The Journal of Microbiology (2011) Vol. 49, No. 6, pp. 1050-1053
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DOI 10.1007/s12275-011-1043-z

NOTE

IL-10 Suppresses Bactericidal Response of Macrophages against *Salmonella* Typhimurium[§]

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(Received January 26, 2011 / Accepted June 20, 2011)

We report, herein, an attempt to determine whether an IL-10-induced immunological state affects the response of macrophages against *Salmonella* Typhimurium (ST). Pretreatment with mrIL-10 induced the intracellular invasion of ST into macrophages in a dose-dependent manner. It also activated AKT phosphorylation, cyclin D1, Bcl-X_L, and COX-2 upon ST infection, which may correlate with Salmonella's survival within the macrophages. However, I-κB phosphorylation was shown to be inhibited, along with the expression of TNF-α and MIP-2α mRNA. Therefore, IL-10 not only suppresses the bactericidal response of macrophages against ST, but also ultimately causes infected macrophages to function as hosts for ST replication.

***Keywords*:** IL-10, macrophage, *Salmonella*

During bacterial infection, macrophages perform a critical function in the removal of bacteria via endocytosis and phagolysosomes, which are important defense mechanisms of macrophages against exogenous pathogens. However, many bacterial pathogens employ their own strategies to avoid these mechanisms and replicate in macrophages. *Salmonella*, *Mycobacterium*, and *Helicobacter* are bacterial pathogens that evidence such abilities (Monack *et al.*, 2004). *Salmonella* spp. are Gram-negative bacteria known to cause various diseases ranging from gastroenteritis and diarrhea to typhoid fever and sepsis. Although *Salmonella* Typhimurium (ST) is the etiologic agent of gastroenteritis in humans, it causes a systemic infection in mice that is similar to human typhoid fever (Santos *et al.*, 2001).

Salmonella harbors two distinct virulence-associated genes, *Salmonella* pathogenicity island (SPI)-1 and SPI-2 (Haraga *et al.*, 2008). Intracellular *Salmonella* releases a variety of "effector" proteins from the SPI system. Among these, SopB/SigD activates AKT/protein kinase B (PKB), a pro-survival kinase, in host cells and suppresses host cell death to promote persistent *Salmonella* infection (Knodler *et al.*, 2005; Fink and Cookson, 2007; Prost *et al.*, 2007).

Cytokines are crucial factors for determining the functional phenotypes of macrophages, and also regulate immune responses. Upon pretreatment with cytokines before LPS stimulation, macrophages produce many different patterns of released cytokines (Stout *et al.*, 2005). Interleukin (IL)-4 and -13 stimulate the endocytosis of murine macrophages, while interferon-γ inhibits this process (Raveh *et al.*, 1998). Interestingly, the *spiC* gene of ST induces an up-regulation of IL-10 in macrophages via protein kinase A (Uchiya and Nikai, 2004). Additionally, two other intracellular pathogens, *Mycobacterium tuberculosis* and *Leishmania major*, require IL-10 for persistent infection (Belkaid *et al.*, 2001; Beamer *et al.*, 2008). These findings imply that IL-10 can play an important function in persistent infections by pathogenic bacteria.

IL-10, an 18 kDa cytokine, is produced in a broad variety of cells, including T-helper type 2 (Th2) cells, regulatory T cells, DCs, B cells, macrophages, and even tumor cells (Mosser and Zhang, 2008). IL-10 also activates AKT phosphorylation via the phosphoinositide 3-kinase (PI3K) pathway (Williams *et al.*, 2004). However, in an IL-10-dominant immunological state, the relationship between macrophages and *Salmonella* remains unknown. In order to address this issue, macrophages pretreated with IL-10 were infected with ST to assess the influence of IL-10 on ST infection. Changes in signal transduction and mRNA expression of various factors related to cell survival and immune responses were assessed.

The M-15 *Salmonella* Typhimurium (ST) was used in this study, and grown in brain heart infusion broth (Merck, Germany), at 37°C overnight, with agitation. The murine macrophage RAW 264.7 cells were purchased from the Korean Cell Line Bank (South Korea) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; PAA Laboratories Inc., Austria) supplemented with 10% fetal calf serum (FCS; PAA Laboratories Inc.), 100 U of penicillin, and 100 μg/ml of streptomycin (Gibco BRL, USA).

First, the effect of murine recombinant IL-10 (mrIL-10) on ST infection of macrophages was evaluated via *in vitro* infection. Cells were seeded at a density of 2×10^4 cells/well in 96-well plates and incubated for 24 h. For mrIL-10 treatment and bacterial infection, seeded RAW cells were washed

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§ Supplemental material for this article may be found at http://www.springer.com/content/120956

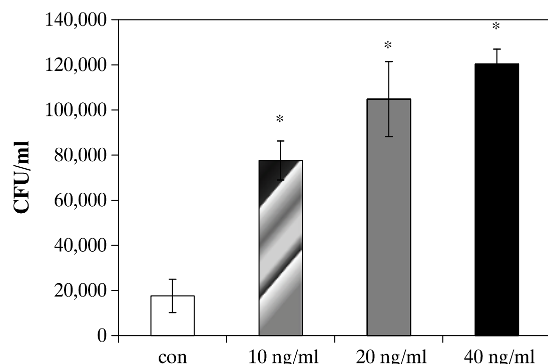

Fig. 1. Effects of mrIL-10 on the invasion of *Salmonella* Typhimurium within macrophages. After pretreatment with the indicated concentrations of mrIL-10, RAW cells were infected with *Salmonella* Typhimurium for 90 min. All treatments were carried out in triplicate. The results shown are representative of two independent experiments. Values for the number of intracellular *Salmonella* Typhimurium are represented as the Mean±SEM. *, $p<0.05$ compared with untreated controls using Student's *t*-test.

with PBS, then incubated in antibiotic-free DMEM supplemented with 5% FCS and mrIL-10 (10, 20, and 40 ng/ml; BD Pharmingen, USA) 4 h before the start of the infection. ST was diluted to a multiplicity of infection (MOI) of 50 in antibiotic-free DMEM containing 1% FCS. ST was added to cell cultures at an MOI of 50 and the cultures were centrifuged briefly for 5 min at 500×g. The plates were subsequently incubated for 90 min at 37°C and washed two times with PBS. To remove extracellular ST, DMEM with 5% FCS and 50 μg/ml gentamicin was added to each well. After 30 min, cells were lysed with 1% Triton X-100 (Bio Basic Inc., Canada) in water, and then serial dilutions of the lysates were spotted onto DHL agar plates (Merck). The amount of intracellular ST in the mrIL-10-pretreated cells was significantly higher ($p<0.05$) than that in the non-pretreated cells (Fig. 1). The numbers of intracellular ST were increased depending on the dosage of mrIL-10. Additionally, in the cell-viability assay using WST-1 (EZ-Cytox, Daeil Lab Co. Ltd., Korea), mrIL-10-pretreated RAW cells evidenced significantly ($p<0.05$) higher survival than the untreated cells after ST infection for 18 h (data not shown). These data indicate that IL-10 contributed to the ST invasion of macrophages.

Next, we attempted to determine whether an mrIL-10-dominant immunological state affects signal transduction, and mRNA expression related to the survival and defense of macrophages during ST infection. RAW cells were seeded at a density of 1×10^6 cells/well in 6-well plates and incubated for 24 h. After mrIL-10 treatment (20 ng/ml), the cells were infected with ST for 90 min at 37°C, and harvested for Western blot and mRNA analyses. For Western blot analysis, the harvested cells were lysed in 0.4 M RIPA buffer (Cell Signaling Technology, USA). The isolated proteins were separated via SDS-PAGE and transferred onto a 0.45 μm nitrocellulose membrane. The membranes were incubated overnight with antibodies against phospho-AKT (1:1,000 dilution), cyclin D1 (1:1,000), Bcl-X_L (1:1,000), phospho-IκBα (1:500), and IκBα (1:1,000) (all from Cell Signaling Technology, USA), GAPDH (1:2,000, Santa Cruz Biotechnology, USA), or COX-2 (1:1,000, Cayman, USA). After 90 min of incubation with secondary antibodies, primary antibody binding was detected using a LumiGLO® chemiluminescent detection system (KPL, USA). For mRNA analysis, total RNA was extracted from the cells lysed with TRIzol reagent (Invitrogen Co., USA) according to the manufacturer's instructions. First strand cDNA was prepared from total RNA (1 μg) via reverse transcription using M-MLV reverse transcriptase (Invitrogen). TaqMan™ fluogenic probes and real-time PCR primers (Table 1) were designed by Metabion Int. (Germany). Real-time PCR was conducted with a Chromo4™ real-time PCR system (Bio-Rad, USA) using the TaqMan-iQ™ supermix kit (Bio-Rad) and analyzed using Opticon Monitor 3 analysis software (Bio-Rad). A microliter volume of cDNA was used for RT-PCR amplification with a PCR premix (Hotstart®; Bioneer Inc., Korea); PCR products were separated via electrophoresis on 1.5% agarose gel.

ST infection significantly induced AKT phosphorylation levels, but reduced the expression of cyclin D1 and Bcl-X_L (Fig. 2). In infected macrophages, treatment with mrIL-10 resulted in enhanced phosphorylated AKT, cyclin D1, and Bcl-X_L relative to untreated cells. AKT phosphorylation is regarded as an important mediator for the persistent intracellular proliferation of ST in epithelial cells (Knodler *et al.*, 2005). The phosphorylation of AKT is also correlated positively with the production of cyclin D1, a critical player in cell cycle progression, and Bcl-X_L, an anti-apoptotic protein (Chang *et al.*, 2003). In particular, cyclin D1 mRNA expression was downregulated upon ST infection (Fig. 3A). We also evaluated the

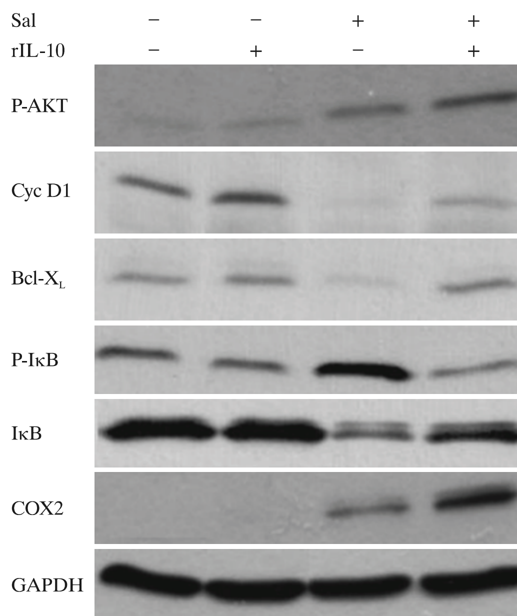

Fig. 2. Western blot analysis of macrophages after *Salmonella* Typhimurium infection. After mrIL-10 treatment (20 ng/ml), whole proteins from *Salmonella* Typhimurium-infected cells (50 MOI) were extracted using 0.4 M RIPA buffer. The expression levels of each protein were determined by Western blotting. The figure shows a representative blot from three independent experiments.

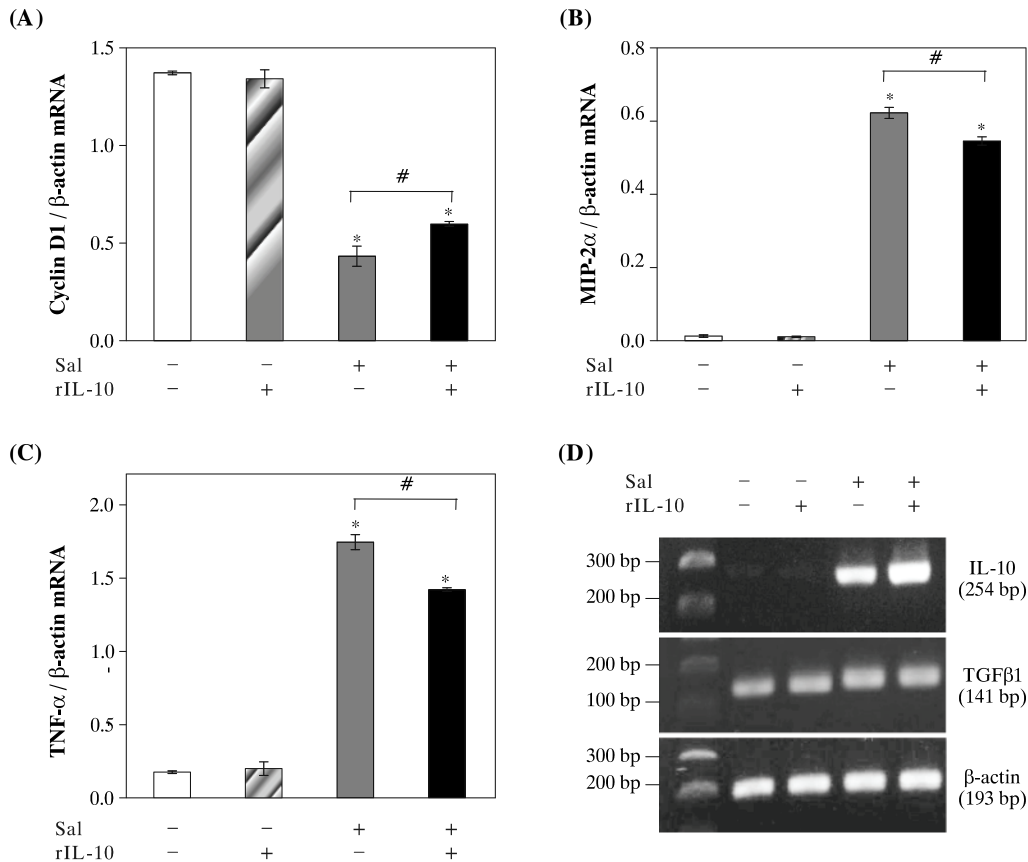

Fig. 3. mRNA expression in *Salmonella* Typhimurium-infected macrophages. After mrIL-10 treatment (20 ng/ml), total mRNA was obtained from *Salmonella* Typhimurium-infected cells (50 MOI), and reverse-transcribed. The expression levels of cyclin D1 (A), MIP-2α (B), and TNF-α (C) mRNAs were determined via real-time quantitative PCR, and normalized against β-actin mRNA. IL-10 and TGF1β were analyzed by RT-PCR (D). The data are expressed as the Mean±SEM of three independent experiments.
*, $p<0.05$ compared with both uninfected controls; #, $p<0.05$ compared with untreated cells using Student's *t*-test.

expression of cyclooxygenase-2 (COX-2), known as prostaglandin synthase, which is essential for the survival of ST within the macrophages (Uchiya and Nikai, 2004). COX-2 expression in infected macrophages was higher than that in uninfected macrophages, and the response was increased as the result of mrIL-10 treatment. Thus, our results indicate that mrIL-10 treatment can protect ST-infected macrophages against cell death via the activation of phosphorylated AKT, cyclin D1, Bcl-X_L, and COX-2.

Nuclear factor-kappa B (NF-κB), activated by the phosphorylation of IkappaB alpha (IκBα), is a key transcriptional modulator of innate and adaptive immunity (Hoffmann and Baltimore, 2006). IL-10 activates the suppressors of cytokine signaling (SOCS) and prevents NF-κB activation (Williams *et al*., 2004). We indirectly assessed the activity of NF-κB after infection by monitoring IκBα phosphorylation (Fig. 2). We determined that IκBα phosphorylation was increased in infected macrophages, and was attenuated by mrIL-10 treatment. We also analyzed the mRNA expression of TNF-α, macrophage inflammatory protein (MIP)-2α, IL-10, and TGFβ1 (Fig. 3). Consistent with a previous study demonstrating that ST and LPS trigger the mRNA expression of these factors in macrophages (Rosenberger *et al.*, 2000), our analysis showed that TNF-α, MIP-2α, and IL-10 mRNA were expressed at high levels by infected macrophages. However, the mRNA levels of TGFβ1, another anti-inflammatory cytokine, were unaffected by ST infection and mrIL-10 treatment. The mrIL-10 treatment slightly alleviated the level of TNF-α and MIP-2α mRNA induced by infection, but enhanced the induction of IL-10 mRNA.

We showed that the response of macrophages against ST can be determined by their immunological state. Macrophages in an mrIL-10-induced immunological state activate AKT, cyclin D1, Bcl-X_L, and COX-2, which permitted intracellular ST survival and replication. Additionally, this study demonstrated that the immunological state down-regulates the immune responses of macrophages by suppressing I-κB phosphorylation as well as the mRNA expression of TNF-α and MIP-2α. All pathogens need to establish a safe site as a replication niche in their respective hosts. Bacterial pathogens have evolved traits for survival in the host. These are classified into three methods (Faherty and Maurelli, 2008): (1) avoiding recognition by the host immune system, (2) inducing apoptosis in immune cells such as macrophages and neutrophils, (3) manipulation and suppression of cell death. Many bacteria that can exist intracellularly, including ST and *Mycobacterium*, utilize

host immune cells for the progress of infection (Prost *et al*., 2007; Lee *et al*., 2009).

Previous studies have demonstrated that cytokines produced by various immune cells, induce macrophages with distinct physiologies, and that macrophages are sequentially altered by their microenvironment (Stout *et al*., 2005; Mosser and Edwards, 2008). It has previously been reported that ST stimulates IL-10 production in macrophages, and that the increased IL-10 levels affect the suppression of bactericidal functions of activated macrophages but not pathogen survival (Uchiya *et al*., 2004). However, in this study we demonstrated that an mrIL-10-induced immunological state increased the invasion of ST into macrophages. The mRNA expression of IL-10 was higher in ST-infected macrophages than in uninfected macrophages, and this response was enhanced by mrIL-10 treatment (Figs. 2 and 3).

Additionally, the mrIL-10 treatment of infected macrophages promoted AKT phosphorylation, thereby inhibiting cell death. This result is consistent with a previous review describing the manner in which IL-10 can activate AKT phosphorylation via the PI3K signaling pathway (Williams *et al*., 2004). Indeed, IL-10 appears to be essential for the persistence of infection with intracellular bacteria and viruses including *Leishmania major*, *Mycobacterium tuberculosis*, hepatitis C virus, and human immunodeficiency virus (Belkaid *et al*., 2001; Ejrnaes *et al*., 2006; Beamer *et al*., 2008). Collectively, mrIL-10 promotes the survival of infected macrophages, which can create a beneficial environment for ST to reside within these cells. ST infection reduced cyclin D1, an activator of cell proliferation, and Bcl-X_L, an anti-apoptotic protein. However, these reductions were attenuated by treatment with mrIL-10. We also determined that mrIL-10 treatment contributed to high COX-2 expression in infected macrophages, which triggers the expression of Bcl-X_L (Oh *et al*., 2007). Interestingly, a pathogenicity island on the ST chromosome, SPI-2, activates the expression of COX-2 in macrophages for intracellular ST survival via the up-regulation of PGE2 or PGI2, which participate in the inhibition of host defense systems (Uchiya and Nikai, 2004).

In conclusion, we assessed the effects of IL-10 on macrophages during ST infection. The results of the present study demonstrated that IL-10 increased the invasion of ST into macrophages in a dose-dependent manner via the suppression of the host immune response, and enhanced the survival of infected macrophages. Based on the results of these studies, it is presumed that mrIL-10-stimulated macrophages can function as carriers of systemic infections. Thus, the immunological state, governed by IL-10, can provide beneficial effects for *Salmonella*, enabling the pathogen to circumvent the defenses of the host.

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (KRF-2007-313-E00532).

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