c-Fos-Deficient Mice Are Susceptible to *Salmonella enterica* Serovar Typhimurium Infection[▽]

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c-Fos is a component of transcription factor AP-1. We show that macrophages lacking c-Fos exhibit enhanced production of proinflammatory cytokines, potentiated NF- κ B phosphorylation, and increased cell death following *Salmonella enterica* serovar Typhimurium infection. Furthermore, mice lacking c-Fos are highly susceptible to infection, suggesting that c-Fos confers resistance to *Salmonella* infection in mice.

Salmonella enterica serovar Typhimurium infection in mice provides a model to study the pathogenesis of typhoid-like systemic disease caused by gram-negative intracellular parasites. Detailed molecular mechanisms by which host cells, particularly macrophages, respond to and clear *Salmonella* infections are still obscure. *Salmonella* activates various Toll-like receptors (TLR) of host cells, such as TLR4 by lipopolysaccharide (LPS) and TLR5 by flagellin. TLR signaling activates transcription factors, including nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), which are responsible for producing proinflammatory cytokines (1, 11).

The transcription factor c-Fos, a component of AP-1, is necessary for osteoclast differentiation. Mice lacking c-Fos (*Fos*^{−/−} mice) are osteopetrotic due to a lack of osteoclasts (3, 18). In contrast to its role in activating osteoclastogenic or antiosteoclastogenic target genes (9, 17, 20), the function of c-Fos in the innate immune system remains unclear (10). c-Fos and AP-1 family genes are induced in macrophages activated by LPS and gamma interferon (1). Several studies reveal that c-Fos also functions in macrophages (2, 12, 15). Using *Fos*^{−/−} mice, we observed that the absence of c-Fos leads to enhanced production of tumor necrosis factor alpha (TNF- α), interleukin-12 p40 (IL-12 p40), and IL-6 but decreased production of IL-10 in response to LPS stimulation (13). However, the responses of *Fos*^{−/−} macrophages or mice to bacterial infection have not been evaluated.

To determine if inflammatory responses of macrophages infected with *Salmonella* are affected by lack of c-Fos, *Fos*^{+/-} mice on a 129 $\times$ C57BL/6 genetic background (18) were backcrossed to C57BL/6 until all progeny carried the *Salmonella*-sensitive *Nramp1* (*Slc11a1*) allele derived from C57BL/6 (Asp¹⁶⁹ mutation) (16). We then prepared macrophage colony-stimulating factor (M-CSF)-dependent macrophages (MDMs) from wild-type and *Fos*^{−/−} littermates and infected them with *Salmonella enterica* serovar Typhimurium strain χ 3306 (4). We harvested both cells and culture supernatants at various time points and measured mRNA and protein levels of TNF- α ,

FIG. 1. Proinflammatory cytokine production by MDMs after *Salmonella* infection. MDMs were generated by culturing nonadherent cells in the presence of 10 ng/ml M-CSF (R&D) for 4 days as described previously (8). Adherent cells were replated at 1×10^5 cells per well in 48-well plates. MDMs from wild-type (WT) and *Fos*^{−/−} (KO) mice were infected with *Salmonella enterica* serovar Typhimurium strain χ 3306 at an MOI of 10 for 30 min. Infected cells were washed and fed with fresh medium containing 25 μ g/ml gentamicin to kill extracellular bacteria. Cellular mRNA (left panels) and supernatant protein levels (right panels) of TNF- α , IL-12 p40, and IL-6 were measured by quantitative reverse transcription-PCR (RT-PCR) and enzyme-linked immunosorbent assay, respectively. Bars represent means $\pm$ standard deviations (SD) ($n = 3$ for each time point).

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FIG. 2. Induction of *Socs1* and *Socs3* mRNA is suppressed in *Fos*^{-/-} macrophages in response to *Salmonella* infection. (A) TLR signaling-associated transcripts in MDMs. MDMs were infected with *Salmonella* as described for Fig. 1. Cellular mRNA levels of *Tlr4*, *Ly96* (*MD2*), *Cd14*, *Myd88*, *Irak4*, *Irak3*, *Socs1*, and *Socs3* were measured by quantitative RT-PCR from triplicate cultures. The transcripts are relative to *Gapdh*. Bars represent means $\pm$ standard errors of the means.

FIG. 3. Western blotting of NF-κB p65 and Mal after *Salmonella* infection. MDMs prepared from wild-type (WT) or *Fos*^{-/-} (KO) mice in the presence of 30 ng/ml M-CSF were plated at 4×10^6 cells per 6-cm dish and infected with *Salmonella* as described for Fig. 1. Total cell lysates were harvested, and Western blotting was performed as previously described (8) using mouse monoclonal anti-phospho (Ser536) NF-κB p65 (phospho-p65) (7F1; Cell Signaling), rabbit polyclonal anti-NF-κB p65 (F-6; Santa Cruz), rabbit polyclonal anti-TIRAP (Mal/TIRAP) (Abcam), and anti-actin (I-19; Santa Cruz).

IL-12 p40, and IL-6. We observed that both mRNA and protein levels of IL-12 p40 and TNF-α in *Fos*^{-/-} MDMs were significantly higher than those seen for wild-type MDMs after *Salmonella* infection, while levels of IL-6 production were comparable between wild-type and *Fos*^{-/-} MDMs (Fig. 1).

To gain insight into molecular mechanisms underlying the observed hyperresponsiveness of *Fos*^{-/-} MDMs, we quantified mRNA levels of *Tlr4* (encoding TLR4), *Ly96* (MD-2), *Cd14* (CD14), *Myd88* (MyD88), *Irak4* (IRAK-4), *Irak3* (IRAK-M), *Socs1* (suppressor of cytokine response-1), and *Socs3* at 0, 2, 4, and 6 h after infecting MDMs with *Salmonella*. Strikingly, *Socs1* and *Socs3* induction was impaired in *Fos*^{-/-} MDMs (Fig. 2A). Consistently, when peritoneal macrophages (PMs) were infected with *Salmonella* or stimulated with LPS purified from *Salmonella*, induction of *Socs1* and *Socs3* mRNA was lower in *Fos*^{-/-} PMs than in wild-type PMs (Fig. 2B). SOCS-1 negatively regulates TLR signaling by mediating degradation of Mal (MyD88-adaptor like, also called TIRAP), an adaptor molecule that relays TLR signaling (7). One might predict that impaired *Socs1* induction in *Fos*^{-/-} MDMs would result in sustained activation of Mal followed by potentiated phosphorylation of the p65 subunit of NF-κB, which enhances proinflammatory cytokine production. We did observe potentiated p65 phosphorylation but not sustained Mal activation (Fig. 3). Therefore, at present, it is unknown how p65 phosphorylation is elevated in the absence of c-Fos.

Salmonella can induce cell death in macrophages via diverse

WT, wild type; KO, *Fos*^{-/-}. (B) *Socs1* and *Socs3* transcripts in PMs. Peritoneal cells were plated at 1×10^5 cells per well in 48-well plates and cultured overnight, and adherent cells were used as PMs (duplicate cultures for each genotype). Cells were infected with *Salmonella* as described for Fig. 1 or stimulated with 100 ng/ml LPS (*S. enterica* serovar Minnesota Re595; Sigma) for 4 h, and *Socs1* and *Socs3* mRNA levels were measured by quantitative RT-PCR from duplicate cultures. The transcripts are relative to *Gapdh*. Bars represent means $\pm$ standard errors of the means.

FIG. 4. Induction of apoptosis by *Salmonella* infection. MDMs prepared as described for Fig. 3 were infected at 3×10^5 cells per 24 wells as in Fig. 1 at an MOI of 5 (upper panel) or 100 (lower panel) for 1 h. After an additional 1 or 8 h of incubation, cells were stained by TUNEL (Promega), and the percentages of TUNEL-positive cells relative to total DAPI (4',6'-diamidino-2-phenylindole)-positive nuclei were calculated. Error bars represent means $\pm$ SD ($n = 3$ each). *, $P < 0.05$ versus wild-type control; **, $P < 0.01$ versus wild-type control.

mechanisms (5). We therefore compared the frequencies of apoptosis for wild-type and *Fos*^{-/-} MDMs after *Salmonella* infection. MDMs of each genotype were infected with *Salmonella*, and after 1 or 8 h, apoptotic cells were stained by a terminal deoxynucleotidyltransferase-mediated biotin-dUTP nick end labeling (TUNEL) method. At a low multiplicity of infection (MOI) of 5, *Fos*^{-/-} cultures contained a greater abundance of TUNEL-positive cells than did wild-type controls 8 h after infection (Fig. 4, upper panel). At a high MOI (100), increased apoptosis in *Fos*^{-/-} MDMs was apparent 1 h after infection (Fig. 4, lower panel).

To determine whether *Fos*^{-/-} mice are resistant or susceptible to *Salmonella* infection, we orally infected wild-type and *Fos*^{-/-} mice with 3.3×10^7 CFU/g body weight of *Salmonella*. We sacrificed infected mice on day 3 and measured levels of TNF- α , IL-12 p40, and IL-6 in sera. Consistent with in vitro data (Fig. 1), TNF- α and IL-12 p40 levels were greatly elevated in *Fos*^{-/-} compared to those in wild-type mice (Fig. 5A). On the other hand, IL-6 levels after infection were similar for both genotypes (Fig. 5A). We next quantified *Salmonella* CFU in these mice on day 3. *Fos*^{-/-} mice showed significantly higher numbers of CFU in blood, spleen, and liver than did wild-type mice (Fig. 5B). Finally, we observed that while wild-type mice survived infection, all *Fos*^{-/-} mice died within 7 days (Fig. 5C). These data show that lack of c-Fos results in increased proliferation of *Salmonella*, elevated production of proinflammatory cytokines, and death.

FIG. 5. *Fos*^{-/-} mice are susceptible to *Salmonella* infection. (A and B) Wild-type (WT) and *Fos*^{-/-} (KO) mice ($n = 4$ for each genotype) were inoculated orally with 3.3×10^7 CFU/g body weight of *Salmonella*. Serum cytokine levels (A) and CFU in blood, liver, and spleen (B) were determined 3 days after infection. Error bars represent means $\pm$ SD ($n = 3$ each). *, $P < 0.05$ versus wild-type control; **, $P < 0.01$ versus wild-type control. Cont., control; Infec., infected. (C) Survival of mice was monitored for 1 week after infection as described for panel A. $n = 4$ for each genotype.

We have shown that serum levels of TNF- α and IL-12 p40 are greatly elevated in *Fos*^{-/-} mice after *Salmonella* infection, most likely due to potentiated NF- κ B p65 phosphorylation. Currently, we cannot explain why c-Fos loss does not affect IL-6 induction after *Salmonella* infection, while it does after LPS stimulation (13). Increased levels of proinflammatory cytokines, particularly TNF- α , cause profound endotoxic shock in *Fos*^{-/-} mice (13). Consistently, mice lacking TNF- α or mice injected with a neutralizing anti-TNF- α antibody show increased survival after endotoxic shock (14). On the other hand, increased mortality observed for *Fos*^{-/-} mice is associated with excessive proliferation of *Salmonella* in various organs. Since increased proinflammatory cytokines are reported to promote bacterial proliferation in vitro (6), elevated proinflammatory cytokines might enhance bacterial proliferation in *Fos*^{-/-} mice, thereby increasing lethality. Alternatively, elevated proinflammatory cytokines may directly enhance apoptotic cell death of macrophages and other immune cells (19), allowing bacterial proliferation. We cannot formally exclude the possibility, however, that elevated proinflammatory cytokines help

clear bacteria in *Fos*^{-/-} mice after limited infection at lower doses.

In this study, we report that *Fos*^{-/-} mice are highly susceptible to *Salmonella* infection and that *Salmonella* replicates more vigorously in *Fos*^{-/-} than in wild-type mice. These findings suggest that c-Fos confers protective immunity against *Salmonella* in mice.

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