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In vitro effects of interleukin-4 on interferon-γ-induced macrophage activation

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SUMMARY

Interferon-γ (IFN-γ) and interleukin-4 (IL-4) have been shown to be secreted by distinct T-helper cell subsets which have different roles in the determination of host resistance to infection. We studied the activity of these two cytokines on effector mechanisms of mouse macrophages. *In vitro* cultured bone marrow-derived macrophages from C57BL/6 mice were treated with IFN-γ, IL-4, or a combination of both cytokines and the ability to secrete superoxide or nitrite or to restrict growth of *Mycobacterium avium* and *Toxoplasma gondii* was then evaluated. We found that IL-4 could inhibit the priming of macrophages for enhanced superoxide production induced by IFN-γ although IL-4 when used alone did have some enhancing effect of its own. This effect of IL-4 on IFN-γ-primed superoxide production was dose dependent and could be observed even if the treatment by IL-4 was done 24 hr after treatment by IFN-γ. IL-4 did not, however, influence the enhanced production of nitrogen reactive intermediates, the induction of bacteriostatic activity against *M. avium*, or the restriction of *T. gondii* by IFN-γ-treated macrophages, and did not have any effect of its own regarding these latter functions.

INTRODUCTION

Recent evidence suggests that the profile of cytokine secretion separates T-helper cells into two broad subpopulations: in Th1 subset that secretes interleukin-2 (IL-2) and interferon-γ (IFN-γ)[1,2] and mediates delayed-type hypersensitivity,[3] and the Th2 subset that produces IL-4 and IL-5 (refs 1, 2 and reviewed in ref. 4). These two subpopulations of T-helper cells apparently serve different roles in acquired resistance to infection, the Th1 subset conferring cell-mediated immunity to intracellular parasites by secreting the powerful macrophage activator IFN-γ, and Th2 cells promoting antibody formation and humoral immunity. In terms of protective antimicrobial immunity, it has been shown in the case of some infections that the resistance of mice can be related to the subset of T-helper cells that is induced. Thus, in the case of *Leishmania major* infection, the induction of Th2 cells is associated with susceptibility to infection whereas the induction of Th1 cells correlates with a resistant phenotype.[5,6] Furthermore, the pattern of host resistance or susceptibility has been related to the production of particular cytokines. IFN-γ is responsible for the induction of resistance to leishmania both *in vivo*[5-8] and *in vitro*[9-11] whereas IL-4 is responsible for *in vivo* susceptibility.[5] Both cytokines might interfere with the immunity developed during infection either by promoting the differentiation of one of the T-helper cell subsets or, in the presence of both types of Th cells, by interfering with the effector mechanisms, namely at the level of the macrophage. Regarding the first hypothesis, it has been found that IL-4 inhibits the production of IFN-γ by mononuclear cells[12,13] and favours the proliferation of Th2 cells[14] whereas IFN-γ inhibits the proliferation of Th2 cells.[14] Regarding the second alternative, it has been shown that these two cytokines have modulatory effects on the macrophage which are in some cases coincidental and in others opposing. Besides the described effects on leishmania infection,[9-11] IFN-γ is known to induce an enhancement of the antimicrobial activity of the macrophage against some mycobacteria,[15,16] toxoplasma,[10,17-19] legionella[20,21] and trypanosoma[22] as well as to prime macrophages for enhanced production of reactive oxygen metabolites (ROI)[18,23,24] and of reactive nitrogen metabolites (RNI),[25] enhanced expression of Ia molecules[26] and induction of tumoricidal activity.[27,28] IL-4 can also activate the macrophage for certain functions such as tumoricidal[29] and trypanocidal[30] activities, respiratory burst activity,[31] up-regulation of Ia[29,32] and complement receptors 3 and 4 expression,[31] and down-regulation of Fc receptors 1 and 2.[32] It has recently been suggested by Liew *et al.*[33] that IL-4 could mediate the enhancing effect of Th2 cells on microbial proliferation during mouse infections by *Leishmania major* by acting at the effector level on macrophage antimicrobial functions.

Here, we examined the effects of IL-4 on four distinct functions of bone marrow-derived macrophages (superoxide and nitrite production and the induction of antimicrobial activity against *Mycobacterium avium* or *Toxoplasma gondii*) as well as on the priming of these functions by IFN-γ. We found

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that IL-4 had divergent effects on the activation of the macrophage according to the function that was evaluated.

MATERIALS AND METHODS

Animals

Female C57BL/6 and male CD-1 mice were purchased from the Instituto Gulbenkian de Ciência (Oeiras, Portugal) and maintained under conventional conditions, given commercial chow and acidified water *ad libitum*.

Reagents and micro-organisms

Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing 4·5 g/l glucose (Gibco, Paisley, Renfrewshire, U.K.) and supplemented with 10% foetal calf serum (FCS) and 10 mM of HEPES buffer (Gibco). No antibiotics were used. Hanks' balanced salt solution (HBSS) was also from Gibco. Recombinant mouse IFN-γ and recombinant mouse IL-4 were purchased from Genzyme (Boston, MA). Polymyxin B, saponin, aminoacridine hydrochloride, horse heart cytochrome *c* (type III), *Escherichia coli* lipopolysaccharide (LPS), and phorbol myristate acetate (PMA) were from Sigma (St Louis, MO). Inoculum of *M. avium* ATCC 25291 was prepared as described previously.[34]

Preparation of toxoplasma tachyzoites

The RH strain of *T. gondii* was maintained by serial passage in CD-1 mice, inoculated intraperitoneally (i.p.). Groups of CD-1 mice were inoculated i.p. with 5×10^5 tachyzoites in 0·5 ml of phosphate-buffered saline (PBS) and 4 days later the peritoneal exudate was collected with HBSS. The parasites were separated from the peritoneal cells by centrifugation of the lavage fluid, washed twice with HBSS and suspended in DMEM medium to the desired concentration, as determined by counting in a haemocytometer. The per cent of living parasites was calculated by the trypan blue exclusion test, and was found to be always above 99%.

Bone marrow-derived macrophage culture

The femurs from C57BL/6 mice were aseptically removed and the bone marrow cells collected by flushing the diaphysis with cold HBSS. The cell suspension was centrifuged at 100 ***g*** and the pellet resuspended in DMEM containing 10% of L929 cell-conditioned medium (LCCM, also in DMEM). The clumps were removed by allowing them to sediment at 1 ***g*** and the cell suspension was plated in 24-well tissue culture plates ($0{\cdot}6 \times 10^6$ cells in 1 ml/well, corresponding to one femur equivalent per plate). The cells were incubated for 10 days at 37° in a 7% CO_2/93% humidified air atmosphere. At Day 4, an additional volume of 0·1 ml of LCCM was added and at Day 7 the medium was removed and fresh medium also containing 10% LCCM was added.

Superoxide assay

The method of Johnston[35] was followed. Briefly, the supernatant culture medium from the macrophage cultures was removed by suction, the monolayer was washed twice with warm phenol red-free HBSS to remove any traces of phenol red and 1 ml of the incubation mixture consisting of 0·8 mM cytochrome *c* and 0·5 μg of PMA/ml in Krebs Ringer phosphate buffer was added to each well. The plates were incubated for 90 min at 37°. The incubation mixture was then collected and the absorbance read at 550 nm. The protein content of the macrophage monolayer was determined using the Lowry assay. The amounts of superoxide produced were calculated by transforming the absorbance values into nmol considering an extinction coefficient of 47·6 nmol/cm. Data are presented as the amount of superoxide produced in nmol/mg of adherent protein.

Assessment of nitrite production

Macrophages were treated with the cytokines for 24, 48, 72 or 96 hr in 0·5 ml of complete DMEM. During the last 24 hr, an additional volume of 0·5 ml of medium containing lipopolysaccharide (LPS) (5 μg/ml final concentration) was added to trigger the production of RNI. The amount of nitrite present in macrophage culture supernatants was quantitated with a colorimetric assay using the Griess reagent as described elsewhere.[25]

Study of in vitro *infection of bone marrow-derived macrophages by* M. avium

Bone marrow-derived macrophages were infected with *M. avium* after 10 days of culture. The medium was removed by aspiration and 200 μl of DMEM with 10% FCS plus 2×10^7 colony-forming units (CFU) of *M. avium* ATCC 25291 was added to each well. The plates were incubated for 4 hr and then washed extensively with warm HBSS to remove unphagocytosed bacteria and 400 μl of fresh medium was added. Every day, 100 μl of fresh medium with or without cytokines was added from Day 0 to Day 6. At time 0 (after the initial 4 hr of infection and the wash) and at different periods of infection, the volume of the wells was completed to 1 ml and saponin was added to a final concentration of 0·1% (w/v) to lyse the macrophages. The supernatant was vigorously pipetted to disrupt cell remnants, serially diluted in a 0·04% Tween 80 solution and plated onto 7H10 agar medium to determine the viable counts as described previously.[34]

Infection of macrophage cultures with toxoplasma

Macrophage monolayers containing about 5×10^5 cells/well of a 24-well tissue culture plate were infected with toxoplasma suspensions to give a ratio toxoplasma/macrophage between 1/5 and 1/10. For that, the medium was removed from the cultures and 1 ml of fresh DMEM containing the parasites was added. The plates were then incubated for 90 min at 37° with 7% CO_2 to allow for the infection of the macrophages. After this incubation, the wells were washed twice with fresh DMEM, and incubated as above. At the indicated times, the coverslips were removed from the wells and washed with saline and air dried. To reveal the intracellular parasites, the coverslips were fixed overnight at 4° in 0·4% aminoacridine hydrochloride in 50% ethanol and stained with the Wright stain. The number of parasites/intramacrophagic vacuole was determined in the infected macrophage cultures by counting at least 200 infected macrophages/coverslip. All countings were carried out in six coverslips per time-point, and the experiments were repeated three times. The values were compared statistically by Student's *t*-test.

RESULTS

Choice of macrophage population

Different populations of macrophages were tested for their ability to be primed for enhanced production of oxygen metabolites. We found that resident peritoneal macrophages produce low amounts of superoxide when triggered by PMA and that this low level of secretion of superoxide was not significantly increased when the cells were treated with either IFN-γ or crude lymphokines from concanavalin A-stimulated spleen cell supernatants (results not shown). Exudative peritoneal macrophages elicited by either thioglycollate broth or 5% sodium caseinate secreted high amounts of superoxide after PMA triggering but these high levels of superoxide production were not further increased by treatment with IFN-γ (results not shown). Bone marrow-derived macrophage showed several advantages for the study of the priming of superoxide production by cytokines: they produced low amounts of superoxide when triggered by PMA; these low levels were increased several-fold by treatment with IFN-γ, the cellular monolayer was constituted by over 99% of macrophages; and their survival was longer than that of the other macrophage populations in terms of cell loss and healthy appearance of the cells (adherent and well spread) even when kept in the absence of conditioned medium. Therefore, in the following experiments, bone marrow-derived macrophages were used.

Table 1. Superoxide production by bone marrow-derived macrophages (nmol of O_2^-/mg of protein/90 min) incubated for 3 days in medium alone or in the presence of cytokines with or without polymyxin B (Pol.B) at a concentration of 10 μg/ml

Treatment	Superoxide produced
Medium alone	5·9 ± 2·9
100 U IFN-γ/ml	64·8 ± 11·6
100 U IFN-γ/ml + Pol.B	58·0 ± 3·6
100 U IFN-γ + 100U IL4/ml + Pol.B	33·6 ± 3·1

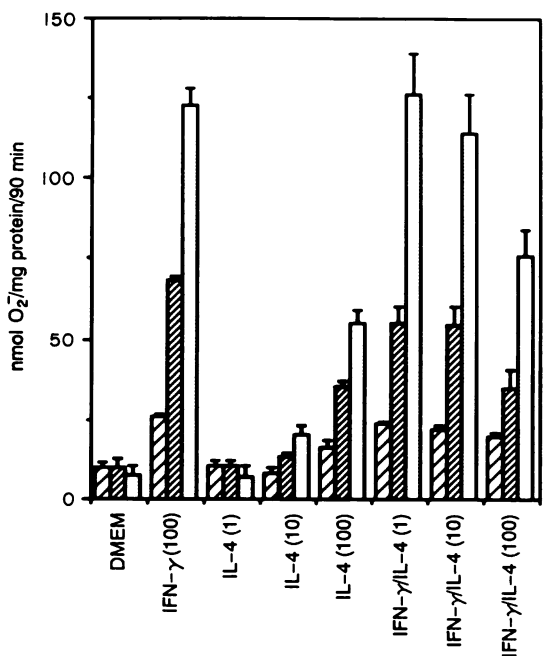

Figure 1. Production of superoxide by PMA-triggered bone marrow-derived macrophages cultured in medium alone (DMEM), and in medium containing 100 U of IFN-γ/ml (IFN-γ 100), 1, 10 or 100 U of IL-4/ml (IL-4 1, 10, and 100, respectively), or 100 U of IFN-γ plus 1, 10 or 100 U of IL-4/ml (IFN/IL-4 1, 10, and 100, respectively) for 24 hr (▨), 48 hr (■), or 72 hr (□). The results represent the mean of three replicate wells ± one standard deviation. The IL-4-induced inhibitory effects were consistent in five independent experiments.

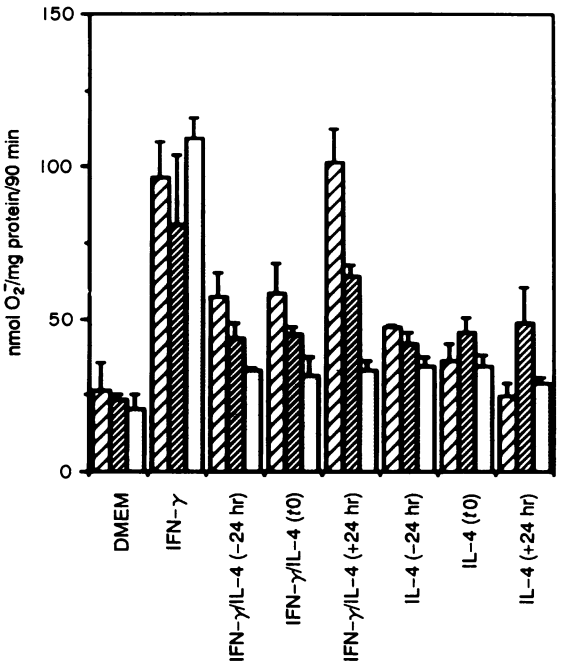

Figure 2. Production of superoxide by PMA-triggered bone marrow-derived macrophages cultured in medium alone (DMEM) or in medium containing IFN-γ, IL-4, or both. IFN-γ was added at time 0 to the wells indicated as IFN-γ or IFN-γ/IL-4. IL-4 was added either 24 hr prior to IFN-γ (−24 hr), at the same time as IFN-γ (t0), or 24 hr later (+24 hr) to wells that were or were not to be treated with IFN-γ. Superoxide production was assayed at 24 hr (▨), 48 hr (■), or 72 hr (□).

Effect of IFN-γ and IL-4 on superoxide production by macrophages

Bone marrow-derived macrophages cultured in medium alone and triggered by PMA produced low levels of superoxide (Fig. 1). When 100 U of IFN-γ/ml was added for 24, 48 or 72 hr, the ability of these macrophages to secrete superoxide after PMA triggering increased more than 10-fold. Previous experiments had shown that this concentration of IFN-γ was optimal for the induction of enhanced superoxide secretion (data not shown). Increasing concentrations of IL-4 were able to prime macrophages for higher levels of superoxide production although with much less efficiency than IFN-γ (Fig. 1). When both cytokines were added together to macrophages at the same concentrations as before, IL-4 exerted a dose-related inhibitory effect on the IFN-γ-induced enhancement of superoxide production (Fig. 1).

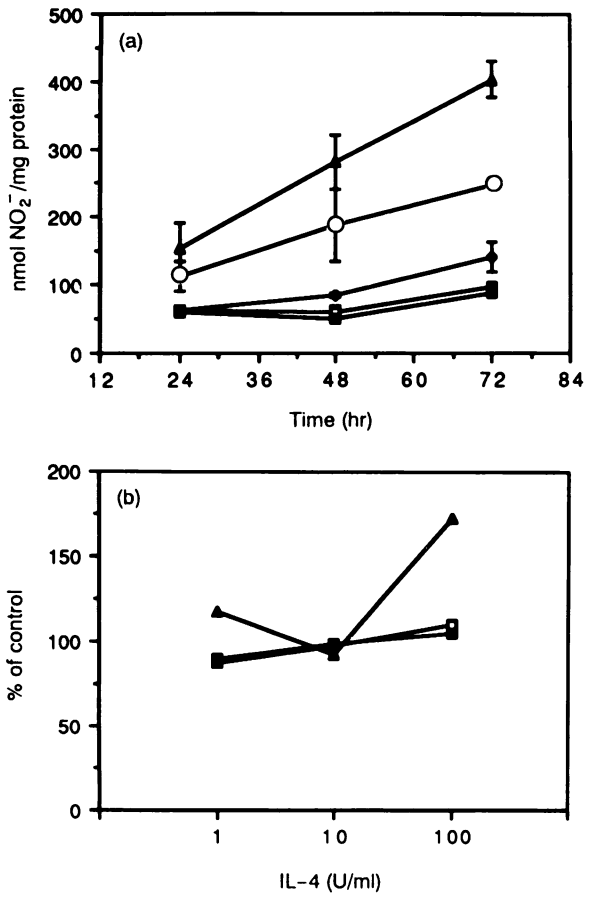

Figure 3. Nitrite production by bone marrow-derived macrophages cultured in the absence or presence of cytokines and with the addition of LPS during the last 24 hr of culture. (a) Macrophages were treated with 1 (●), 10 (○) or 100 (▲) U IFN-γ/ml or IL-4 (100 U/ml) (□) for 24–72 hr or left untreated (control) (■). (b) Amounts of nitrite produced at 72 hr of culture with 100 U of IFN-γ/ml and IL-4 (1, 10 or 100 U/ml) added at the same time (▲), 24 hr prior to (□), or 24 hr after (■) the addition of IFN-γ. Results are expressed as percentage of the production by IFN-γ-treated macrophages. There were no statistically significant differences between controls and IL-4-treated cells. The results are representative of three independent experiments.

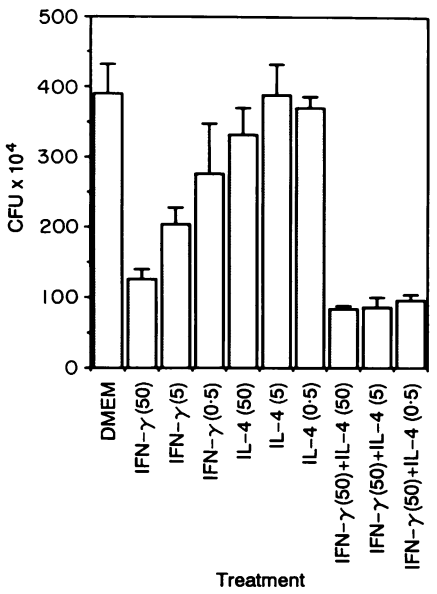

Figure 4. Number of CFU/well of bone marrow-derived macrophages infected 7 days before with $60 \times 10^4 \pm 5 \times 10^4$ CFU of *M. avium*. Macrophage cultures were treated daily with 100 μl of fresh medium containing IFN-γ, IL-4, or a combination of both. The amount of cytokine given daily was 0·5, 5·0, or 50·0 U/day as indicated. The results represent the mean of three replicate wells ± 1 standard deviation. The results are representative of five independent experiments.

The inhibitory effect of IL-4 (100 U/ml) was seen when this cytokine was added before, at the same time as, or after the addition of IFN-γ (Fig. 2). The inhibitory effect of IL-4 was observed when polymyxin B was added to the cultures (Table 1) showing that the inhibitory effect was not due to endotoxin contamination.

Effects on nitrogen metabolism

Bone marrow-derived macrophages were cultured with the cytokines for different periods of time and LPS was added to the cultures during the last 24 hr of incubation to trigger the production of RNI. Other experiments showed that this protocol did not show different results from another where LPS was present throughout the incubation period (not shown). IFN-γ was able to prime macrophages for enhanced release of nitrite in a dose-dependent way and with maximum amounts detected at 72–96 hr of incubation (Fig. 3a). IL-4, at doses effective on oxidative metabolism and shown by others to be effective on other macrophage functions,[33] was unable to affect nitrogen metabolism (Fig. 3a) and had no inhibitory action on the IFN-γ-induced activation when added at the same time, 24 hr prior, or 24 hr after IFN-γ (Fig. 3b).

Effect of IFN-γ and IL-4 on the antimycobacterial activity of macrophages

It was then questioned whether the inhibitory effects of IL-4 might influence the ability of IFN-γ to restrict the growth of *M. avium* in infected macrophages. To test this possibility, bone marrow-derived macrophages were infected with *M. avium* for 4 hr and after washing out the extracellular mycobacteria, they were cultured in medium in the presence or absence of cytokines for another 7 days. The protocol of addition of cytokines was modified from the one used above since the assay took longer to perform. Thus, the cytokines were added daily to the cultures to mimick the constant activity of immune cells in the *in vivo* situation. *M. avium* was able to proliferate in bone marrow-derived macrophages cultured in medium alone (Fig. 4). When IFN-γ was added daily at the doses of 0·5, 5·0 or 50·0 U/day there was a dose-related induction of bacteriostasis (Fig. 4). IL-4 given in the same regimen was unable by itself to promote bacteriostasis or to inhibit the bacteriostatic activity induced by IFN-γ given at the dose of 50·0 U/day (Fig. 4).

Anti-toxoplasma effect of IFN-γ and IL-4 on macrophages

Bone marrow-derived macrophage cultures were treated with the cytokines for 48 hr and were subsequently infected with toxoplasma tachyzoites. The proliferation of the protozoa was

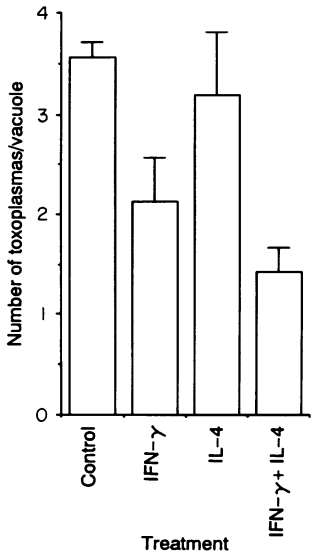

Figure 5. Proliferation of *T. gondii* tachizoytes in bone marrow-derived macrophages left untreated (control) or treated with 100 U of IFN-γ/ml, or 100 U of IL-4/ml, or both, 48 hr prior to the infection. Growth in both groups treated with IFN-γ is statistically lower than in the other two groups ($P < 0{\cdot}01$).

assessed at 20 hr post-infection by counting the number of parasites/vacuole. As shown in Fig. 5, the growth of the parasite was reduced by treatment with 100 U of IFN-γ/ml but not by treatment with 100 U of IL-4/ml. Furthermore, IL-4 had some potentiating effect on the IFN-γ-mediated anti-toxoplasma activity (Fig. 5). In other experiments, where a more marked reduction in *T. gondii* growth was achieved by treatment of the macrophages with IFN-γ, no effect of IL-4 was found. The addition of IL-4 5 hr prior to or 24 hr after the addition of IFN-γ did not affect the induction of anti-toxoplasma activity by IFN-γ (results not shown).

DISCUSSION

The macrophages are major cellular mediators of immunity at the effector level. Their activity is modulated by cytokines produced by T cells that affect metabolic and functional activities of the phagocyte through events collectively regarded as activation. Different cytokines have in some cases opposing effects suggesting that the pattern of cytokine production in a given situation may lead to different pathways in macrophage differentiation and/or activity. Since the data regarding the effects of IL-4 on macrophage activation are still controversial, we studied the effects of this cytokine on the macrophage activity of untreated cells and of IFN-γ-treated cells, by evaluating four different parameters.

In the present study we found that IL-4 had distinct effects on oxidative metabolism when it acted on unprimed versus IFN-γ-primed responsive bone marrow-derived macrophages being able to induce a low enhancement of ROI production in the former but inhibiting the activity of IFN-γ. Other reports on the interactions between IFN-γ and IL-4 and their effects on macrophage functions have revealed contradictory results.[31,32,36-38] This could be related either to the heterogeneity of the macrophage populations studied, to whether they were of human or mouse origin, or to the state of macrophage activation of the cells responding to IL-4.

In contrast to the effects on oxidative metabolism, IL-4 did not induce the production of nitrogen metabolites, anti-*M. avium* or anti-toxoplasma activity in macrophages and was not able to revert the effects of IFN-γ, which had the ability to prime all three of these macrophage functions. Our data are thus different from those of Lehn *et al.*[36] and Liew *et al.*[33] who found an inhibitory effect of IL-4 on the activation of macrophages for the killing of leishmania induced by IFN-γ. Furthermore, we could not find an inhibitory effect of IL-4 on nitrogen metabolism as described by Liew *et al.*[33] The difference in the effects on RNI production could also be explained by the use of different macrophage populations differing in their responsiveness to cytokines.

Our data suggest that IFN-γ is one of the mediators of acquired resistance to infection by *M. avium* since it was able to induce bacteriostasis *in vitro* in macrophages infected with that microbe. The protective effects of IFN-γ on toxoplasma infections is well documented.[10,17-19] Since IL-4 was able to inhibit the effect of IFN-γ on macrophage oxidative metabolism but had no effect on anti-mycobacterial or anti-toxoplasma activities we suggest, as others did for *M. tuberculosis*,[39] that ROI are not involved in these antimicrobial effects in IFN-γ-activated macrophages. Killing of ingested toxoplasma tachizoytes during ingestion could still be mediated by ROI since we did not quantitate this parameter but rather looked at microbistatic mechanisms. Although others have shown that reactive nitrogen metabolites are involved in the killing of some mycobacteria,[40,41] we have found (R. Appelberg and I. M. Orme, submitted for publication) that these metabolites are not involved in the bacteriostasis of the virulent strain of *M. avium* used here. Here we showed that IL-4 did not affect either the nitrogen metabolism or the mechanism responsible for mycobacteriostasis. Our data, however, are compatible with a role for RNI in the anti-toxoplasma activity as shown by others.[42]

In the leishmania model, IL-4 has been found to promote microbial growth, an effect that could be partly due to a down-modulation of the oxidative metabolism of the macrophage since it is known that ROI mediate some of the antimicrobial activity of macrophages against leishmania.[43] The finding of extensive polyclonal and specific B-cell activation in human and experimental mycobacterial infections[44] might suggest that B-cell-acting factors such as IL-4 could be associated with diminished resistance to infection by mycobacteria. A Th2 type profile of cytokine expression was found in lepromatous leprosy[45] confirming this hypothesis. Our data suggest that the susceptibility to mycobacteria associated with IL-4 would not involve a direct effect of this cytokine on the effector mechanisms of immunity by macrophages but rather an effect at the level of the immunoregulation of T-cell function.

In conclusion, we found an inhibitory effect of IL-4 over the IFN-γ-mediated activation of the macrophage for the metabolism of reactive oxygen radicals. This inhibitory activity may account for the anti-protective effects of IL-4 in the infections caused by pathogens to which ROI are important antimicrobial effector molecules. This inhibitory effect of IL-4 was not a general effect on macrophage functions since it did not affect the IFN-γ-mediated activation for the generation of nitrogen metabolites, mycobacteriostasis or anti-toxoplasma activity.

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