Reduced interleukin-18 secretion in *Brucella abortus* 2308-infected murine peritoneal macrophages and in spleen cells obtained from *B. abortus* 2308-infected mice

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Th1 immune responses in which gamma interferon (IFN-γ) production predominates are associated with protective immunity against intracellular bacteria. Following infection, interleukin-18 (IL-18) may contribute, in association with IL-12, to optimal IFN-γ production. In this study, the secretion of IL-18 following intracellular infection with virulent *Brucella abortus* 2308 in CD-1 cultured peritoneal macrophages and splenocyte cultures was investigated. The production of IL-18 was reduced in both CD-1 mouse peritoneal macrophages infected with *B. abortus* 2308 and splenocyte cultures obtained from *B. abortus* 2308-infected mice at 3, 6 and 10 days post-infection (p.i.). In contrast, splenocyte cultures obtained from *B. abortus* 2308-infected mice at 3 days p.i. secreted significant amounts of IFN-γ. Stimulation of these cells with recombinant IL-18 (rIL-18) and/or rIL-12 did not significantly increase IFN-γ secretion at the splenocyte level. These data suggest that once the infection has been established, *B. abortus* 2308 selectively limits IL-18 secretion without affecting endogenous IFN-γ production.

INTRODUCTION

*Brucella abortus* is a facultative intracellular bacterium and is one of the causative agents of brucellosis in animals and humans (Fernández-Lago et al., 1999). Professional phagocytes are the first target of *B. abortus* invasion and the bacteria are able to survive and multiply within these cells (Pizarro-Cerdá et al., 1998). Resistance to *B. abortus* depends on the effective generation of a T-cell-mediated response (Araya et al., 1989) as well as on an effective activation of macrophages by gamma interferon (IFN-γ)-producing CD4 T-lymphocytes (Zhan et al., 1993). IFN-γ is a key mediator in conferring protection against *Brucella* spp. infections both in vivo (Zhan & Cheers, 1995) and in vitro (Jiang & Baldwin, 1993), and an elevated IFN-γ production, mainly in the early phase of the infection, could be crucial in their control (Pasquali et al., 2002). In consequence, cytokines secreted in this initial response, which can affect IFN-γ secretion, can strongly affect the outcome of infection.

Interleukin-18 (IL-18) is a proinflammatory cytokine produced by several cell types, including activated macrophages, dermal keratinocytes, osteoblasts, adrenal cortex cells and intestinal epithelial cells (Gerdes et al., 2002), that is synthesized as a biologically inactive 24 kDa precursor protein. Cleavage to an 18 kDa active mature protein, mediated by the interleukin-1 (IL-1)-converting enzyme, also called caspase-1, is essential for IL-18 to become biologically active (Ghayur et al., 1997). Although IL-18 alone does not induce significant IFN-γ production, it synergistically enhances IL-12-stimulated IFN-γ production (Yoshimoto et al., 1998) and promotes cell-mediated immunity (Takeda et al., 1998). Besides its IFN-γ inducing effect, IL-18 has a direct proinflammatory effect on T and natural killer cells, enhancing proliferation and cytotoxicity and stimulating the production of cytokines such as tumour necrosis factor alpha (TNF-α), IL-1β, IL-6 and IL-8 (Netea et al., 2000).

Previous studies have demonstrated the role of IL-18 in the host’s response to infection. Endogenously synthesized IL-18 has been demonstrated to be required in mice for an adequate host defence against mycobacterial infections (Sugawara et al., 1998), disseminated candidiasis (Stuyt et al., 2004), pneumococcal pneumonia (Lauw et al., 2002), and *Pseudomonas aeruginosa* (Schultz et al., 2003), *Yersinia enterocolitica* (Bohn et al., 1998), *Salmonella typhimurium* (Mastroeni et al., 1999), *Leishmania major* (Ohkus et al., 2000) and *Listeria monocytogenes* (Neighors et al., 2001) infections. Recently, it has been shown that only a combined administration of

Abbreviations: HK, heat-killed; i.p., intraperitoneal; p.i., post-infection; S-LPS, smooth lipopolysaccharide.
recombinant IL-18 and IL-12 induces protection against *B. abortus* 2308 infection in mice, and this effect is possibly due to an increase in IFN-γ production in the early phases of infection (Pasquali et al., 2001, 2002). However, at present the dynamics of endogenous IL-18 production in response to a *B. abortus* infection have not been thoroughly investigated.

The aim of the present study was to investigate the capacity of *B. abortus* 2308 to stimulate the secretion of IL-18 by cultured CD-1 peritoneal macrophages and splenocyte cultures, and to evaluate the role of this cytokine in IFN-γ production in response to *B. abortus* 2308 infection.

**METHODS**

**Mice.** Female outbred CD-1 mice (Charles River, Barcelona, Spain) were purchased at 8 weeks of age and kept for 1 week before use. The animals were housed in microisolation cages in Horsefall units, and were cared for in accordance with standard guidelines.

**Bacteria and infection of animals.** Virulent *B. abortus* 2308, used as the challenge strain, was cultured on tryptic soy agar (TSA; BBL) enriched with 0.3 % (w/v) yeast extract (YE; Difco) for 72 h at 37 °C under a 5 % (v/v) CO2 atmosphere in air. To infect the mice, the bacteria were suspended in sterile PBS (pH 7.4) and adjusted turbidimetrically to a concentration of 10^6 cells ml^-1. Macrophages were derived from female outbred CD-1 mice, purchased at 8 weeks of age and kept for 1 week before use. Sigma. Recombinant mouse IL-18 and IL-12 were obtained from Pharmingen.

**Phagocytosis.** Virulent *B. abortus* 2308, and S-LPS from *E. coli* O157 was obtained, as a percentage of dry weight, 1.5 % protein and 1.1 % 2-keto-3-deoxyoctulosonic acid. S-LPS from *E. coli* O157 (1 μg ml^-1; Sigma). Macrophage supernatants were also obtained at 48 h from each well, filtered, and kept at −70 °C until assayed.

**Cytokine ELISA.** Cytokine contents in macrophage and splenocyte supernatants were determined by ELISA, using commercial kits for IL-18 (biologically active and non-active forms) and IFN-γ (OptEIA; Pharmingen). Protocols were as recommended by the manufacturer. Known concentrations of each cytokine were used to generate standard curves in each assay and used as references to cytokine concentrations. Samples were assayed in duplicate and values are expressed as means ± SD. The lowest limit of detection for IL-18 was 30 pg ml^-1.

**Statistical methods.** All experiments in this study were performed at least twice. Experimental values are given as means ± SD. The data obtained were analysed by one-way analysis of variance, comparing the mean values with the Fisher’s protected LSD (least significant differences, *P* < 0.05).
E. coli from cultured peritoneal macrophages were exposed to S-LPS abortus 2308 (LPS-Ba; 20 μg ml⁻¹) or a B. abortus 2308 culture extract (Extract-Ba; 1:10 dilution). After 2 days of culture, supernatants were collected at 48 h for cytokine analysis. The level of sensitivity for both preparations (data not shown). These findings suggest, as happens with wild-type Salmonella dublin infections (Elhofy & Bost, 1999), that this decrease in IL-18 synthesis in mouse macrophages is possibly dependent on the intracellular replication of B. abortus 2308, although the mechanism responsible for this remains to be elucidated.

**IFN-γ production in B. abortus 2308-infected splenocyte cultures is not dependent on the presence of IL-18**

In subsequent experiments we analysed the effect that the reduction in IL-18 secretion, observed in cultured macrophages as a result of B. abortus 2308 infection, had on IFN-γ secretion in B. abortus 2308-infected mice at the splenocyte level. As shown in Table 1, a 48 h splenocyte culture supernatant obtained from uninfected CD-1 mice constitutively expressed IL-18. A significant decrease (P < 0.05) in the secretion of this cytokine was observed in 48 h splenocyte cultures obtained from B. abortus 2308-infected mice on days 3, 6 and 10 p.i., and stimulated in vitro with 10⁷ HK B. abortus 2308 cells. In contrast, a statistically significant (P < 0.05) increase in IFN-γ secretion by the splenocyte cultures was observed on days 3, 6 and 10 p.i. (Table 1). These results show that the limited secretion of IL-18, as a result of B. abortus 2308 infection, does not significantly affect IFN-γ production, and therefore that IL-18 would not be involved in B. abortus-induced IFN-γ secretion at the splenocyte level. To investigate this further, splenocytes were obtained from B. abortus 2308-infected mice at 3 days p.i. and, once stimulated

![Graph showing IL-18 and IFN-γ levels after infection](image)

**Fig. 1.** B. abortus 2308-infected peritoneal macrophages secrete reduced amounts of IL-18. Cultured peritoneal macrophages were uninfected, infected with virulent B. abortus 2308 (Ba-infected), or treated with S-LPS from E. coli (LPS-Ec; 1 μg ml⁻¹), S-LPS from B. abortus 2308 (LPS-Ba; 20 μg ml⁻¹) or a B. abortus 2308 culture extract (Extract-Ba; 1:10 dilution). After 2 days of culture, supernatants were harvested and assayed for IL-18 by ELISA. Results are expressed as means ± SD (five wells per point). *Statistically different from uninfected macrophage cultures (P < 0.005).

**Table 1.** Production of IL-18 and IFN-γ in stimulated spleen cells from B. abortus 2308-infected mice

CD-1 mice were i.p.-infected with 5 × 10⁷ c.f.u. B. abortus 2308. Mice, five per group, were killed at 3, 6 and 10 days after infection. Data are mean values of five animals ± SD. An asterisk indicates that the results are statistically significant (P < 0.05) as compared to those for the uninfected control group.

<table>
<thead>
<tr>
<th>Time after infection that splenocytes were obtained (days)†</th>
<th>Spleen wt (mg)</th>
<th>Cytokine level (pg ml⁻¹)‡</th>
<th>IL-18</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected control</td>
<td>100 ± 17</td>
<td>125 ± 10</td>
<td>&lt; 30</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>164 ± 20*</td>
<td>60 ± 8*</td>
<td>137 ± 20*</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>204 ± 15*</td>
<td>35 ± 3*</td>
<td>120 ± 15*</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>404 ± 47*</td>
<td>&lt; 30</td>
<td>130 ± 12*</td>
<td></td>
</tr>
</tbody>
</table>

†Splenocytes were cultured in RPMI medium and stimulated with 10⁷ HK B. abortus 2308 cells as described in Methods. Supernatants were collected at 48 h for cytokine analysis.

‡Culture supernatants were tested for IL-18 and IFN-γ by ELISA. The level of sensitivity for both cytokines was 30 pg ml⁻¹.

Reduced IL-18 secretion by B. abortus
Cytokines (10 ng ml\(^{-1}\)) were added to splenocyte cultures. Concanavalin A was used at 5 μg ml\(^{-1}\).

With 10\(^7\) HK B. abortus cells, were treated with rIL-12 (10 ng ml\(^{-1}\)) and/or rIL-18 (10 ng ml\(^{-1}\)) or concanavalin A (5 μg ml\(^{-1}\)). The levels of IFN-γ were then measured in the culture supernatants 48 h after stimulation. As shown in Table 2, the addition to splenocyte cultures of rIL-12, rIL-18 or both cytokines simultaneously did not significantly affect IFN-γ secretion as compared to untreated cells. A significant \((P < 0.05)\) increase in IFN-γ production was only seen in the concanavalin A-treated splenocyte cultures (Table 2). It may therefore be concluded that once the infection has been established, the exogenous addition of IL-12, IL-18 or both cytokines simultaneously does not contribute to IFN-γ production in response to B. abortus 2308 infection. Thus it is possible that both cytokines are active, inducing a protective endogenous IFN-γ production only when pharmacologically effective levels of them in a biologically active form are present in the first stages of infection (Pasquali et al., 2002).

**ACKNOWLEDGEMENTS**

This work was financed by project G03/204 Red para la Investigación de la Brucelosis, Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Spain.

**REFERENCES**


Reduced IL-18 secretion by *B. abortus*


