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The wild-derived inbred mouse strain SPRET/Ei is resistant to LPS and defective in IFN-β production

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Running title: LPS hyporesponsiveness of SPRET/Ei mice.

Abbreviations used: LPS, lipopolysaccharide; TLR, Toll-like receptor; STAT, signal transducer and activator of transcription; IFN, interferon; IFNAR, interferon (alpha and beta) receptor; BMDM, bone marrow-derived macrophages; PAMP, pathogen-associated molecular pattern; DCs, dendritic cells; TNF, tumor necrosis factor; IL, interleukin; TIR, Toll/IL-1 receptor domain; MyD88, myeloid differentiation factor 88; TIRAP, TIR-containing adaptor protein; MAL, MyD88-adaptor like; TRIF, TIR domain-containing adaptor inducing IFN; TICAM, Toll-like receptor adaptor molecule; TRAM, TRIF-related adaptor molecule; IRAK, IL-1 receptor associated kinase; TRAF, TNF receptor-associated factor; IKK, I-κB kinase; NF-κB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; poly(I-C), polyinosine-polycytidylic acid; IRF, IFN regulating factor; CBP, CREB-binding protein; TBK, TANK-binding kinase; JAK, janus kinase; TYK2, tyrosine kinase 2; ISGF-3, IFN-stimulated gene factor-3; ISRE, IFN-stimulated response element; OAS, 2'-5' oligoadenylate synthetase; B, C57BL/6; S, SPRET/Ei; MEF, mouse embryonic fibroblast; CXCL10, chemokine (C-X-C motif) ligand 10 10; ISG15, interferon-stimulated gene 15; I-κB, inhibitor kappaB.

Abstract.

Although activation of TLR4-positive cells is essential for eliminating Gram-negative bacteria, over-activation of these cells by the TLR4 ligand lipopolysaccharide (LPS) initiates a systemic inflammatory reaction and shock. Here we demonstrate that SPRET/Ei mice, derived from *Mus spretus*, exhibit a dominant resistance against LPS-induced lethality. This resistance is mediated by bone marrow-derived cells. Macrophages from these mice have normal MyD88-dependent responses, but they are impaired for Interferon-β (IFN-β) production and downstream signaling. IRF-3 phosphorylation and gene induction are normal. The defect appears to be specific for IFN-β, although the SPRET/Ei IFN-β promoter is normal. *In vivo* IFN-β induction by LPS or influenza virus are very low in SPRET/Ei, IFN-β-treatment restores the sensitivity to LPS and IFNAR1-/- mice indeed also resist LPS. Due to the defective induction of IFN-β, these mice are completely resistant to *Listeria monocytogenes* and highly sensitive to *Leishmania major* infection. Stimulation of SPRET/Ei macrophages leads to rapid downregulation of the IFNAR1 mRNA expression, which is reflected in poor induction of IFN-β-dependent genes. This indicates that the resistance of SPRET/Ei to LPS is due to disruption of the positive-feedback loop for amplifying IFN-β production. In contrast to TLR4-deficient mice, SPRET/Ei mice resist both LPS and sepsis induced with *Klebsiella pneumoniae*.

Introduction.

Bacterial lipopolysaccharide (LPS), a Gram-negative cell wall component, is a potent activator of macrophages and dendritic cells (DCs)(1-4). Genetic studies identified that TLR4 is the LPS receptor (5), and that two LPS resistant mouse strains, C3H/HeJ and C57BL10/ScCr, are defective in TLR4. Activation of macrophages by LPS results in the release of a variety of inflammatory cytokines, interferons, and chemokines(6). These molecules initiate inflammation and both innate and adaptive immune responses aimed at clearing the infection. However, cytokine production must be kept tightly controlled, because excessive production may lead to an exaggerated inflammatory response and consequent lethal shock(7).

Signal transduction through TLRs requires the presence of a Toll/IL-1R (TIR) domain found in the cytoplasmic domain of TLRs and the IL-1 receptor. Several TIR domain-containing adaptor proteins can be recruited to the TIR domains of TLRs: MyD88, TIRAP/MAL, TRIF/TICAM-1 and TRAM/TICAM-2(8-12). The association of MyD88 and TIRAP with the TIR domain of TLR4 results in recruitment of IL-1R-associated kinase (IRAK), which associates with TNF receptor-associated factor (TRAF)6(13, 14). This leads to activation of the canonical IKK-αβγ complex, and ultimately to activation of Rel/NF-κB transcription factors and mitogen-activated protein (MAP) kinase family members(11). TLR3 and TLR4 can signal independently of MyD88 to induce IFN-β and, to a lesser extent, NF-κB and MAPK activation(15-17). Genetic studies on mice demonstrated that TRIF is crucial in the MyD88-independent pathway that is shared by TLR3 and TLR4, and that TRIF is involved in the activation of interferon responsive factor-3 (IRF-3)(9, 18). IRF-3 is activated by phosphorylation by the non-canonical IκB kinases (IKKs), TANK-binding kinase 1

(TBK1) and IKK-ε (also called IKK-ι)(19-21). This causes IRF-3 to homodimerize and interact with the co-activators CREB-binding protein (CBP) and p300(22, 23). The complex then translocates to the nucleus, where it activates promoters containing IRF-3 binding sites(24), such as the type I IFN promoters. Secreted IFN binds and stimulates the two receptor sub-units IFNαR1 and IFNαR2(25). This leads to activation of the tyrosine kinases, JAK-1 and TYK2 and the signal transducer and activators of transcription-1 (STAT1) and STAT2 to form a STAT1/STAT2 heterodimer(26). STAT1/STAT2 complexes associate with IRF-9 to form the interferon-stimulated gene factor-3 (ISGF-3). ISGF-3 recognizes interferon stimulated response elements (ISREs) in promoter regions of interferon responsive genes, e.g. *Isg15* (encoding the protein IP17), *Cxcl10*, *Irf7* and *Oas*(27). IRF-7, alone or in combination with IRF-3, can bind to the promoter region of type I IFNs, IFN-α/β. So a positive feedback loop is responsible for the *en masse* induction of IFN-α/β after exposure to LPS(28). Although first characterized by their potent antiviral functions, IFN-α and -β also mediate a variety of immune regulatory effects. These immune modulating functions indicate that type I IFNs may form an important link between innate and adaptive immune responses(29). Recently, targeted mutations have generated mice, deficient in their ability to produce type I IFNs or to respond to them, and several of these mutant mice have proved to resist in models of inflammation. This was the case for TRIF(18), IFN-β and Tyk2 knockouts(30), all of which resist against LPS. These data illustrate that type I IFNs play an important mediating role in inflammatory processes.

Several spontaneous and induced mutant mouse strains defective in LPS signaling have been described. Most of them are extremely resistant to LPS-induced lethal shock but hypersensitive to bacterial infection, for example C3H/HeJ and

C57BL/10ScCr, both of which carry mutations in *Tlr4*(5). Despite their endotoxin-resistance, TLR4-deficient C3H/HeJ mice are extremely susceptible to infection by Gram-negative bacteria, most probably because the mutant TLR4 protein fails to sense the bacteria, thereby failing to activate innate immune responses. We here describe our findings concerning the LPS resistance of the strain SPRET/Ei, an inbred strain derived from *Mus spretus*, to LPS-induced lethal shock.

Results.

SPRET/Ei mice are very resistant to LPS.

To determine the response of SPRET/Ei, C57BL/6 and F_1 mice to LPS, we injected mice intraperitoneally (i.p.) with increasing amounts of LPS. Compared to C57BL/6, SPRET/Ei mice were highly resistant to the lethal effects of LPS (Fig. 1A). For C57BL/6 mice, LD_{50} is 25 μg and LD_{100} is 50 μg, but all SPRET/Ei and F_1 mice readily survived a dose of 500 μg LPS, which also shows that the LPS hypo-responsiveness is a dominant trait.

In vivo response of SPRET/Ei, F_1 and C57BL/6 mice to LPS.

To study the resistance of SPRET/Ei mice in more detail, a dose of 250 μg of LPS was given i.p. After injection of LPS, the body temperature of C57BL/6 mice decreased dramatically. SPRET/Ei and F_1 mice were significantly protected against LPS-induced hypothermia (Fig. 1B). Injection of LPS induces high concentrations of IL-6 in serum(31). After injection of LPS, serum IL-6 levels were determined. LPS induced high amounts of IL-6 in C57BL/6, SPRET/Ei and F_1 mice. However, the serum concentrations in SPRET/Ei and F_1 mice remained significantly lower than those in C57BL/6 mice, especially at later time points (Fig. 1C). Based on the dominant nature of the phenotype, F_1 mice were used to generate a set of 140 (C57BL/6 x SPRET)F_1 x C57BL/6 backcross mice (N2). The mice were injected with a high dose of LPS. All mice were also genotyped using a set of 70 different polymorphic microsatellites and a linkage study was performed. One third of the N2 mice died and significant linkage of the phenotype was found with 5 different chromosomes. So, inheritance is complex, non-Mendelian. After we had generated and studied consomic mice by isolating the relevant SPRET/Ei chromosomes in a

C57BL/6 background, we had to conclude that the very strong phenotype entirely depends on epistatic interactions between the different loci, and that cloning of the responsible genes by a positional cloning strategy was virtually impossible.

Bone marrow-derived cells are critically involved in the LPS resistance of F_1 mice.

Macrophages are the first targets of LPS(32). To study whether macrophages are relevant for the LPS resistance, F_1 mice were lethally irradiated and reconstituted with bone marrow from C57BL/6 or F_1 mice. Unfortunately, lethally irradiated C57BL/6 mice could not be reconstituted with F_1 bone marrow due to transplant rejection. The mice where challenged with 250 µg of LPS 8 weeks after the bone marrow transplant. All of the F_1 mice reconstituted with F_1 bone marrow survived (n = 5), while all of those reconstituted with C57BL/6 bone marrow (n = 4) died within less than 24 h (Fig. 1D). Furthermore, LPS induced high amounts of IL-6 in F_1 mice reconstituted with C57BL/6 bone marrow (3852 ± 2152 ng/ml), while the serum concentrations in F_1 mice reconstituted with F_1 bone marrow were significantly lower (812 ± 1247 ng/ml) (P = 0.03). The data suggest that the LPS-resistant phenotype of F_1 depends critically on bone marrow derived cells, probably macrophages.

The MyD88-dependent and MyD88-independent pathways in SPRET/Ei macrophages *in vitro*.

Bone marrow derived macrophages (BMDMs) from C57BL/6 and SPRET/Ei mice were stimulated with 100 ng/ml LPS. At different time points, total RNA was prepared from the cells for real-time Q-PCR analysis, and cell lysates were prepared for Western blot. The phosphorylation of p38 MAP kinase and JNK, and the transient degradation of I-κB were comparable in BMDMs from SPRET/Ei and C57BL/6 (Fig.

2A). Hence, the MyD88-dependent early NF-κB and MAP kinase activation in SPRET/Ei is intact. This was confirmed by the absence of a significant difference between SPRET/Ei and C57BL/6 BMDMs in LPS-induced TNF-α and IL-6 mRNA expression as measured 1h and 4h after stimulation with LPS (not shown). Furthermore, there were no significant differences between the two mouse strains in LPS-induced mRNA expression of other genes, e.g. *Il1a*, *Il1b*, *Il12a*, *Il12b*, *Bcl2*, *Birc4* (encoding XIAP) and *Tnfaip3* (encoding A20*)* (data not shown).

Induction of IFN-β mRNA by LPS was largely impaired in SPRET/Ei-derived BMDMs (Fig. 2B). The same was observed when SPRET/Ei- and C57BL/6-derived BMDMs were stimulated with 10 μg/ml poly(I-C) (not shown). As a consequence, LPS-induced STAT1 phosphorylation was impaired in SPRET/Ei BMDMs (Fig. 2C). This in turn impaired the induction of IRF-7 in LPS-stimulated SPRET/Ei BMDMs (Fig. 2D). LPS-induced phosphorylation of IRF-3 was comparable in SPRET/Ei BMDMs and C57BL/6 BMDMs, and IRF-3 knockout BMDM had broader gene defects than SPRET/Ei macrophages (data not shown). Furthermore, there were no significant differences in LPS-mediated induction of *Cxcl10* and *Isg15* mRNA, two genes that are also induced by TRIF-mediated induction of IRF-3 (data not shown). Hence, the defect seems to be specific for IFN induction. Finally, the sequences of the IFN-β promoters (150 bp before transcription start) in SPRET/Ei and C57BL/6 mice were identical. These data suggest that direct IRF-3-dependent IFN-β induction in SPRET/Ei is intact, and that the defect must be in the second wave of IFN induction.

SPRET/Ei has a defect in IFN-β production *in vivo*.

SPRET/Ei mice are extremely resistant to the lethal effects of LPS, and their macrophages are defective in IFN-β induction *in vitro*. We reasoned that pretreatment

with IFN-β would restore LPS sensitivity to SPRET/Ei mice. Indeed, we found that F_1 mice can be sensitized for LPS by pretreatment with IFN-β. Two hours before injection of 200 μg LPS, we pretreated F_1 mice (n = 4) with 20 μg IFN-β. All of the pretreated F_1 mice died, but all of the control F_1 mice (n = 6) survived. All of the control C57BL/6 mice (n =10) also died after injection of 200 μg of LPS. Furthermore, IFNAR1 knockout mice (n = 4) resisted the dose of 200 μg LPS, while 129/SvEv control mice (n = 4) died within 48 h after challenge (Fig. 3A). So, IFN-β is a critical factor in the resistance of SPRET/Ei mice to LPS.

To examine whether the impairment of LPS-induced IFN-β induction in SPRET/Ei was also evident *in vivo*, a dose of 500 μg of LPS was injected i.p. in C57BL/6 and F_1 mice, and lungs were collected at different times after challenge. High concentrations of biologically active IFN were found in lungs of C57BL/6 mice (n = 4), and significantly less in F_1 mice (n = 4) (Fig. 3B). Furthermore, C57BL/6, F_1 and SPRET/Ei mice were infected with influenza virus strain X47 and biologically active IFN measured in lung lysates 2 days or 5 days after infection. Very large amounts of IFN were induced in C57BL/6 mice, but only minute amounts in F_1 mice, and no IFN at all could be detected in SPRET/Ei (Fig. 3C).

Consequences of a defective IFN-β production.

It is well known that IFN-β is an important factor in immunity, e.g. it can sensitize macrophages for cell death induced by *Listeria monocytogenes*(33) and can protect against progressive leishmaniasis(34). We therefore examined the responses to infection with *Listeria monocytogenes*. SPRET/Ei mice (n = 12) and C57BL/6 mice (n = 6) were infected with 8.5 10^3 cfu *L. monocytogenes*; SPRET/Ei mice were completely resistant to the infection (Fig. 3D). We also infected F_1 mice in the

footpad with 2.10^6 stationary phase *Leishmania major* promastigotes. Parasite burdens in the draining lymph nodes were determined by limiting-dilution assays (n = 6). Due to the defective IFN-β response, F_1 mice were significantly more sensitive to *Leishmania major* infection (Fig. 3E).

TLR4 deficiency leads to extreme sensitivity to Gram-negative sepsis. We tested the response of F_1 mice to infection with *Klebsiella pneumoniae,* a model of Gram-negative septic pneumonia. C3H/HeJ mice, which express a mutant *Tlr4* gene, are extremely sensitive to Gram-negative infections(5). F_1 (n = 9), C57BL/6 (n = 16), C3H/HeN (n = 5) and C3H/HeJ (n = 5) mice were infected intranasally (i.n.) with an LD_{50} (for C57BL/6) of *K. pneumoniae,* and mortality was monitored. About 60% of the C57BL/6 and C3H/HeN mice succumbed to this infection, C3H/HeJ mice were extremely sensitive and died very soon after infection, but F_1 mice were relatively resistant to Gram-negative infections (Fig. 3F).

IFN-β production is reduced by a defect in the positive feedback loop of IFN-β induction.

To determine if there is a defect in the intracellular signaling pathway activated by type I IFN, BMDMs from SPRET/Ei and C57BL/6 were stimulated with LPS or IFN-β. We studied the mRNA induction of *Oas*, an IFN-dependent gene, using semi-quantitative RT-PCR. Significantly less OAS mRNA was induced in SPRET/Ei BMDMs than in C57BL/6 BMDMs 1 h after LPS treatment (not shown) or IFN-β stimulation (Fig. 4A). Thus, a defect in the positive feedback loop of IFN-β induction appears likely. In addition, after IFN-β stimulation, induction of IL-6 was significantly lower in SPRET/Ei-BMDM than in C57BL/6 BMDM (data not shown).

We then used semi-quantitative RT-PCR to study the IFNAR1 mRNA expression in BMDMs after stimulation with LPS or IFN-β. We found that the basal expression of this gene was identical in SPRET/Ei and C57BL/6 BMDM, but that it was down-regulated in SPRET/Ei cells very soon after LPS or IFN-β stimulation. No similar effect was seen in C57BL/6 cells (Fig. 4 B-D). These data suggest that hyporesponsiveness to IFN-β in SPRET/Ei, as described above regarding low induction of OAS and IL-6, may be due to rapid downregulation of the IFNAR1 by IFN-β itself.

Discussion

To identify pathogens, the innate immune system relies, a.o. on the TLR receptor family(1, 35) which recognize PAMPs. One of the PAMPs is LPS, an essential component of the outer membrane of Gram-negative bacteria(2). LPS causes many of the inflammatory effects observed in Gram-negative septic shock patients, and considerable research has been devoted to elucidating the mechanisms by which LPS functions. A major breakthrough was achieved by the identification of two LPS resistant mouse strains, C3H/HeJ and C57BL10/ScCr, and the identification of *Tlr4* as the gene encoding the major receptor for LPS, TLR4(5).

We here describe a novel LPS resistant mouse strain, SPRET/Ei, an inbred strain derived from *Mus spretus*. We found that both SPRET/Ei and F_1 mice resist doses of LPS at least 10 times higher than those tolerated by C57BL/6 mice. We demonstrated, by bone marrow transplantation, that bone marrow-derived cells, most probably macrophages, are critical in the resistance of F_1 mice. In contrast to the resistance of two other LPS-resistant inbred lines, C3H/HeJ and C57BL10/ScCr, which resist thanks to mutations in the *Tlr4* gene encoding the LPS receptor TLR4,

the LPS resistant phenotype of SPRET/Ei is not a monogenic, simple, Mendelian trait, but a polygenic, complex, non-Mendelian trait. Moreover, the loci envolved in the phenotype interact with each other in a strictly epistatic way. We found that SPRET/Ei-derived macrophages have a defect in IFN-β production, and in subsequent STAT1 phosphorylation and IRF-7 induction. The MyD88-dependent pathway leading to early NF-κB and MAPK activation seems to be intact. The defect in IFN-β production is also observed *in vivo* in SPRET/Ei mice treated with LPS or influenza virus. Furthermore, the resistance of SPRET/Ei mice can be reversed by exogenous IFN-β. IFNAR1 deficient mice proved resistant to LPS as well. As expected(33,34) the defect in IFN-β production caused F_1 mice to be more sensitive to *Leishmania major* infection and SPRET/Ei mice to show complete resistance to *Listeria monocytoges* infection.

The phenotype that we observed in SPRET/Ei and F_1 mice is very comparable to that observed in mice with induced mutations in signaling molecules of the JAK-STAT-dependent pathway. Macrophages derived from TYK2-null mice show impaired expression of IFN-β mRNA 30 min to 2 h after LPS stimulation(30). Also, IRF-7 mRNA expression, which is positively regulated by IFNAR signaling, is reduced in TYK2-null cells. NF-κB and MAPK activation are normal in macrophages derived from TYK2-null mice. IFN-β deficient mice are resistant to LPS but they produce normal amounts of TNF, IFN-γ and NO after LPS injection. In addition, STAT1-null mice are also resistant to LPS(30). Furthermore, IRF-$3^{-/-}$ mice are resistant to LPS and show defects in the induction of IFN-β and CXCL10 mRNA(36). SPRET/Ei seems to be unique in that the defect is specific to IFN-β induction, because other IRF-3 responsive genes, such as *Cxcl10* and *Isg15,* are induced with

comparable kinetics in SPRET/Ei and C57BL/6, and phosphorylation of IRF-3 appears normal in SPRET/Ei macrophages. In this respect, the phenotype observed in SPRET/Ei closely resembles that of IFN-β deficient mice. Also, induction of OAS mRNA expression (by LPS or IFN-β), which is positively regulated by IFNAR signaling, was found to be severely reduced in SPRET/Ei-derived macrophages. Thus, it appears that the defect is not situated in the TRIF-dependent signaling pathway, but in the autocrine/paracrine loop following IFN-β induction. We found that LPS and IFN-β caused rapid down-regulation of the IFNAR1 mRNA expression in SPRET/Ei-derived macrophages but not in C57BL/6 macrophages. Mice lacking the interferon receptor 1 subunit are completely unresponsive to type I IFNs(37). Indeed, probably due to the downregulation of the IFNAR1 we found less induction of IL-6 by IFN-β in SPRET/Ei macrophages, and less induction of the *Oas* gene. Furthermore, just like SPRET/Ei, IFNAR1 knockout mice exhibit profound resistance to LPS and to infection by the Gram-positive intracellular bacterium *Listeria monocytogenes(38)*. It has been reported that TYK2 slows down IFNAR1 degradation, a process that is at least partly due to inhibition of IFNAR1 endocytosis(39). Future work will need to address the extent to which TYK2 is directly responsible for the IFNAR1 (mRNA) degradation observed in SPRET/Ei macrophages, or whether another mechanism is responsible for this phenomenon. A profound study on mRNA stability of the mRNA's of IFNAR1, IFN-β and other genes will be needed, along with a detailed expression profiling of SPRET/Ei versus C57BL/6 macrophages.

We believe that the phenotypes that we describe here are very relevant in the search for new therapeutic interventions for sepsis. SPRET/Ei, in contrast to the TLR4-deficient LPS-resistant C3H/HeJ and C57BL10/ScCr strains, not only resists the toxic effects of LPS, but can also resist infection by Gram-negative pathogens,

such as *Klebsiella pneumoniae*. SPRET/Ei mice exhibit the characteristics of a perfect therapy for sepsis: unresponsiveness to the toxic effects of LPS and preservation of recognition and destruction of pathogens. Probably, the considerable reduction in IFN-β in SPRET/Ei mice enables them to resist endotoxic shock, but the small amount that is still produced (about 10% of normal) is sufficient to stimulate innate and adaptive immunity. Therefore, detailed identification of the defect that leads to the downregulation of the IFNAR1 could lead to a major breakthrough in sepsis research.

Material and methods

Mice. C57BL/6JIco mice, hereafter called C57BL/6, originated from The Jackson Laboratory (Bar Harbor, MN, USA) and were bred further at Iffa Credo (Brussels, Belgium), from which they were purchased. SPRET/Ei mice were obtained from The Jackson Laboratory. It should be noted that only about 10 SPRET/Ei mice were available each year, and that, in our animal house, SPRET/Ei mice were extremely poor breeders or did not breed at all. Hybrid F_1 mice obtained by crossing C57BL/6 female mice with SPRET/Ei males are designated as F_1. C3H/HeJOlaHsd-Lpsd and C3H/HeNHsd mice were purchased from Harlan (Oxon, UK). IFNAR1 KO mice and 129 SvEv control mice were purchased from B&K Universal Limited (Aldbrough, Grimston, UK). Mice were housed in individually ventilated cages in an air conditioned, temperature controlled conventional animal house, and received food and water *ad libitum*. They were used at the age of 8 weeks and had comparable bodyweights. All experiments were approved by the local ethics committee.

Agents. LPS from *Salmonella abortus equii* was purchased from Sigma-Aldrich (St. Louis, MD, USA). IFN-β ($4.6\ 10^6$ U/mg protein) was expressed in and purified from *E. coli* in our laboratory (DMBR, Ghent, Belgium). Poly(I-C) was purchased from Amersham Biosciences (Uppsala, Sweden). Antibodies directed against IκBα, phospho-JNK, phospho-p38α, p38α, STAT1 and NF-κB p65 were purchased from Santa Cruz Biotechnology (California, USA). Antibody against phospho-STAT1 was from Upstate (Charlottesville, USA). Antibody against JNK was from Pharmingen (Leiden, The Netherlands).

Isolation of Mouse Embryonic Fibroblasts. MEFs were isolated from 18-day old embryos and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, gentamycin and L-glutamine.

Immunoblot analysis. Whole cell, cytoplasmic, and nuclear extracts for immunoblot analysis were prepared and subjected to SDS-PAGE. Proteins transferred to nitrocellulose membrane were probed with rabbit antiserum against phospho-Stat1 and Stat1, and the immune complexes were visualized with the ECL Western blot reagent (Pierce Chemical Co, Rockford, USA).

Injection, blood collection, and measurement of body temperature. LPS was diluted in endotoxin-free PBS immediately before injection. All injections were given i.p. in a volume of 250 µl. Blood was collected by retro-orbital bleeding, allowed to clot for 1 h at 37 °C, and for another hour at 4 °C. Serum was prepared and stored at -20 °C. Rectal body temperatures were measured with an electronic thermometer (Comarks, Littlehampton, U.K.). To prepare lung extracts, mice were anesthetized with avertin (tribromoethanol), the thorax was opened, and the left and right lungs were isolated, washed and kept in 1 ml ice-cold, sterile PBS. Lungs were homogenized in a tissue homogenizer (Heidolph Homogenizer, model RZR 2020; Heidolph-Elektro, Kelheim, Germany) and centrifuged 15 min at 15,000g. The clear supernatant was centrifuged once more and kept at -80 °C until needed.

Infection models. *Klebsiella pneumoniae* (ATCC 43816) was inoculated i.n. (50 µl) under light isoflurane anesthesia. Before each experiment the LD_{50} was determined in C57BL/6 mice and this dose was used in the experiment. Mortality was monitored for at least 8 days. For infection with influenza type A virus strain X47, the $2xLD_{50}$ dose was first determined in C57BL/6 mice. The virus was inoculated i.n. (50 µl)

under light isoflurane anesthesia. Just before infection and on days 2 and 5 after infection, mice were anesthetized with avertin, and lungs were isolated and processed as described. For infection with *L. monocytogenes* EGD (serotype 1/2a) were washed with PBS, viable bacteria were counted by plating serial dilutions. Bacteria were diluted in PBS to a final concentration of 2 x 10^5 cfu/ml, and mice were infected with 100 µl in a lateral tail vein. For infection with *Leishmania major* (MHOM/TN/95/GLC94), the parasites were maintained by continuous passage in BALB/c mice. Mice were infected s.c. in one hind footpad with 2x10^6 stationary phase promastigotes in a volume of 25 µl. Parasite burden in the draining lymph nodes was determined by a limiting dilution assay of single-cell suspensions made from individual lymph nodes. Each sample was plated in quadruplicate in 96-well flat-bottom microtiter plates. The number of viable parasites was determined from the reciprocal of the highest dilution at which promastigotes could be detected after 10 days of incubation at 26°C. Four mice were used for each parasite burden determination. The results presented are the averaged values (± standard errors) of the parasite burdens found for each group.

Determination of IL-6 in the serum. IL-6 was determined using an IL-6 dependent 7TD1 hybridoma cell line as previously described(40).

Determination of IFN biological activity in lung lysates. IFN biological activity was determined using an encephalomyocarditis (EMC) virus-dependent biological assay. In the presence of serially diluted lung lysates or IFN as a standard, 8x10^3 L929 cells were cultured in 96-well plates in DMEM supplemented with 2% heat-inactivated FCS. After 1 night of incubation at 37°C, EMC virus was added at a concentration of 10^3/ml (50 pfu/well). After overnight incubation, cell death was

measured by a colorimetric technique (MTT assay), which is based on the reduction of a yellow tetrazolium salt to a purple formazan. The assay has a detection limit of 1 pg/ml.

Bone marrow transplantation. Mice were lethally irradiated (10 Gy) and then maintained under sterile conditions. Drinking water was supplemented with 2 mg/ml neomycin sulfate. Bone marrow cells were isolated from tibias and femurs. $7x10^6$ bone marrow cells were injected intravenously (i.v.) one day after lethal irradiation. As a control, several irradiated mice did not receive donor cells. These mice died within 2 weeks of irradiation. The bone marrow-reconstituted mice were challenged with 250 μg of LPS 8 weeks after the bone marrow transplant.

Real-time Q-PCR analysis. Total RNA was isolated from triplicate cultures of BMDMs using the RNAwiz reagent (Ambion). cDNAs were synthesized using the Superscript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.2 μg of total RNA was amplified through 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. Output was monitored using SYBR Green core reagents and the ABI Prism 7700 System (PE Applied Biosystems). The results were normalized to the level of cyclophilin mRNA. Individual primer sequences are available upon request.

Semi-quantitative RT-PCR. Total RNA was isolated from BMDMs using the RNeasy Mini Kit (Qiagen). cDNAs were synthesized using the Superscript II reverse transcriptase system (Invitrogen). The cDNA equivalent of 0.1 μg of total RNA was amplified using primers for OAS and IFNAR1. The results were normalized to the level of β-actin.

Statistical Analysis. Mortality data were analyzed using a χ^2 test. Kaplan Meyer survival curves were compared by means of a Log-rank test, and all other data were

analyzed using a one-tailed Student's *t* test. Significance levels are as follows: *, $P<0.05$; **, $P<0.01$; ***, $P<0.0001$.

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Legends

Fig. 1. Response of C57BL/6, (B x S)F_1, and SPRET/Ei mice to LPS. (A) Response of C57BL/6 (■), F_1 (▲), SPRET/Ei (▼) (n = at least 5 for each dose) to increasing doses of LPS. Mortality was monitored up to 1 week after challenge (no further deaths occurred). **(B)** Induction of hypothermia after injection of 250 μg LPS. C57BL/6 (■, n = 5), F_1 (▲, n = 5), SPRET/Ei (▼, n = 5). (**C**) IL-6 concentrations in serum after injection of 250 μg LPS. Black bars, C57BL/6 mice (n =5); hatched bars, F_1 mice (n =5); white bars, SPRET/Ei mice (n =5). **(D)** Survival of C57BL/6 mice (▲; n = 4), F_1 mice (▼; n = 5), lethally irradiated F1 mice reconstituted with C57BL/6 bone marrow (■; n = 4) or lethally irradiated F1 mice reconstituted with F1 bone marrow (♦; n = 5) after challenge with 250 μg of LPS. Significance was calculated for difference from C57BL/6.

Fig. 2. The MyD88-dependent signaling pathway is intact in SPRET/Ei BMDM and SPRET/Ei BMDM have a defect in IFN-β production *in vitro*. (**A**) BMDMs were stimulated with 100 ng/ml LPS for the indicated time intervals. IκBα degradation, and JNK1/2 and p38 expression and phosphorylation were analyzed by Western Blot. (**B**) LPS-induced expression of the IFN-β gene in BMDM. BMDM from C57BL/6 and SPRET/Ei mice were stimulated with 100 ng/ml LPS for the indicated durations, and induction of IFN-β mRNA was analyzed by real-time Q-PCR in triplicate cultures: ■, C57BL/6; □, SPRET/Ei. (**C**) Phosphorylation of STAT1 2 h after stimulation with 100 ng/ml LPS. (**D**) Induction of IRF-7 mRNA after stimulation with 100 ng/ml for 4 h. Black bars, C57BL/6; white bars, SPRET/Ei.

Fig. 3. SPRET/Ei has a defect in IFN-β production *in vivo*, shows IFN-β deficient phenotypes and resist a lethal challenge with *Klebsiella pneumoniae.* (A) Survival of IFNAR1$^{-/-}$ mice after injection of 200 μg LPS. IFNAR1$^{-/-}$ (▲, n = 4), 129Sv mice (■, n = 4). **(B)** IFN biological activity in lung extracts at the indicated time points after injection of 500 μg of LPS. Black bars, C57BL/6 (n = 4); hatched bars F_1 (n = 4). **(C)** IFN biological activity in lung lysates after influenza virus infection. Black bars, C57BL/6 (n = 12); hatched bars, F_1 (n = 10); white bars, SPRET/Ei (n = 2). Significance was calculated for difference from C57BL/6. **(D)** Mortality following intravenous injection with 8.5 10^3 cfu *Listeria monocytogenes*: SPRET/Ei (**▲,** n = 12), C57BL/6 (■, n = 6). **(E)** Response to infection with *Leishmania major*. Parasite burdens in the draining lymph nodes were determined by limiting-dilution assays (4 mice/group/time point, dilutions tested in quadruplicate). C57BL/6 (black bars) and F_1 (hatched bars) mice. **(F)** Lethal response of C57BL/6 (■, n = 16), F_1 (♦, n = 9), C3H/HeJ (▲, n = 5), and C3H/HeN mice (▼, n = 5) after infection i.n. with 10.000 cfu *Klebsiella pneumoniae*. Mortality was monitored for 8 days. No further deaths occurred. Significance was calculated for difference from C57BL/6.

Fig. 4. SPRET/Ei has a defect in the autocrine/paracrine loop of IFN-β induction. (**A**) IFN-β-induced expression of the *Oas* gene in BMDM. Cells from C57BL/6 and SPRET/Ei mice were either left stimulated or stimulated with 200 U/ml IFN-β for 1 h, and induction of OAS mRNA was analyzed by semi-quantitative RT-PCR. Black bar, C57BL/6; white bar, SPRET/Ei. (**B**) Relative IFNAR1 mRNA level in unstimulated BMDM, **(C)** 1 h after stimulation with 100 ng/ml LPS, and **(D)** 1 h after treatment with 200 U/ml IFN-β. Black bars, C57BL/6; white bars SPRET/Ei (n

= 3). Significance was calculated for difference from C57BL/6. See ethidium bromide stained gels below the figures.

References.

1. Akira, S., Takeda, K. & Kaisho, T. (2001) *Nat Immunol* **2,** 675-80.
2. Ulevitch, R. J. & Tobias, P. S. (1995) *Annu Rev Immunol* **13,** 437-57.
3. Meijer, A. H., Gabby Krens, S. F., Medina Rodriguez, I. A., He, S., Bitter, W., Ewa Snaar-Jagalska, B. & Spaink, H. P. (2004) *Mol Immunol* **40,** 773-83.
4. Beutler, B. & Rietschel, E. T. (2003) *Nat Rev Immunol* **3,** 169-76.
5. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., *et al.* (1998) *Science* **282,** 2085-8.
6. Sweet, M. J. & Hume, D. A. (1996) *J Leukoc Biol* **60,** 8-26.
7. Beutler, B. (2001) *Biochem Soc Trans* **29,** 853-9.
8. Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K. & Akira, S. (2003) *Nat Immunol* **4,** 1144-50.
9. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. & Akira, S. (2003) *Science* **301,** 640-3.
10. Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., *et al*. (2001) *Nature* **413,** 78-83.
11. Janssens, S. & Beyaert, R. (2002) *Trends Biochem Sci* **27,** 474-82.
12. Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., *et al*. (2002) *Nature* **420,** 324-9.
13. Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., *et al*. (1999) *Genes Dev* **13,** 1015-24.
14. Cao, Z., Henzel, W. J. & Gao, X. (1996) *Science* **271,** 1128-31.
15. Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M. & Golenbock, D. T. (2003) *J Exp Med* **198,** 1043-55.
16. Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Muhlradt, P. F., Sato, S., Hoshino, K. & Akira, S. (2001) *J Immunol* **167,** 5887-94.
17. Kawai, T., Adachi, O., Ogawa, T., Takeda, K. & Akira, S. (1999) *Immunity* **11,** 115-22.
18. Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., *et al*. (2003) *Nature* **424,** 743-8.
19. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M. & Maniatis, T. (2003) *Nat Immunol* **4,** 491-6.
20. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R. & Hiscott, J. (2003) *Science* **300,** 1148-51.
21. Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K. & Akira, S. (2003) *J Immunol* **171,** 4304-10.
22. Hiscott, J., Pitha, P., Genin, P., Nguyen, H., Heylbroeck, C., Mamane, Y., Algarte, M. & Lin, R. (1999) *J Interferon Cytokine Res* **19,** 1-13.
23. Yang, H., Lin, C. H., Ma, G., Orr, M., Baffi, M. O. & Wathelet, M. G. (2002) *Eur J Biochem* **269,** 6142-51.

24. Servant, M. J., ten Oever, B., LePage, C., Conti, L., Gessani, S., Julkunen, I., Lin, R. & Hiscott, J. (2001) *J Biol Chem* **276,** 355-63.
25. Novick, D., Cohen, B. & Rubinstein, M. (1994) *Cell* **77,** 391-400.
26. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. (1998) *Annu Rev Biochem* **67,** 227-64.
27. Taniguchi, T. & Takaoka, A. (2002) *Curr Opin Immunol* **14,** 111-6.
28. Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T. & Tanaka, N. (1998) *FEBS Lett* **441,** 106-10.
29. Smith, P. L., Lombardi, G. & Foster, G. R. (2005) *Mol Immunol* **42,** 869-77.
30. Karaghiosoff, M., Steinborn, R., Kovarik, P., Kriegshauser, G., Baccarini, M., Donabauer, B., Reichart, U., Kolbe, T., Bogdan, C., Leanderson, T., *et al*. (2003) *Nat Immunol* **4,** 471-7.
31. Brouckaert, P. G., Libert, C., Everaerdt, B., Takahashi, N. & Fiers, W. (1989) *Lymphokine Res* **8,** 269-74.
32. Guha, M. & Mackman, N. (2001) *Cell Signal* **13,** 85-94.
33. Stockinger, S., Materna, T., Stoiber, D., Bayr, L., Steinborn, R., Kolbe, T., Unger, H., Chakraborty, T., Levy, D. E., Muller, M. & Decker, T. (2002) *J Immunol* **169,** 6522-9.
34. Mattner, J., Wandersee-Steinhauser, A., Pahl, A., Rollinghoff, M., Majeau, G. R., Hochman, P. S. & Bogdan, C. (2004) *J Immunol* **172,** 7574-82.
35. Takeda, K., Kaisho, T. & Akira, S. (2003) *Annu Rev Immunol* **21,** 335-76.
36. Sakaguchi, S., Negishi, H., Asagiri, M., Najima, C., Mizutani, T., Takaoka, A., Honda, K. & Taniguchi, T. (2003) *Biochem Biophys Res Com* **306,** 860-866.
37. Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. & Aguet, M. (1994) *Science* **264,** 1918-21.
38. O'Connell, R. M., Saha, S. K., Vaidya, S. A., Bruhn, K. W., Miranda, G. A., Zarnegar, B., Perry, A. K., Nguyen, B. O., Lane, T. F., Taniguchi, T., *et al*. (2004) *J Exp Med* **200,** 437-45.
39. Ragimbeau, J., Dondi, E., Alcover, A., Eid, P., Uze, G. & Pellegrini, S. (2003) *Embo J* **22,** 537-47.
40. Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P. G., Rubira, M. R. & Simpson, R. J. (1986) *Proc Natl Acad Sci U S A* **83,** 9679-83.