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Phosphorylation of the Stat1 Transactivation Domain Is Required for Full-Fledged IFN-γ-Dependent Innate Immunity

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Summary

Stat1 is phosphorylated on serine 727 within its transactivating domain (TAD) in response to interferons or other immunological signals. We generated gene-targeted mutant mice expressing a serine727-alanine mutant of Stat1. These animals showed increased mortality upon infection with *Listeria monocytogenes* and impaired clearance of the bacteria from spleen and liver. The Stat1S727A mice were more resistant to the LPS-induced septic shock syndrome, suggesting that Stat1 serine phosphorylation promotes inflammatory responses. Expression of IFN-γ-induced genes was strongly reduced in macrophages expressing $Stat1_{S727A}$. While mutation of Stat1 at S727 did not reduce its binding to chromatin, association with the coactivator CBP and histone acetylation at the interferon-responsive GBP promoter was strongly reduced, suggesting defective recruitment of histone acetylases as the mechanism underlying IFN-γ hyporesponsiveness. Our data demonstrate that the increase in transcription factor activity caused by Stat serine phosphorylation contributes to macrophage activation and to IFN-γ-dependent immune responses in vivo.

Introduction

Cytokines and their cell surface receptors regulate adaptive and innate immunity. To reprogram cellular gene expression, many cytokine receptors make use of Jak-Stat signal transduction (Levy and Darnell, 2002). Cytokine binding activates receptor-associated Janus kinases (Jaks), which phosphorylate one or more receptor chains to create docking sites for signal transducers and activators of transcription (Stats). Receptor-bound Stats are phosphorylated on a single tyrosine residue by Jaks, whereupon they dissociate from the receptor and dimerize. Stat dimers acquire the ability to traverse the nuclear envelope and to associate with a short sequence of promoter DNA, the γ interferon-activated site (GAS).

While tyrosine phosphorylation represents an essential step of the linear Jak-Stat paradigm, it is not the only posttranslational modification regulating Stat activity. Mammalian Stats 1, 3, 4, and 5 contain a serine phosphorylation site in their C-terminal transactivation domain (TAD). The location of the serine phosphorylation site in this part of the molecule is consistent with the assumption that it plays a role in transcriptional activation (Decker and Kovarik, 2000).

Stat1 is essential for the biological effects of interferons (IFN), foremost innate immunity to virus and bacteria (Durbin et al., 1996; Meraz et al., 1996). A large variety of signals cause phosphorylation of Stat1 at the C-terminal S727 (Decker and Kovarik, 2000). They can be divided into two categories. Interferons cause serine phosphorylation in addition to tyrosine phosphorylation, and CaM kinase II and PKCδ were presented as IFN-responsive, candidate serine 727 kinases (Deb et al., 2003; Nair et al., 2002; Uddin et al., 2002; see also Pilz et al., 2003). Signals like bacteria, or mediators of stress and inflammation, stimulate exclusively the phosphorylation of S727 through the p38MAPK pathway (Kovarik et al., 1999; Ramsauer et al., 2002). The two categories of signals may synergize in eliciting a maximal transcriptional response through Stat1, as in the case of macrophage activation, which is caused by bacterial signals together with IFN-γ. Alternatively, the serine-phosphorylated Stat1 may activate a group of target genes that do not require tyrosine phosphorylation, as first proposed by Stark and colleagues for genes mediating an apoptotic response of fibrosarcoma cells to TNF-α (Kumar et al., 1997).

All conclusions about the consequences of phosphorylation at the TAD were either derived from correlations between serine phosphorylation and a particular biological response, or by studying Stat1-deficient cell lines, engineered to reexpress Stat1 serine727-alanine (S727A) mutants. Therefore, available evidence about the relevance of Stat1 serine phosphorylation is limited to either transformed human fibrosarcoma cells or to immortalized mouse fibroblasts. In these cells, serine phosphorylation of the Stat1 dimer is required for a full-fledged transcriptional response to IFN-γ (Kovarik et al., 2001; Wen et al., 1995). These studies clearly document a potential of TAD phosphorylation to increase transcription factor activity of Stat1 dimers, but they do not establish whether the increase in gene expression stimulated by Stat1 serine phosphorylation is really important for IFN-γ-dependent immune responses, particularly since

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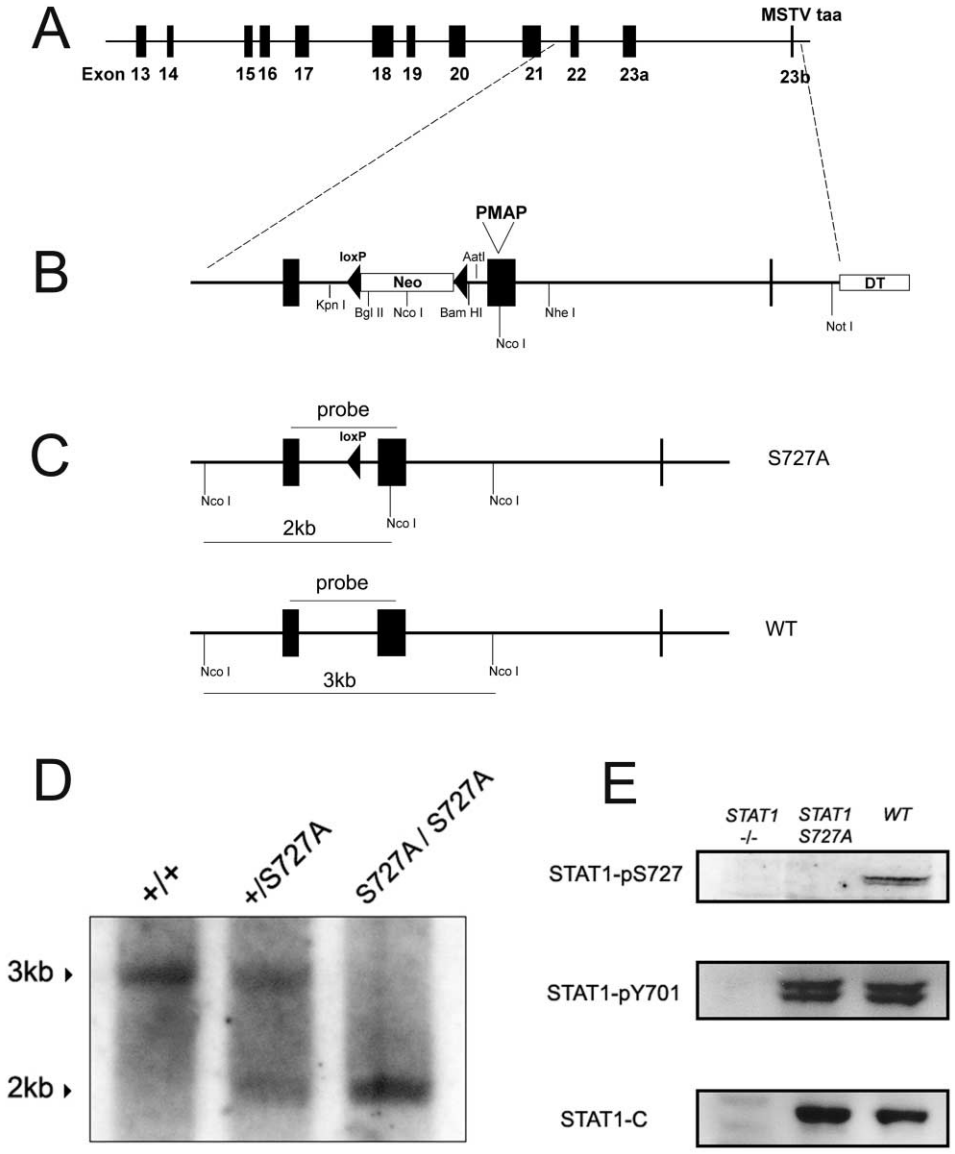

Figure 1. Mutation of the Murine Stat1 Locus to $STAT1_{S727A}$

(A) Intron-exon arrangement of the murine STAT1 locus.

(B) The STAT1 S727A targeting construct. The neomycin (Neo) selection marker flanked by two lox P sites was inserted in the intervening intron between exons 22 and 23a, and the diphtheria toxin (DT) selection marker was cloned at the 3′ end.

(C) The STAT1 locus of the S727A targeted mutant mice. The A727 mutation creates an additional NcoI restriction site within exon 23a.

(D) Southern blot of genomic DNA digested with NcoI produces a 2 kb band in homozygous S727A/S727A targeted mutants and a 3 kb band in wild-type (+/+) animals.

(E) STAT1 expression and phosphorylation in bone marrow-derived macrophages from wild-type, $STAT1_{S727A}$, and STAT1-deficient mice as determined by Western blotting with phosphospecific (Stat1pY701, Stat1pS727) or $STAT1\alpha$ antibodies (Stat1-C).

a main target of IFN-γ in the immune system is the macrophage. To address whether signaling of the immune system to the Stat1 TAD is an essential part of establishing innate immunity, we have generated mice expressing a $Stat1_{S727A}$ mutant. We show that S727 phosphorylation increases IFN-γ-dependent innate immunity to bacteria and the inflammatory response to bacteria-derived signals like lipopolysaccharide (LPS). We also demonstrate a role of Stat1S727 phosphorylation in stimulating histone acetylation and the expression of IFN-γ-induced genes.

Results

Generation of Mice Expressing Stat1S727A

We cloned the murine Stat1 locus starting from exon 13 (GenBank Accession Number AF349678). Its exon-intron organization is generally very similar to that of the human locus, but the coding region corresponding to human exon 23 is split into murine 23a and 23b exons with 23b encoding only four amino acids and the stop codon (Figure 1A). The S727 phosphorylation site of the TAD is encoded by exon 23a. The base triplet for S727 was

mutated to encode A, and the Stat1 locus of ES cells was targeted for homologous recombination with a construct containing the mutated genomic DNA, starting with intron 21 and ending within the 3′UTR. A Neo^R cassette flanked by loxP recombination sequences was inserted between exons 22 and 23, and a diphtheria toxin gene at the 3′ end was used for negative selection of nonhomologous recombinants (Figure 1B). Clones of targeted ES cells were identified by PCR and used for injection into blastocysts. Two clones produced chimeric mice that transmitted the mutant locus to their offspring. Excision of the Neo cassette in intron 22 was achieved by mating homozygous mutant animals to mice expressing an X-linked CMV-Cre transgene. Deletion of the Neo gene in the resulting offspring and the presence of the S727A mutation was verified by Southern blotting (Figures 1C and 1D). Macrophages from homozygous Stat1S727A mice were analyzed by Western blotting and showed that Stat1 expression and IFN-γ-induced tyrosine phosphorylation were comparable to that of wt mice (Figure 1E). As expected, phosphorylation at S727 occurred in wt mice, but not in those with two $Stat1_{S727A}$ alleles (Figure 1E).

Response of Cells and Animals Expressing $Stat1_{S727A}$ to Pathogens

Antiviral assays are an established method for testing the biological activity of interferons, hence the output of Jak-Stat signaling. The lack of Stat1 serine phosphorylation significantly reduced the antiviral activity of IFN-γ on vesicular stomatitis virus (VSV)-infected primary fibroblasts. A cytopathic effect of VSV on $Stat1_{S727A}$ cells was observed at IFN-γ concentrations causing full protection of cells from wt animals, and complete lysis of $Stat1_{S727A}$ cells occurred when cells from wt animals were still partially protected (Figure 2A). This suggests that the lack of Stat1 serine phosphorylation diminishes the antiviral response to IFN-γ but does not completely abolish it. As previously reported (Meraz et al., 1996), cells from Stat1-deficient mice failed to establish an antiviral state.

The prevalent activity of IFN-γ in the immune system is to activate macrophages for an enhanced antimicrobial activity. We tested the ability of IFN-γ-pretreated macrophages from $Stat1_{S727A}$ mice to clear intracellular *Listeria monocytogenes*. Compared to macrophages from wt mice, increased numbers of the bacteria were present in $Stat1_{S727A}$ macrophages throughout infection (Figure 2B). Stat1-deficient macrophages showed a complete defect in killing intracellular *L. monocytogenes*.

Remarkably, infection of mice with *L. monocytogenes* confirmed the in vitro observations. All $Stat1_{S727A}$ mice infected with 10^6 bacteria died, whereas 80% of the wt littermates survived (Figure 3A). Following infection with 10^5 bacteria 80% of the $Stat1_{S727A}$ mice and all of the wt controls survived. All Stat1-deficient mice succumbed to infection with either 10^5 (Figure 3A, lower panel) or 10^4 bacteria (data not shown). Consistent with the reduced ability to survive infection, the clearance of *L. monocytogenes* from liver and spleen 3 days after infection was reduced 10-fold on average in $Stat1_{S727A}$ mice, compared to wt littermates (Figure 3B).

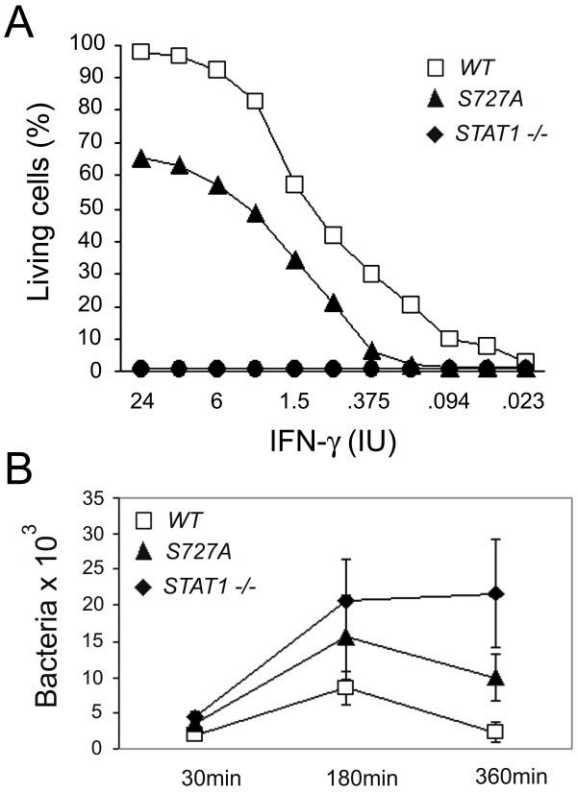

Figure 2. Reduced Antiviral and Antibacterial Activity in Cells from $STAT_{S727A}$ Mice

(A) Primary fibroblasts derived from isogenic wild-type and $STAT_{S727A}$ mice were primed with serial dilutions of IFN-γ for 24 hr and subsequently infected with vesicular stomatitis virus at a multiplicity of infection (MOI) of 0.1. Cell viability was determined 24 hr later with crystal violet staining.

(B) Bone marrow-derived macrophages were primed with IFN-γ (240 U/ml) for 24 hr and subsequently infected with *L. monocytogenes* at an MOI of 10. The ability of macrophages to clear *L. monocytogenes* was assessed by detecting the titer of intracellular bacteria after 30, 180, and 360 min of infection by colony-forming unit assay.

Resistance of $Stat1_{S727A}$ Mice to the LPS-Induced Septic Shock Syndrome

IFN-γ enhances the susceptibility of mice to the toxic effects of LPS (Kohler et al., 1993). Mice lacking the IFN-γ receptor or Stat1 show an increased resistance to LPS (Car et al., 1994; Karaghiosoff et al., 2003). To test whether serine phosphorylation increases the ability of Stat1 to sensitize mice to LPS, we injected 25 mg/kg LPS into $Stat1_{S727A}$ mice or isogenic wt controls. Forty-six percent of wild-type mice died between 22–46 hr after injection, whereas only 9% of $Stat1_{S727A}$ mice died 70 hr after LPS administration (Figure 4A). At 37 mg/kg of LPS, a dose survived by 100% Stat1-deficient mice (Karaghiosoff et al., 2003), 80% $Stat1_{S727A}$ mice died, albeit with a significant delay with respect to wild-type controls (data not shown). Together, the data suggest that serine phosphorylation increases the activity of Stat1 in the LPS-induced septic shock. In addition to its proinflammatory activity, Stat1 was reported to exert a suppressive effect on IL-12/Stat4-induced IFN-γ production by NK and T cells (Nguyen et al., 2000). In line

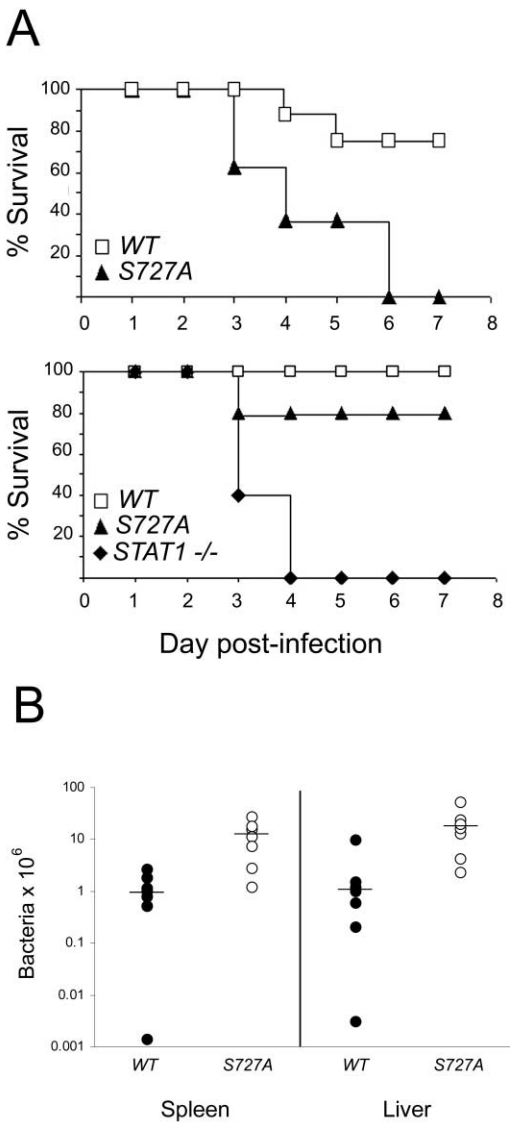

Figure 3. STAT1$_{S727A}$ Mice Have Increased Susceptibility to *Listeria monocytogenes*

(A) Upper panel: Survival of wild-type and STAT1$_{S727A}$ mice intraperitoneally infected with 10^6 cfu *L. monocytogenes*. Lower panel: Survival of wild-type, STAT1 S727A, and STAT1-deficient mice infected intraperitoneally with 10^5 cfu *L. monocytogenes*. For all experiments, eight mice of each genotype were infected.

(B) Bacterial loads in spleen and liver of wild-type and Stat1$_{S727A}$ mice infected with *L. monocytogenes*. Groups of eight wild-type and eight STAT1$_{S727A}$ mice were infected intraperitoneally with 5×10^5 cfu *L. monocytogenes*. The bacterial load in spleens and livers from the infected animals was detected on the third day of *L. monocytogenes* infection by colony-forming unit assay.

with this, Stat1-deficient mice produced higher amounts of IFN-γ after LPS treatment than their wt controls (Figure 4B). By contrast, IFN-γ production in LPS-treated Stat1$_{S727A}$ mice was similar to that seen in wt mice. This suggests that serine phosphorylation is not required for the suppressive effects of Stat1 on IFN-γ production.

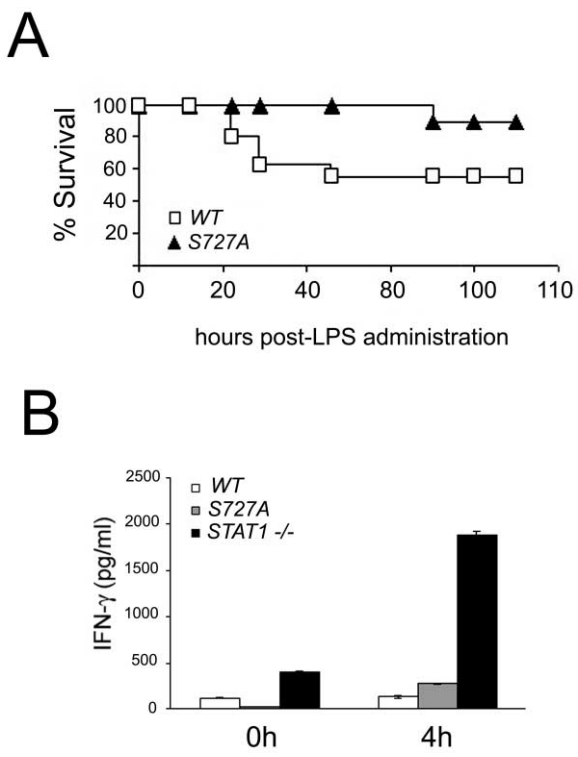

Figure 4. Increased Survival and IFN-γ Production of Stat1$_{S727A}$ Mice in Response to LPS

(A) Survival of wt or STAT1$_{S727A}$ mice after intraperitoneal injection of 25 mg/kg of *E. coli* LPS. Eleven mice of each genotype were scored. Statistical analysis of the data was performed with the log rank test ($p = 0.035$).

(B) Serum levels of IFN-γ 4 hr after intraperitoneal injection of 50 mg/kg of *E. coli* LPS, as determined by ELISA.

Expression of IFN-γ-Responsive Genes in the Absence of Stat1 TAD Phosphorylation

To assess whether the reduced IFN-γ responsiveness of Stat1$_{S727A}$ mice reflected a general decrease in the nuclear response to the cytokine, a panel of previously characterized, IFN-γ-induced genes were investigated in macrophages. Quantitative PCR of the GBP1, IRF1, SOCS1, and TAP1 genes showed reduced induction by IFN-γ in all cases. In accordance with previous results obtained with fibroblast lines, the dependence on S727 phosphorylation was variable (Kovarik et al., 2001). GBP1 and IRF1 were strongly affected (up to 90% reduction 4 hr after IFN-γ addition), whereas SOCS1 and TAP1 showed a lower dependence on serine phosphorylation (Figure 5A). Additional genes related to antigen presentation and to macrophage bactericidal activity were investigated by flow cytometry or RT-PCR (Figures 5B and 5C). MHC I expression (Figure 5B) was reduced at 4 and 6 hr after IFN-γ induction, but by 12 hr after treatment there was no apparent effect of S727 phosphorylation. Similarly, the rather weak induction of MHC II was decreased at 6 hr after IFN-γ treatment, but not at later stages of induction (Figure 5B). Upon treatment with IFN-γ alone, induction of the iNOS and LRG47 genes was markedly inhibited by the lack of Stat1 phosphoryla-

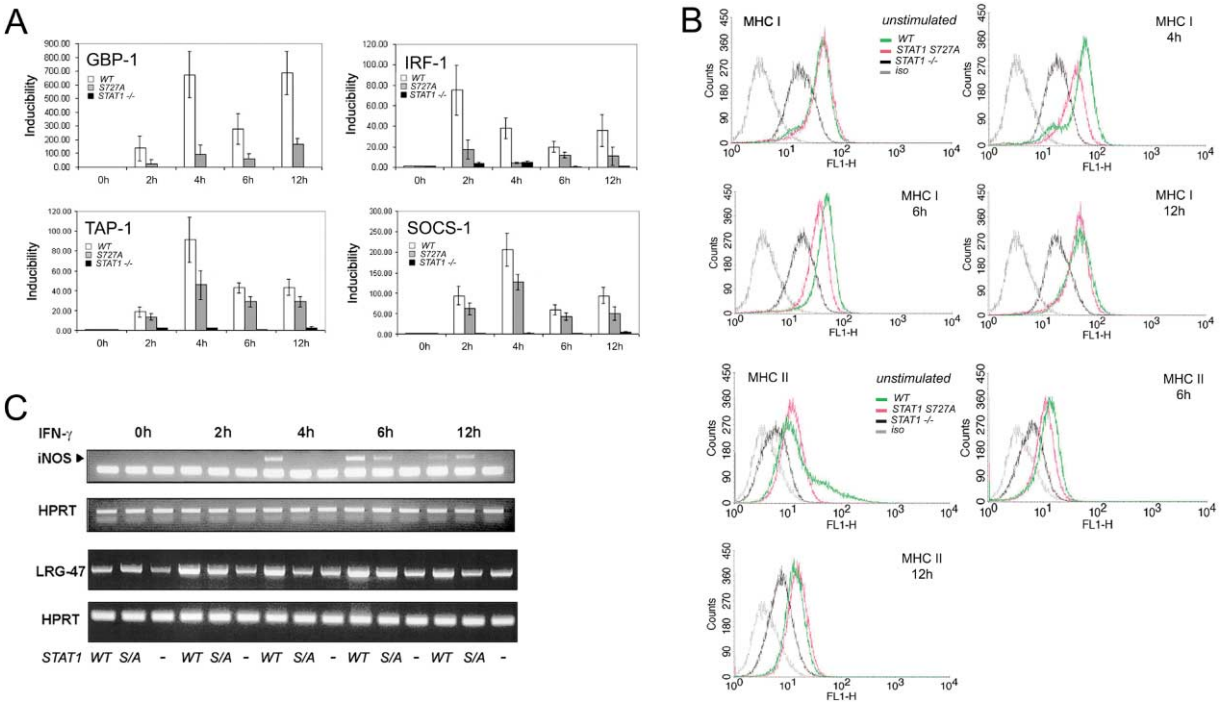

Figure 5. Reduced Expression of IFN-γ-Induced Genes in $STAT1_{S727A}$ Primary Macrophages

(A) Bone marrow-derived macrophages were stimulated with 10 ng/ml (240 IU/ml) IFN-γ for the indicated time points, and RNA was extracted and reverse transcribed. Inducibility of the IFN-γ-inducible GBP1, IRF1, SOCS1, and TAP1 genes was detected by real-time RT-PCR and normalized to endogenous HPRT.

(B) Bone marrow-derived macrophages stimulated for 2–12 hr with 240 IU/ml IFN-γ were stained with anti-MHC I (panels 1–4) or anti-MHC II (panel 5–7) monoclonal antibody, and the MHC cell surface expression in wild-type, $STAT1_{S727A}$, and STAT1-deficient macrophages was detected by flow cytometry.

(C) Bone marrow-derived macrophages were stimulated with 10 ng/ml (240 IU/ml) IFN-γ for the indicated time points, and RNA was extracted and reverse transcribed. Inducibility of the IFN-γ-inducible iNOS and LRG47 genes was detected by RT-PCR and normalized to endogenous HPRT.

tion at S727 (Figure 5C). Both genes mediate resistance to *L. monocytogenes* (Collazo et al., 2001; MacMicking et al., 1995). Moreover, iNOS contributes to the LPS-induced toxic shock syndrome (MacMicking et al., 1995).

Many infection-related genes respond not only to cytokines but also to pathogen signals directly. Therefore, we tested whether LPS, added in combination with IFN-γ, would compensate for the absence of Stat1 serine phosphorylation. Compared to IFN-γ alone, the iNOS and LRG47 genes responded more strongly to combined LPS/IFN-γ treatment in macrophages from wt mice, and both were affected by the $Stat1_{S727A}$ mutation (Figure 6). The kinetics of iNOS induction was delayed, but 12 hr after induction similar levels were reached as in wt macrophages. By contrast, expression of LRG47 was reduced at all time points after IFN-γ addition.

Decreased Acetylation of Histones at the GBP Promoter of IFN-γ-Treated Macrophages

To test whether the lack of serine phosphorylation impedes the ability of the Stat1 TAD to recruit coactivator/histone acetylase complexes to target promoters, we performed chromatin immunoprecipitation (ChIP) assays from macrophage nuclei. The GBP promoter was chosen for these experiments because the two GBP genes, GBP-1 and GBP-2, show a particularly strong dependence on S727 phosphorylation (Figure 5A and data not shown). The promoter of both genes appears to be identical (Anderson et al., 1999; Briken et al., 1995; Nicolet and Paulnock, 1994). The IFN response region contains a proximal ISRE sequence and distal GAS and ISRE elements in close proximity (Figure 7A). Transcriptional induction depends on both Stat1 and IRF1 (Briken et al., 1995; Kimura et al., 1994, 1996; Meraz et al., 1996). ChIP with a C-terminal antibody to Stat1 showed that IFN-γ stimulated rapid association of Stat1 with both IFN-responsive subregions of the GBP promoter. Recruitment by the part of the distal promoter containing GAS and ISRE was stronger compared to that seen at the proximal ISRE. Binding to either region remained unchanged in macrophages expressing $Stat1_{S727A}$ (Figure 7B). This finding was confirmed by showing identical binding of Stat1wt and $Stat1_{S727A}$ to the GAS sequence of the nuclear IRF1 promoter (data not shown). IFN-γ also stimulated acetylation of histone 4 at the GBP promoter in a Stat1-dependent manner (Figure 7C, panel 2). Contrasting the ability to bind nuclear target sequences, the $Stat1_{S727A}$ mutant showed a strongly reduced potential to mediate this histone modification (Figure 7C, panel

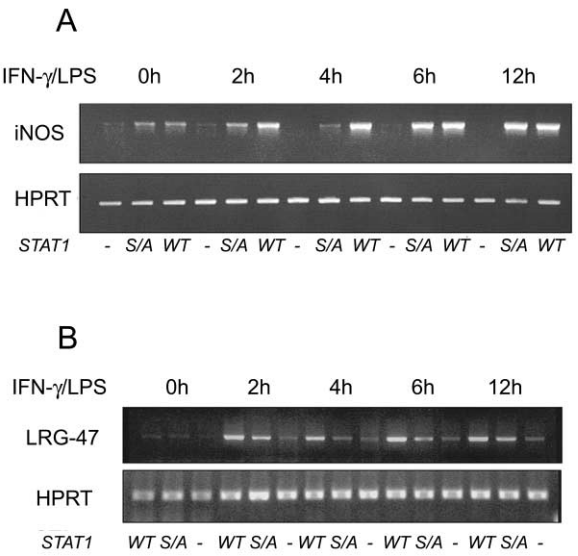

Figure 6. Reduced Expression of iNOS and LRG-47 after IFN-γ/LPS Costimulation in $STAT1_{S727A}$ Primary Macrophages

Bone marrow-derived macrophages were stimulated with 10 ng/ml (240 IU/ml) IFN-γ and 10 ng/ml LPS for the indicated time points, and RNA was extracted and reverse transcribed. Inducibility of the genes encoding iNOS (A) and LRG47 (B). Transcript levels were analyzed by RT-PCR and normalized to endogenous HPRT.

1). Our data thus suggest an impairment of the unphosphorylated Stat1 TAD to recruit histone acetylases to the GBP promoter.

CBP, a coactivator protein with histone acetyl transferase activity was shown to associate with the Stat1 C terminus (Zhang et al., 1996). In an in vitro system with recombinant proteins, binding was shown to be reduced upon mutation of S727 to A (Zakharova et al., 2003). To test whether reduced CBP binding might be in part responsible for the decreased Stat1-dependent histone acetylation,we performed coimmunoprecipitation assays using macrophage extracts. As shown in Figure 7D, IFN-γ stimulated association between CBP and Stat1, which was strongly reduced in extracts containing Stat1S727A.

Discussion

While the signaling pathways and kinases causing serine phosphorylation of Stats have received considerable attention, their significance in the context of the whole organism and specifically during challenge with pathogens has remained obscure. Introduction of Stat SA mutants into cell lines, or, as in the case of Stat4, primary cells, strongly suggested a contribution of serine phosphorylation to the transcription factor activity of Stats 1, 3, and 4 (Kovarik et al., 2001; Morinobu et al., 2002; Wen et al., 1995; Shen et al., 2003). Whether this contribution is essential in the context of an organism depends on the importance of gene dosage, i.e., the threshold of expression required for proper function. The relevance of this question has been emphasized by the recent analysis by Jim Darnell's group of mice expressing a $Stat3_{S727A}$ mutant, where the 50% transcriptional decrease observed with the $Stat3_{S727A}$/$Stat3_{S727A}$ genotype failed to cause phenotypical alterations (Shen et al, 2003). Our studies addressing the IFN-γ-dependent clearance of bacteria and amplification of LPS toxicity document the importance of Stat serine phosphorylation for innate immunity and inflammatory responses in vivo. The comparison with Stat1-deficient mice shows that $Stat1_{S727A}$ animals can cope with a low pathogen burden, which is lethal in the complete absence of Stat1. Similarly, in the context of inflammation, Stat1 serine phosphorylation is required only for a part of the IFN-γ-mediated increase of LPS toxicity. It thus appears that the engine Stat1 can function without its turbo charger phospho-S727, but it cannot successfully withstand a strong or maximal challenge for IFN-γ-dependent immunity. Serine phosphorylation of Stat1 provides an extra supply of the IFN-γ-induced gene products required for maximal protection against microbes. From this perspective the IFN-independent bacteria or stress-induced serine phosphorylation of Stat1 makes perfect sense because it links the transcriptional activity of the protein to pathogen quantity and the intensity of an inflammatory response (Kovarik et al., 1998).

The analysis of gene expression in $Stat1_{S727A}$ macrophages demonstrated a variable effect of the mutation on nuclear responses to IFN-γ. Some IFN-γ/Stat1 target genes were reduced to only about 10% maximal mRNA synthesis, and genes relevant for antibacterial responses were strongly affected by the absence of serine phosphorylation. This might explain why, unlike Stat3, the Stat1 mutation on both chromosomal alleles causes functional defects. The concomitant presence of IFN-γ and bacterial signals, i.e., the activation of additional transcription factors like NFκB and AP1, which impinge on antibacterial genes, only partially compensated for the lack of serine phosphorylation. Induction of iNOS eventually reached the levels found in wt macrophages, but the S727A mutation still caused a strong delay of expression. Induction of the infection-related gene LRG47 remained below wt levels also in the presence of LPS. These results strongly suggest that the reduced antibacterial immunity of $Stat1_{S727A}$ mice is due to a reduced or delayed expression of IFN-γ/Stat1 target genes.

The presence of the serine phosphorylation site within the TAD and the identification of proteins preferentially associating with the serine-phosphorylated TAD (Ouchi et al., 2000; Zhang et al., 1998) support the notion that phosphorylation might influence the interaction of Stat1 with coactivator complexes. Although the relevant complexes remain to be identified, our results demonstrating that the lack of serine phosphorylation strongly reduces histone acetylation at the GBP promoter and the association of Stat1 with the coactivator/histone acetylase CBP suggest this hypothesis to be true. Separate analysis of the two IFN-responsive subregions showed a strong decrease of acetylation at the proximal ISRE sequence. This sequence, shown to be dominant for IFN inducibility of the isolated GBP promoters during transient transfection (Briken et al., 1995; Nicolet and Paulnock, 1994), can stimulate IFN-γ-induced transcription by binding IRF1. Moreover, Stat1-containing complexes were proposed to participate in IFN-γ-induced transcriptional responses through the ISRE sequence (Bluyssen et al., 1995; Matsumoto et al., 1999). We show

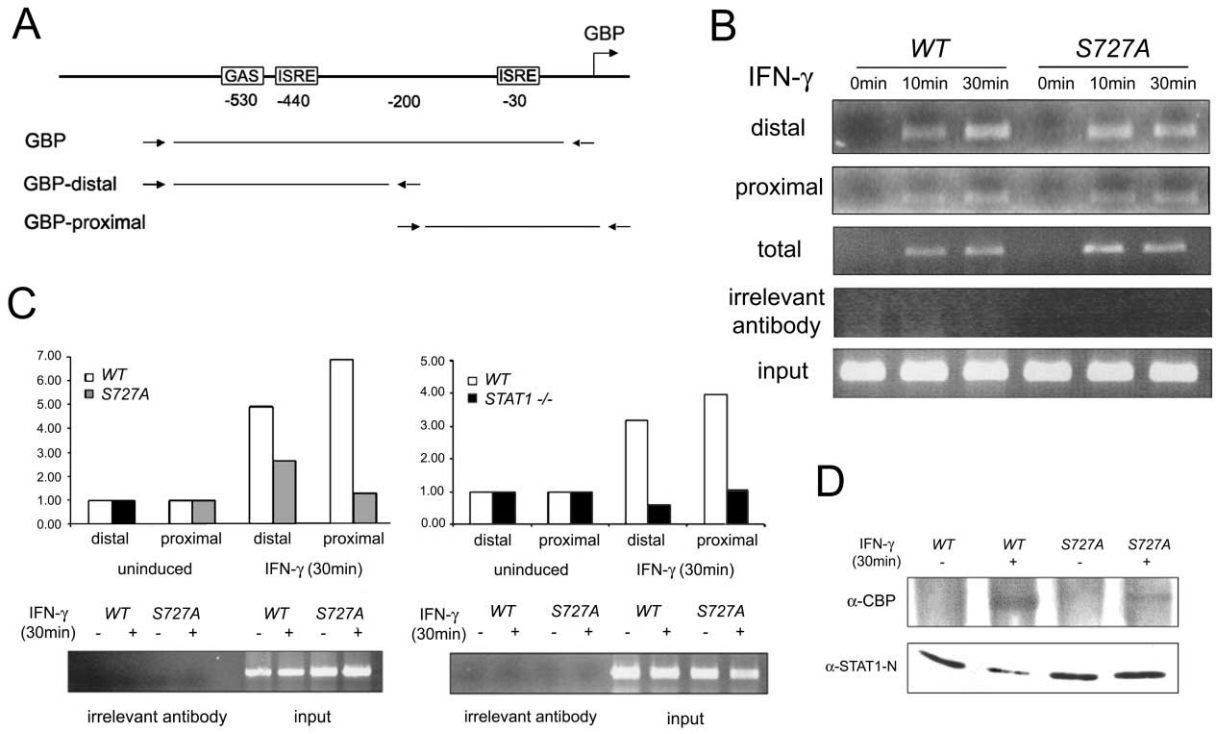

Figure 7. Mutation of Stat1 on S727 Decreases Histone Acetylation and Association with CBP, but Does Not Affect Association with Chromatin

(A) Schematic representation of the GBP-1 promoter depicting distal and proximal GAS and ISRE elements and the transcription initiation site. For the amplification of the distal and proximal promoter parts, the indicated primer pairs were used in chromatin immunoprecipitation (ChIP) assays.

(B) Wild-type and $STAT1_{S727A}$ bone marrow-derived macrophages were treated for 10 and 30 min with 240 IU IFN-γ. The cell extracts were analyzed for the effect of S727 phosphorylation on STAT1 DNA binding by ChIP assay. Immunoprecipitation was performed with a rabbit anti-STAT1 C-terminal antibody. As a control, serum from nonimmunized rabbits was used (irrelevant antibody). Quantitative PCR of the precipitate with the irrelevant antibody did not produce a specific amplification signal for the GBP promoter DNA (data not shown).

(C) Bone marrow-derived macrophages were used to detect histone acetylation levels of the distal and proximal region of the GBP promoter in ChIP assay using an acetyl-histone 4 antibody. Acetylation levels were determined by quantitative real-time PCR. Irrelevant antibody controls were performed as described for (B). Wild-type and $STAT1_{S727A}$ cells (panel 1), or wild-type and $STAT1^{-/-}$ (panel 2) macrophages were treated with IFN-γ for 30 min or left untreated.

(D) Bone marrow-derived macrophages were stimulated for 30 min with IFN-γ or left untreated. Immunoprecipitation was performed with a STAT1 C-terminal antibody, and the blot was probed with a CBP polyclonal antibody (upper panel) and a STAT1 N-terminal antibody (lower panel).

that, at least in the early stages of promoter activation, Stat1-containing complexes really bind nuclear ISRE sequences in the context of IFN-γ responses. Possibly, IRF1 binding to the ISRE requires Stat1-dependent chromatin remodeling. We do not know at present why serine phosphorylation is much less required for some IFN-γ target genes like SOCS1 or MHC I. One possibility is that coactivator complexes requiring serine phosphorylation for tight association with Stat1 are gene specific and are not necessary to the same extent for all target genes. A further open question for future studies concerns the repressive activity of Stat1 observed for some target genes. The lack of effect of $Stat1_{S727A}$ mutation on the expression of the IFN-γ gene after LPS administration suggests that, at the very least, not all repressive Stat1 activity requires S727 phosphorylation. Finally, cells derived from $Stat1_{S727A}$ mice will also be useful tools for identifying genes induced by the serine phosphorylated Stat1 in the absence of tyrosine phosphorylation. It will be of considerable interest to determine whether such genes mainly function in the context of apoptosis, as suggested by some recent publications (Agrawal et al., 2002; Kumar et al., 1997; Stephanou et al., 2001).

Experimental Procedures

Generation of S727A-Targeted Mutant Mice

After screening the λFix II 129Sv mouse genomic library (Stratagene), we isolated and fully sequenced a 17.3 kb genomic clone (GenBank Number AF349678), which included 12 exons from the 3′ end genomic locus of the Stat1 gene. This genomic sequence encoded amino acids 378–750. An 8.5 kb fragment including exons 22, 23a, and 23b from the 3′ end of the clone was subcloned into the pBlueskript vector. The neomycin selection cassette (kindly provided by Erwin Wagner, Institute of Molecular Pathology, Vienna Biocenter, Austria) flanked by two loxP sites was cloned into Bam HI-Kpn I sites between exons 22 and 23a. A fragment incorporating the A727 mutation in exon 23a was generated by PCR. The fragment was sequenced and cloned into the Aat I-Nhe I sites of the subcloned 8.5 kb Stat1 genomic sequence, replacing the nonmutated original exon. A diphtheria toxin (DT) gene for negative selection (kindly provided by Erwin Wagner) was cloned into the NotI site at the 3′ end of the 8.5 kb genomic fragment. The targeting construct was linearized after cleavage with PvuI, and electroporated into E14.1 ES cells. Transfected ES cells were cultured in the presence of 400

μg/ml G418 (Gibco). Cells resistant to G418 were expanded and identified for homologous recombination by PCR. The presence of the A727 mutation was verified by PCR-RFLP analysis, made possible by a NcoI site created by the mutated sequence. The Stat1$^{A727/+}$, Neo^{+} cells were reconfirmed by Southern blot and injected into C57/BL6 blastocysts. The male chimeric offspring were mated with C57BL/6 females. Heterozygous offspring were interbred to generate homozygous Stat1 A727, Neo^{+} animals. In order to delete the neomycin cassette, Stat1 A727 Neo^{+} mice were mated with female 129/C57/BL6 deleter mice carrying the Cre gene on the X chromosome (Schwenk et al., 1995). The progeny were screened for absence of the neomycin cassette and presence of the A727 mutation by PCR and Southern blot. Finally, heterozygous Stat1$^{A727/+}$ mice were interbred to generate homozygous Stat1 A727 mice.

Cells

Macrophages were derived from liquid bone marrow cultures as previously described (Baccarini et al., 1985). Fibroblasts were obtained from day 11.5 embryos by digestion with trypsin and cultured in DMEM, supplemented with 10% FCS. The EGD strain of *Listeria monocytogenes* (kindly provided by Martin Wagner, Veterinary Medical University, Vienna, Austria) was used for infection experiments. To assure virulence, the bacteria were reisolated from livers of infected mice. The bacteria were grown in brain heart infusion broth to the desired optical densities.

Cytokines, Reagents, Antibodies

Interferon-γ (kindly provided by G. Adolf, Boehringer Ingelheim, Austria) was used at a concentration of 240 U/ml. For the in vitro experiments *Salmonella minnesota* LPS (Sigma) was used at a concentration of 10 ng/ml. Antisera against the Stat1 C terminus and phospho-S727-STAT1 have been described recently (Kovarik et al., 1998). The phospho-Y701-Stat1 antibody was purchased by New England Biolabs (Beverly, MA) and used at a 1:1000 dilution. A polyclonal antiserum to the N terminus of CBP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bone marrow-derived macrophage single-cell suspensions were stained with FITC-labeled MHC I (clone M1/42AL) and FITC-labeled MHC II (clone 2G9) monoclonal antibody (kindly provided by K. Lingnau, Intercell, Austria) and analyzed by flow cytometry.

Gene Expression Analysis

Total RNA was isolated from bone marrow-derived macrophages using a nucleo-spin reagent kit (Clontech). Reverse transcription was performed using Mu-MLV reverse transcriptase (MBI Fermentas) according to the manufacturer's protocol. Primers and Taqman probes for HPRT, IRF1, SOCS1, TAP1 real-time PCR and also for iNOS RT-PCR have been described recently (Karaghiosoff et al., 2000; Kovarik et al., 2001). For the LRG-47 RT-PCR the following primers were used: LRG-47-f 5′-GGAACTGGTCTACGGAATCA AGG-3′ and LRG47-r 5′-GCTATTCTCTGCTTCCAGACTGC-3′.

Immunoprecipitation

5×10^6 primary macrophages were treated with 240 IU IFN-γ for 30 min or left untreated. Cells were lysed in IP buffer (10% glycerol, 20 mM Tris/HCl [pH 8], 135 mM NaCl, 1% NP-40). The binding of first antibody was performed overnight at 4°C with a Stat1 C-terminal antibody at a 1:500 dilution. Protein A sepharose beads were added for 2 hr, and immune complexes were washed twice with IP buffer. Beads were boiled in Laemmli Buffer for 10 min and subjected to SDS gel electrophoresis.

Chromatin Immunoprecipiation Assays

1×10^7 primary macrophages were treated with 240 IU IFN-γ for 10 and 30 min or left untreated. Chromatin immunoprecipitation assays were performed according to the acetyl-histone immunoprecipitation kit manufacturer's instructions (Upstate Biotech). Sonication was optimized to get fragments of approximately 500 bp (90% duty cycle, 40% output; 7×12 s). Chromatin was precipitated with 4 μl of acetyl histone H4 antibody (Upstate Biotech) or 2 μl of Stat1 C-terminal antibody (Kovarik et al., 1998), respectively. As a control for antibody specificity, precipitation was performed with rabbit serum (irrelevant antibody). Analysis of immunoprecipitated DNA was performed either with PCR using primers for the GBP promoter (5′-AGCTAGCTGATTTCCCAGCA-3′ and 5′-TTGTGAGGAGTTCGCT CTTT-3′) or by real-time PCR using two primer pairs covering proximal and distal regions to the GBP2 transcription initiation site, respectively (proximal for 5′-GCTGGCAACTTCACAAAACA-3′, rev 5′-TGCCAGAGAACTTGTGAGGA-3′; distal for 5′-TGATTTCCCAGCAT TTGACA-3′, rev 5′-AGGGTGAAAAGGGTGTGGTT-3′). Primers for histone 4 used as endogenous controls in real-time PCR, were described previously (Lagger et al., 2002).

In Vitro Assays

Antiviral activity assays with vesicular stomatitis virus (Indiana strain) were performed as described previously (Meraz et al., 1996). For the determination of intracellular bacteria in macrophage cultures, bone marrow-derived macrophages were cultured in 96-well plates at a density of 2×10^5 cells/well in penicillin/streptomycin-free medium containing 10% FCS and 15% L cell conditioned medium. Cells were stimulated with IFN-γ for 18 hr and subsequently infected with wild-type *L. monocytogenes* EGD strain at an MOI of 10. To measure phagocytic uptake, cells were incubated at 37°C, 5% CO2 for 30 min. Extracellular bacteria were removed by extensive washing with PBS, followed by macrophage lysis in distilled water. The cell lysates were transferred immediately to PBS, serially diluted, and plated onto Oxford *Listeria*-selective agar plates. Numbers of intracellular bacteria were determined by counting colony-forming units (cfu) in the cell lysates. To quantify intracellular bacteria at later time points, the cell supernatants were aspirated after the phagocytosis period and replaced with fresh penicillin/streptomycin-free medium containing 50 μg/ml gentamycin. Following incubation at 37°C for 1 hr, the cells were washed again and finally reincubated in medium containing 10 μg/ml gentamycin. At the appropriate time points the supernatants were removed, and the cells were thoroughly washed and lysed as outlined above.

In Vivo Experiments

For the in vivo bacterial challenge, mice were intraperitoneally injected with wild-type *L. monocytogenes* EGD strain at the indicated cell numbers per animal. The mice were monitored twice per day for survival over a period of 7 days. To determine the bacterial loads in spleen and liver, mice were injected with 5×10^5 live bacteria and sacrificed on the third day postinfection. The organs were homogenized, and the homogenates were serially diluted and plated onto Oxford *Listeria*-selective agar plates. The bacterial load/organ was determined by counting cfus in the organ homogenates.

For the in vivo LPS challenge, mice were injected intraperitoneally with 25 mg/kg of body weight with chromatographically purified *E. coli* LPS (Sigma) as described recently (Karaghiosoff et al., 2003).

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