Targeted Disruption of the *MyD88* Gene Results in Loss of IL-1- and IL-18-Mediated Function

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Summary

MyD88, originally isolated as a myeloid differentiation primary response gene, is shown to act as an adaptor in interleukin-1 (IL-1) signaling by interacting with both the IL-1 receptor complex and IL-1 receptor–associated kinase (IRAK). Mice generated by gene targeting to lack MyD88 have defects in T cell proliferation as well as induction of acute phase proteins and cytokines in response to IL-1. Increases in interferon-γ production and natural killer cell activity in response to IL-18 are abrogated. In vivo Th1 response is also impaired. Furthermore, IL-18-induced activation of NF-κB and c-Jun N-terminal kinase (JNK) is blocked in MyD88$^{-/-}$ Th1-developing cells. Taken together, these results demonstrate that MyD88 is a critical component in the signaling cascade that is mediated by IL-1 receptor as well as IL-18 receptor.

Introduction

Interleukin-1 (IL-1) is a proinflammatory cytokine involved in local and systemic inflammatory reaction (reviewed by Dinarello, 1989, 1996). IL-1 induces a variety of inflammatory proteins, such as acute phase proteins, cytokines, and adhesion molecules, whose genes are mainly regulated by NF-κB and/or AP-1 (reviewed by Dinarello, 1989, 1996). IL-1 signaling is initiated by the formation of the high-affinity complex composed of IL-1, IL-1 receptor (IL-1R), and IL-1R accessory protein (IL-1R-AcP). This is followed by the recruitment of IL-1 receptor–associated kinase (IRAK) to the receptor complex (Greenfeder et al., 1995; Cao et al., 1996a; Huang et al., 1997; Korherr et al., 1997; Wesche et al., 1997a). Then, IRAK leaves the receptor complex and interacts with TRAF6, which finally results in NF-κB activation (Cao et al., 1996b). IL-18, originally identified as an interferon-γ (IFNγ)-inducing factor, promotes the production of IFNγ and enhances natural killer cell activity (Okamura et al., 1995a, 1995b). IL-18 synergizes with IL-12 in inducing IFNγ production from T cells and plays an important role in Th1 response (Robinson et al., 1997; Takeda

et al., 1998). Despite the functional similarity, IL-18 is not structurally related to IL-12. IL-18 has a structural similarity with IL-1 (Bazan et al., 1996). IL-18 is synthesized as an inactive precursor that requires cleavage by IL-1β-converting enzyme (ICE)/caspase 1 for its maturation, as in the case of IL-1β (Ghayur et al., 1997; Gu et al., 1997). IL-18 also activates IRAK and NF-κB (Matsumoto et al., 1997; Robinson et al., 1997).

The Toll protein determines dorsal–ventral polarity in *Drosophila melanogaster* embryos and also participates in an anti-fungal immune response (Hashimoto et al., 1988; Morisato and Anderson, 1994, 1995; Lemaitre et al., 1996). The cytoplasmic domain of Toll is homologous to that of IL-1R (Gay and Keith, 1991). The signaling pathway through Toll in *Drosophila* shows striking structural and functional similarities with the signaling pathway induced by the IL-1Rs in mammals (Wasserman, 1993; Belvin and Anderson, 1996). Toll activates the transcription factor Dorsal, a *Drosophila* homolog of NF-κB, upon binding to its ligand Spatzle (Morisato and Anderson, 1994, 1995). Transduction of the intracellular signal generated by Toll requires the adaptor molecule called Tube for the association of Toll with the Pelle protein kinase, a *Drosophila* homolog of IRAK (Letsou et al., 1993; Shelton and Wasserman, 1993; Grosshans et al., 1994; Galindo et al., 1995; Norris and Manley, 1996). Until recently, a mammalian homolog of Tube had not been identified.

MyD88 was originally isolated as a myeloid differentiation primary response gene, which is rapidly induced upon IL-6-stimulated differentiation to macrophages in M1 myeloleukemic cells (Lord et al., 1990). This immediate–early activation profile suggested that MyD88 functions in the regulated progression of myeloid differentiation. MyD88 is subsequently found to be related to the IL-1R family, particularly Toll (Hultmark, 1994; Hardiman et al., 1996; Bonnert et al., 1997). Different from the IL-1R family, MyD88 does not harbor the transmembrane portion. The death domain is present in its N terminus. The C-terminal domain of the protein is highly homologous to the cytoplasmic segments of the IL-1R family. Recently, MyD88 has been cloned as an adaptor molecule that recruits IRAK to the IL-1R complex after IL-1 stimulation and activates NF-κB (Muzio et al., 1997; Wesche et al., 1997b). MyD88 is therefore a functional homolog of Tube, although these two molecules are not structurally related. To know the in vivo functional role of MyD88 in IL-1 as well as IL-18 signaling, we have generated MyD88-deficient mice. In MyD88-deficient mice, all of the IL-1- and IL-18-mediated functions examined were impaired, demonstrating that MyD88 is a functional molecule critical for IL-1- and IL-18-mediated signaling.

Results and Discussion

Generation of MyD88-Deficient Mice
The mouse *MyD88* gene was disrupted by homologous recombination in E14.1 embryonic stem (ES) cells. A targeting vector was designed to replace two exons

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A

1.0kb
probe
4.3kb

wild-type
allele

targeting
vector

neo

HSV-tk

mutated
allele

neo

1.9kb

B; BamHI; P; PstI

B

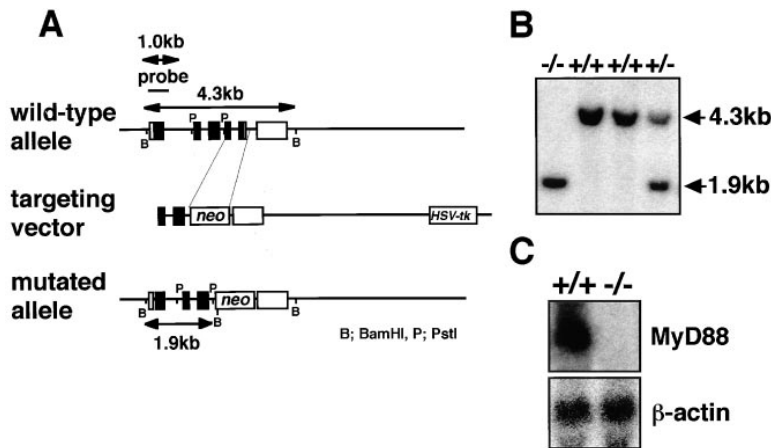-/- +/+ +/+ +/-

◄4.3kb

◄1.9kb

C

+/+ -/-

MyD88

β-actin

Figure 1. Targeted Disruption of the Mouse *MyD88* Gene

(A) Maps of the MyD88 wild-type genome, targeting vector, and predicted targeted gene. Open and closed boxes denote the noncoding and coding exons, respectively. Restriction enzymes: B, BamHI; P, PstI.
(B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with BamHI, electrophoresed, and hybridized with the probe indicated in (A). The approximate size of the wild-type band is 4.3 kb, and the mutated band is 1.9 kb.
(C) Northern blot analysis of liver cells. Total RNA was extracted from liver, electrophoresed, transferred to nylon membrane, and hybridized with the mouse MyD88 cDNA probe. The same membrane was rehybridized with a β-actin probe.

encoding the C-terminal portion of MyD88 with the neomycin resistance gene (Figure 1A). This region of the *MyD88* gene shows high similarity with the cytoplasmic domain of the IL-1R. The corresponding region in the human IL-1R includes the residues critical for its signaling (Hultmark, 1994; Hardiman et al., 1996; Bonnert et al., 1997). Homologous recombination was achieved in 33 out of 176 ES cell clones doubly resistant to G418 and gancyclovir. Three targeted ES cell clones were microinjected into C57BL/6 blastocysts, two of which successfully transmitted the disrupted *MyD88* gene through the germline (Figure 1B). MyD88-deficient (MyD88$^{-/-}$) mice were born at the expected Mendelian ratios (+/+: +/−: −/− = 52: 93: 53). The mice grew healthy and showed no obvious abnormalities until 20 weeks of age. We performed Northern blot analysis to confirm that the mutation causes inactivation of the *MyD88* gene. MyD88 mRNA could not be detected in the liver and spleen of MyD88$^{-/-}$ mice (Figure 1C). Flow cytometric analysis of the expression of CD3, B220, CD4, and CD8 in thymus, spleen, and lymph node showed that lymphocyte composition was not altered in MyD88$^{-/-}$ mice when compared with wild-type mice (data not shown).

**Impaired IL-1-Mediated Functions
in MyD88$^{-/-}$ Mice**

We first examined IL-1-mediated response in the mice. IL-1 is shown to be a costimulant for T cell growth (Mannel et al., 1985; Dinarello, 1989; Suda et al., 1989). Thymocytes of MyD88$^{-/-}$ mice and their wild-type littermate controls were cultured with IL-1β plus several stimuli. In wild-type mice, thymocytes displayed enhanced proliferation when cultured with phytohemagglutinin (PHA), concanavalin A (ConA), or a low concentration of IL-2 in the presence of IL-1β. However, MyD88$^{-/-}$ thymocytes did not show any enhanced proliferation in response to IL-1β (Figure 2). Similarly, splenocytes from MyD88$^{-/-}$ mice did not proliferate in response to IL-1β (data not shown). Thymocytes from wild-type and MyD88$^{-/-}$ mice showed almost equal proliferative response to IL-2 plus Phorbol 12-Myristate 13-Acetate PMA or IL-2 plus ConA, indicating that IL-1-mediated

growth signal was specifically impaired in the lymphocytes from MyD88$^{-/-}$ mice (Figure 2).

IL-1 is also shown to induce production of acute phase proteins and inflammatory cytokines such as TNF-α and IL-6. We next analyzed the effect of MyD88 deficiency on these functions. Mice were intravenously injected with 1 μg of IL-1β, and 2 hr later liver and sera were taken. Total RNA was extracted from the liver and subjected to Northern blot analysis for expression of serum amyloid A (SAA-I), serum amyloid P (SAP), and haptoglobin (HP). In wild-type mice, there was a dramatic increase in mRNA expression for SAA-I, SAP, and HP after IL-1β injection (Figure 3A). However, IL-1-induced increase in mRNA expression of SAA-I, SAP, or HP was not observed in MyD88$^{-/-}$ mice. Serum concentrations of TNF-α and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). Serum TNF-α and IL-6 concentrations were significantly increased after IL-1β injection in wild-type mice (Figure 3B). In contrast, neither serum TNF-α nor IL-6 level was elevated after IL-1β treatment in MyD88$^{-/-}$ mice. Thus, IL-1-mediated
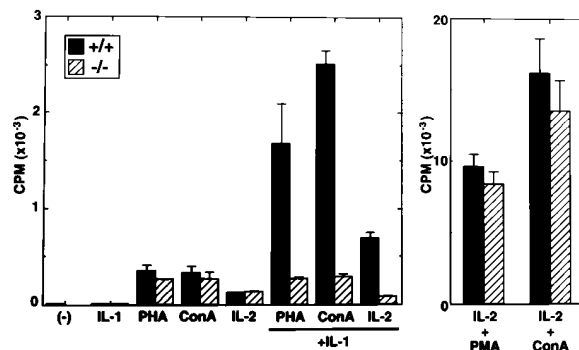
Figure 2. Impaired IL-1-Mediated T Cell Proliferation in MyD88$^{-/-}$ Mice

Thymocytes from wild-type and MyD88$^{-/-}$ mice were incubated with 100 U/ml IL-1β in the presence of the indicated mitogens for 72 hr. [³H]thymidine was pulsed for the last 12 hr, and [³H] uptake was measured. Thymocytes were also incubated with 20 ng/ml IL-2 in the presence of 10 ng/ml PMA or 2.5 μg/ml ConA for 72 hr. [³H] uptake was measured. Representative from three independent experiments.
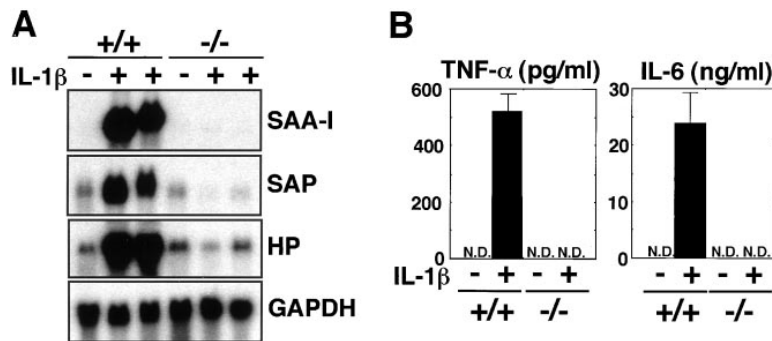
A

B

Figure 3. Impaired IL-1-Induced Expression of Acute Phase Proteins and Production of Inflammatory Cytokines in MyD88$^{-/-}$ Mice

(A) Mice were intravenously injected with IL-1β. Total RNA was extracted from liver after 2 hr of injection and subjected to Northern blot analysis using probes for acute phase proteins such as SAA-I, SAP, and HP. The figure is the representative of results from three independent experiments.

(B) Mice were intravenously injected with 1 μg of IL-1β. Sera were taken 2 hr after injection. Concentration of TNF-α and IL-6 was measured by ELISA. The data are mean ± S.D. of six serum samples. N.D., not detected.

major biological functions were severely impaired in MyD88$^{-/-}$ mice.

Impaired IL-18-Mediated Functions in MyD88$^{-/-}$ Mice

IL-18, originally identified as an IFNγ-inducing factor, has a structural similarity to IL-1, and the receptor for IL-18 belongs to the IL-1R family (Okamura et al., 1995a, 1995b; Bazan et al., 1996; Parnet et al., 1996; Torigoe et al., 1997). Therefore, we examined IL-18-mediated biological functions in MyD88$^{-/-}$ mice. IL-18 has been shown to be a potent activator of natural killer (NK) cells (Okamura et al., 1995a, 1995b; Tsutsui et al., 1996; Ushio et al., 1996). Splenocytes were cultured in the presence or absence of IL-18 for 24 hr, and NK lytic activity was analyzed by ^{51}Cr release from YAC-1 target cells. In vitro culture of splenocytes with IL-18 dramatically enhanced the lytic activity against YAC-1 cells in wild-type mice (Figure 4A). However, IL-18 did not enhance NK lytic

A

B

Figure 4. Impaired IL-18-Mediated NK Cell Activation in MyD88$^{-/-}$ Mice

(A) Splenocytes from wild-type and MyD88$^{-/-}$ mice were cultured in the presence of IL-18 or IL-2 for 24 hr. NK lytic activity against YAC-1 target cells was analyzed. Representative results from three independent experiments.

(B) Splenocytes were cultured with or without 20 ng/ml IL-18 for 24 hr. Concentration of IFNγ in the culture supernatants was measured by ELISA. N.D., not detected.

activity in MyD88$^{-/-}$ mice. Stimulation of splenocytes with IL-2 led to almost equal augmentation of the NK lytic activity against YAC-1 cells in both wild-type and MyD88$^{-/-}$ mice, indicating that IL-18-mediated activation of NK cells was specifically disturbed in MyD88$^{-/-}$ mice (Figure 4A). Besides the activation of NK cells, in vitro stimulation of splenocytes with IL-18 induced IFNγ production in wild-type mice (Figure 4B). In contrast, IFNγ production in response to IL-18 was not observed in MyD88$^{-/-}$ mice (Figure 4B). When wild-type mice were treated with anti-asialo GM1 antibody, which reduces NK cell population in vivo, splenocytes did not produce IFNγ in response to IL-18 (data not shown). These data suggest that NK cells in MyD88$^{-/-}$ mice have a defect in their capacity to promote IFNγ production in response to IL-18.

Additionally, IL-18 has been recently shown not to drive Th1 development but to potentiate IL-12-induced Th1 development (Matsui et al., 1997; Robinson et al., 1997; Takeda et al., 1998). Naive splenic T cells were cultured with IL-12 on anti-CD3 antibody-coated plates to induce Th1 cell development. After 4 days of culture, these Th1-developing cells were washed and restimulated with IL-18 or IL-12 on anti-CD3 antibody-coated plates for 24 hr. Culture supernatants were analyzed for IFNγ production by ELISA. Both wild-type and MyD88$^{-/-}$ T cells produced almost equal levels of IFNγ by stimulation with anti-CD3 antibody, indicating that IL-12-induced Th1 cell development in vitro was not impaired in MyD88$^{-/-}$ mice. T cells from wild-type mice produced an elevated level of IFNγ in response to IL-18 (Figure 5A). In contrast, T cells from MyD88$^{-/-}$ mice did not show any enhanced production of IFNγ in response to IL-18.

It has been shown that IL-18-deficient mice displayed defective Th1 cell development after injection of Propionibacterium acnes (P. acnes) and Bacillus Calmette-Guérin (BCG) (Takeda et al., 1998). MyD88$^{-/-}$ mice were injected with heat-killed P. acnes, and 7 days later splenic T cells were purified and stimulated with immobilized anti-CD3 antibody in the presence or absence of IL-18. Wild-type T cells produced significant levels of IFNγ, and the presence of IL-18 resulted in enhanced production of IFNγ. In contrast, IFNγ production from MyD88$^{-/-}$ T cells was severely reduced when compared with wild-type. In addition, increased IFNγ production in response to IL-18 was not observed in MyD88$^{-/-}$ T cells (Figure 5B). T cells of BCG-infected wild-type mice also produced high amounts of IFNγ and more enhanced
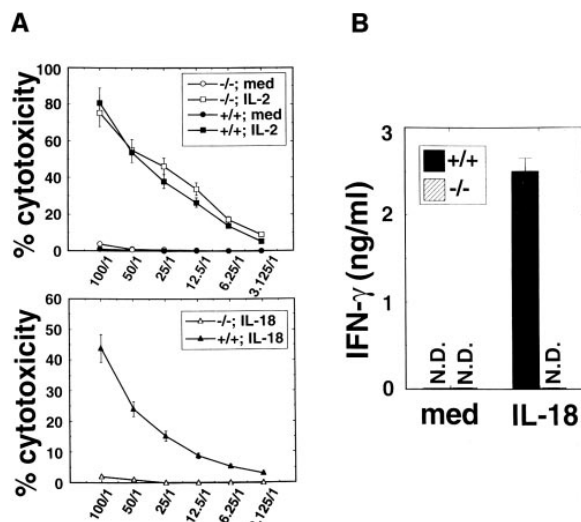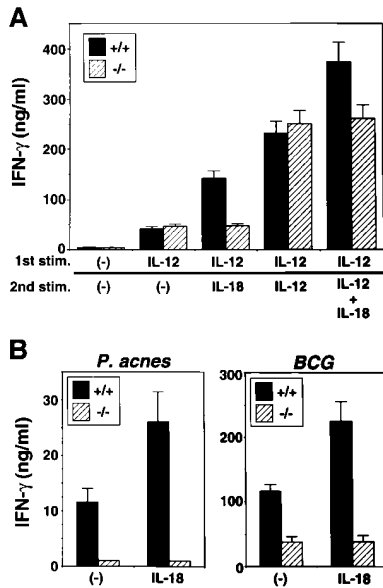
A

B

P. acnes   BCG

Figure 5. Impaired IL-18-Mediated Th1 Response in MyD88$^{-/-}$ Mice

(A) Splenic T cells were purified and incubated with or without 2 ng/ml IL-12 on anti-CD3 Ab-coated plates. After 4 days of culture, T cells were washed and restimulated with 20 ng/ml IL-18 and/or 2 ng/ml IL-12 on anti-CD3 Ab-coated plates for 24 hr. Production of IFNγ from T cells was analyzed by ELISA.

(B) Mice were intraperitoneally injected with 500 μg of heat-killed P. acnes. Seven days after injection, splenic T cells were purified and stimulated with immobilized anti-CD3 antibody in the presence or absence of 20 ng/ml IL-18 for 24 hr. The concentration of IFNγ in culture supernatants was measured by ELISA. In the case of BCG infection, mice were intravenously injected with 2 mg of BCG. Production of IFNγ from splenic T cells was analyzed by ELISA 14 days later.

levels of IFNγ in response to IL-18. However, IFNγ production from T cells of BCG-infected MyD88$^{-/-}$ mice was about 3-fold lower than that from wild-type T cells (Figure 5B). These results demonstrate that MyD88$^{-/-}$ mice are defective in Th1 cell development in vivo as is

the case with IL-18-deficient mice. Thus, major biological activities mediated by IL-18 were completely abolished in MyD88$^{-/-}$ mice.

Dominant Negative MyD88 Blocks IL-18-Induced Activation of NF-κB and AP-1

MyD88$^{-/-}$ mice displayed severely impaired IL-18-mediated functions. Therefore, we examined whether the IL-18R complex interacts with MyD88 as shown in the case of the IL-1R complex (Muzio et al., 1997; Wesche et al., 1997b). We transiently coexpressed FLAG-tagged MyD88 and Myc-tagged IL-18R into COS-7 cells. The cell lysates were immunoprecipitated with the anti-Myc antibody and blotted with the anti-FLAG antibody. FLAG-tagged MyD88 was coimmunoprecipitated with the anti-Myc antibody (Figure 6A). Reciprocally, Myc-tagged IL-18R was also coimmunoprecipitated with the anti-FLAG antibody. Thus, MyD88 specifically associates with IL-18R.

The C-terminal domain of MyD88 (amino acids 152 to 296) has been shown to act as a dominant negative inhibitor of IL-1R complex–induced NF-κB activation (Muzio et al., 1997; Wesche et al., 1997b). In addition, IL-18 is shown to induce NF-κB activation as does IL-1 (Matsumoto et al., 1997; Robinson et al., 1997). Therefore, we studied whether the dominant negative MyD88 mutant blocks IL-18-induced NF-κB activation as well. COS-7 cells were transiently transfected with MyD88 (152–296) expression vector together with NF-κB-dependent luciferase reporter gene, and luciferase activity after IL-18 treatment was measured. Coexpression of MyD88 (152–296) almost completely blocked IL-18-induced NF-κB activation (Figure 6B, upper). IL-18 is also shown to activate AP-1-dependent gene expression (Barbulescu et al., 1998). We examined whether MyD88 (152–296) also acts as a dominant negative mutant of IL-18-induced AP-1 activation. Stimulation with IL-18 induced an approximately 3- to 4-fold increase in AP-1 activity, and this activation was blocked by coexpression of MyD88 (152–296) (Figure 6B, lower). Taken together, these results show that MyD88 is involved in IL-18-induced activation of both NF-κB and AP-1.
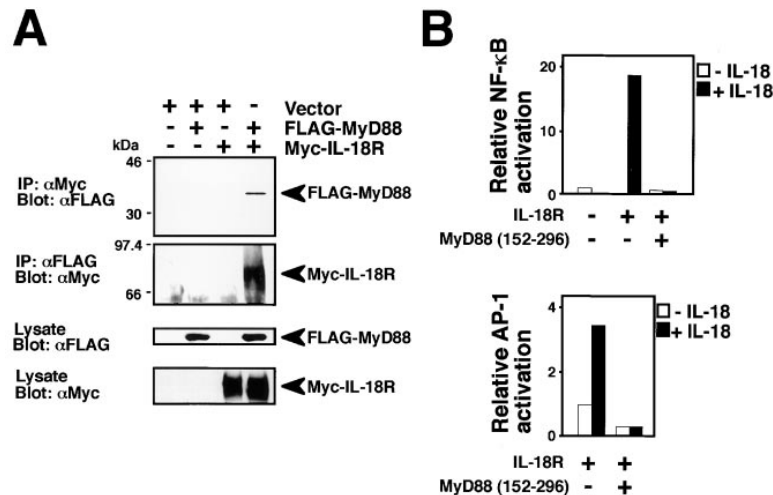
A

B

Figure 6. MyD88 Interacts with IL-18 Receptor and Induces NF-κB and AP-1 Activation

(A) COS-7 cells were transiently cotransfected with the indicated combination of expression vectors for FLAG-tagged MyD88, Myc-tagged IL-18R, or empty vector (Vector). After 36 hr, cell lysates were prepared and immunoprecipitated with either anti-Myc antibody or anti-FLAG antibody. Coprecipitating FLAG-MyD88 or Myc-IL-18R protein was detected by Western blotting with either anti-FLAG antibody or anti-Myc antibody, respectively. The total level of protein expression was monitored by Western blot analysis of the same lysates with the relevant antibodies. The molecular weight (in kilodaltons) is listed on the left.

(B) COS-7 cells were transiently cotransfected with pNF-κB-Luc or pAP-1-Luc reporter gene plasmid together with expression plasmid for IL-18R or MyD88 (152–296) as indicated. Cells were left untreated (open bars) or stimulated with 1.0 μg/ml IL-18 (closed bars) 24 hr after transfection. After 3 hr, relative luciferase activity was determined. Similar results were obtained from three independent experiments.
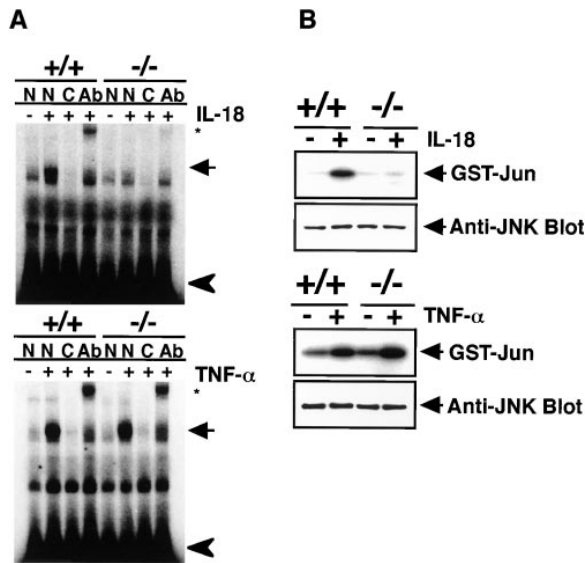
A **B**

Figure 7. Loss of NF-κB and JNK Activation in Response to IL-18 in MyD88$^{-/-}$ Cells

(A) T cells cultured with IL-12 plus anti-CD3 antibody for 4 days were stimulated with 100 ng/ml IL-18 for 30 min, and thymocytes were stimulated with 100 ng/ml TNF-α for 30 min. Nuclear extracts were then prepared and incubated with a specific probe containing NF-κB-binding sites, and NF-κB activity was determined by a gel mobility shift assay. Specificity was determined by adding none (N), 50-fold molar excess of specific competitor (C), or 1.0 μg of specific antibody to p65 (Ab). Inducible NF-κB complex was indicated by the arrow. The asterisks show the supershift. The free probe was indicated by the arrow head.

(B) Cells (Th1-developing cells used for IL-18 stimulation and thymocytes for TNF-α) were left untreated or stimulated with 100 ng/ml IL-18 or 100 ng/ml TNF-α for 20 min. Cell lysates were prepared and immunoprecipitated with anti-JNK antibody. The kinase activity was measured using GST-c-Jun-fusion protein as a substrate (upper). The lower panels show Western blot analysis of the same lysates with anti-JNK antibody.

Loss of IL-18-Induced NF-κB DNA Binding Activity and JNK Activation in MyD88$^{-/-}$ Th1-Developing Cells

We next investigated whether IL-18-induced activation of NF-κB was observed in MyD88$^{-/-}$ cells. Splenic T cells cultured with IL-12 plus anti-CD3 antibody for 4 days were starved for 3 hr and then stimulated with IL-18. Nuclear extracts from the stimulated cells were analyzed by a gel mobility shift assay using a specific probe containing NF-κB binding site. As shown in Figure 7A, IL-18-induced NF-κB DNA binding activity was detected in the nuclear extracts from wild-type cells but not from MyD88$^{-/-}$ cells. On the other hand, treatment of wild-type or MyD88$^{-/-}$ thymocytes with TNF-α resulted in almost the same levels of NF-κB DNA binding activity, demonstrating that the impaired IL-18-induced NF-κB activation in MyD88$^{-/-}$ cells was not due to the abnormal function or down-regulation of NF-κB.

In addition to induction of NF-κB activation, IL-1 is also shown to activate c-Jun N-terminal kinase (JNK) (Wesche et al., 1997a). To test whether IL-18 induces JNK activation, we carried out an in vitro kinase assay using GST-c-Jun-fusion protein as a substrate (Figure 7B). Treatment with IL-18 induced JNK activation in

Th1-developing cells of wild-type mice. However, IL-18-induced JNK activation was not observed in MyD88$^{-/-}$ cells. By contrast, normal activaion of JNK was observed in MyD88$^{-/-}$ cells treated with TNF-α. Thus, IL-18-induced NF-κB and JNK activation was abrogated in MyD88$^{-/-}$ mice, although TNF-α-induced activation was not affected. Taken together, these results demonstrate that MyD88 is essential for IL-18-induced activation of both NF-κB and JNK.

In the present study, we demonstrated that MyD88 is essential for IL-1 and IL-18 signaling, because the major functions mediated by these cytokines were almost completely abolished in the MyD88$^{-/-}$ mice. The family of IL-1R/Toll related proteins is now expanding in mammals (Medzhitov and Janeway, 1997; Medzhitov et al., 1997). These include IL-1R-related protein (IL-1Rrp), IL-1Rrp2, T1/ST2, and recently identified human Toll-like molecules (TLR1-5) (Yanagisawa et al., 1993; Lovenberg et al., 1996; Mitcham et al., 1996; Parnet et al., 1996; Rock et al., 1998). Recently, IL-1Rrp was found to be the receptor for IL-18 (Torigoe et al, 1997). The ligands and biological functions of several IL-1R-related molecules have yet to be clarified. MyD88 may also participate in the signaling of these orphan receptors such as IL-1Rrp2, T1/ST2, and TLR1-5. Thus, the use of MyD88$^{-/-}$ mice may help to elucidate the functions of these orphan receptors. In this respect, it is noteworthy that MyD88$^{-/-}$ mice are viable when we consider that the Toll pathway in *Drosophila* is analogous to the IL-1 pathway in mammals and that MyD88 is a functional mammalian homolog of *Drosophila* Tube, whose loss of function results in early embryonic lethality due to defective dorsoventral polarity in *Drosophila* (Letsou et al., 1993; Belvin and Anderson, 1996; Norris and Manley, 1996). Based on these findings, mammalian Toll-like molecules appear to utilize another Tube-related molecule or do not play a major role in mammalian development. Future studies will be required to answer this question. Finally, agents that block the function of MyD88 will be therapeutically useful against a variety of pathological conditions associated with excessive production of the IL-1 family of cytokines.

T Cell Proliferation Assay

Thymocytes (1×10^5) were cultured in 96-well plates for 72 hr with 100 U/ml IL-1β (Genzyme) in the presence of 2 μg/ml PHA, 2.5 μg/ml ConA, or 2 ng/ml IL-2. [³H]thymidine was pulsed for the last 12 hr, and [³H] uptake was measured. In the case of the response to IL-2, thymocytes were cultured with 20 ng/ml IL-2 (Genzyme) in the presence of 10 ng/ml PMA or 2.5 μg/ml ConA.

Induction of Inflammatory Cytokines and Acute Phase Proteins

Mice were intravenously injected with 1 μg of recombinant mouse IL-1β (Genzyme). After 2 hr of injection, mice were bled for serum cytokine and sacrificed. Total RNA was extracted from the liver using the TRIZOL reagent (GIBCO). RNA (10 μg) was electrophoresed, transferred to a nylon membrane, and hybridized with ³²P-labeled cDNA probes for SAA-I, SAP, and HP. Serum concentrations of TNF-α and IL-6 were determined by ELISA (Genzyme).

Analysis of NK Cell Activity

Splenocytes were cultured for 24 hr with or without 20 ng/ml IL-18 or 100 U/ml IL-2. Then, cultured cells were incubated with ⁵¹Cr-labeled YAC-1 target cells at the indicated effector/target ratios. After 4 hr of incubation, supernatants were counted for ⁵¹Cr release using a gamma counter. IL-18 was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Japan).

Induction of Th1 Cell Differentiation

Splenic T cells were purified as described previously (Takeda et al., 1998). In brief, splenic T cells were enriched by two rounds of nylon wool column passage. Enriched T cells were incubated with anti-I-A^b antibody for 30 min on ice and then washed and incubated with rabbit complement (Low-Tox-M, Cedar Lane) for 45 min at 37°C. Cell purity was analyzed by flow cytometry using fluorescein isothicyanate–conjugated anti-CD3 antibody (PharMingen). T cells purified to more than 95% were cultured on anti-CD3 Ab (20 μg/ml)-coated plates in the presence of 2 ng/ml IL-12. After the 4 days of culture, cells were harvested and washed with Hanks' balanced salt solution. Cells (2×10^5) were stimulated on anti-CD3 Ab (20 μg/ml)-coated 96-well plates for 24 hr in the presence or absence of 20 ng/ml IL-18 and/or 2 ng/ml IL-12. Concentration of IFNγ in culture supernatants was determined by ELISA (Genzyme).

In the case of in vivo induction of Th1 cell development, mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes*. Seven days after injection, T cells were purified from spleen and stimulated on anti-CD3 Ab (20 μg/ml)-coated 96-well plates for 24 hr in the presence or absence of 20 ng/ml IL-18. For BCG treatment, mice were intravenously injected with 2 mg of BCG (Kyowa, Japan). T cells were purified and assayed for IFNγ production, as described above, 14 days after injection.

Transfection, Immunoprecipitation, and Western Blot Analysis

COS-7 cells (1×10^6) were transiently cotransfected with a total of 10 μg of the indicated plasmids by Lipofection according to the manufacturer's instructions (Mirus Corporation). After 36 hr, cells were lysed in 500 μl of lysis buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.2). Half of the cell lysate was incubated with 2 μg of anti-FLAG antibody (Eastman Kodak Company) and 40 μl of protein-G Sepharose (Pharmacia) for 12 hr at 4°C, and the other half was incubated with 2 μg of anti-Myc antibody (Santa Cruz) and 40 μl of protein-A Sepharose (Pharmacia). After washing with the lysis buffer, the immunocomplex was eluted by boiling in SDS-PAGE sample buffer, separated on SDS-PAGE, and transferred onto a nitrocellulose filter. The filter was blotted with either the anti-FLAG antibody or the anti-Myc antibody and visualized using the enhanced chemiluminescence system (Dupont).

Expression Plasmids

The C-terminal FLAG-tagged human MyD88 and MyD88 (152–296) (amino acid 152 to 296) cDNAs were obtained by PCR from a human placenta cDNA library and ligated into mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990). The C-terminal Myc-tagged human IL-18R cDNA was obtained by PCR from human IL-18R cDNA as a template and ligated into pEF-BOS. Human IL-18R cDNA was kindly provided by Hayashibara Biochemical Laboratories, Inc.

Reporter Assay

COS-7 cells (1×10^5) seeded on 6-well plates were transiently cotransfected with 1.0 μg of reporter plasmid (pNF-κB Luc or pAP-1 Luc) (Stratagene) together with expression plasmids for IL-18R (0.4 μg) and MyD88 (152–296) (0.6 μg) by Lipofection. The total amounts of DNA were kept constant by supplementation with empty vector (pEF-BOS). After 24 hr of transfection, cells were stimulated with 1 μg/ml of recombinant human IL-18 for 3 hr. The relative NF-κB or AP-1 activity was determined and normalized on the basis of seapansy luciferase activity (pRL-SV40) (Promega). The recombinant human IL-18 was kindly provided by Hayashibara Biochemical Laboratories, Inc.

Gel Mobility Shift Assay

Splenic T cells were purified and cultured with 2 ng/ml IL-12 plus 100 ng/ml anti-CD3 antibody. After 4 days of culture, T cells were washed and starved with RPMI 1640 containing 2% FCS for 3 hr. Cells (1×10^7) were stimulated with 100 ng/ml IL-18 for 30 min. In the case of TNF-α-induced NF-κB activation, freshly isolated thymocytes (1×10^7) were stimulated with 100 ng/ml TNF-α for 30 min. Cells were washed once with phosphate-buffered saline and resuspended in 400 μl of buffer A (10 mM HEPES [pH 7.8], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 μg/ml Aprotinin, 0.5 mM PMSF, and 0.5% Triton X-100). Nuclei were pelleted, and the cytoplasmic proteins were carefully removed. The nuclei were then resuspended in buffer C (50 mM HEPES [pH 7.8], 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 10% Glycerol, 1 mM DTT, 2 μg/ml Aprotinin, and 0.5 mM PMSF). After vortexing and stirring for 30 min at 4°C, the samples were centrifuged, and the nuclear proteins in the supernatant were transferred to a fresh vial. Protein concentration of nuclear extracts was determined by BCA Protein Assay Reagent (Pierce). Double-stranded, NF-κB-specific oligonucleotide probe containing two tandemly positioned NF-κB-binding sites (5'-ATCAGGGACTTTCCGCT GGGGACTTTCCG-3' and 5'-GGCGGAAAGTCCCCAGCGGAAAGTC CCTGAT-3') were labeled with [α-³²P]dCTP by Klenow fragment. Nuclear extract (3 μg) was incubated with a 300 fmol probe in a total of 25 μl of binding buffer (10 mM HEPES [pH 7.8], 50 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, and 2 μg of poly-dIdC) for 20 min at room temperature. For the competition assay, a 50-fold molar excess of unlabeled oligonucleotide probe was added to nuclear extracts for 15 min before addition of the labeled probe. The supershift assay was performed by preincubating with 1 μg of anti-p65 antibody (Santa Cruz) for 60 min at 4°C before addition of the labeled probe. After incubation, samples were fractionated on a 4% polyacrylamide gel in 25 mM Tris-Cl (pH 8.5), 190 mM glycine, and 1 mM EDTA. The gel was subsequently dried and visualized by autoadiography.

In Vitro Kinase Assay

Th1-developing cells prepared as described above (5×10^6) were stimulated with 100 ng/ml IL-18 for 20 min. Freshly isolated thymocytes (5×10^6) were stimulated with 100 ng/ml TNF-α for 20 min. Cells were then lysed in an ice-cold lysis buffer (20 mM Tris-Cl [pH 8.0], 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 20 μg/ml Aprotinin, 20 μg/ml Leupeptin, 1 mM Na₃VO₄, 1 mM EGTA, 10 mM NaF, 1 mM Na₄P₂O₇, and 10 mM β-glycerophosphate). Cell lysates were precleared for 1 hr with protein A-Sepharose and then mixed with 0.5 μg of rabbit anti-JNK1 antibody (Santa Cruz) and protein A-Sepharose for 12 hr. The beads were washed four times with the lysis buffer and once with the kinase assay buffer (25 mM HEPES [pH 7.6], 20 mM MgCl₂, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, and 2 mM DTT). Kinase reactions were carried out in 20 μl of the kinase buffer containing 1 μg of GST-c-Jun (1–79)-fusion protein (Santa Cruz) and 10 μCi [γ-³²P] ATP for 30 min at 30°C. Reactions were terminated by adding 20 μl of 2× SDS-PAGE sample buffer. Proteins were separated on SDS-PAGE and visualized by autoradiography.

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