HOST RESPONSE TO INFECTION

Differential secretion of interleukin-12 (IL-12) subunits and heterodimeric IL-12p70 protein by CD-1 mice and murine macrophages in response to intracellular infection by *Brucella abortus*

LUIS FERNÁNDEZ-LAGO, ELISA RODRÍGUEZ-TARAZONA and NIEVES VIZCAÍNO

Departamento de Microbiología y Genética, Edificio Departamental, Universidad de Salamanca, Avda Campo Charro s/n, 37007 Salamanca, Spain

The secretion of interleukin-12 (IL-12) following intracellular infection with virulent *Brucella abortus* strain 2308 was investigated in CD-1 mice and in CD-1 cultured peritoneal macrophages. Bioactive IL-12p70 and free non-immunoactive p40 subunits (IL-12p40) were determined by enzyme-linked immunosorbent assays. In CD-1 mice, *B. abortus* 2308 was a potent inducer of IL-12p40 (maximum levels were 5.9 and 3.4 ng/ml in sera and spleen homogenates, respectively). Secretion of IL-12p70 was also demonstrated *in vivo*, although at much lower levels (216.6 and 198.9 pg/ml in sera and spleen homogenates, respectively). Production of IL-12 over the first 7 days after infection was accompanied by active multiplication of *B. abortus* in the spleens of infected mice. CD-1 cultured peritoneal macrophages secreted only IL-12p40 (878.4 pg/10⁷ macrophages) in response to *B. abortus* infection and no production of IL-12p70 was observed. In contrast, CD-1 peritoneal macrophages secreted detectable amounts of IL-12p70 (16.2 pg/10⁷ macrophages) in response to purified lipopolysaccharide (S-LPS) from *B. abortus* 2308. The macrophages also secreted significant amounts of interferon- γ (IFN- γ) (520.1 pg/10⁷ macrophages) in response to intracellular *B. abortus*. These results indicate that *B. abortus* 2308 is not a potent inducer of IL-12p70 production, whereas purified S-LPS from *B. abortus* 2308 induces the secretion of this bioactive form of IL-12 in cultured peritoneal macrophages. CD-1 peritoneal macrophages were able to secrete IFN- γ , as well as high amounts of IL-12p40, in response to intracellular infection by *B. abortus*.

Introduction

Brucella abortus is a facultative intracellular bacterium and is one of the causative agents of brucellosis in animals and man [1]. Professional phagocytes are the first target for *B. abortus* invasion [1, 2] and the bacteria are able to survive and multiply within these cells [1–3]. Immunity to *B. abortus* depends on the effective generation of a cell-mediated response involving both CD4⁺ and CD8⁺T cells as well as a T-cell-dependent activation of macrophages [4, 5]. The cytokines secreted during this response may strongly influence this process and the outcome of the infection [6–11].

Interleukin-12 (IL-12) is a heterodimeric cytokine composed of a heavy chain of 40 kDa and a light chain of 35 kDa linked by a disulphide bond [12]. In this bioactive form, with a molecular mass of 70 kDa (IL-12p70), it is secreted mainly by monocytes and macrophages, but also by several other cell types including dendritic cells, B cells [12] and polymorphonuclear leucocytes [13]. Furthermore, the 40-kDa subunit of this cytokine is secreted at much higher levels than the p70 bioactive form [14, 15]. It has also been demonstrated that both the p40 monomer (p40) and the disulphide-linked p40 homodimer (p40)₂ strongly antagonise the activity of IL-12p70 [14, 16, 17], possibly representing a novel form of self-regulating cytokine response [17]. IL-12 is a potent inducer of interferon- γ (IFN- γ) production by both resting and activated T cells and natural killer (NK) cells; moreover, it directs the generation of T-helper type 1 responses [16, 18, 19]. This cytokine also

Received 20 Oct. 1998; revised version accepted 9 April 1999.

Corresponding author: Dr L. Fernández-Lago (e-mail: lrlago@gugu.usal.es).

enhances the cytolytic activity of a number of effector cells, including T cells, NK cells and macrophages, and stimulates the proliferation of activated T and NK cells [12, 20, 21]. Recently, IL-12 has also been reported to act directly on activated B lymphocytes to enhance immunoglobulin secretion [22].

Early production of IL-12 within the first hours of infection has been reported, particularly in the case of intracellular bacteria and parasites [23]. In murine models, endogenously produced IL-12 has been observed to contribute to the control of the host response against infections with intracellular organisms such as *Mycobacterium tuberculosis* [24], *M. avium* [25], *Legionella pneumophila* [26], *Borrelia burgdorferi* [27], group B streptococci [28], *Salmonella dublin* [29], *Listeria monocytogenes* [30], *Leishmania major* [31], *B. abortus* [9–11] and *Toxoplasma gondii* [32]. A possible common mechanism could be associated with the production of IFN- γ [10] and the subsequent activation of phagocytic cells, increasing their capacity to destroy the intracellular pathogen [23]. However, neither the dynamics of endogenous IL-12 production during the course of *B. abortus* infection nor its relationship with the evolution of the infectious process have been thoroughly investigated.

An earlier study established that inoculation of CD-1 mice with the virulent 2308 strain of *B. abortus* leads to chronic infection, with the micro-organism being detected up to 12 weeks after infection, and accompanied by production of IFN- γ and IL-10 [33]. In the present study, the production of endogenous IL-12 in response to infection with virulent *B. abortus* was investigated in the murine model described previously. The capacity of *B. abortus* 2308 to stimulate secretion of IL-12 and IFN- γ by cultured CD-1 peritoneal macrophages was also explored.

Materials and methods

Mice

Female outbred CD-1 mice (Charles River, Barcelona, Spain) were purchased at 9 weeks of age and kept for 1 week before use. The animals were housed in micro-isolator cages in Horsfall units and were cared for in accordance with standard guidelines.

Bacterial strain and culture medium

B. abortus 2308 was used as the challenge strain. Virulent *B. abortus* 2308 was passaged in CD-1 mice and isolated in pure culture from spleens. Stock cultures were prepared from cultures grown for 72 h at 37°C in Tryptic Soy Broth (TSB; BBL Microbiology Systems) enriched with yeast extract (Difco) 0.3% w/v and stored at –70°C in glycerol 50% v/v. For challenge, the contents of freshly thawed vials were cultured on Tryptic Soy Agar (TSA; BBL) for 72 h at

37°C in an atmosphere of CO₂ 5% v/v in air. The bacteria were suspended in sterile phosphate-buffered saline (PBS; pH 7.4) and adjusted turbidimetrically to the desired concentration. The exact dose was established retrospectively by viable counting.

Experimental infection of mice

Mice were inoculated intravenously (i.v.) with 0.7×10^6 cfu of *B. abortus* 2308 in 0.1 ml of PBS. To evaluate cytokine production, mice in groups of three were killed by cervical dislocation daily during the first week after inoculation and then weekly from the first to the seventh week after inoculation. Control groups received 0.1 ml of PBS i.v. Before the mice were killed, blood samples were obtained from the tail vein and were stored at –70°C until used.

Preparation of spleen homogenates for bacterial counts and cytokine analysis

A minor modification of a previously described process was used [33, 34]. Briefly, spleens obtained from infected and control mice were homogenised individually at a concentration of 100 mg of spleen/ml of Hanks's balanced salts solution (Gibco) containing 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (Sigma) 1% w/v. A sample of the resulting cell suspension was diluted appropriately in PBS and plated out in triplicate on TSA. After incubation for 72 h in CO₂ 5% v/v, bacterial colonies were counted and expressed as the log₁₀/organ value. Previous studies had shown that the procedure used to prepare the samples does not affect the viability of the organisms present [33, 34]. After extraction of cytokines for 1 h at 4°C, samples were centrifuged (10 000 g for 30 min) and the supernates were held at –70°C until assayed.

Isolation and infection of CD-1 mouse peritoneal macrophages

Macrophages were isolated as described previously [20], with some modifications. Briefly, 8–10-week-old female CD-1 mice (Charles River) were inoculated intraperitoneally with 0.3 ml of incomplete Freund's adjuvant [35]. Three days later, mice were killed by cervical dislocation and peritoneal cells were harvested by lavage with cold RPMI-1640 containing 2 mM L-glutamine and supplemented with heat-inactivated fetal calf serum (Gibco) 10% v/v without antibiotics (complete medium). The endotoxin levels of all medium components were < 25 pg/ml (final concentration). The cell suspensions (3–4 ml) were placed in 25-cm² tissue-culture flasks (Costar, Cambridge, MA, USA) and macrophages were allowed to adhere for 24 h in a humidified CO₂ 5% v/v incubator at 37°C. Non-adhering cells were then washed off and adhering cells were washed three times with 3 ml of complete medium. The adhering cells were then incubated in

3 ml of complete medium for 2 h. The number and viability of macrophages obtained by this procedure were determined by morphological examination and Trypan blue exclusion [35]. Bacteria from overnight cultures were opsonised at 37°C for 60 min in PBS containing a subagglutinating dilution (1 in 200) of the mouse monoclonal antibody BmE10-5 with specificity against the lipopolysaccharide (S-LPS) of *Brucella* spp. [36]. Opsonised bacteria were then centrifuged, washed and diluted in complete medium. Opsonisation had no effect on bacterial survival *in vitro*. To infect macrophages, different numbers of opsonised *B. abortus* 2308 in complete medium were added for 1 h. After incubation for 1 h, non-phagocytosed bacteria were washed off. Following this, 3 ml of complete medium containing gentamicin (Sigma) 100 µg/ml were added to the macrophages to kill any remaining extracellular bacteria [37]. Then, the cells were cultured at 37°C in a CO₂ 5% v/v incubator and, at the indicated time points, culture supernates were obtained from each flask and held at -70°C until used for cytokine analysis. Supernates obtained at the same times from uninfected macrophage cultures were used as negative controls.

In some experiments, macrophage cultures were incubated in complete medium plus gentamicin with purified S-LPS obtained from *B. abortus* 2308 (20 µg/ml) in the presence or absence of IFN-γ (1500 U/ml; Boehringer Mannheim). This *B. abortus* antigen contained, as percentage of dry weight protein 1.5% and 2-keto-3-deoxyoctulosonic acid (KDO) 1.1% [36]. S-LPS from *Escherichia coli* O26:B6 (Sigma) 5 µg/ml in the presence or absence of IFN-γ (1500 U/ml) was also used to stimulate macrophage cultures. At the indicated time points, culture supernates were obtained from each flask and held at -70°C until assayed. Supernates obtained at the same times from non-treated macrophage cultures were used as negative controls.

Cytokine analysis

IFN-γ and IL-12 levels in serum, whole spleen homogenates and macrophage culture supernates were measured by commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (Inter-test-γ, Inter-test-IL-12X total mouse IL-12 and Inter-test-IL-12X mouse IL-12p70, respectively; Genzyme, Cambridge MA, USA). Total mouse IL-12 ELISA detects all three forms of IL-12, i.e., p40 monomer, (p40)₂ homodimer and p70 heterodimer. 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 the cytokine concentration present in the samples. Whole spleen homogenates obtained from PBS-treated mice or supernates obtained from uninfected macrophage cultures were used as negative controls in each assay. Results were expressed as the level of each cytokine

per ml of sample (spleen extract, serum or macrophage culture supernate) or per 0.1 g of organ.

Cytotoxicity assay

This was performed with CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay (Promega) kits. Briefly, macrophages were cultured for 24 h in 96-well flat-bottomed cell culture plates at 2×10^5 cells/well (100 µl) in complete medium without antibiotics. Non-adhering cells were washed off and the macrophage culture was maintained at 37°C for 2 h in a humidified CO₂ 5% v/v incubator. Bacteria, opsonised as described above, were added to each well and, after incubation for 1 h, non-phagocytosed bacteria were washed off and macrophages were cultured for 24 or 48 h in complete medium with gentamicin 100 µg/ml. Macrophage viability was determined by measuring the A₄₉₀ values of eight wells containing cells of the same multiplicity of infection. Results are presented as the means of eight determinations and standard deviations were <10% of the mean values.

Statistical methods

Statistical analyses were performed with Student's *t* test and the StatView computer program. A *p* value <0.05 was considered statistically significant.

Results

IL-12 production during the course of *B. abortus* infection of mice

As shown in Fig. 1a, total IL-12 was significantly enhanced in sera and in whole spleen homogenates between the first and seventh day after inoculation, reaching maximum levels on the fifth (5.9 ng/ml) and fourth (3.4 ng/ml) day, respectively. Endogenous production of this cytokine during the first week after inoculation was correlated with a significant increase (*p* < 0.001) in the number of viable *B. abortus* 2308 in the spleens of inoculated mice (Fig. 1a). An increase in the number of bacteria in the spleens was also observed during the second week (Fig. 1b), together with an increase in the production of total IL-12 in serum and spleen homogenates. Between the second and seventh weeks after inoculation the numbers of *B. abortus* in the spleens decreased significantly (*p* < 0.001), along with a significant diminution in the levels of total IL-12 in serum and spleen homogenates (Fig. 1b). The levels of total IL-12 in sera and whole spleen homogenates obtained at the same times from control PBS-treated mice were always <15 pg/ml.

When the levels of the bioactive p70 heterodimer were measured by a sensitive and specific ELISA (Fig. 2), this immunoactive form of IL-12 was detected only during the first 4 days after inoculation and in much lower amounts than the monomeric p40 and dimeric

Fig. 1. Levels of total IL-12 in serum (○) and whole spleen homogenates (●) and growth curve (□) of *B. abortus* in spleens of CD-1 mice after i.v. injection of 0.7×10^6 cfu of *B. abortus* strain 2308. (a) First 7 days after infection (b) First 7 weeks after infection. Each symbol represents the mean of three mice. SDs were <15% of mean values. The levels of total IL-12 in samples from PBS-treated mice (control group) were always <15 pg/ml. The lowest detection limit of total IL-12 was 10 pg/ml.

(p40)₂ forms of this interleukin. Maximum levels of IL-12p70 in serum and spleen homogenates were 216.6 and 198.9 pg/ml, respectively, on the first and the third day. The levels of IL-12p70 in sera and whole spleen homogenates obtained at the same times from control PBS-treated mice were always <8 pg/ml.

B. abortus-induced secretion of IL-12p40 and IL-12(p40)₂ by CD-1 macrophages

During the course of infection, professional phagocytes are the first target for invasion by *Brucella* spp. and the

pathogen may survive within these cells [1, 2]. Moreover, together with B cells, phagocytic cells are the main producers of IL-12 [12, 13]. To determine the effect that intracellular infection by the bacterium might have on the production of this cytokine, it was first necessary to determine the number of *B. abortus* cells that could be added to macrophage cultures without significantly altering their viability. Table 1 shows that at 24 h after exposure, once extracellular bacteria had been eliminated by the addition of gentamicin to the macrophage culture, intracellular bacteria induced a dose-dependent decrease in the

Fig. 2. Levels of IL-12 p70 in serum (■) and whole spleen homogenates (▨) of CD-1 mice after i.v. injection of 0.7×10^6 cfu of *B. abortus* 2308. Results are the means of three mice and SDs were <15% of mean values. The levels of IL-12p70 in samples from PBS-treated mice (control group) were always <8 pg/ml. The lowest detection limit of IL-12p70 was 5 pg/ml.

Table 1. Determination of macrophage viability after infection with *B. abortus* 2308

Challenge dose [†]	Macrophage viability (%) [*] at	
	24 h	48 h
100:1	37	17
30:1	56	51
10:1	69	61
3:1	>98	89
1:1	>98	>98
0.3:1	>98	>98

*Macrophage viability was determined by the CellTiter 96TMAQueous Non-Radioactive Cell Proliferation Assay (see text). Results are presented as means of eight determinations. SD values were <10% of mean values.

[†]*B. abortus*: macrophage ratio.

number of viable macrophages. This effect was greater at 48 h after exposure.

The ability of *B. abortus* 2308 to induce secretion of bioactive p70 heterodimer and inactive p40 monomeric and (p40)₂ dimers in cultured CD-1 macrophages was assessed by a sensitive ELISA assay. CD-1 macrophages (10^7 cells) were infected with different numbers of opsonised *B. abortus* 2308 cells ranging from 0.3×10^7 to 3×10^8 cfu. After incubation for 1 h, extracellular bacteria were eliminated by successive washings and the addition of gentamicin, and infected cultures were incubated for 48 h. Fig. 3 shows that, at 48 h after exposure, macrophages secreted substantial amounts of total IL-12 in response to intracellular bacteria. The secretion of the cytokine was dose-dependent, with a maximum secretion of 1010.1 pg/ml at a challenge dose of 30:1 (bacteria:macrophage). However, macrophages did not secrete detectable

amounts of IL-12p70, the immunoactive form of IL-12 (detection limit of the ELISA assay 5 pg/ml). Different assays were performed with up to 10^9 cfu of *B. abortus*/ 10^7 macrophages, but no secretion of IL-12p70 was detected. The challenge dose of 30:1 was selected to assess the secretion kinetics of total IL-12 at 24, 48 and 72 h after exposure (Fig. 4). Maximum secretion (878.4 pg/ml) was observed at 24 h after exposure. Again, no IL-12p70 secretion was detected.

The inability of *B. abortus* 2308 to induce IL-12p70 secretion in cultured macrophages cannot be explained by the incapacity of the macrophages to secrete this form of IL-12. Macrophages cultured with S-LPS (20 µg/ml) from *B. abortus* 2308 or S-LPS (5 µg/ml) from *E. coli* O26:B6 secreted detectable amounts of IL-12p70 (16.2 pg/ml and 24.7 pg/ml, respectively; data not shown). These S-LPS preparations were also potent inducers of IL-12 p40 and (p40)₂ secretion (Figs. 3 and 4).

B. abortus induced secretion of IFN-γ by CD-1 macrophages

A possible mechanism through which IL-12 contributes to resistance to *B. abortus* infection would be via an IFN-γ-dependent pathway [9, 10]. As shown in Fig. 5, *B. abortus* 2308 induced the secretion of IFN-γ by CD-1 peritoneal macrophages. Maximum secretion (520.1 pg/ml) was demonstrated at 24 h after infection. Similarly, in response to stimulation by S-LPS (20 µg/ml) from *B. abortus* 2308, CD-1 peritoneal macrophages also released substantial amounts of IFN-γ (105.1 pg/ml at 48 h) (Fig. 5).

Fig. 3. Secretion of total IL-12 by cultured CD-1 peritoneal macrophages (10^7 cells) in response to different numbers of opsonised *B. abortus* 2308 cells and to S-LPS preparations. Results obtained at 48 h after infection are mean values of triplicate ELISA determinations and SDs were <15% of mean values. Negative controls of total IL-12 production included supernates obtained at 48 h from macrophages in culture medium only. The lowest detection limit of total IL-12 was 10 pg/ml. Similar results were observed in at least two other experiments.

Fig. 4. Kinetics of total IL-12 secretion by cultured CD-1 peritoneal macrophages (10^7 cells) in response to infection with 3×10^8 cfu of opsonised *B. abortus* 2308. Results obtained at 24, 48 and 72 h after infection are mean values of triplicate ELISA determinations and SDs were <15% of mean values. Negative controls of total IL-12 production included supernates obtained at each time point from macrophages in culture medium only. Positive controls were supernates from macrophages treated with *B. abortus* S-LPS (20 μ g/ml) with or without IFN- γ (1500 U/ml). The lowest detection limit of total IL-12 was 10 pg/ml. Similar results were obtained in at least two other experiments.

Discussion

Previous reports have indicated that endogenous IL-12 is produced in the course of *B. abortus* infection in a

murine model, contributing to resistance to infection [9, 10]. In the present study, these aspects have been broadened and CD-1 mouse and CD-1 cultured peritoneal macrophages were used to analyse the

Fig. 5. Kinetics of IFN- γ secretion by cultured CD-1 peritoneal macrophages (10^7 cells) in response to infection with 3×10^8 cfu of opsonised *B. abortus* 2308. Results obtained at 24, 48 and 72 h after infection are mean values of triplicate ELISA determinations and SDs were <15% of mean values. Negative controls of IFN- γ production included supernates obtained at each time point from macrophages in culture medium only. Positive controls were supernates from macrophages treated with *B. abortus* S-LPS (20 μ g/ml). The lowest detection limit of IFN- γ was 5 pg/ml. Similar results were observed in at least two other experiments.

kinetics of production of the different biological forms of this cytokine (immunoactive IL-12p70 and free inactive p40 subunits), to determine their relationship to the development of an infection with virulent *B. abortus* 2308 and its tendency to become chronic.

In the assays performed *in vivo* and *in vitro*, quantitative differences in the secretion of the free inactive p40 subunits of IL-12 and the bioactive form p70 of this cytokine were observed. Thus, by ELISA on the culture supernates from CD-1 peritoneal macrophages infected with *B. abortus* 2308, significant levels of only the non-immunoactive monomeric (p40) and dimeric (p40)₂ forms of IL-12 were detected. In contrast, with the same ELISA on serum and whole spleen homogenates from mice infected *i.v.* with *B. abortus* 2308, much higher levels of these biologically inactive forms of IL-12 were detected, as well as the production of the p70 immunoactive form, mainly during the first days after infection. A similar picture has been observed *in vivo* and *in vitro* in the secretion of IL-12 induced by *S. dublin*, another facultative intracellular micro-organism [29, 35]. In view of these results, at present it is not possible to rule out the notion that different factors, hitherto unidentified and generated *in vivo* during the course of infection by *B. abortus*, would be required to induce the synthesis of the immunoactive form of this interleukin more efficiently. However, the biological relevance of these results is considerable, as it has been established experimentally that the secreted IL-12p40 subunit and IL-12p40 homodimers may function as antagonists of

IL-12p70 bioactivity [14, 16, 17]. Thus *B. abortus* may interfere with the establishment of immunity induced by IL-12 by stimulating mainly the secretion of the non-immunoactive forms of this interleukin and down-regulating the secretion of the bioactive form IL-12p70.

In murine models of infection with *B. abortus* [9] and with other intracellular pathogens [24–27, 29, 30], the neutralisation of endogenously synthesised IL-12 is associated with an exacerbation of the infection. In the present study, the low levels of the bioactive form of IL-12p70 demonstrated in serum and whole spleen homogenates of mice infected with *B. abortus* could be sufficient to control the multiplication of the micro-organism (Figs. 1a and 1b), possibly *via* an IFN- γ -dependent route [8, 10]. It should also be noted that the heterodimeric IL-12p70 may not necessarily be secreted in a soluble form and may function as a membrane-bound protein on macrophages [38]. Under these circumstances, the levels of soluble IL-12p70 would be low, accounting for the results obtained here with the specific ELISA. In any case, and regardless of the mechanism of secretion of the heterodimer p70, the bacteria persisted in the spleens of the mice for at least seven weeks. Others have reported persistence of the organism at 14 [33] and 20 [39] weeks after infection. In consequence, pro-inflammatory and regulatory cytokines synthesised in the course of infection of CD-1 mice by *B. abortus* 2308 are not sufficient *per se* to induce an effective protective response and to prevent the infection from becoming chronic. Possibly, as with other micro-organisms [24, 28, 29, 40–43], the admin-

istration of exogenous IL-12 before or during infection by *B. abortus* might favour the induction of different effector mechanisms and afford better protection against the infectious agent. Therefore, further research should be conducted to establish the possible beneficial effects of cytokine therapy on the course of *B. abortus* infections.

The results obtained in the present study also demonstrate that, in addition to IL-12, cultures of CD-1 peritoneal macrophages also secrete substantial amounts of IFN- γ in response to *B. abortus* 2308 infection. Similar findings have been reported recently [44] for cultured human alveolar macrophages stimulated with *M. tuberculosis*. As it has been established that this secreted IL-12 is responsible for the induction of IFN- γ production by human alveolar and murine peritoneal macrophages [44, 45], it is possible that the IL-12 secreted by CD-1 peritoneal macrophages in response to *B. abortus* 2308 might play a role in the secretion of IFN- γ by these cells. Further research will also be required to elucidate the role of the secreted IFN- γ in the activation and in the microbicidal activities of *B. abortus*-infected peritoneal macrophages.

This work was supported by a grant from the 'Junta de Castilla y León', Spain.

References

- Baldwin CL, Winter AJ. Macrophages and *Brucella*. *Immunol Ser* 1994; **60**: 363–380.
- Liautard JP, Gross A, Doonand J, Kohler S. Interactions between professional phagocytes and *Brucella* spp. *Microbiologia* 1996; **12**: 197–206.
- Pizarro-Cerdá J, Moreno E, Sanguedolce V, Mege J-L, Gorvel J-P. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. *Infect Immun* 1998; **66**: 2387–2392.
- Araya LN, Elzer PH, Rowe GE, Enright FM, Winter AJ. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. *J Immunol* 1989; **143**: 3330–3337.
- Zhan Y, Yang J, Cheers C. Cytokine response of T-cell subsets from *Brucella abortus*-infected mice to soluble *Brucella* proteins. *Infect Immun* 1993; **61**: 2841–2847.
- Fernandes DM, Baldwin CL. Interleukin-10 downregulates protective immunity to *Brucella abortus*. *Infect Immun* 1995; **63**: 1130–1133.
- Jiang X, Baldwin CL. Effect of cytokines on intracellular growth of *Brucella abortus*. *Infect Immun* 1993; **61**: 124–134.
- Zhan Y, Cheers C. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. *Infect Immun* 1993; **61**: 4899–4901.
- Zhan Y, Cheers C. Endogenous interleukin-12 involved in resistance to *Brucella abortus* infection. *Infect Immun* 1995; **63**: 1387–1390.
- Zhan Y, Liu Z, Cheers C. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. *Infect Immun* 1996; **64**: 2782–2786.
- Zhan Y, Cheers C. Control of IL-12 and IFN- γ production in response to live or dead bacteria by TNF and other factors. *J Immunol* 1998; **161**: 1447–1453.
- Stern AS, Podlaski FJ, Hulmes JD *et al.* Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci USA* 1990; **87**: 6808–6812.
- Cassatella MA, Meda L, Gasperini S, D'Andrea A, Ma X, Trinchieri G. Interleukin-12 production by human polymorphonuclear leukocytes. *Eur J Immunol* 1995; **25**: 1–5.
- Gillessen S, Carvajal D, Ling P *et al.* Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur J Immunol* 1995; **25**: 200–206.
- Skeen MJ, Miller MA, Shinnick TM, Ziegler HK. Regulation of murine macrophage IL-12 production. Activation of macrophages *in vivo*, restimulation *in vitro*, and modulation by other cytokines. *J Immunol* 1996; **156**: 1196–1206.
- Gately MK, Carvajal DM, Connaughton SE *et al.* Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer *in vitro* and *in vivo*. *Ann N Y Acad Sci* 1996; **795**: 1–12.
- Heinzel FP, Hujer AM, Ahmed FN, Rerko RM. *In vivo* production and function of IL-12 p40 homodimers. *J Immunol* 1997; **158**: 4381–4388.
- Manetti R, Parronchi P, Guidizi MG *et al.* Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4 producing Th cells. *J Exp Med* 1993; **177**: 1199–1204.
- Trinchieri G. Interleukin-12 and its role in the generation of Th1 cells. *Immunol Today* 1993; **14**: 335–337.
- Gately MK, Gazzinelli RT. Effects of IL-12 on immune responses to microbial infections: a key mediator in regulating disease outcome. *Curr Opin Immunol* 1995; **7**: 485–496.
- Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 1994; **84**: 4008–4027.
- Jelinek DF, Braaten JK. Role of IL-12 in human B lymphocyte proliferation and differentiation. *J Immunol* 1995; **154**: 1606–1613.
- Trinchieri G. Proinflammatory and immunoregulatory functions of interleukin-12. *Int Rev Immunol* 1998; **16**: 365–396.
- Cooper AM, Roberts AD, Rhoades ER, Callahan JE, Getzy DM, Orme IM. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. *Immunology* 1995; **84**: 423–432.
- Saunders BM, Zhan Y, Cheers C. Endogenous interleukin-12 is involved in resistance of mice to *Mycobacterium avium* complex infection. *Infect Immun* 1995; **63**: 4011–4015.
- Brieland JK, Remick DG, LeGendre ML, Engleberg NC, Fantone JC. *In vivo* regulation of replicative *Legionella pneumophila* lung infection by endogenous interleukin-12. *Infect Immun* 1998; **66**: 65–69.
- Anguita J, Samanta S, Barthold SW, Fikrig E. Ablation of interleukin-12 exacerbates Lyme arthritis in SCID mice. *Infect Immun* 1997; **65**: 4334–4336.
- Mancuso G, Cusumano V, Genovese F, Gambuzza M, Beninati C, Teti G. Role of interleukin 12 in experimental neonatal sepsis caused by group B streptococci. *Infect Immun* 1997; **65**: 3731–3735.
- Kincy-Cain T, Clements JD, Bost KL. Endogenous and exogenous interleukin-12 augment the protective immune response in mice orally challenged with *Salmonella dublin*. *Infect Immun* 1996; **64**: 1437–1440.
- Emoto Y, Emoto M, Kaufmann SHE. Transient control of interleukin-4-producing natural killer T cells in the livers of *Listeria monocytogenes*-infected mice by interleukin-12. *Infect Immun* 1997; **65**: 5003–5009.
- Mattner F, Di Padova K, Alber G. Interleukin-12 is indispensable for protective immunity against *Leishmania major*. *Infect Immun* 1997; **65**: 4378–4383.
- Walker W, Roberts CW, Ferguson DJR, Jebbari H, Alexander J. Innate immunity to *Toxoplasma gondii* is influenced by gender and is associated with differences in interleukin-12 and gamma-interferon production. *Infect Immun* 1997; **65**: 1119–1121.
- Fernández-Lago L, Monte M, Chordi A. Endogenous gamma interferon and interleukin-10 in *Brucella abortus* 2308 infection in mice. *FEMS Immunol Med Microbiol* 1996; **15**: 109–114.
- Nakajima R, Brubaker RR. Association between virulence of *Yersinia pestis* and suppression of gamma interferon and tumor necrosis factor alpha. *Infect Immun* 1993; **61**: 23–31.
- Bost KL, Clements JD. Intracellular *Salmonella dublin* induces substantial secretion of the 40-kilodalton subunit of interleukin-12 (IL-12) but minimal secretion of IL-12 as a 70-kilodalton protein in murine macrophages. *Infect Immun* 1997; **65**: 3186–3192.
- Vizcaino N, Chordi A, Fernández-Lago L. Characterization of smooth *Brucella* lipopolysaccharides and polysaccharides by monoclonal antibodies. *Res Microbiol* 1991; **142**: 971–978.

37. Elsinghorst EA. Measurement of invasion by gentamicin resistance. *Methods Enzymol* 1994; **236**: 405–420.
38. Fan X, Sibalic V, Niederer E, Wüthrich RP. The proinflammatory cytokine interleukin-12 occurs as a cell membrane-bound form on macrophages. *Biochem Biophys Res Commun* 1996; **225**: 1063–1067.
39. Stevens MG, Olsen SC, Pugh GW, Palmer MV. Immune and pathologic responses in mice infected with *Brucella abortus* 19, RB51, or 2308. *Infect Immun* 1994; **62**: 3206–3212.
40. Bohn E, Schmitt E, Bielfeldt C, Noll A, Schulte R, Autenrieth IB. Ambiguous role of interleukin-12 in *Yersinia enterocolitica* infection in susceptible and resistant mouse strains. *Infect Immun* 1998; **66**: 2213–2220.
41. Hunter CA, Slifer T, Araujo F. Interleukin-12-mediated resistance to *Trypanosoma cruzi* is dependent on tumor necrosis factor alpha and gamma interferon. *Infect Immun* 1996; **64**: 2381–2386.
42. Kobayashi K, Kasama T, Yamazaki J *et al.* Protection of mice from *Mycobacterium avium* infection by recombinant interleukin-12. *Antimicrob Agents Chemother* 1995; **39**: 1369–1371.
43. Wang Z-E, Zheng S, Corry DB *et al.* Interferon γ -dependent effects of interleukin 12 administered during acute or established infection due to *Leishmania major*. *Proc Natl Acad Sci USA* 1994; **91**: 12932–12936.
44. Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. *Infect Immun* 1997; **65**: 5149–5156.
45. Puddu P, Fantuzzi L, Borghi P *et al.* IL-12 induces IFN-gamma expression and secretion in mouse peritoneal macrophages. *J Immunol* 1997; **159**: 3490–3497.