doi: 10.1111/j.1365-3083.2008.02212.x

Susceptibility to *Yersinia pseudotuberculosis* Infection is Linked to the Pattern of Macrophage Activation

A. Tansini & B. M. M. de Medeiros

Department of Biological Sciences, School of Pharmaceutical Sciences, UNESP – São Paulo State University, Araraquara, SP, Brazil

Received 18 June 2008; Accepted in revised form 5 December 2008

Correspondence to: B. M. M. de Medeiros, Departamento de Ciências Biológicas, UNESP – Universidade Estadual Paulista, Faculdade de Ciências Farmacêuticas, Rodovia Araraquara – Jaú, km 1, 14801-902, Araraquara, SP, Brasil. E-mail: medeiros@fcfar.unesp.br

Abstract

T helper 1 cells play a crucial role in the clearance of *Yersinia pseudotuberculosis* infection. By producing cytokines and presenting antigens to T cells, activated macrophages can orientate the adaptive immune response. The pathway used by macrophages to metabolize arginine has been employed as an important parameter to discriminate their activation state. In this study, the pattern of macrophage activation in *Y. pseudotuberculosis*-infected BALB/*c* (*Yersinia*-susceptible) and C57BL/6 (*Yersinia*-resistant) mice and their immunostimulatory capacity were analysed. In the early phase of infection, macrophages obtained from C57BL/6 mice produced higher levels of NO, lower arginase activity, and larger amounts of IL-12 and TNF-α than macrophages from BALB/*c* mice. On the other hand, macrophages derived from BALB/*c* mice produced higher levels of IL-10 and TGF-β than C57BL/6 mice. The *Y. pseudotuberculosis* infection leads to a fall in the macrophage immunostimulatory capacity of both strains of mice, with T-cell proliferation significantly reduced 12 h after infection. Moreover, we observed in the supernatant of co-culture of macrophages from infected mice with T lymphocytes from heat-killed *Yersinia*-immunized mice lower IFN-γ production by cells from BALB/*c* mice than by C57BL/6 mice, and IL-4 was produced only by BALB/*c* mice on the first- and third-day post-infection. These results suggest that the pattern of macrophage activation is associated with susceptibility and resistance to *Y. pseudotuberculosis* infection in BALB/*c* and C57BL/6 mice.

Introduction

Yersinia pseudotuberculosis is an enteropathogen that causes gastrointestinal disorders, usually self-limiting enteritis and mesenteric lymphadenitis [1]. In the mouse oral infection model, enteropathogenic yersiniae produce systemic disease, the bacteria replicating in the small intestine, invading the Peyer's patches and disseminating to the liver and spleen [2, 3].

Several virulence factors have been identified that promote resistance to mouse serum, coordinate gene expression and enable the acquisition of iron by the bacterium [4]. *Yersinia* contains an extrachromosomal 70 Kb plasmid, essential for pathogenicity, which encodes the type III protein secretion system (TTSS), the Yop effector proteins and non-fimbrial adhesin Yad A [5, 6]. The type III secretion system is used to inject effector proteins from the bacterial cytoplasm into the cytosol of a host cell. Once inside the cell, the Yop effectors interfere with signalling pathways involved in the regulation of the actin cytoskeleton, phagocytosis, apoptosis and the inflammatory response, thus favouring the survival of the bacteria [6].

The early control of infection with *Yersinia* is mediated by mechanisms of innate immunity, involving macrophages, NK cells and neutrophils [7–9]. This early response is followed by an adaptive immune response, and the resolution of the infection is mediated by $CD4_+$ Th1 cells that produce cytokines such as IFN-γ and IL-2 [8, 9]. An effective way for *Yersinia* to subvert this immune response would be to inhibit the presentation of its antigens by antigen-presenting cells (APC) and thus impair T-cell stimulation [10].

By producing diverse molecules and presenting antigens to T cells, macrophages influence the type of adaptive immune response, leading to the expansion and differentiation of specific lymphocytes [11]. Microbial antigens, tumour products, effector T cells and their secretory products influence the heterogeneity and the state of activation of macrophage populations [12, 13].

SCANDINAVIAN JOURNAL OF Immunology AN INTERNATIONAL MONTHLY JOURNAL

Classical macrophage activation is characterized by: high interleukin-12 (IL-12) production, activating a Th1 cell response, and high production of toxic intermediates, such as nitric oxide (NO) and reactive oxygen intermediates [14]. Many have referred to cells activated in this way as M1 [15]. Macrophages exposed to immune complexes, IL-4, IL-13 and IL-10, undergo an alternative form of activation, characterized by an IL-10^{high} and IL-12^{low} phenotype, and promote a type II response [14, 16]. These alternatively activated macrophages are called M2 [15].

L-Arginine metabolism in macrophages has been used as an important parameter to discriminate between classically and alternatively activated macrophages [12]. The activation by the classical pathway results in the production of NO, synthesized from arginine by inducible NO synthase (iNOS) [13, 17]. The alternative metabolic pathway for arginine is catalysed by arginase, which converts arginine to ornithine and urea [13]. It has been shown that macrophages from Th1 strains of mice (C57BL/6, B10D2) preferentially use the iNOS pathway and macrophages from Th2 strains of mice (BALB/*c*, DBA/2) preferentially use the arginase pathway [15].

It is possible that susceptibility or resistance to *Y. pseudotuberculosis* infection correlates with the activation state of macrophages. We have recently demonstrated that the predominant macrophage population (M1 or M2) at the early period of infection seems to be important in determining *Y. enterocolitica* susceptibility or resistance in mice [18]. However, little is known about the responses of M1 and M2 macrophages to *Y. pseudotuberculosis* infection. Thus, this study was designed to reveal the pattern of macrophage activation in *Y. pseudotuberculosis* infection of BALB/*c* (*Yersinia*-susceptible) and C57BL/6 (*Yersinia*-resistant) mice and the immunostimulatory capacity of these cells.

Material and methods

Bacterial strain. *Yersinia pseudotuberculosis* YpIIIpIB1, bearing the virulence plasmid, was generously provided by Dr Hans Wolf-Watz (Umeå University, Sweden).

Animals. Six-week-old C57BL/6 and BALB/*c* female mice were purchased from CEMIB (Centro Multidisciplinar para Investigação Biológica), UNICAMP, SP, Brazil. All mice were maintained in isolators under specific-pathogen-free conditions and provided with sterilized food and water. The study was approved by the School Committee for Ethics in Animal Experimentation, UNESP at Araraquara.

Experimental infection of mice. Groups of 16 mice were infected by gavage with 0.25 ml of a bacterial suspension containing 10^7 CFU/ml. Groups of 16 uninfected mice were used as controls. Four mice from each group were sacrificed at intervals of 12 h, 1 day, 3 days and 5 days after infection, with carbon dioxide.

Preparation of heat-killed Yersinia antigen. Viable cells of *Y. pseudotuberculosis* were killed by incubation for 1 h at 60 °C. The heat-killed bacteria were collected by centrifugation, washed twice and resuspended in PBS. A suspension of heat-killed *Y. pseudotuberculosis* equivalent to 10 μg of protein per ml was used in the cultures.

Peritoneal macrophage culture. Macrophages were collected from the peritoneal cavities of infected and control mice in 5.0 ml of sterile PBS, pH 7.4. The cells were washed twice by centrifugation at 200 ×*g* for 5 min at 4 °C and 100 μl were plated in microplates at 2.5×10^6 cells/ml in RPMI-1640 (Gibco, Carlslad, CA, USA), supplemented with HEPES (12.5 mM), sodium bicarbonate (2 g/l), L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), 5×10^{-2} M 2-mercaptoethanol (Sigma, Steinheim, Germany) and 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil). Adherent and non-adherent cells were separated by plate inversion after 1 h incubation at 37 °C, in an atmosphere of 5% CO_2 and 95% relative humidity. Macrophages were then cultured in supplemented RPMI-1640 in the presence of either 10 μg/ml of lipopolysaccharide (LPS) (*Escherichia coli* O111 B4; Sigma) or 10 μg/ml of heat-killed *Yersinia* (HKY) or without antigenic stimulation. Supernatants were removed after 48 h and tested for NO and cytokine (IL-10, IL-12, TNF-α, TGF-β) production. Arginase activity was measured in macrophage lysates.

NO production. Nitric oxide production by macrophages was determined by measuring nitrite, employing the method of Green *et al.* [19]. Briefly, 50 μl of cell supernatant was removed from each well and incubated with an equal volume of Griess reagent (1% w/v sulfanilamide, 0.1% w/v naphthylethylene diamine dihydrochloride, 3.0% H_3PO_4) at room temperature for 10 min. Absorbance at 550 nm was determined with an ELISA microplate reader (Model 550; BioRad, Hercules, CA, USA). Nitrite concentration was calculated from an analytical curve for $NaNO_2$.

Determination of arginase activity. Arginase activity was measured in cell lysates [20]. Briefly, cells were lysed with 100 μl of 0.1% Triton X-100. After 30 min on a shaker, 100 μl of 25 nm Tris–HCl was added to 100 μl of this lysate, 10 μl of 10 mm $MnCl_2$ was added and the enzyme was activated by heating for 10 min at 56 °C. Arginine hydrolysis was conducted by incubating the lysate with 100 μl of 0.05 M L-arginine at 37 °C for 60 min. The reaction was stopped with 900 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7). The urea concentration was measured at 540 nm after addition of 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95 °C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of urea per min.

IL-4, IL-10, IL-12, TNF-α, TGF-β and IFN-γ determination. Cytokines in cell culture supernatants were

measured with commercially available kits from BD Biosciences/Pharmingen, San Diego, CA, USA (IL-10, IL-12, TNF-α, TGF-β and IL-4) or BioLegend, San Diego, CA, USA (IFN-γ).

Immunization of mice. BALB/*c* and C57BL/6 mice were immunized with an intraperitoneal injection of 10 μg of HKY mixed with 1 mg of aluminium hydroxide in 0.15 M NaCl. Fourteen days later, the mice received a reinforcement dose of 10 μg of the antigen in saline solution. Lymphocytes were collected 7 days later and co-cultured with macrophages from mice infected with *Y. pseudotuberculosis* or uninfected.

T-cell enrichment. Lymphocytes were obtained from the spleens of immunized mice using the Pan T-Cell Isolation Kit (Miltenyi Biotec, Bergisch Galdbach, Germany), according to manufacturer's instructions. The cell enrichment process was accompanied by flow cytometry, using anti-CD3-FITC, anti-CD4-PE and anti-CD8-SPRD (BD/PharMingen). The final cell suspension contained approximately 80% CD3, 50% CD4 and 35% CD8.

T-cell antigen-specific activation. T-cell enriched fractions were seeded in 96-well plates, at a density of 5×10^5 cells/well, and co-cultured with macrophages (2×10^4 cells/well) from mice infected with *Y. pseudotuberculosis* or uninfected, in the presence of HKY, in supplemented RPMI-1640, at 37 °C, in an atmosphere of 5% CO_2 and 95% relative humidity. After 4 days, 100 μl of supernatant was removed for cytokine analyses and 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) was added to the cultures (5 mg/ml; 10 μl/well), which were incubated for an additional 4 h. After dissolving the formazan crystals by incubating with isopropanol, the plates were read at 540 nm.

Statistical analysis. Results are representative of two independent experiments and are presented as mean ± SD of triplicate observations. Data were analysed statistically by Student's *t*-test, using the Origin statistical program, version 5.0 (Northampton, MA, USA), with the level of significance set at $P < 0.05$.

Results

NO production by macrophages from C57BL/6 and BALB/*c* mice

Macrophages from C57BL/6 control mice produced 1.2-fold more NO than macrophages from BALB/*c* controls (Fig. 1). *Yersinia pseudotuberculosis* infection led to a decrease in the production of NO by macrophages from BALB/*c* mice, 12 h post-infection, when these cells were stimulated with LPS or HKY. On the first-day post-infection, there was a significant decrease in the production of NO, in both strains of mice, C57BL/6 macrophages producing around 1.5 more NO than those from BALB/*c* mice.

From the third-day post-infection, an increase in NO production was seen; however, the increase became significant only on the fifth-day post-infection, in both C57BL/6 and BALB/*c* mouse cells. The amount of NO produced by macrophages from infected C57BL/6 mice on the fifth-day post-infection was greater than that for BALB/*c* mice (about 1.2-fold more in the cells stimulated with LPS or HKY, and 1.5-fold in macrophages incubated with RPMI medium alone).

Arginase activity

Macrophages from BALB/*c* control mice exhibited higher arginase activity than macrophages from C57BL/6 controls (Fig. 2). There was no significant difference in arginase activity between macrophages from C57BL/6 and BALB/*c* mice, 12 h after infection.

Yersinia pseudotuberculosis infection led to a significant increase in the arginase activity on the first-day post-infection, macrophages from BALB/*c* mice showing higher levels than those from C57BL/6 mice (around 1.3-fold more in the cells stimulated with LPS or HKY).

The arginase activity of the macrophages collected from BALB/*c* mice on the third- and fifth-day post-infection

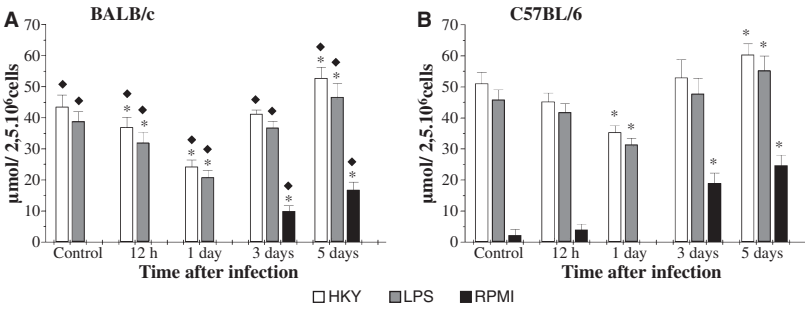

Figure 1 Nitrite (NO_2^-) production by murine peritoneal macrophages collected from BALB/*c* (A) and C57BL/6 (B) mice during *Yersinia pseudotuberculosis* infection. Cells (2.5×10^6/ml) were cultured with HKY, LPS or RPMI-1640 medium, and the supernatants were collected after 48 h of culture. Nitrite was assayed with Griess reagent. Control refers to average values obtained from uninfected mice throughout the experiment. Results are representative of two independent experiments and are presented as mean ± SD of triplicate observations. *$P < 0.05$ versus control and ♦$P < 0.05$ versus C57BL/6.

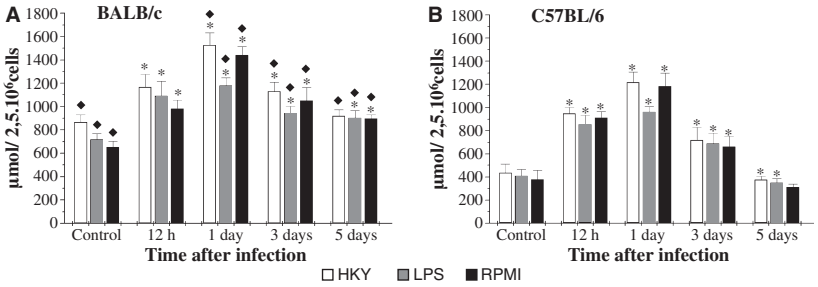

Figure 2 Arginase activity in murine peritoneal macrophages collected from BALB/*c* (A) and C57BL/6 (B) mice during *Yersinia pseudotuberculosis* infection. Cells (2.5×10^6/ml) were cultured with HKY, LPS or RPMI-1640 medium for 48 h and then macrophages were lysed and arginase activity determined. Control refers to average values obtained from uninfected mice throughout the experiment. Results are representative of two independent experiments and are presented as mean ± SD of triplicate observations. *$P < 0.05$ versus control and ♦$P < 0.05$ versus C57BL/6.

stayed high, although at lower levels than on the first-day post-infection. On the other hand, in macrophages from C57BL/6 mice, a significant decrease was observed in the arginase activity on the fifth-day post-infection, in the cells stimulated with LPS or HKY, the levels being 2.5 times lower than those obtained in BALB/*c* mice in the same period.

Cytokine production

Figure 3 represents the levels of IL-10, IL-12, TNF-α and TGF-β in culture supernatants of macrophages from uninfected or *Y. pseudotuberculosis*-infected mice.

Macrophages from C57BL/6 control mice produced higher levels of IL-12 and TNF-α than macrophages from BALB/*c* mice. There was no detectable production of TGF-β by macrophages from uninfected C57BL/6 mice. Macrophages from BALB/*c* control mice produced larger amounts of IL-10, and appreciable levels of TGF-β.

In the early period of *Y. pseudotuberculosis* infection, there was an increase in the IL-10 and TGF-β production, followed by a decrease in the IL-12 and TNF-α production, in both strains of mice. At 12-h and 1-day post-infection, macrophages from BALB/*c* mice produced significantly higher amounts of IL-10 (around 1.9-fold more at 12 h and 1.8-fold at 1 day post-infection) and TGF-β (about fivefold more when stimulated with HKY and eightfold when incubated with LPS, 12 h post-infection) than macrophages from C57BL/6 mice. A small amount of TGF-β was produced by macrophages from C57BL/6 mice at 12-h and 1-day post-infection. The levels of IL-12 and TNF-α produced by macrophages from BALB/*c* were lower than those by C57BL/6 mouse cells: BALB/*c* cells produced levels of IL-12 around seven times lower than C57BL/6 in the early period of infection, and levels of TNF-α 3.2 times lower at 12 h post-infection.

On the third- and fifth-day post-infection, an increase in IL-12 and TNF-α levels was observed, followed by a decrease in IL-10 production, in both strains of mice. At these times, macrophages from C57BL/6 mice produced significantly higher levels of IL-12 (about 3.6-fold more on the third-day and twofold on the fifth-day post-infection, in the cells stimulated with LPS or HKY) and TNF-α (approximately threefold more in the macrophages stimulated with HKY) than macrophages from BALB/*c* mice, and lower levels of IL-10. There was no TGF-β production by C57BL/6 cells on the third- and fifth-day post-infection, while a non-significant amount was produced by BALB/*c* mouse cells on the fifth-day post-infection.

T-cell antigen-specific activation

The capacity of macrophages from uninfected or infected mice to present the antigen HKY to T lymphocytes was investigated by using the MTT assay to measure cell proliferation. This method is based on reduction of the tetrazolium salt by living cells to produce a blue formazan.

The results show that macrophages obtained from C57BL/6 control mice induced higher proliferation of T cells than macrophages obtained from BALB/*c* control mice (Fig. 4). The formazan production was reduced at 12-h and 1-day post-infection for both strains of mice, and the level of proliferation of T cells obtained from BALB/*c* mice remained lower than for the C57BL/6 strain. On the third- and fifth-day post-infection, the formazan concentration increased to a level exceeding the control, with greater production by cells from C57BL/6 mice.

Figure 5 represents the levels of IFN-γ and IL-4 in the supernatants of the co-cultures of T cells obtained from HKY-immunized BALB/*c* and C57BL/6 mice with macrophages from infected or uninfected mice. Cells from C57BL/6 control mice produced higher levels of IFN-γ than cells from BALB/*c* control mice. There was no detectable production of IL-4 in supernatants of cells from BALB/*c* and C57BL/6 control mice.

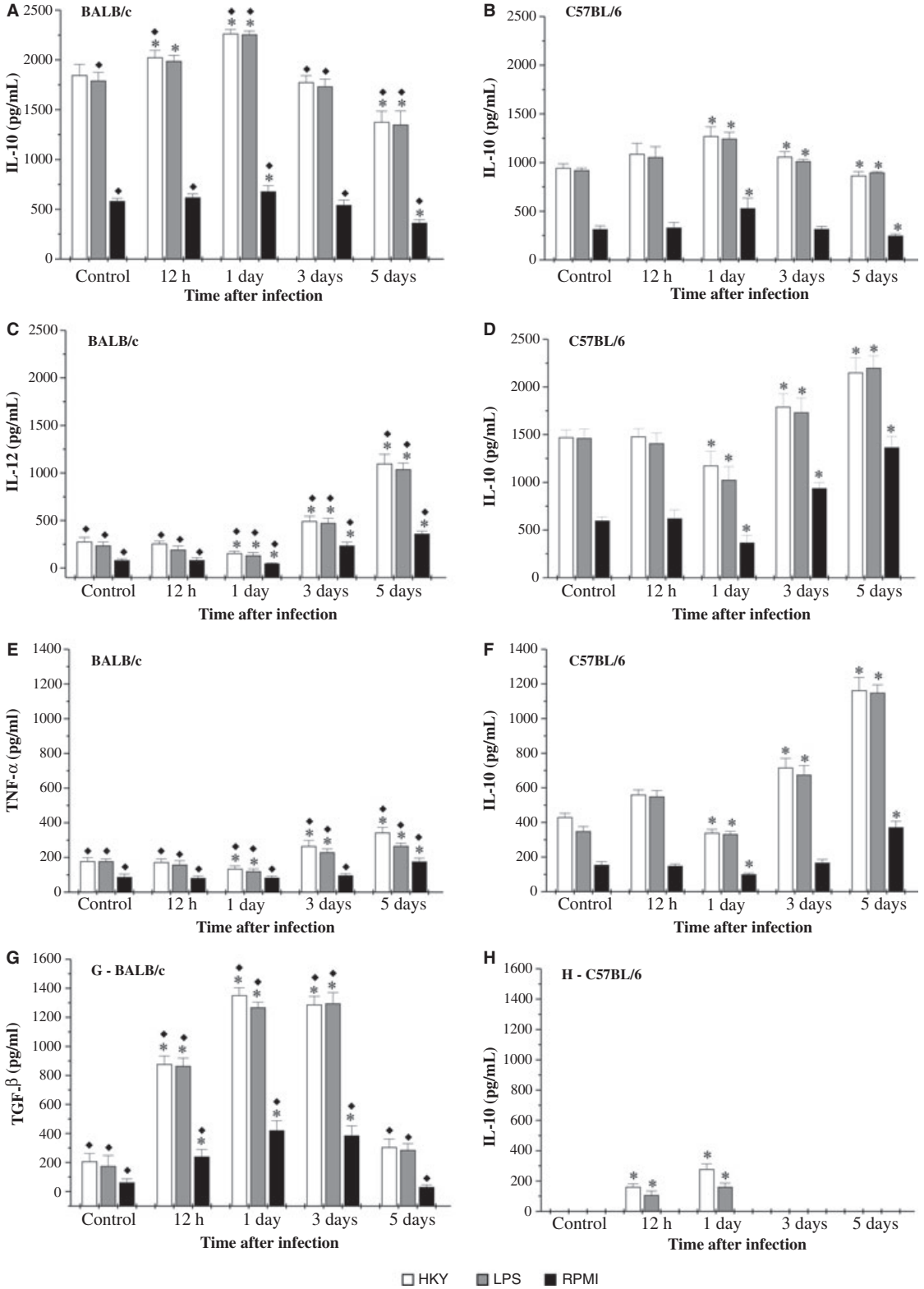

Figure 3 Cytokine production by murine peritoneal macrophages collected from BALB/*c* and C57BL/6 mice during *Yersinia pseudotuberculosis* infection. Cells (2.5×10^6/ml) were cultured with HKY, LPS or RPMI-1640 medium, the supernatants were collected after 48 h of culture and the concentrations of IL-10 (A,B), IL-12 (C,D), TNF-α (E,F) and TGF-β (G,H) assayed by ELISA. Control refers to average values obtained from uninfected mice throughout the experiment. Results are representative of two independent experiments and are presented as mean ± SD of triplicate observations. *$P < 0.05$ versus control and ♦$P < 0.05$ versus C57BL/6.

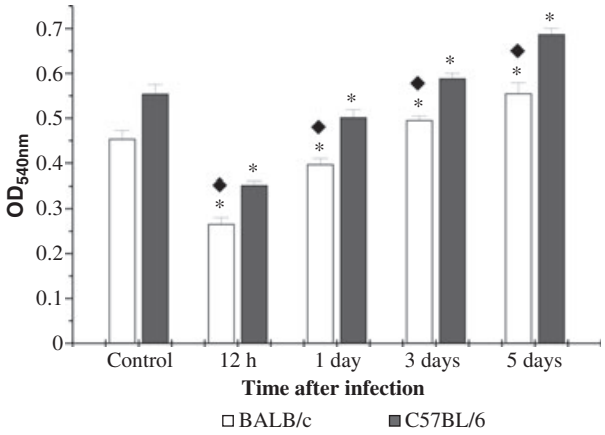

Figure 4 Influence of *Yersinia pseudotuberculosis* infection on the capacity of macrophages to stimulate T-cell proliferation. Peritoneal macrophages obtained from BALB/*c*- and C57BL/6-infected mice were co-cultured with T cells from immunized mice of the same strain, for 4 days in the presence of HKY. T-cell proliferation was measured by the MTT assay. Results are expressed as the OD at 540 nm, representing the production of formazan, and are presented as mean ± SD of quadruplicate observations. *$P < 0.05$ versus control and ♦$P < 0.05$ versus C57BL/6.

There was a decrease in the IFN-γ production in the co-culture with macrophages obtained in the early period of *Y. pseudotuberculosis* infection, in both strains of mice. The amount of IFN-γ detected in the supernatants of the co-culture of BALB/*c*-infected mice was lower than that detected in co-culture of C57BL/6-infected mice on all days post-infection. On the fifth-day post-infection, a significant increase in the production of IFN-γ by cells from C57BL/6-infected mice was observed (twofold more in relation to BALB/*c*).

There was no detectable production of IL-4 in the supernatants of the co-culture of C57BL/6-infected mice. A small amount of IL-4 was detected in the co-culture of BALB/*c* mice on the first- and third-day post-infection.

Discussion

Our results showed that *Yersinia*-resistant and -susceptible strains of mice have different patterns of macrophage activation, during *Y. pseudotuberculosis* infection.

The NO levels produced by macrophages from C57BL/6 control mice were significantly higher than those produced by macrophages from BALB/*c* mice. Conversely, the arginase activity of cells from C57BL/6 control mice was significantly lower than that observed for the BALB/*c* mice. Similarly, macrophages from C57BL/6-infected mice, stimulated with HKY or LPS or unstimulated, produced higher levels of NO and lower arginase activity than equivalent macrophages from BALB/*c* mice. These results corroborate reports showing that macrophages from Th1 strains of mice are more easily activated for NO production, and macrophages from Th2 strains of mice use the arginase pathway of arginine metabolism [21, 22].

At 12 h and 1 day after infection, macrophages from C57BL/6- and BALB/*c*-infected mice showed decrease in the NO production when compared with macrophages from control mice. These results suggest that *Y. pseudotuberculosis* infection can interfere with NO production, as described by Pujol and Bliska [23] in experiments where macrophages were infected *in vitro* with *Y. pseudotuberculosis* and *Yersinia pestis*.

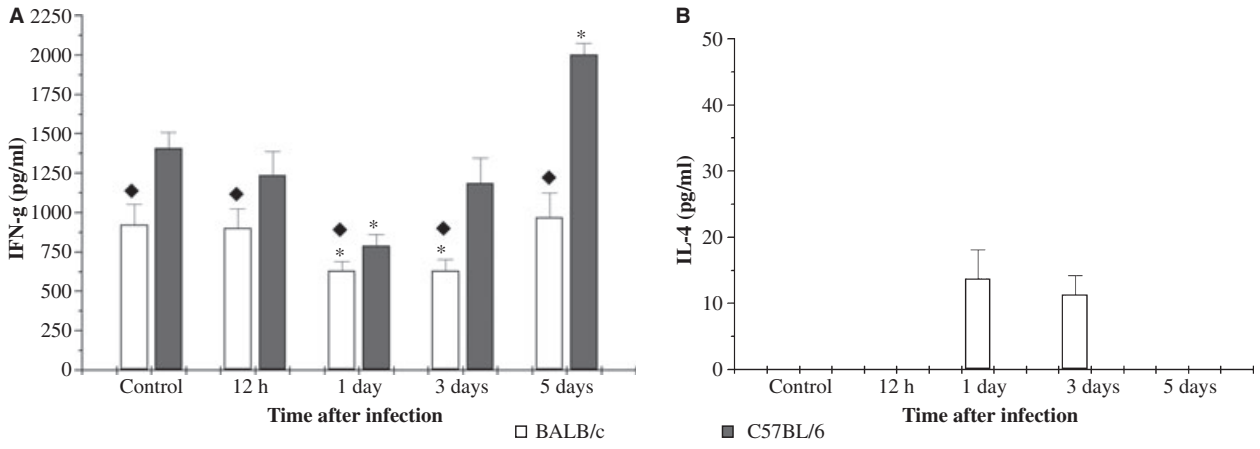

Figure 5 Cytokine production in supernatants of co-cultures of T cells with macrophages from uninfected or infected mice. Peritoneal macrophages obtained from infected BALB/*c* and C57BL/6 mice were co-cultured with T cells from immunized mice of the same strain, for 4 days in the presence of HKY, and the concentrations of IL-4 and IFN-γ were assayed in supernatants by ELISA. Control refers to average values obtained from uninfected mice throughout the experiment. Results are presented as mean ± SD of triplicate observations. *$P < 0.05$ versus control and ♦$P < 0.05$ versus C57BL/6.

Macrophages activated in different patterns display distinct profiles of cytokine secretion. In general, M1 cells have an IL-12^{high}, IL-10^{low} phenotype, and are efficient producers of proinflammatory cytokines (IL-1β, TNF-α, IL-6). M2 macrophages share an IL-12^{low}, IL-10^{high} phenotype, with low production of proinflammatory cytokines [14]. Consistent with these previously published data, our study showed that macrophages from C57BL/6 control mice produced higher levels of IL-12 and TNF-α than macrophages from BALB/*c* mice. On the other hand, macrophages from BALB/*c* control mice produced higher amounts of IL-10 and considerable levels of TGF-β.

During *Y. pseudotuberculosis* infection, macrophages from C57BL/6 mice produced significantly higher amounts of IL-12 and TNF-α, while macrophages from BALB/*c* mice produced significant higher amounts of IL-10 and TGF-β. These observations suggest that the infection does not change the characteristic pattern of activation of macrophages in these mice.

At 12 h and 1 day after infection, there was an increase in the production of IL-10 by macrophages of both strains of mice. IL-10 is a cytokine with broad anti-inflammatory properties that results from its ability to inhibit functions of both macrophages and dendritic cells, including secretion of their proinflammatory cytokines [24] and NO production [25]. Sing *et al.* [26] reported that low calcium response V (LcrV) or antigen V, a secreted antihost protein with strong immunomodulatory effects that is associated *Yersinia* virulence, inhibits zymosan-induced production of TNF-α by inducing IL-10. The increase of IL-10 production, in the early period of infection, was consistent with the observed decrease of proinflammatory cytokines and NO production in the same period.

In this study, we verified that infection with *Y. pseudotuberculosis* exerts a suppressor role on TNF-α and IL-12 production by macrophages from BALB/*c* and C57BL/6 mice, corroborating *in vitro* studies that reported the function of LcrV and YopB in the inhibition of the production of TNF-α and IL-12 in murine peritoneal macrophages [27] and revealing one survival mechanism used by this bacterium.

In the early period of infection, significant production of TGF-β was seen, with higher levels for BALB/*c* mice. TGF-β is an immunoregulatory cytokine that inhibits the activation of macrophages [28]. Possibly the most important deactivating effect of TGF-β on macrophages is its ability to limit the production of cytotoxic reactive oxygen and nitrogen intermediates [29]. This is coherent with the decrease in NO production seen 12 h and 1 day after infection, mainly in macrophages from BALB/*c* mice.

On the third- and fifth-day post-infection, there was a decrease in the production of TGF-β by macrophages of BALB/*c* mice and the absence of TGF-β production by macrophages of C57BL/6 mice. In the same period, a concomitant increase of NO levels was observed in both strains of mice.

In the course of infection, a decrease of IL-10 production and increase of TNF-α and IL-12 concentrations were observed in macrophages from BALB/*c* and C57BL/6 mice. TNF-α and IL-12 are two major mediators of inflammatory responses in mammals [30]. IL-12 is a heterodimer cytokine that induces IFN-γ production, activates TNF-α and increases NK-cell cytotoxicity, as well as T-cell proliferation [31]. The high TNF-α and IL-12 production and lower IL-10 levels that occurred on the fifth-day post-infection, in macrophages from C57BL/6 mice, were coherent with the increase of NO and decrease of arginase activity.

The T cells play a crucial role in the defence of the immune system against *Yersinia*; an effective way for yersiniae to subvert the host immune response is to inhibit the presentation of their antigens and thus impair T-cell stimulation [10, 32]. In the early *Y. pseudotuberculosis* infection, we observed a decrease in the immunostimulatory capacity of macrophages, which led to a lower proliferation of T-cells from BALB/*c* mice. On the third- and fifth-day post-infection, macrophages acquired the capacity to stimulate T-cells, higher T-cell proliferation occurring in C57BL/6 mice. These results corroborated the published reports [32] and show that macrophages influence the susceptibility of BALB/*c* mice to *Yersinia* infection.

It is known that T lymphocytes from different strains of mice tend to produce different profiles of cytokines [33]. In this study, we observed that the C57BL/6 uninfected mice produced higher amounts of IFN-γ than BALB/*c*-uninfected mice in supernatants of co-cultures. However, neither strain of mice produced IL-4. It is possible that BALB/*c* control mice produced an amount that was below the limit of detection of the test. The results obtained with C57Bl/6 mice agree with reports of Heinzel *et al.* [34], who verified that T cells from this strain expressed high levels of IFN-γ mRNA and that IL-4 mRNA was not detectable.

We did not detect IL-4 in supernatants of co-culture of C57BL/6-infected mice. Cells from BALB/*c*-infected mice produced detectable IL-4 levels on the first- and third-day post-infection. Our results corroborate reports of IL-4 production by T lymphocytes from BALB/*c* mice infected with species of enteropathogenic *Yersinia* [15, 34, 35]. C57BL/6-infected mice also produced higher amounts of IFN-γ than BALB/*c*-infected mice, but no IL-4. Therefore, the response of C57BL/6 mice is typically Th1. Autenrieth *et al.* [36] showed that T cells isolated from C57BL/6 mice infected with *Y. enterocolitica*, produced IFN-γ levels significantly larger than T cells from BALB/*c* mice. The neutralization of IFN-γ *in vivo* abolishes the resistance to *Yersinia* infection and leads to

disease progression [36, 37]. It is known that IFN-γ stimulates C57BL/6 mice to produce NO [38], justifying the high levels of NO produced by macrophages of this strain of mice.

On the other hand, we observed that BALB/*c*-infected mice produced a typically Th2 response, with IL-4 production on the first- and third-day post-infection and lower production of IFN-γ. IL-4 does not protect against *Yersinia* infection. The administration of anti-IL-4 antibodies before *Y. enterocolitica* infection transforms BALB/*c* susceptible mice into resistant animals, while the same treatment does not affect C57BL/6 mice significantly [36].

Macrophages from *Yersinia*-resistant and *Yersinia*-susceptible strains not only differ in their ability to be activated in the classical sense, but also respond differently to the same stimuli. It may well be that the susceptibility of BALB/*c* mice to *Yersinia* is a consequence not only of the weak and delayed T-lymphocyte response [36], but also of the deficient activation of macrophages observed early in the infection.

Our results suggest that the deficient activation of macrophages in BALB/*c* mice (weak levels of NO, high levels of IL-10 and TGF-β1 and a reduced immunostimulatory capacity) may contribute to the susceptibility of this strain of mice to *Yersinia pseudotuberculosis*.

Acknowledgment

We thank Valéria Aparecida de Araújo Mallavolta for technical support. The work reported here was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP/Brazil (grant number 06/06964–8), Programa de Apoio ao Desenvolvimento Científico da Faculdade de Ciências Farmacêuticas-UNESP/Brazil (PADC 2006/13-I) and by a fellowship provided by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES/Brazil (Aline Tansini).

References

1 Barnes PD, Bergman MA, Mecsas J, Isberg RR. *Yersinia pseudotuberculosis* disseminates directly from a replicating bacterial pool in the intestine. *J Exp Med* 2006;203:1591–601.

2 Oellerich MF, Jacobi CA, Freund S *et al. Yersinia enterocolitica* infection of mice reveals clonal invasion and abscess formation. *Infect Immun* 2007;75:3802–11.

3 Viboud GL, Bliska JB. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annu Rev Microbiol* 2005;59:69–89.

4 Carniel E. Plasmids and pathogenic islands of *Yersinia*. *Curr Top Microbiol Immunol* 2002;264:89–108.

5 Navarro L, Alto NM, Dixon JE. Functions of the *Yersinia* effector proteins in inhibiting host immune responses. *Curr Opin Microbiol* 2005;8:21–7.

6 Heesemann J, Sing A, Trülzsch K. *Yersinia*'s stratagem: targeting innate and adaptive immune defense. *Curr Opin Microbiol* 2006;9:55–61.

7 Hanski C, Naumann M, Grutzkau A *et al.* Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. *Infect Immun* 1991;59:1106–13.

8 Autenrieth IB, Hantschmann P, Heymer B, Heesemann J. Immunohistological characterization of the cellular immune response against *Yersinia enterocolitica* in mice: evidence for the involvement of T lymphocytes. *Immunobiology* 1993;187:1–16.

9 Autenrieth IB, Vogel U, Preger S, Heymer B, Heesemann J. Experimental *Yersinia enterocolitica* infection in euthymic and Tcell- deficient athymic nude C57BL/6 mice: comparison of time course, histomorphology, and immune response. *Infect Immun* 1993;61: 2585–95.

10 Kramer U, Wiedig CA. *Y. enterocolitica* translocated Yops impair stimulation of T-cells by antigen presenting cells. *Immunol Lett* 2005;100:130–8.

11 Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G *et al.* Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology* 2006;211:487–501.

12 Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by $CD4_+$ T cells correlates with Th1/Th2 phenotype. *J Immunol* 1998;160:5347–54.

13 Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nature Rev Immunol* 2005;5:641–54.

14 Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverses forms of macrophages activation and polarization. *Trends Immunol* 2004;25:677–86.

15 Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 2000; 164:6166–73.

16 Gordon S. Alternative activation of macrophages. *Nature Rev Immunol* 2003;3:23–35.

17 Kröncke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. *Biol Chem Hoppe Seyler* 1995;376:327–43.

18 Tumitan ARP, Monnazzi LGS, Ghiraldi FR, cilli EM, Medeiros BMM. Pattern of macrophage activation in *Yersinia*-resistant and *Yersinia*-susceptible strains of mice. *Microbiol Immunol* 2007;51:1021–8.

19 Green LCD, Wagner DDA, Glogowski J, Skepper PL, Wishnok J, Tannebaum SR. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Ann Biochem* 1982;126:131–8.

20 Corraliza IM, Campo ML, Soler G, Modollel M. Determination of arginase activity in macrophages: a micromethod. *J Immunol Methods* 1994;174:231–5.

21 Liew FY, Li Y, Moss D, Parkinson M, Rogers V, Moncada S. Resistance to *Leishmania major* infection correlates with the induction of nitric oxide synthase in murine macrophages. *Eur J Immunol* 1991;21:3009–14.

22 Stenger S, Thuring H, Rollinghoff M, Bogdan C. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *J Exp Med* 1994;180:783–93.

23 Pujol C, Bliska JB. Turning *Yersinia* pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. *Clin Immunol* 2005;117:216–26.

24 O'Garra A, Vieira P. Th1 cells control themselves by producing interleukin-10. *Nature* 2007;7:425–8.

25 Ter Steege JC, Van de Ven WC, Forget PP, Buurman WA. Regulation of lipopolysaccharide-induced NO synthase expression in the major organs in a mouse model. The roles of endogenous interferon-gamma, tumor necrosis factor-alpha and interleukin-10. *Eur Cytokine Netw* 2000;11:39–46.

26 Sing A, Roggenkamp A, Geiger AM, Heesemann J. *Yersinia enterocolitica* evasion of the host innate immune response by V

antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice. *J Immunol* 2002;168:1315–21.

27 Sharma RK, Sodhi A, Batra HV, Tuteja U. Effect of rLcrV and rYopB from *Yersinia pestis* on murine peritoneal macrophages *in vitro*. *Immunol Lett* 2004;93:179–87.

28 Tsunawaki S, Spora M, Ding A, Nathan C. Deactivation of macrophages by transforming growth factor beta. *Nature* 1998;334:260–2.

29 Bogdan C, Nathan C. Modulation of immune macrophages function by transforming growth factor beta, interleukin-4 and interleukin-10. *Ann Acad Sci* 1993;685:713–39.

30 Ma X. TNF-α and IL-12: a balancing act in macrophage functioning. *Microbes Infect* 2001;3:121–9.

31 Del Vecchio M, Bajetta E, Canova S *et al.* Interleukin-12: biological properties and clinical application. *Clin Cancer Res* 2007;13:4677–85.

32 Erfuth SE, Grobner S, Kramer U *et al. Yersinia enterocolitica* induces apoptosis and inhibits surface molecule expression and cytokine production in murine dendritic cells. *Infect Immun* 2004;72:7045–54.

33 Hsieh CS, Macatonia SE, O'Garra A, Murphy MK. T cell genetic background determines default T helper phenotype development in vitro. *J Exp Med* 1995;181:713–21.

34 Heinzel FP, Sadick MD, Mutha SS, Locksley RM. Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by $CD4_+$ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proc Natl Acad Sci USA* 1991;88:7011–5.

35 Bohn E, Heesemann J, Ehlers S, Autenrieth IB. Early gamma interferon mRNA expression is associated with resistance of mice against *Yersinia enterocolitica*. *Infect Immun* 1994;62:3027–32.

36 Autenrieth IB, Beer M, Bohn E, Kaufmann SH, Heesemann J. Immune responses to *Yersinia enterocolitica* in susceptible BALB/c and resistant C57BL/6 mice: an essential role for gamma interferon. *Infect Immun* 1994;62:2590–9.

37 Autenrieth IB, Heesemann J. In vivo neutralization of tumor necrosis factor-alpha and interferon-gamma abrogates resistance to *Yersinia enterocolitica* infection in mice. *Med Microbiol Immunol* 1992;18:333–8.

38 Dileepan KN, Page JC, Stechchulte JD. Direct activation of murine peritoneal macrophages for nitric oxide production and tumor cell killing by interferon-γ. *J Interferon Cytokine Res* 1995;15:387–94.