-4-deficient mice appeared to be normal (data not shown). In addition, there were no apparent morphological abnormalities in the spleen of the knockout mice (data not shown). To examine differentiation of Th1 and Th2 cells, $CD4^+$ T cells purified from spleen and lymph nodes of IRAK-4-deficient and wild-type mice were stimulated in anti-CD3-coated plates in the presence of IL-2, IL-12, and anti-IL-4 Ab for Th1 differentiation, or IL-2, IL-4, anti-IFN-γ Ab, and anti-IL-12 Ab for Th2 differentiation. Abs specific for CD25 and CD44 were used to stain enriched $CD4^+$ T cells before and after 6 days in differentiation cultures, and flow cytometric analysis revealed that both CD25 and CD44 expression are induced in all cell types (Fig. 1*A*).

We then examined PMA/ionomycin-induced IFN-γ production by wild-type and IRAK-$4^{-/-}$ Th1 cells, as well as IL-4 production by wild-type and IRAK-$4^{-/-}$ Th2 cells. As the results in Fig. 1*B* indicate, there is no observable difference in cytokine production between wild-type and IRAK-4-deficient cells. Thus, Th1 and Th2 cells from IRAK-4 deficient mice differentiate as well as wild-type cells do.

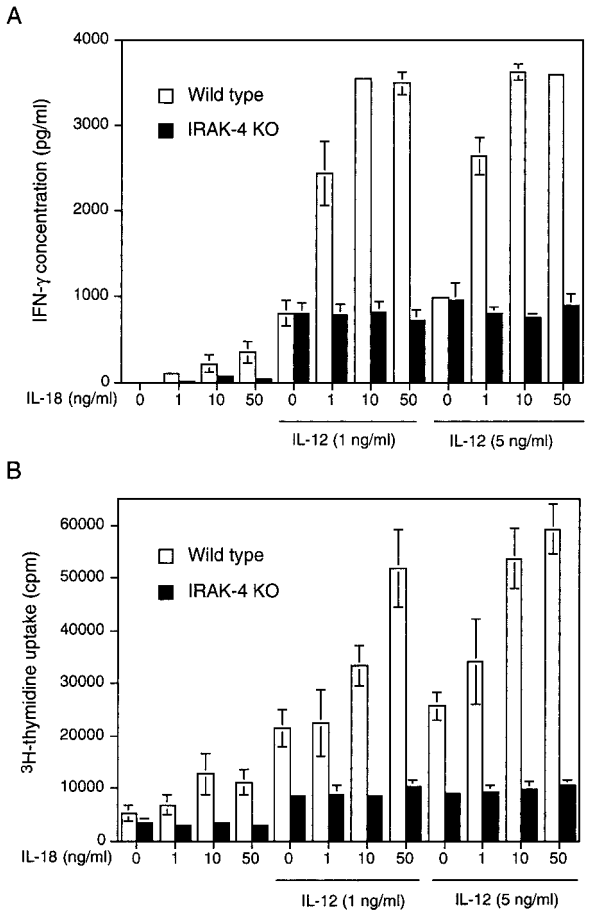

FIGURE 2. Defective IL-18-induced IFN-γ production by and proliferation of IRAK-4-deficient splenocytes. *A*, IFN-γ production of splenocytes in response to IL-18 stimulation. Splenocytes were harvested and activated with immobilized anti-CD3 mAb (1 μg/ml) at 10^5 cells/well for 3 days. Activated splenocytes were then washed with medium, transferred to a 96-well plate (10^5 cells/well), and stimulated with the indicated concentrations of murine IL-18 in the absence or the presence of 1 or 5 ng/ml murine IL-12 for 24 h. Culture supernatants were collected and assayed for IFN-γ production by ELISA. *B*, Proliferation of splenocytes in response to IL-18 stimulation. Activated splenocytes (10^5 cells/well) were washed and then plated on a 96-well plate and stimulated with the indicated concentrations of IL-18 alone, or IL-18 plus IL-12 for 24 h. Cell proliferation was measured by [^{3}H]thymidine incorporation.

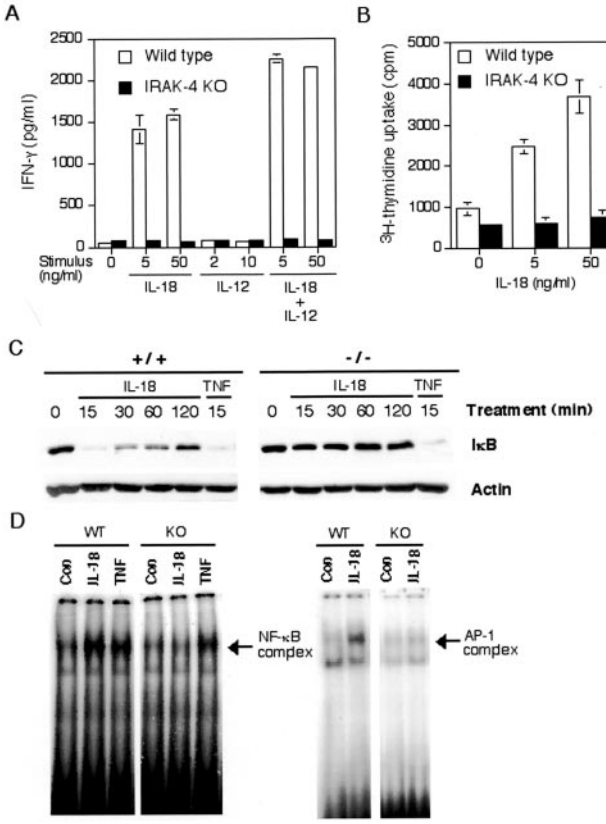

FIGURE 3. Impaired IL-18 signaling of IRAK-4-deficient Th1 cells. *A*, Impaired IFN-γ production in response to IL-18 of IRAK-4-deficient Th1 cells. IRAK-4 deficient and wild-type Th1 cells were stimulated with various amounts of IL-18 and/or IL-12 as indicated. After 24 h, supernatants were collected and assayed for IFN-γ production by ELISA. Each data point on the graph represents the average IFN-γ concentration of supernatants from Th1 cells derived from three mice of each genotype in two separate experiments. *B*, Impaired proliferation in response to IL-18 of IRAK-4-deficient Th1 cells. Th1 cells (10^5 cells/well) were plated on a 96-well plate and cultured in RPMI medium with the indicated concentrations of IL-18 for 24 h. Cell proliferation was determined by an 8-h pulse with [^{3}H]thymidine. *C*, Defective IL-18-mediated IκB degradation in IRAK-4-deficient Th1 cells. Th1 cells were stimulated with IL-18 (50 ng/ml) for different time points as indicated. IκB was detected by Western blot analysis. Amounts of protein loading were determined by probing the same filter with anti-actin Ab. *D*, Impaired DNA binding activity of NF-κB and AP-1 induced in response to IL-18 in IRAK-4-deficient Th1 cells. Wild-type and knockout Th1 cells were left untreated (Con) or treated with IL-18 (50 ng/ml) or TNF (10 ng/ml) for 30 min, and gel mobility shift assays were performed on nuclear extracts.

Requirement of IRAK-4 for IL-18-induced IFN-γ production and splenocyte proliferation

IFN-γ production by Th cells can be induced by IL-18 alone or synergistically by the combination of IL-18 and IL-12 (3, 15). To determine the role of IRAK-4 in IL-18-induced production of IFN-γ, total splenocytes from IRAK-4-deficient and wild-type mice were stimulated with different concentrations of IL-18 or IL-18 in combination with IL-12, and the production of IFN-γ was measured by ELISA. In wild-type cells, there was significant induction of IFN-γ by IL-18 alone and IL-18 plus IL-12, but in cells lacking IRAK-4, this induction was severely diminished (Fig. 2*A*). IL-12 alone induced minimal IFN-γ production in both wild-type and IRAK-4-deficient cells. These results indicate that IRAK-4 is essential for the induction of IFN-γ by IL-18 stimulations.

We next investigated the effect of IL-18, and its synergism with IL-12 on stimulating proliferation of IRAK-4-deficient splenocytes. Total splenocytes from wild-type and IRAK-deficient mice were stimulated with different concentrations of IL-18, or IL-18 plus IL-12. Proliferation of splenocytes after 24 h of stimulation was determined by [^{3}H]thymidine uptake. As shown in Fig. 2*B*, proliferation of wild-type splenocytes was significantly enhanced by IL-18 in a dose-dependent manner. However, the effect of IL-18 on proliferation of IRAK-4-deficient cells was minimal. Although IL-12 induced proliferation of both wild-type and IRAK-4-deficient cells to the same extent, the combination of IL-18 and IL-12 resulted in a synergistic proliferative response in wild-type splenocytes but not in IRAK-4-deficient cells.

Requirement of IRAK-4 in Th1 cell signaling

To further determine the role of IRAK-4 specifically in Th1 cells, IRAK-4-deficient and wild-type Th1 cells were prepared and stimulated with different concentrations of IL-18, or IL-18 in combination with IL-12, and the production of IFN-γ was measured by ELISA. In wild-type Th1 cells, significant induction of IFN-γ by IL-18 or IL-18 plus IL-12 was observed, but in cells lacking IRAK-4, this induction was severely diminished (Fig. 3*A*). In addition, proliferation of wild-type Th1 cells was enhanced by IL-18 in a dose-dependent manner, whereas IL-18-induced proliferation of IRAK-4-deficient Th1 cells was negligible (Fig. 3*B*). Taken together, these results suggest that IRAK-4 plays a critical role in IL-18-mediated cellular responses.

To investigate whether IRAK-4 is required for specific signaling cascades downstream of IL-18, we first examined the activation of

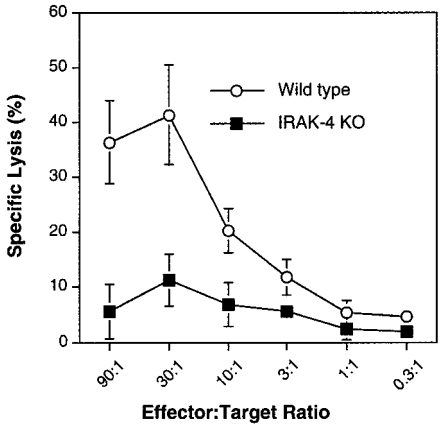

FIGURE 4. Impaired IL-18-induced NK cytotoxicity in IRAK-4 deficient mice. IRAK-4 knockout (KO) and wild-type mice were injected i.p. with 1 μg of IL-18 for two consecutive days. Then spleen cells were assayed for their NK cytotoxic activity against ^{51}Cr-labeled YAC-1 target cells.

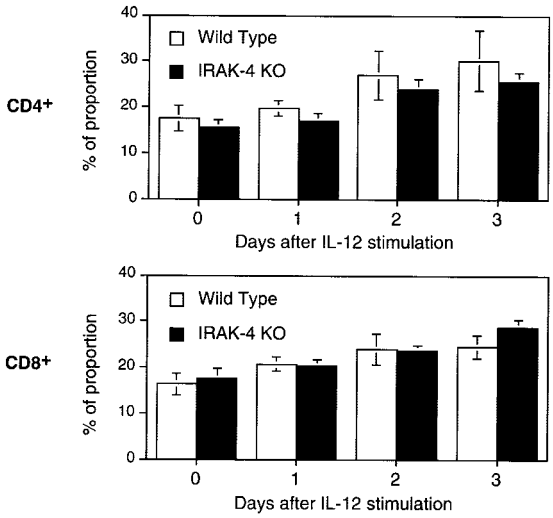

FIGURE 5. IL-18R expression on T cells. Splenic cells were cultured with RPMI 1640 plus 10% FCS plus 10 ng/ml IL-2 for 3 days. Splenic nonadherent cells were stained with anti-IL-18R mAb followed by FITC-conjugated anti-rat IgG1, together with PE-conjugated anti-CD4 or anti-CD8. The proportion of IL-18R$^+$ cells gated with the CD4$^+$ or CD8$^+$ cells is shown before and after culture with 5 ng/ml IL-12 for the indicated days

NF-κB. The transcription factor NF-κB, which plays a critical role in transducing cytokine signals (16), is normally sequestered in the cytoplasm as an inactive complex associated with IκB, its inhibitory partner. Upon cell activation, the IκB kinases (IKKs) IKKα and IKKβ phosphorylate IκB (e.g., IκBα at residues 32 and 36), resulting in the degradation of IκB by proteosomes (17, 18). We first examined degradation of the IκB protein in wild-type and IRAK-4-deficient Th1 cells upon IL-18 treatment. Although wild-type cells showed a typical course of IκB degradation peaking at 15 min, almost no IκB degradation was observable in IRAK-4-deficient Th1 cells following IL-18 stimulation (Fig. 3*C*). However, stimulation with TNF induced IκB degradation in both wild-type and IRAK-4-deficient cells. Similarly, when NF-κB activity was examined by DNA binding gel mobility shift assay, cells lacking IRAK-4 were defective in IL-18-induced but not in TNF-induced NF-κB activation (Fig. 3*D*). Another important IL-18 downstream signaling pathway is the activation of the stress kinase JNK that regulates the activation of the transcription factor AP-1. We examined the activation of AP-1 by gel mobility shift assay and found that IL-18-induced AP-1 is completely abolished in IRAK-4-deficient cells. Together, these results indicated that IRAK-4 is indispensable in IL-18-mediated NF-κB and AP-1 activation.

IL-18-induced NK cytotoxicity is impaired in IRAK-4-deficient mice

IL-18 has been shown to enhance NK cell cytotoxicity (3). To determine whether IRAK-4 is important for IL-18-induced NK activity, mice were injected i.p. with IL-18 daily for two consecutive days, and the NK activity of splenocytes was assayed using ^{51}Cr-labeled YAC-1 cells as targets. Basal NK cell activities in PBS-injected wild-type and IRAK-4-deficient mice were comparable (data not shown). In vivo challenge with IL-18 resulted in significant increase in NK activity in wild-type animals, but its effect in IRAK-4-deficient mice was minimal, suggesting that IRAK-4 is required for IL-18-mediated induction of NK cytotoxic activity (Fig. 4).

Expression of IL-18R is not impaired in IRAK-4-deficient mice

The Stat4-deficient T cell is reported not to express any IL-18R on its surface (19). It is also reported that up-regulation of the IL-18R induced by IL-12 is completely abrogated in Tyk2-deficient T cells

(20). To investigate the mechanism of IL-18 unresponsiveness in IRAK-4-deficient cells, we examined the cell surface IL-18R. The proportion of IL-18R-positive $CD4^+$ and $CD8^+$ T cells (Fig. 5) and the intensity of positive stains (data not shown) are comparable between wild-type and IRAK-4-deficient mice, suggesting that the IL-18R expression level is normal in the knockout mice. IL-18R on T cells is also up-regulated by the treatment of IL-12 in IRAK-4-deficient mice as in wild-type mice (Fig. 5). Therefore, IL-18R expression and its induction are intact in IRAK-4-deficient mice.

Discussion

In this study, we demonstrated that IRAK-4 is required for the majority of IL-18-mediated functions both in vitro and in vivo. IL-18, also known as IFN-γ-inducing factor, is an important molecule in the stimulation of Th1 and NK cell responses (21). Th1 cells lacking IRAK-4 failed to produce IFN-γ and undergo proliferation in response to IL-18. We have shown previously that lymphocytic choriomeningitis virus infection resulted in defective IFN-γ production by IRAK-4-deficient NK cells, which was consistent with a potential defect in IL-18 signaling in the mutant mice (5). In this study, we showed that specific challenge by IL-18 in IRAK-4-deficient mice failed to induce NK cytotoxic activity.

IL-18-mediated activation of IFN-γ promoter activity depends on the binding of AP-1 and NF-κB sites, whereas IL-12 acts on both Stat4 and AP-1 sites (22). Therefore, the combination of IL-18 and IL-12 is anticipated to result in a synergistic effect on IFN-γ expression at the transcriptional level. In cells lacking IRAK-4, IL-18-induced NF-κB and AP-1 activations are severely impaired, and therefore, the induction of IFN-γ production by IL-18 or IL-18 plus IL-12 is severely compromised. In contrast, IRAK-1-deficient cells showed residual activities of NF-κB and JNK (which leads to AP-1 activation), and IFN-γ induction was still detectable upon IL-18 stimulation.

Based on our study and published reports, it appears that IRAK-4 and MyD88 are required for IL-18 signaling, whereas IRAK-1 is not absolutely required but necessary for the optimization of IL-18 signals. Similar conclusions can be drawn regarding the signaling cascades triggered by IL-1 and at least some TLR ligands. Although all IRAK family members can be recruited to MyD88 and the receptor signaling complex, and mediate signals downstream to TNFR-associated factor 6, it may be that IRAK-4 functions upstream of other IRAK family members. For example, IRAK-1 can be phosphorylated by IRAK-4 in vitro (23), which may lead to the activation and autophosphorylation of IRAK-1, which would then trigger a downstream signaling cascade. Further investigations into the interaction between IRAK-1 and IRAK-4 will be required to elucidate the signaling mechanisms of IL-18R and related receptors.

The relationship between IRAK-4 and two other IRAK family members, IRAK-2 or IRAK-M, also remains unclear. Future studies may reveal whether IRAK-2 or IRAK-M can also act as substrates for IRAK-4, and how these two kinase-inactive IRAK family members might contribute in a positive manner to signaling cascades and partially replace IRAK-1 function. Interestingly, IRAK-M-deficient mice did not show a defect in signal transduction, but instead demonstrated enhanced responses when stimulated by IL-1 or TLR ligands (10). This finding further illustrates the potential for unique, nonredundant functions of individual IRAK family members in signaling cascades. Future studies comparing mutant mice and cells lacking individual or combinations of IRAK molecules will help clarify the molecular mechanisms of signal transduction pathways involving IRAK molecules.

The study of IL-18 and its signaling transduction stands to contribute to the understanding and treatment of many severe disease states. For example, certain autoimmune diseases are characterized by the production of IL-18 and its enhancement of Th1 cell development in the lesion (24). Moreover, a potential role of IL-18 in tumor immune therapy (25) has also generated considerable interest, because IL-18 is critical for stimulating the cytotoxic activities of T cells and NK cells to clear tumor cells (26). Our finding that IRAK-4 is critical for IL-18R-mediated signaling may provide an additional target for therapeutic intervention in these areas.

Acknowledgments

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