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

Luis Fernández-Lago,1 Antonio Orduña2 and Nieves Vizcaíno1

1Departamento de Microbiología y Geneética, Edificio Departamental, Universidad de Salamanca, Plaza Doctores de la Reina s/n, 37007 Salamanca, Spain
2Departamento de Microbiología, Facultad de Medicina, Universidad de Valladolid, Valladolid, Spain

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 (Neta 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.*, 1999), 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* (Ohkusu *et al.*, 2000) and *Listeria monocytogenes* (Neighbors *et al.*, 2001) infections. Recently, it has been shown that only a combined administration of
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 microisoler 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 the desired concentration. The exact dose was established retrospectively by viable cell counting. Mice were infected by the intraperitoneal (i.p.) route with 0.2 ml containing 5 × 10⁸ c.f.u. of the B. abortus 2308 virulent strain. Control groups received 0.2 ml PBS. At the indicated time in each experiment, five mice per group were killed by cervical dislocation.

Reagents. B. abortus 2308 smooth lipopolysaccharide (S-LPS) and culture supernatants obtained were described previously (Vizcaíno et al., 1991; Joubier-Maurin et al., 2001). The S-LPS contained, as a percentage of dry weight, 1.5% protein and 1.1% 2-keto-3-deoxyoctulosonic acid. S-LPS from Escherichia coli O157 was obtained from Sigma. Recombinant mouse IL-18 and IL-12 were obtained from Pharmingen.

Isolation and infection of CD-1 mouse peritoneal macrophages. Macrophages were isolated as described previously (Fernández-Lago et al., 1999), with some modifications. Briefly, CD-1 mice were inoculated i.p. with 0.3 ml incomplete Freund’s adjuvant (Sigma). Three days later, the mice were killed and peritoneal cells were harvested by washing with cold RPMI (Gibco) with 10% (v/v) fetal calf serum (FCS; Gibco). The peritoneal cells thus collected were washed twice by centrifugation at 1200 g for 5 min, and resuspended in RPMI, 20 mM HEPES (Life Technologies) supplemented with 2 mM t-glutamine (Life Technologies), 10⁻³ M β-mercaptoethanol and 10% (v/v) FCS. The cell suspensions were adjusted to 10⁶ cells ml⁻¹, seeded on 24-well plates (0.5 ml per well), and macrophages were allowed to adhere for 90 min at 37 °C under a 5% (v/v) CO₂ atmosphere. Non-adhering cells were washed off and adhering cells were washed three times with 1 ml of the same medium. The number and viability of the macrophages obtained by this procedure were determined by morphological examination and trypan blue exclusion (Fernández-Lago et al., 1999). For macrophage infection, bacteria from overnight cultures were opsonized at 37 °C for 1 h in PBS containing a subagglutinating dilution (1:200) of the monoclonal antibody BmE10-5, which reacts specifically with the S-LPS of Brucella spp. (Vizcaíno et al., 1991). Opsonized B. abortus 2308 cells were then centrifuged, washed and diluted in RPMI supplemented as described above. For infection, macrophage cells were incubated with 1 ml of the opsonized bacterial suspension at a m.o.i. of 30:1 (bacteria: macrophage) for 1 h. After incubation, non-phagocytosed bacteria were washed off. Following this, 1 ml RPMI supplemented as described above and containing 50 µg gentamicin ml⁻¹ was added to the macrophages to kill any remaining extracellular bacteria. The cells were then cultured in the same medium with gentamicin (50 µg ml⁻¹) at 37 °C under a 5% (v/v) CO₂ atmosphere in air for 48 h, and culture supernatants were obtained from each well, filtered through 0.45 µm pore-size filters, and kept at −70 °C until used for cytokine analysis. Supernatants obtained at the same time from uninfected macrophage cultures were used as negative controls. Uninfected macrophage cultures (five wells per sample) were also incubated, in the same medium with gentamicin (50 µg ml⁻¹), in the presence of either purified S-LPS (20 µg ml⁻¹) or a 1/10 dilution of culture supernatant obtained from B. abortus 2308, and 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.

Splenocyte cultures. Infected CD-1 mice were killed on days 3, 6 and 10 p.i. The spleens were removed aseptically, and individual cell suspensions were prepared by gentle teasing through cell strainers (Becton Dickinson). Erythrocytes were lysed by adding lysis buffer (0:155 M NH₄Cl, 10 mM KHCO₃, 10 mM EDTA, pH 7.4) to the pellet for 3 min. The cells were then washed three times in RPMI, 20 mM HEPES supplemented with 2 mM t-glutamine, 10⁻³ M β-mercaptoethanol and 10% (v/v) FCS. Spleen cells (10⁶) were cultured in 24-well plates (0:5 ml per well) and stimulated with 0:5 ml heat-killed (HK) B. abortus 2308 at 10³ cells ml⁻¹. Cultures were incubated at 37 °C under a 5% (v/v) CO₂ atmosphere in air. Supernatants were harvested at 48 h, filtered through a 0:45 µm pore-size filter, and frozen at −70 °C for use in cytokine assays. Controls including cultures of splenocytes obtained from uninfected mice or from infected mice stimulated with the mitogen concanavalin A (5 µg per well; Sigma) were also established. In some experiments, supernatants obtained at 3 days p.i. from mice infected with 5 × 10⁵ c.f.u. B. abortus 2308 were cultured, once stimulated with 10³ HK B. abortus 2308 cells, in the presence of recombinant IL-18 (10 ng ml⁻¹) and/or IL-12 (10 ng ml⁻¹). Supernatants were also removed after 48 h, filtered, and tested for IFN-γ production.

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 calculate cytokine concentrations. Samples were assayed in duplicate and values are expressed as means ± SD. The lowest limit of detection for IL-18 and IFN-γ 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).

RESULTS AND DISCUSSION

IL-18 secretion is reduced in B. abortus 2308-infected macrophages

To investigate the effect of B. abortus 2308 infection on macrophage-derived IL-18 secretion, CD-1 mouse elicited-peritoneal macrophage cultures were used. As shown in Fig. 1, 24 h culture supernatants of uninfected macrophages constitutively secreted IL-18. A significant (P < 0.05) increase in the secretion of this cytokine was observed when
Reduced IL-18 secretion by *B. abortus*

**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 < 0.05).

Cultured peritoneal macrophages were exposed to S-LPS from *E. coli* O157 (1 μg ml⁻¹) (Fig. 1). In contrast, when infected with opsonized *B. abortus* 2308 for 90 min, followed by removal of extracellular bacteria, supernatants of these macrophage cultures showed no IL-18 activity (<30 pg ml⁻¹; Fig. 1). These results demonstrate that *B. abortus* 2308 infection induces a reduction of IL-18 secretion in mice at the macrophage level. It has previously been demonstrated that *Brucella* culture supernatants contain a protein factor(s) that is able to inhibit the secretion in LPS-activated human macrophages of other macrophage-derived cytokines, such as TNF-α (Joubier-Maurin et al., 2001). Accordingly, we also analysed the capacity of both the culture supernatants (1:10 dilution) and the S-LPS (20 μg ml⁻¹) obtained from *B. abortus* 2308 to induce the secretion of IL-18 mediated by CD-1 mouse elicited-peritoneal macrophages. As shown in Fig. 1, neither *B. abortus* 2308 culture supernatants nor *B. abortus* 2308 S-LPS had a significant effect on IL-18 secretion. Similar results were obtained upon using different concentrations of 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

<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.

### 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 × 10⁶ 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 2. Effect of IL-18 and IL-12 on IFN-γ production by stimulated spleen cells from B. abortus 2308-infected mice

CD-1 mice were i.p.-infected with 5 × 10^5 c.f.u. B. abortus 2308. Mice, five per group, were killed at 3 days after infection. Data are mean values of five animals ± SD.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>IFN-γ level (pg ml^-1)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>130 ± 26</td>
</tr>
<tr>
<td>IL-18</td>
<td>145 ± 60</td>
</tr>
<tr>
<td>IL-12</td>
<td>142 ± 33</td>
</tr>
<tr>
<td>IL-18 + IL-12</td>
<td>145 ± 50</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>550 ± 125</td>
</tr>
</tbody>
</table>

*Cytokines (10 ng ml^-1) were added to splenocyte cultures. Concanavalin A was used at 5 μg ml^-1.
†Splenocytes were cultured in RPMI medium and stimulated with 10^7 HK B. abortus 2308 cells as described in Methods. Supernatants were collected at 48 h and tested for IFN-γ by ELISA. The level of sensitivity was 30 pg 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


