

Impaired Cytokine Signaling in Mice Lacking the IL-1 Receptor-Associated Kinase¹

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Stimulation of the type 1 IL-1R (IL-1R1) and the IL-18R by their cognate ligands induces recruitment of the IL-1R-associated kinase (IRAK). Activation of IRAK leads in turn to nuclear translocation of NF- κ B, which directs expression of innate and adaptive immune response genes. To study IRAK function in cytokine signaling, we generated cells and mice lacking the IRAK protein. IRAK-deficient fibroblasts show diminished activation of NF- κ B when stimulated with IL-1. Immune effector cells without IRAK exhibit a defective IFN- γ response to costimulation with IL-18. Furthermore, mice lacking the *Irak* gene demonstrate an attenuated response to injected IL-1. Deletion of *Irak*, however, does not affect the ability of mice to develop delayed-type hypersensitivity or clear infection with the intracellular parasite, *Listeria monocytogenes*. These results demonstrate that although IRAK participates in IL-1 and IL-18 signal transduction, residual cytokine responsiveness operates through an IRAK-independent pathway. *The Journal of Immunology*, 1999, 163: 978–984.

Mammals respond to a variety of stressful and pathologic insults with a series of coordinated measures to neutralize the challenge. Infection, trauma, ischemia, neoplasia, and allogeneic organ transplantation all threaten the host's integrity and elicit a complex, multistage protective response. Cytokine signaling regulates this host response to injury. In concert with other cytokines such as TNF- α , IL-1 initiates the innate host immune response, including induction of fever and synthesis of hepatic acute phase proteins (reviewed in Ref. 1). It stimulates neutrophil release into the peripheral circulation (2) and activates vascular endothelium to increase vessel wall adhesiveness to circulating effector cells in the general area of injury (3, 4). IL-1 also directs the secretion of other cytokines, such as TNF- α , IL-6, and IFN- γ .

The IL-1 family of cytokines includes the agonists IL-1 β , IL-1 α , and the IL-1R antagonist (5). Both IL-1 agonists signal through the IL-1R type 1 (IL-1R1)⁴ (6, 7) and initiate a number of downstream events, including nuclear translocation of NF- κ B, a *rel*-related transcription factor that activates expression of many inflammatory and immune response genes (7–9).

IL-1 binding to the IL-1R1 induces the formation of a receptor complex that includes the IL-1R accessory protein (10–12). An

intracellular adapter molecule, MyD88, is then recruited to the complex (13), providing a platform for the IL-1R-associated kinase (IRAK) (14). IRAK subsequently undergoes phosphorylation and interacts with the TNFR-associated factor-6, a downstream transducer required for NF- κ B activation induced by IL-1 (14–16).

IRAK is also involved in IL-18 signal transduction. Though its amino acid sequence and chromosomal localization differ from those of IL-1 α , IL-1 β , and the IL-1Ra, IL-18 shares structural features with these IL-1 family members (17, 18). In conjunction with IL-12, IL-18 supports Th1 lymphocyte development and proliferation (17, 19), enhances NK cell proliferation and activity (20, 21), and induces IFN- γ production in both types of cells (17, 19, 21–23). The ligand binding subunit of the IL-18R is the IL-1R-related protein (24), and IL-18 stimulation of cells also leads to IRAK activation and NF- κ B nuclear translocation (19, 25, 26).

Although the role of IRAK in NF- κ B activation mediated by either IL-1 or IL-18 has not been defined, two lines of evidence suggest that it may be required. First, IRAK is a vertebrate homologue of the *Drosophila* protein kinase Pelle. Pelle is required in a signaling cascade that specifies body axis formation; genetic inactivation of *pelle* completely blocks nuclear localization of Dorsal, the *Drosophila rel* protein that establishes dorsoventral polarity in the embryo (27, 28). Second, experiments involving mutant forms of the IL-1R1 have demonstrated a close association between IRAK activity and NF- κ B activation. Mutations in the cytoplasmic tail of the receptor that block NF- κ B activation also cannot recruit IRAK to the receptor complex (29).

In this report we describe the generation and initial characterization of IRAK-deficient cells and mice. We show that cells lacking IRAK fail to activate NF- κ B in response to IL-1 stimulation. We also show that IL-18 signaling is compromised in IRAK-deficient cells. We further demonstrate that IRAK-deficient animals exhibit an impaired response to IL-1 stimulation. Finally, *Irak*-null mice retain a normal response to *Listeria monocytogenes* infection.

Materials and Methods

Chromosomal mapping of *Irak* locus

Genomic DNA from five mouse YACs spanning the telocentric murine X chromosome from positions 29.4 to 30.5 (C39D5, D7413C, H864F2,

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⁴ Abbreviations used in this paper: IL-1R1, IL-1R type 1; IRAK, IL-1R-associated kinase; MEF, murine embryonic fibroblast; DNFB, 2,4-dinitrofluorobenzene; CBC, complete blood count; WT, wild type; KO, knockout; YAC, yeast artificial chromosome; ES cell, embryonic stem cell.

C176B11, and B7S6, provided by A. Chatterjee, Houston, TX) (30) were screened using PCR. The upstream primer corresponded to intron 5 and had the sequence TTAAGAAGCTCTGTTT. The downstream primer was from exon 6 and had the sequence CCTCCATAGATTTGG. These primers amplified a 137-bp fragment. PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide.

Generation of IRAK-deficient mice

Two overlapping clones spanning the murine *Irak* locus were isolated from a 129 Sv genomic library using the human IRAK cDNA as a probe. A targeting vector was constructed which replaced 7 kb of the *Irak* gene, including 3.5 kb of the 5' regulatory region, exons 1–8, and most of exon 9 with a neomycin resistance gene and included the HSV-*tk* gene in the vector sequence.

E1.1C ES cells (from K. Graves, Dallas, TX) were cultured on mitotically inactivated STO cells (from J. Herz, Dallas, TX) in DMEM supplemented with 15% FCS, penicillin-streptomycin, L-glutamine, nonessential amino acids, and 2-ME. ES cells were electroporated in medium with linearized targeting vector (50 μ g/ml). Transformants were selected in G418 and ganciclovir, and doubly resistant clones were screened by Southern blot hybridization of *Nde*I-digested DNA and a random-primed 3'-flanking probe and were confirmed using both a 5'-flanking probe and neomycin resistance gene probe.

Chimeric mice were produced from embryos injected with targeted ES cells. Male chimeras were bred with C57BL/6 females. Germline transmission of injected ES cells was determined by agouti coat color and the presence of the targeted locus determined by Southern blotting of tail genomic DNA. Heterozygous females were then crossed back to chimeric founders to yield homozygous and hemizygous null mice. Subsequent generations were produced by sibling-sibling intercrosses.

Generation of IRAK-deficient fibroblasts

IRAK-deficient murine embryonic fibroblasts (MEFs) were obtained from 11.5 day postconception embryos and selected and transformed as described previously (31). Neomycin-resistant MEFs derived from the same ES cell subclone (provided by J. Herz) and prepared using the same methods served as controls for all experiments.

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

Spleens were harvested from IRAK-deficient mice and heterozygous littermates and transferred to medium (RPMI 1640 supplemented with 10% fetal bovine serum, 50 μ M 2-ME, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, and 50 μ g/ml streptomycin). Spleen cells were dispersed by grinding the spleens between two frosted glass slides. After filtration of the ground spleen through a cell strainer, RBC were removed by hypotonic lysis. Spleen cells (4×10^6 cells/ml) were washed with medium and stimulated with immobilized α -CD3 mAb (1 μ g/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.

Immunoprecipitation and Western blot hybridization

MEFs grown to confluence in 100-mm plates were collected and incubated in 1 ml of lysis buffer on ice with occasional rocking for 30 min. The suspension was centrifuged at $2000 \times g$ for 10 min. Immunoprecipitation and immunoblotting of murine IRAK protein using rabbit polyclonal antisera raised against human IRAK were performed essentially as described for human IRAK (12).

In vitro response to IL-1 β and EMSA

IRAK-deficient and control MEFs were grown to confluence in 100-mm plates. Recombinant human IL-1 β (10 ng/ml), TNF- α (10 ng/ml), or no cytokine was added to the medium. Nuclear extracts were prepared 30 min after incubation with the cytokine as previously described (32) and were tested for the ability to shift the electrophoretic mobility of a 32 P-radio-labeled oligonucleotide containing an NF- κ B binding site derived from the human Ig κ -chain promoter.

Delayed-type hypersensitivity response

Mice were sensitized to 2,4-dinitrofluorobenzene (DNFB) and challenged later, essentially as previously described (33). For sensitization, shaved abdomens of mice were treated with 25 μ l of 0.5% DNFB in acetone/olive oil (4/1). Seven days later, baseline ear thickness was measured, and one

ear from each mouse was challenged with 0.2% DNFB (10 μ l/side of ear pinna). Twenty-four hours later, ear thickness was measured three times, and the average change from baseline was recorded.

In vivo response to IL-1 β injection

Before cytokine injection, 20 μ l of whole blood was obtained from each animal for complete blood counts (CBC) and differential leukocyte count. Animals were then injected with recombinant murine IL-1 β i.p. (R&D Systems, Minneapolis, MN). One and a half hours after cytokine injection, serum was obtained from half the animals for subsequent cytokine analysis. Three hours postinjection, serum was obtained from the remaining half of the injected animals. Six hours after injection, an additional 20 μ l of whole blood was drawn from each animal for CBC and differential count determinations. Twenty-four hours after injection, animals were euthanized, and serum was collected. Hybrid (C57BL/6 \times 129 Sv/J) wild-type (WT) and knockout (KO) mice from F₃ and F₄ generations were used in these experiments. Serum IL-6 and TNF- α concentrations were measured using ELISA (R & D Systems).

CBC was measured using an automated cell counter (Coulter Electronics, Hialeah, FL). Differential counts were determined manually, and absolute numbers of polymorphonuclear leukocytes were calculated by multiplying the total white blood cell count by the fraction of neutrophils plus the fraction of band forms.

L. monocytogenes infection

Virulent *L. monocytogenes* isolated from homogenized mouse liver (provided by C. Lu, Dallas, TX) was grown in brain-heart infusion broth and frozen at -70°C in 200- μ l aliquots in 25% glycerol at a density of 3×10^9 CFU/ml.

Mice were injected i.p. with live *Listeria* organisms. For determination of lethal dose, six groups of five WT mice each were injected with 3×10^3 , 3×10^4 , 3×10^5 , 3×10^6 , and 3×10^7 CFU/25 g and monitored daily for survival. The WT and KO mice were then injected with 2×10^4 , 2×10^5 , 1.2×10^6 , and 1.2×10^7 CFU/25 g and were monitored daily for survival. For determination of chronic infection, mice surviving infection with 2.5 – 5.0×10^6 CFU/25 g were sacrificed 45–60 days after infection, and bacterial organ content was determined. After sacrifice, livers and spleens were removed, weighed, and homogenized in PBS. Lysates were plated as serial 10-fold dilutions on trypticase soy agar plates, and bacterial colonies were counted to determine organ bacterial load (CFU per 100 mg of tissue). For sterilizing immunity experiments, mice surviving and initial infection of 2×10^5 CFU/25 g were rechallenged with a lethal dose (3×10^7 CFU/25 g), and survival was monitored for 2 wk.

Results

Targeted disruption of Irak

Previous mapping of a panel of hamster-human radiation-induced hybrids (J. L. Allen, unpublished observations) as well as sequencing of four overlapping cosmids covering the human MeCP2 locus by others (GenBank accession no. AF030876) localized *Irak* to Xq28. This region is syntenic to positions 29.5–29.7 on the murine X chromosome (34).

As a first step in making an IRAK-deficient mouse, we wished to learn whether murine *Irak* also mapped to the X chromosome. Using DNA from five contiguous YACs spanning Xq29.4 to Xq30.5 (30), we assigned murine *Irak* to a 50-kb region of overlap between the telomeric end of YAC D741C3 and the centromeric end of B7S6, between the genes for the type 2 vasopressin receptor (Avp2r; Xq29.52) and red-sensitive visual pigment (Rsvp; Xq29.7; Fig. 1). The amplified region contains sequence from the N-terminal region of the kinase domain and an adjacent intron. This location falls within the broader map position for *Irak* recently defined using interspecific backcross analysis (35). Examination of known X-linked human and mouse diseases and syndromes in this region failed to identify any phenotypes expected for a mutation affecting a cytokine signaling molecule.

IRAK-deficient ES cells were then generated using standard homologous recombination techniques. The targeting vector substituted a neomycin resistance gene (*neo*^r) in place of 7 kb of the *Irak* gene, including upstream regulatory regions, transcriptional and

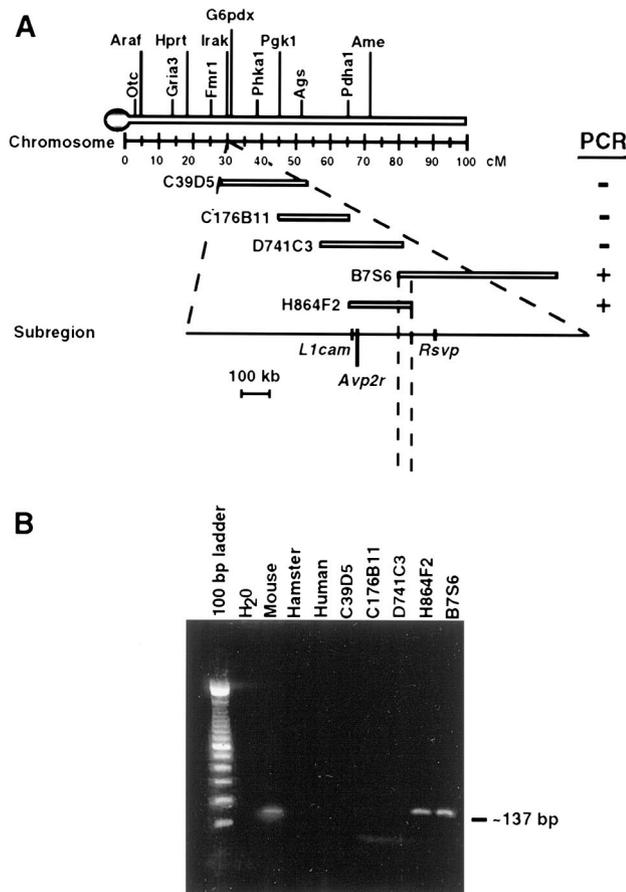


FIGURE 1. *Irak* maps to a 50-kb region in murine Xq29. **A**, Chromosomal localization of the YAC contig spanning the *Irak* locus. The top line represents the telocentric mouse X chromosome. The centromere is at the left end of the figure. Genes with assigned loci are shown above the chromosome. The scale beneath the figure is in centimorgans. The second line depicts the segments of the chromosome contained in the five YACs. The YACs and their positions relative to the chromosomal subregion are located above the second line. DNA from the five overlapping YACs was screened for the presence of the *Irak* gene using PCR primers complementary to sequence within the kinase domain and an adjacent intron. Amplification of the target sequence occurred with YACs B7S6 and H864F2. **B**, Agarose gel showing products of PCR reaction with YAC and control templates, including mouse, hamster, and human genomic DNA.

translational start sites, and exonic sequence (Fig. 2A). The targeting plasmid was transfected into E1.1C ES cells, which were then cultured in the presence of G418 and ganciclovir. A single clone bearing the targeted allele was identified among 1100 doubly resistant transformants screened by Southern blot hybridization with the downstream flanking probe (Fig. 2B, lane 16). Targeted *Irak* gene disruption was confirmed with Southern hybridization using the upstream flanking probe and a probe to the *neo^r* gene (data not shown). The mutant ES clone was expanded and injected into C57BL/6 blastocysts.

Deletion of *Irak* blocks IL-1 signaling to NF- κ B

Because most ES cells, including the ones we used, have an XY karyotype, mutating a gene on the X chromosome leaves these cells and their descendants without a WT copy. To determine the effect of deleting *Irak* on IL-1 signaling, we isolated MEFs from chimeric embryos, using G418 to select for *Irak*-null MEFs. For control cells, we used MEFs derived from E1.1C ES cells bearing a *neo^r* gene flanked by two *loxP* sites adjacent to an unrelated

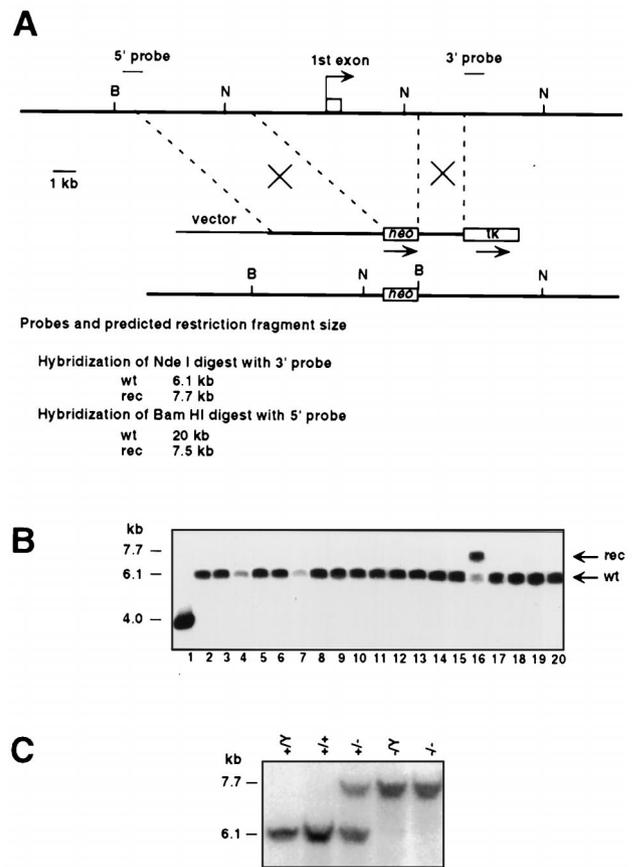


FIGURE 2. Targeted disruption of *Irak* gene. **A**, Targeting strategy. The top line shows the WT *Irak* locus with selected restriction sites. The middle line depicts the targeting vector that replaces 7 kb of the *Irak* gene with a neomycin resistance gene. A single HSV-*tk* gene was included for negative selection with ganciclovir. The bottom line represents the correctly targeted *Irak* locus. Probes used to screen for and confirm homologous recombination between the WT *Irak* locus and the targeting vector are also shown. Restriction enzyme sites: B, *Bam*HI; N, *Nde*I. **B**, Targeted disruption of *Irak*. Southern blot of ES cell genomic DNA digested with *Nde*I and hybridized with ³²P-labeled 3'-flanking probe. Lane 16 contains DNA from the correctly targeted ES cell clone. The presence of a faint band with WT mobility in the same lane probably represents residual feeder cell DNA. Lane 1 contains probe control DNA. All other lanes exhibit a WT restriction pattern. **C**, Southern blot analysis of mice showing wild-type (lanes 1 and 2), heterozygous (lane 3), and *Irak*-null (lanes 4 and 5) mice. DNA was prepared from tails of 3-wk-old animals, digested, and hybridized as in panel B.

autosomal locus (36). MEFs obtained from chimeric embryos and surviving G418 selection contained the targeted allele, whereas control cells exhibited a WT restriction pattern (Fig. 3A). Furthermore, KO MEFs do not express the IRAK protein, while control cells do (Fig. 3B).

We assayed the effect of recombinant murine IL-1 β or TNF- α on NF- κ B activity using EMSA (Fig. 3C). Control WT cells have little or no detectable NF- κ B activity in the resting state. Stimulation with either IL-1 β or TNF- α leads to NF- κ B activation in these cells. IRAK-deficient MEFs, like control cells, show little baseline NF- κ B activity. When stimulated with IL-1 β , however, the IRAK-deficient cells showed no increase in NF- κ B activity. Furthermore, KO fibroblasts retain the ability to respond to TNF stimulation with NF- κ B activation, although the intensity of the response was attenuated compared with that in control cells. These

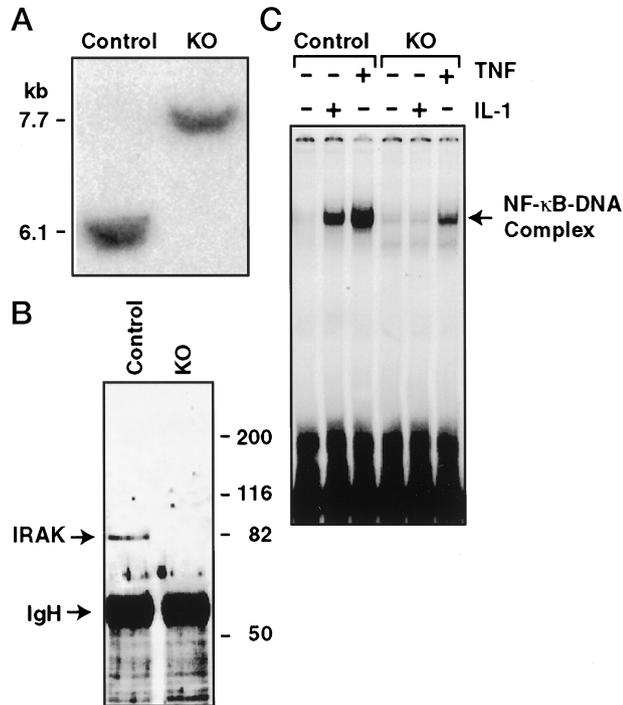


FIGURE 3. IRAK is necessary for IL-1 signaling to NF- κ B. Control and KO MEF lines were prepared and immortalized as described in *Materials and Methods*. *A*, Southern blot analysis of genomic DNA from different MEF lines showing WT and KO restriction patterns. *B*, KO MEFs do not express IRAK protein. Cell extracts from control and KO MEF lines were immunoprecipitated using antiserum against human IRAK protein, fractionated by SDS-PAGE, blotted, and probed with the anti-IRAK antiserum. *C*, IL-1 activation of NF- κ B is blocked in IRAK-deficient cells. Control and KO MEF lines were stimulated with saline, IL-1, or TNF, and nuclear extracts were examined for NF- κ B-DNA binding activity using EMSA.

results suggest that the IRAK protein transmits the IL-1 signal to NF- κ B at the doses assayed.

IRAK-deficient mice are viable and fertile

Injection of mutant ES cells into host blastocysts yielded seven high percentage chimeric mice, as determined by agouti coat color. Two male chimeras transmitted the allele through the germline. F₁ females bearing the targeted allele were backcrossed to the male chimeras to produce homozygous and hemizygous null mice. Fig. 2C shows a typical Southern blot identifying WT, heterozygous, and KO genotypes.

Mice lacking a wild-type *Irak* gene appear healthy when housed in clean conditions (filter-top cages, autoclaved bedding and water, and irradiated food). They are indistinguishable from WT and heterozygous littermates and grow normally. To date, KO animals have reached 24 mo of age without apparent ill effects. Their organs appear normal in size, morphology, and relation. Histologic examination of lymphoid organs from immunologically naive animals as well as heart, liver, and kidney uncovered no differences between WT and IRAK-deficient animals. Flow cytometric analysis of major leukocyte subpopulations from bone marrow, lymph nodes, Peyer's patches, thymus, spleen, and peripheral blood showed normal numbers of lymphocytes, macrophages, and granulocytes in both WT and KO mice. IRAK-deficient animals breed well and have normal-sized litters.

Irak-deficient mice exhibit an attenuated response to IL-1

In humans and other mammals IL-1 causes fever, cytokine secretion (including IL-6 and TNF- α), and reactive neutrophilia among other responses (5). Inhibition of IL-1 signaling by passive immunization against IL-1, IL-1R1 antagonism (37, 38), or genetic deletion of the cytokine or the type I receptor (39–41) decreases or abolishes these responses. To determine whether IRAK mediates these IL-1 responses, we challenged WT and IRAK-deficient mice with IL-1 β and measured serum cytokine concentrations and neutrophils in peripheral blood.

We injected animals with three i.p. doses of murine IL-1 β (1, 10, and 20 μ g/kg) and assayed serum IL-6 and TNF- α concentrations at 1.5 and 3 h after injection. Before injection of IL-1, we performed baseline CBCs in animals receiving the highest IL-1 dose. We avoided sampling more than 200 μ l of whole blood (10% of the estimated blood volume of a 25-g mouse) during the 6-h test period to prevent introducing a stress response associated with hypovolemic or hemorrhagic shock. Neither WT nor KO mice had detectable circulating IL-6 or TNF- α before IL-1 stimulation (data not shown). One and a half hours after IL-1 injection, WT mice showed a marked increase in the serum IL-6 response (Fig. 4A). IRAK-deficient mice also exhibited an IL-6 response to IL-1 administration, but this response was significantly attenuated compared with that of WT animals at the three doses tested (Fig. 4A). By three hours after injection, IL-6 concentrations in WT animals had declined and no longer differed from those in KO animals (data not shown).

We also examined the TNF response to IL-1 β in these animals at the two higher doses. One and a half hours after IL-1 β injection, WT mice exhibited moderate increases in serum TNF- α concentrations, whereas KO animals showed a diminished TNF response at both doses (Fig. 4B). In fact, TNF was undetectable in one of six animals tested with the 10 μ g/kg dose and barely exceeded the ELISA detection threshold (>23.4 pg/ml) for four of the remaining five animals (Fig. 4B). We did not assay TNF- α concentrations in the mice treated with the lowest IL-1 dose. Therefore, IRAK also appears to mediate a modest, but definite, increase in serum TNF- α concentrations following systemic IL-1 β administration. Together, these data demonstrate that IRAK-deficient mice display impaired early cytokine responsiveness to parenterally administered IL-1 β .

IL-1 mediates the translocation of neutrophils from the bone marrow to the peripheral circulation in response to infection or injury. We therefore examined reactive neutrophilia, the acute elevation of neutrophils in circulating blood, in response to IL-1 stimulation to determine whether deletion of *Irak* affected this process. Six hours after IL-1 injection, we determined the CBC and calculated the percent change in neutrophils from baseline. The WT animals responded to 20 μ g/kg of IL-1 β with an average 10-fold increase in circulating neutrophils, whereas KO animals exhibited a significantly smaller increase, approximately one-third that of WT animals (Fig. 4C). The attenuation of IL-1-induced neutrophilia in KO animals further supports the hypothesis that deletion of *Irak* impairs IL-1 signaling in vivo and suggests that IRAK-deficient mice may have impaired defenses against a variety of environmental insults.

IRAK-deficient immune effector cells exhibit reduced responsiveness to IL-18

To determine the function of IRAK in IL-18 signaling, we isolated WT and KO splenocytes and measured their ability to produce IFN- γ in response to stimulation with IL-12 and IL-18. The WT and KO splenocytes produced no detectable IFN- γ when treated

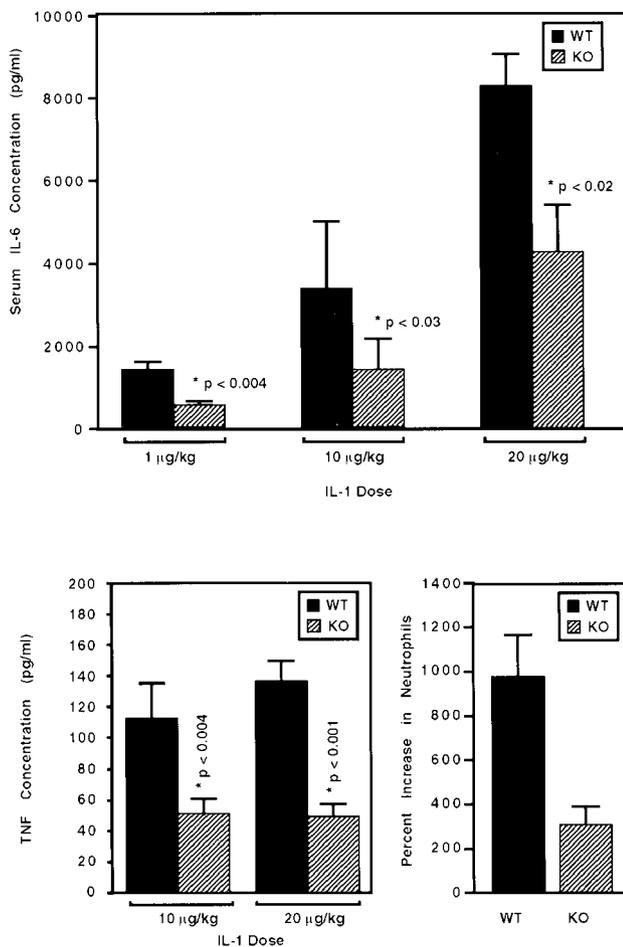


FIGURE 4. Attenuation of IL-1-mediated responses in IRAK-deficient mice. Mice were injected i.p. with recombinant murine IL-1 β , and serum cytokine and peripheral blood neutrophil concentrations were measured at 1.5 and 6 h, respectively. Data are represented as the mean \pm SEM. *A*, IL-6 response to IL-1 at 1.5 h. *B*, TNF- α response to IL-1 at 1.5 h. *C*, Neutrophil response to IL-1 at 6 h. Peripheral neutrophil counts were calculated from whole blood obtained from 12 WT and 12 KO mice treated with 20 μ g/kg of IL-1 β . *, $p < 0.005$.

with IL-18 alone (Fig. 5). Treatment with IL-12 alone resulted in the production of equal amounts of IFN- γ by both WT and KO cells. When stimulated with both IL-12 and increasing doses of IL-18, however, WT splenocytes produced increasing amounts of IFN- γ . Splenocytes from KO mice also secreted increasing amounts of IFN- γ in response to IL-12 and IL-18 costimulation, but only approximately half that produced by WT cells at each dose assayed (Fig. 5). These findings assign IRAK a role in the response of immune effector cells to IL-18.

IRAK-deficient mice display normal delayed-type hypersensitivity

We have also begun to investigate the role played by IRAK in the immune response. We first examined the development of delayed-type hypersensitivity, using contact hypersensitivity as an experimental model. Development of a measurable reaction to a contact immunogen depends on several distinct steps, including Ag processing and presentation, T lymphocyte presence and activation in the lymph node draining the sensitized skin, T cell movement to skin where rechallenge occurs, and the movement of other effector cells to the site of rechallenge (33). IRAK-deficient mice exhibit a

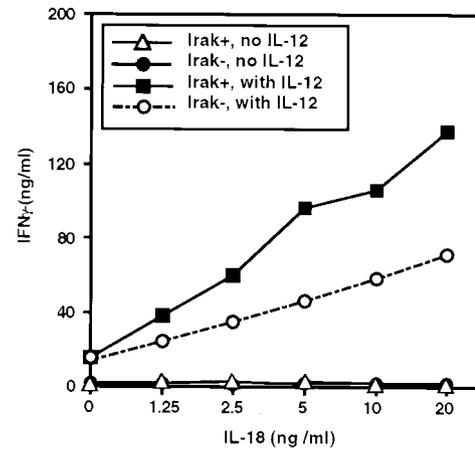


FIGURE 5. Reduced IL-18 responsiveness of IRAK-deficient splenocytes. Splenocytes from WT and KO mice were harvested and activated with anti-CD3 mAb. Activated splenocytes were treated with increasing doses of IL-18 alone or IL-18 and IL-12. After 24 h, supernatants were removed and assayed for IFN- γ activity using ELISA. Each data point on the graph represents the average IFN- γ concentration of supernatants from splenocytes derived from two mice of each genotype in two separate experiments performed on different dates.

brisk contact hypersensitivity response to DNFB, indistinguishable from that of WT controls (data not shown). IRAK is thus not essential in the aforementioned processes involving identification of this Ag and response to repeated exposures.

IRAK-deficient mice have a normal response to *Listeria* infection

Because both IL-1 and IL-18 have been implicated in the host response to infection with intracellular parasites (41–45), we examined the responses of IRAK-deficient mice to *Listeria* infection. *L. monocytogenes* is a facultative, Gram-positive intracellular bacterium. When administered a sublethal inoculum of *Listeria*, WT mice clear the infection and develop the ability to eliminate a second infection much more quickly (sterilizing immunity). We injected WT and KO animals with sublethal (1.2×10^6 CFU/25 g) and lethal (1.2×10^7 CFU/25 g) doses and recorded mortality. No difference in mortality, either in time to death or total numbers of animals per group, were found between WT and IRAK-deficient mice (Table I). Furthermore, we observed no difference in numbers of live *Listeria* recovered from the livers and spleens from either WT or IRAK-deficient mice 3 days after infection (Table II).

We also examined the livers and spleens of infected animals surviving >1 mo to determine whether IRAK-deficient mice had cleared the infection or remained chronically infected. No *Listeria*

Table I. Mortality in mice infected with *L. monocytogenes*^a

Inoculum (cfu/25 g)	Mortality			
	Naive		Sensitized	
	WT	KO	WT	KO
10^7	4/5	5/5	6/10	4/10
10^6	0/5	0/5	ND	ND
10^5	0/10	1/10	ND	ND
10^4	0/10	0/10	ND	ND

^a Naive mice were infected with increasing doses of virulent bacteria and mortality recorded at 7 and 10 days. All mice had died by day 7. Mice previously infected with sublethal inoculum (10^5 cfu/25 g) were rechallenged with a lethal dose, and mortality was monitored over a 10-day period. Organs were harvested from surviving animals at 10 days, and no *Listeria* was recovered. ND, not determined.

Table II. Bacterial organ load in mice infected with *Listeria*^a

	Bacterial Organ Load	
	WT	KO
Spleen	$4.5 \times 10^6 (\pm 3.1 \times 10^6)$	$7.5 \times 10^6 (\pm 1.9 \times 10^6)$
Liver	$2.5 \times 10^5 (\pm 1.9 \times 10^5)$	$4.9 \times 10^5 (\pm 1.7 \times 10^5)$

^a Mice were inoculated with $2.5\text{--}5.0 \times 10^6$ cfu/25 g of virulent *Listeria* in two separate experiments and sacrificed 24 h after infection. Livers and spleens were recovered, homogenized, and plated in serial dilutions on trypticase soy agar, and colonies were counted. Values represent mean cfu/g tissue (\pm SEM) from six different animals. Differences between WT and KO animals are not statistically significant by Student's *t* test.

grew from organ homogenates from either WT or KO mice (data not shown). Finally, we tested the ability of mice immunized with a sublethal dose (2×10^5 CFU) of *Listeria* to withstand a subsequent lethal challenge of the same organism. When injected with 2.3×10^7 CFU (approximately twice the dose causing 100% mortality in previously unchallenged WT mice), 6 of 10 KO animals and 4 of 10 WT mice survived (Table I). Thus, IRAK neither mediates mortality to overwhelming *Listeria* infection nor is it required for the development of sterilizing immunity to *Listeria* in mice.

Discussion

IRAK was first identified as a kinase that is recruited to the IL-1R complex after IL-1 treatment of cells (14, 29). Stimulation of responsive cells with IL-1 or a related cytokine, IL-18, induces rapid IRAK phosphorylation (14, 15, 19), suggesting that IRAK is a likely participant in the transduction of both cytokine signals. The studies reported here confirm the role of IRAK in IL-1- and IL-18-induced responses. IRAK-deficient mice exhibit impaired TNF- α and IL-6 secretion when administered IL-1. Mutant animals are also less capable of mobilizing neutrophils in response to injected IL-1 than their WT counterparts. Furthermore, in acute experiments, IL-18-treated splenocytes from IRAK-deficient mice produce approximately half the IFN- γ of those produced by their WT counterparts.

We generated IRAK-deficient mice from a single mutant ES cell clone, raising the possibility that the impaired cytokine responsiveness could be due to other mutations introduced into ES cells during transfection and selection and not to specific inactivation of the *Irak* gene. Two circumstances argue against this eventuality. First, another independent research group has targeted the *Irak* locus and described essentially identical findings. They report impaired IL-1 responsiveness in fibroblasts isolated from IRAK-deficient embryos and postnatal mice (46) and diminished biochemical and biological responses to IL-18 in mice, Th1 lymphocytes, and NK cells lacking IRAK (47). Furthermore, defective cytokine responses persist in our mice at the F₆ generation, suggesting, although not confirming, that only the mutant *Irak* locus is responsible for the phenotype seen in our animals.

The defective cytokine and neutrophil responses in IRAK-deficient animals and the impaired IFN- γ production by splenocytes isolated from mice lacking IRAK may be due partly or entirely to lack of optimal NF- κ B activation induced by IL-1 and IL-18. We have shown that fibroblasts lacking IRAK do not activate NF- κ B in response to IL-1, but retain the ability to do so with TNF treatment. Kanakaraj et al. (46) also demonstrate attenuation of IL-1-induced NF- κ B activation in IRAK-deficient fibroblasts, but report overriding this diminished responsiveness with increasing doses of IL-1. At 10 ng/ml (the maximum dose used by Kanakaraj et al.), we still see marked down-regulation of signal-induced NF- κ B ac-

tivation, although we have also observed partial restoration of NF- κ B responsiveness to IL-1 in cells that have undergone prolonged passage in culture (data not shown).

Deletion of IRAK, therefore, attenuates, but does not eliminate, the cytokine responsiveness of mutant mice and cells. These results contrast with those seen in mice lacking MyD88, the adapter protein that provides a platform for IRAK recruitment to the activated receptor complex. MyD88-deficient animals produce no IL-6 or TNF when given IL-1, and cells from these mice fail to respond to IL-18 (48). These results suggest that while MyD88 mediates the known biological functions of IL-1 and IL-18, IRAK is not strictly required for these same functions. The presence of IRAK results in optimal signaling through both receptors, but its absence does not abolish signal transduction. Therefore, signal initiated at the IL-1R1, IL-18R, and human Toll-like receptor-4 (49) must pass through MyD88. In IRAK-deficient mice, the signal proceeds through an IRAK-independent pathway but loses strength. The signaling mechanism operative in mice lacking IRAK may represent a compensatory response to IRAK deletion or may reflect a bifurcation of the signal that occurs in the WT downstream of MyD88. Furthermore, this alternate route may be mediated by an IRAK-like molecule, such as IRAK2 (50), or may operate through an unrelated mechanism. Both redundancy and compensation could explain why mice can still produce IL-6 and TNF and mobilize neutrophils in response to IL-1 as well as retain delayed hypersensitivity and clear *Listeria* infections.

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