TNF-α and IL-10 Modulate the Induction of Apoptosis by Virulent *Mycobacterium tuberculosis* in Murine Macrophages

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TNF-α and IL-10 Modulate the Induction of Apoptosis by Virulent Mycobacterium tuberculosis in Murine Macrophages

Mauricio Rojas,* † Martin Olivier, † Philippe Gros, ‡ Luis F. Barrera,* and Luis F. García²*

The Bcg/Nramp1 gene controls early resistance and susceptibility of macrophages to mycobacterial infections. We previously reported that Mycobacterium tuberculosis-infected (Mtb) B10R (Bcg') and B10S (Bcg') macrophages differentially produce nitric oxide (NO), leading to macrophage apoptosis. Since TNF-α and IL-10 have opposite effects on many macrophage functions, we determined the number of cells producing TNF-α and IL-10 in Mtb-infected or purified protein derivative-stimulated B10R and B10S macrophages, and Nramp1+/+ and Nramp1−/− peritoneal macrophages and correlated them with Mtb-mediated apoptosis. Mtb infection and purified protein derivative treatment induced more TNF-α/Nramp1+/+ and B10R, and more IL-10/Nramp1−/− and B10S cells. Treatment with mannosylated lipoarabinomannan, which rescues macrophages from Mtb-induced apoptosis, augmented the number of IL-10 B10R+ cells. Anti-TNF-α inhibited apoptosis, diminished NO2− production, p53, and caspase 1 activation and increased Bcl-2 expression. In contrast, anti-IL-10 increased caspase 1 activation, p53 expression, and apoptosis, although there was no increment in NO2− production. Murine rTNF-α induced apoptosis in noninfected B10R and B10S macrophages that was reversed by murine rIL-10 in a dose-dependent manner with concomitant inhibition of NO2− production and caspase 1 activation. NO2− and caspase 1 seem to be independently activated in that aminoguanidine did not affect caspase 1 activation and the inhibitor of caspase 1, Tyr-Val-Ala-Asp-acylooxymethylketone, did not block NO2− production; however, both treatments inhibited apoptosis. These results show that Mtb activates TNF-α and IL-10-dependent opposite signals in the induction of macrophage apoptosis and suggest that the TNF-α/IL-10 ratio is controlled by theNramp1 background of resistance/susceptibility and may account for the balance between apoptosis and macrophage survival. The Journal of Immunology, 1999, 162: 6122–6131.

During the early stages of Mycobacterium tuberculosis infection, the control of the intracellular bacterial survival and proliferation is dependent on the macrophage innate resistance. Macrophages display several mechanisms, to control intracellular bacteria, including production of reactive nitrogen intermediates, reactive oxygen intermediates, and changes in phagolysosomal pH, among others (1). Additionally, there is a complex circuit of cytokines able to modulate macrophage functions and T cell responses.

TNF-α and IL-10 play important and opposite roles during mycobacterial infections. TNF-α has been associated with tuberculous static macrophage functions (2, 3). In vitro and in vivo TNF-α blockade or the use of knockout mice for the p55 TNFR increased mycobacterial proliferation and inhibited nitric oxide production and granuloma formation in mice infected with Mycobacterium bovis bacillus Calmette-Guérin or M. tuberculosis, demonstrating that TNF-α is essential for protection against mycobacteria (4–6). Conversely, IL-10 produced by either activated T cells or macrophages has effects opposite to those of TNF-α regarding macrophage functions (7). IL-10 suppresses macrophage cytokine release (8) and Ag presentation by maintaining the class II complexes in intracellular vesicles (9). During mycobacterial infections IL-10 inhibits macrophage function (10), resulting in enhanced bacterial intracellular growth and inhibition of nitric oxide production (11). The blockade of IL-10 increases TNF-α and IL-1β production (12). Interestingly, IL-10 is able to block the endogenous production of TNF-α in macrophages activated with IFN-γ (13).

In different experimental systems, it has been shown that TNF-α and IL-10 have opposite roles in the induction of programmed cell death (14). Signals transduced through TNFR1 (p55) can induce an activation of proteases (15), including ICE-like cysteine proteases (caspases), which are recognized mediators of apoptosis (16) by proteolytic cleavage of the “death substrates” poly(ADP-ribose) polymerase (17) and lamin (18–21). TNF-α also increases synthesis of nitric oxide in different cells, and this molecule has been extensively associated with induction of DNA damage and apoptosis (22–25). Additionally, TNF-α may activate sphingomyelin breakdown into ceramide, which has a recognized role in apoptosis (26).

On the other hand, the effects of IL-10 on cell survival have been associated with increased expression of the anti-apoptotic factor Bcl-2. In humans, IL-10 prevents lymphocyte activation-induced apoptosis (27) and spontaneous death of germinal center B cells (28) by induction of the Bcl-2 protein. Conversely, when lymphocytes are grown in the presence of neutralizing anti-IL-10, there is an increase in apoptosis. In human alveolar macrophages, the costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 Abbreviations used in this paper: ICE, IL-1β-converting enzyme; Nramp1, natural resistance-associated macrophage protein 1; AMG, aminoguanidine; Pl, propidium iodide; PPD, purified protein derivative; ManLAM, mannosylated lipoarabinomannan; Mtb, Mycobacterium tuberculosis H37Rv; mr, murine recombinant; YVAD-, Tyr-Val-Ala-Asp-acylooxymethylketone-acmk.

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IL-10 reduces LPS- and IFN-γ-induced apoptosis (14), and endogenous IL-10 prevents apoptosis in murine macrophages infected with Salmonella choleraesuis (29), antagonizing macrophage activation and its functions during inflammation (9–11, 30, 31). Primary human monocytes infected with HIV-1 showed increased IL-10 production, up-regulating Bcl-2 expression, and decreased IL-12 production, leading altered macrophage-accessory cell function (32). Recently, it has been reported that IL-10 down-regulates apoptosis in human alveolar macrophages infected with M. tuberculosis by inducing the release of TNF-α2 leading to the formation of nonactive TNF-α-TNF-RII complexes (33). Taken together, these observations suggest that alterations in the balance of TNF-α and IL-10 production may influence both accessory and effector macrophage functions and the induction of apoptosis and cell survival.

There is evidence that the Nramp1 gene, responsible for the early control of different intracellular microorganisms (Refs. 34 and 35; reviewed in Ref. 36), influences TNF-α production (37). Resistant macrophages produce more TNF-α in response to Candida albicans infection or LPS than do susceptible macrophages (38). During, infection with Salmonella typhimurium, Pierce et al. (39) reported that Bcg/Lsh/Ity-susceptible mice produce more IL-10 than do resistant mice. We have previously reported (40) that B10R macrophages were more prone to undergo apoptosis than B10S macrophages after infection with M. tuberculosis H37Rv. In our system, apoptosis correlates with a higher production of nitric oxide by B10R macrophages. Treatment with anti-TNF-α Abs inhibited both nitric oxide production and apoptosis. Interestingly, Mannsylated ManLAM, a virulent mycobacterial cell wall glycolipid that stimulates TNF-α release (reviewed in Ref. 41), induced apoptosis without affecting nitric oxide production.

The preceding information led us to postulate that the balance between TNF-α and IL-10 produced by macrophages in response to infection with M. tuberculosis may modulate the induction of apoptosis during the infection. The results presented here confirmed this hypothesis. Infection with M. tuberculosis or treatment with PPD resulted in more B10R and Nramp1-/- cells producing TNF-α and more B10S and Nramp1+/+ cells producing IL-10. Blockade of either TNF-α or IL-10 by mAbs inversely affected nitric oxide production, caspase 1 activation, the expression of Bcl-2 and p53, and the loss of cell viability. These results suggest that the balance between IL-10 and TNF-α, influenced by the Nramp-1 genetic background of resistance or susceptibility, may account for the outcome of apoptosis or macrophage survival during infection with M. tuberculosis.

Materials and Methods

Reagents

MEM, RPMI 1640, FBS, glycerol, mTLN-α, and mIL-10 were purchased from Life Technologies (Grand Island, NY). TH10 medium was obtained from Difco Laboratories (Detroit, MI). Oleic acid, PMSF, and aprotinin were from ICN Biochemicals (Cleveland, OH). Tetrapeptides to inhibit caspase 1, biotin-Tyr-Val-Ala-Asp-acyclooxymethylketone (biotin-YVAD-acmk) and Tyr-Val-Ala-Asp-acyclooxymethylketone (YVAD-acmk), and the enhanced chemiluminescence kit were from Amersham (Little Chalfont, U.K.). Actinomycin D, RNase, AMG, nonidet P-40, glycerol, sodium o-vanadate, sodium fluoride, PI, Tween 20, and E-Toxate kit were from Sigma (St. Louis, MO). Anti-TNF-α-FITC and anti-TNF-α clone MP6XT22, anti-IL-10-FITC and anti-IL-10, clone JES5-16E3; anti-Bcl-2, clone 3F11; anti-hamster IgG (mixture)-FITC labeled, clones G70-204 and G94-56; anti-p53 and anti-p53-PE, clone PA2B40; IgG-FITC and IgG-PE isotype controls, clone UC9-4B3; anti-CD16/CD32 (FcγRII/II) receptor, clone 2.4G2; and avidin-FITC were purchased from PharMingen (San Diego, CA). The kit for TUNEL assay was obtained from MEBSTAIN (Medical & Biological Laboratories, Nagoya, Japan). Detergent-compatible protein assay and nitrocellulose membranes were from Bio-Rad (Richmond, CA). FPD from Mtb was from Connaught (Willowdale, Ontario, Canada). ManLM was prepared by G.P. from M. bovis bacillus Calmette-Guérin as previously described (42, 43).

Culture and maintenance of murine macrophage

Inbred 129sv mice were originally purchased from Taconic Farms (Germantown, NY) and subsequently bred in the laboratory of one of the authors (PG). Isogenic 129sv mice bearing a null allele at Nramp1 were generated by homologous recombination as previously described (44). Resident peritoneal macrophages were obtained from Nramp1+/+ and Nramp1-/- mice by lavage with 10 ml of cold PBS. Cells were plated on petri dishes during 1 h at 37°C, and nonadherent cells were washed with DMEM. Adherent cells (>90% CD14+) were scraped, their concentration was adjusted at 2 x 10^7 viable cells/ml, and the cells were used in different experiments, as described below.

Macrophage lines derived from the bone marrow of mice congenic at the Bcg/Nramp1 locus, B10A.Bcg+/+ (B10R) and B10A.Bcg-/- (B10S), (45), were provided by Dr. D. Radzioch (Department of Medicine, McGill University, Montreal, Quebec, Canada) and maintained as described (40, 46) in endotoxin-free medium. The levels of endotoxin for complete culture media were <0.05 endotoxin U/ml as measured by the E-Toxate kit.

Culture and maintenance of Mtb

Mtb (provided by Laboratorio de Mibocacibias, Instituto Nacional de Salud, Santafe de Bogota, Colombia) were cultured in Proskauer-Beck liquid media (47) and collected as described (40, 46).

Infection of macrophages with M. tuberculosis

Cells (400,000/well) were cultured by triplicates in DMEM supplemented with 10% FBS in flat-bottom 24-well culture plates (Nunc, Roskilde, Denmark) and infected and or not with M. tuberculosis-macrophage (5:1) or stimulated with PPD (10 μg/ml) or ManLM (20 μg/ml) and incubated at 37°C, 5% CO2, during variable periods of time, depending on experimental conditions. After culture, apoptosis was determined by PI staining and TUNEL assay, and cell viability was determined by trypan blue exclusion as previously reported (40). Nitrite accumulation was assessed by mixing 50 μl of Griess reagent with 50 μl of culture supernatants as described (48, 49).

Intracellular measurement of TNF-α and IL-10

Macrophages were infected with M. tuberculosis (5:1) or stimulated with PPD or ManLM for 20 h before treatment with 1 μg/ml brefeldin A for 1 h at 37°C and 5% CO2. Then, cells were washed twice with PBS (pH 7.2) and fixed with 4% paraformaldehyde in 0.1 M KH2PO4 during 20 min at room temperature. Thereafter, cells were permeabilized with 0.1% saponin plus 1% BSA and incubated with anti-murine CD16/CD32 (FcγRII/IR) Ab during 30 min at 4°C. Anti-TNF-α-Ab or anti-IL-10-Ab (5 μl) was added to the cell suspension for 30 min at 4°C. Cells were washed three times with cold PBS containing 1% BSA. The specificity of Abs was determined by incubating the cells with anti-TNF-α-anti-TNF-α-FITC or anti-IL-10-anti-IL-10-FITC (1:1). Stained cells were counted by flow cytometry with a FACSort (Becton Dickinson, San Jose, CA). Cytometric analysis was done with LYSIS II software (Becton Dickinson).

TNF-α bioassay

TNF-α bioactivity was determined by a modified cytoxicity assay using L929 cells. Briefly, L929 cell monolayers cultured for 48 h on flat-bottom 24-well plates (1 x 10^4 cells/well) were overlaid with 1 ml of 2-fold serial dilutions of supernatants from macrophages infected with M. tuberculosis (5:1), stimulated with PPD or ManLM for 24 h, or with growing concentrations of mTNF-α (1–100 U/ml) in RPMI 1640 plus 10% PBS and 1 μg/ml actinomycin D. After incubation at 37°C for 24 h, the cells were resuspended in 1.5 ml of PBS containing 50 μg/ml PI and incubated for 5 min at room temperature in the dark. Thereafter, cells were washed twice with cold PBS, and the percentage of PI-fluorescent cells was determined with FACSort and LYSIS II software. The concentration of bioactive TNF-α was calculated by regression analysis by the mlTNF-α standard curve.

Bcl-2 and p53 expression

B10R macrophages were infected in the presence or absence of anti-TNF-α, anti-IL-10, or 1 mM AMG for 24 h. Macrophages were washed with PBS (pH 7.2), and 20 μl of 0.5% Tween 20, 0.1% BSA in PBS were added to the cells and incubated for 30 min at room temperature. Two microliters of anti-CD16/CD32 (FcγRII/IR) were added to the cell suspension for 30 min at 4°C; then, the cells were washed three times with cold
0.1% BSA in PBS. Ten microliters of hamster anti-Blc-2 or rat anti-p53-PE labeled were added to the cell suspension, incubated in the dark for 30 min at 4°C, and washed with cold PBS. Ten microliters of anti-hamster IgG-FITC was added to the cells treated with anti-Bcl-2. Samples were incubated for 30 min at 4°C and washed twice. IgG-FITC and IgG-PE were used as isotypic marker. Stained cells were counted by flow cytometry. The cells present in the gate defined by light scatter of isotypic marker that comprised >95% cells were counted. Cytometric analysis was done with LYSIS II software.

Detection of p53 by Western blot

Macrophages infected with \textit{M. tuberculosis} (5:1) for 24 h were washed twice with cold PBS and lysed with 200 μl of cold lysis buffer (40 mM Tris-HCl (pH 8.0), 275 mM NaCl, 20% glycerol, 2% Nonidet P-40, 1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml sodium o-vanadate, 50 mM sodium fluoride). After 1 h on ice with occasional gentle mixing, the lysate was spun at 15,000 x g during 30 min. Cell lysates were adjusted to 2 μg/ml total protein as measured by detergent-compatible assay (Bio-Rad), following the manufacturer’s instructions, and 20 μg were submitted to 7% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes that were then blocked overnight in Tris-buffered saline/Tween containing 1% gelatin. Membranes were washed and incubated with anti-p53 (1:5,000), washed again, and incubated with anti-mouse IgG-HRP-conjugated Ab (1:20,000). The Western blots were developed with the enhanced chemiluminescence kit.

Activated caspase 1 measurement

Macrophages were cultured in 24-well dishes until complete adherence. Then cells were infected or stimulated with PPD for 24 h. Cultures were washed twice with cold PBS, and macrophages were permeabilized with 0.1% Nonidet P-40, 2% BSA in PBS and incubated with biotin-YVAD-acmkm for 20 min. Cells were washed with cold PBS and stained with avidin-FITC; the excess avidin-FITC was removed by two washings. Cells were resuspended in 0.5 ml of PBS, and the percentage of FITC fluorescent cells was determined by flow cytometry with LYSIS II software. Nonspecific binding was determined by incubating macrophages with biotin-YVAD-acmkm and an excess of nonlabeled YVAD-acmkm.

Blockade experiments

B10R and B10S infected macrophages were plated at \(2 \times 10^6\) viable cells/well in flat-bottom 6-well culture dishes and infected with \textit{M. tuberculosis}-macrophage (5:1) in the presence of 2.5 μg/ml anti-TNF-α or 2.0 μg/ml anti-IL-10 for 24 h. Thereafter, nitrite accumulation was measured in supernatants; the cells were washed and cell viability was determined by trypan blue exclusion. Cells were split for intracellular determination of activated caspase 1, TNF-α, IL-10, and TUNEL as described (40).

Effect of TNF-α and IL-10 on noninfected macrophages

B10R macrophages were plated at \(2 \times 10^6\) viable cells/well in flat-bottom 6-well culture dishes and treated with mrTNF-α (0, 5, 15, or 30 U/ml) for 24 h. Then, the number of hypoploidic cells was determined with PI staining as described (40). Macrophages, were also treated with 30 U/ml mrTNF-α in the presence of growing concentrations of IL-10 (3–150 U/ml) for 24 h. Thereafter, nitrite accumulation in the supernatant, the percentage of cells with activated caspase 1, and cell viability were determined as described above.

Statistical analysis

All experiments were done in triplicate and independently repeated at least three times. Data were analyzed by ANOVA type III of square sum. Interactions above the second level were excluded. Statistical significance was tested at \(p < 0.05\) as critical value, calculated by the interactions between the factors. Data are presented as the mean ± 95% confidence interval for mean. For all analyses, we used Statgraphics Plus, release 2, 1996 (Statgraphics, Rockville, MD).

Results

B10R and B10S macrophages behave similarly to resident peritoneal macrophages

We have previously reported that B10R and B10S macrophages differentially undergo apoptosis after infection with \textit{M. tuberculosis} (40). However, it is not know whether B10R and B10S macrophage lines behave similarly to primary tissue macrophages regarding \textit{M. tuberculosis}-induced apoptosis. For this purpose, resident peritoneal macrophages from \textit{Nramp1}^{+/+} and \textit{Nramp1}^{-/-} mice, B10R and B10S macrophages, were infected (\textit{M. tuberculosis}-macrophage (5:1)) or not for 24 h. As shown in Fig. 1A, there were <1% apoptotic cells in noninfected B10R and B10S cells or

FIGURE 1. Induction of apoptosis and nitric oxide production by \textit{M. tuberculosis} in murine macrophage lines B10R and B10S, compared with peritoneal macrophages. \textit{Nramp1}^{+/+} and \textit{Nramp1}^{-/-} resident peritoneal macrophages and B10R and B10S cells were adjusted at \(2 \times 10^6\) viable cells/ml, infected or not at a \textit{M. tuberculosis}-macrophage ratio of 5:1, and incubated for 24 h at 37°C, 5% CO₂. Cultures were collected and stained with PI to determine by flow cytometry the number cells with both low cell size and low DNA content (hypoploidic cells) that were defined as apoptotic cells. n = 3 independent experiments. B, Cell viability and nitrite accumulation (C) by \textit{M. tuberculosis}-infected or PPD-treated. FSC, forward light scatter; FL-2A, fluorescent light-2 area.
peritoneal macrophages from Nramp1+/+ and Nramp1−/− mice. However, infection with M. tuberculosis resulted in a similar increase in the percentage of apoptotic B10R and Nramp1+/+ macrophages that was higher than the percentages found in B10S and Nramp1−/− macrophages (p < 0.002).

We also compared cell viability and nitric oxide production by the macrophage lines and primary macrophages in response to infection with M. tuberculosis, or stimulation with PPD. Cell viability was significantly (p < 0.02) decreased in B10R and Nramp1+/+ macrophages exposed to these treatments (Fig. 1B). B10S macrophages showed a smaller, but significant (p < 0.02), reduction in the cell viability, and Nramp1−/− cells did not have any significant decrease in the percentage of viable cells after these treatments. The differences between B10R and Nramp1+/+ compared with B10S and Nramp1−/− were significant (p < 0.01). The production of nitric oxide (Fig. 1C) was higher in B10R and Nramp1+/+ macrophages compared with B10S and Nramp1−/− cells (p < 0.001), and there was no significant differences between B10R and Nramp1+/+ or B10S and Nramp1−/− macrophages. Taken together, these results demonstrate that B10R and B10S macrophages behave similarly to Nramp1+/+ and Nramp1−/− macrophages in the induction of apoptosis and in the production of nitric oxide in response to M. tuberculosis infection or PPD stimulation.

B10R and B10S macrophages differ in the percentage TNF-α- and IL-10-producing cells in response to M. tuberculosis, PPD, and ManLAM

Since TNF-α and IL-10 play opposite roles during mycobacterial infection, it was interesting to compare the production of these cytokines in resistant and susceptible macrophages after M. tuberculosis infection or exposure to PPD and ManLAM. The number of B10R and B10S macrophages with intracellular TNF-α was significantly increased (p < 0.0001) after infection with M. tuberculosis, stimulation with PPD, or ManLAM (Fig. 2A). However, the percentage of cells producing this cytokine was always higher in B10R than in B10S macrophages (p < 0.001). There were 70–75% B10R cells producing TNF-α as compared with 25–35% B10S cells infected or stimulated with PPD or ManLAM (p < 0.0001). Since we have previously reported (40) that ManLAM rescued macrophages from M. tuberculosis- or PPD-induced apoptosis in murine macrophages (40), we also determined whether ManLAM affect the production of TNF-α and IL-10 by M. tuberculosis-infected or PPD-treated cells. The addition of ManLAM to M. tuberculosis-infected or PPD-treated cells increased the percentage of TNF-α-positive cells (p < 0.01 for B10R and p < 0.001 for B10S macrophages). There were 90 and 93% TNF-α+B10R and 75 and 72% TNF-α+B10S cells after the double treatment (Fig. 2A).

Because the number of TNF-α-producing cells do not necessarily correlate with the amount of cytokine secreted by the macrophages, we measured the bioactive TNF-α in culture supernatants using L929 cells. There was a clear correlation between the percentage of TNF-α+ cells and the amount of TNF-α present in the supernatants (compare Fig. 2A and Fig. 2B). B10R macrophages produced more TNF-α than B10S macrophages in response to infection with M. tuberculosis, PPD, ManLAM, bacteria plus ManLAM, or PPD plus ManLAM.

The number of IL-10-positive B10R and B10S macrophages was also significantly increased (p < 0.001) after infection with M. tuberculosis, stimulation with PPD or ManLAM (Fig. 2C). Contrary to our observations with TNF-α, the number of B10S macrophages producing IL-10 was higher than the number of B10R cells infected with M. tuberculosis, or stimulated with PPD. There were 70–75% B10S cells producing IL-10 compared with 25–36% B10R cells (p < 0.001). ManLAM significantly augmented the percentage of B10R and B10S cells producing IL-10 (p < 0.001, compared with nonstimulated controls). Double treatment with M. tuberculosis or PPD plus ManLAM did not further increase the percentage observed with ManLAM alone. There were 70–80% IL-10+B10R and 72–75% IL-10+B10S cells after double stimulation.

The number of Nramp1+/+ and Nramp1−/− peritoneal macrophages producing cytokines was also tested. As shown in Fig. 3, there were more Nramp1+/+ TNF-α+ cells (73%) than Nramp1−/−–TNF-α+ cells (20%). On the contrary, there were more Nramp1+/+ IL-10+ cells (71%) than Nramp1−/−/IL-10+ cells (23%). Thus, in the next experiments, the mechanisms by which these cytokines modulate apoptosis were explored using the B10R and B10S macrophage lines.
Anti-TNF-α and anti-IL-10 have opposite effects on M. tuberculosis-induced apoptosis and nitric oxide production

The preceding results demonstrated a differential production of TNF-α and IL-10 by B10R and B10S macrophages in response to M. tuberculosis infection. In the next experiments, we tested whether these differences may be involved in the regulation of apoptosis subsequent to the infection with M. tuberculosis. For this purpose, we used mAbs to block TNF-α and IL-10 produced by the macrophages infected with M. tuberculosis. As we previously reported (40), the blockade of TNF-α rescued the cells from apoptosis and inhibited nitric oxide production in both B10R (Fig. 4A) and B10S (Fig. 4B) macrophages (p < 0.001) at the levels observed in uninfected cells. Conversely, in both macrophage lines, treatment with anti-IL-10 significantly increased cell mortality compared with nontreated infected cells (p < 0.002), although it did not modify the production of nitric oxide (Fig. 4A). These results indicate that IL-10 is clearly an anti-apoptotic factor on M. tuberculosis-infected B10R and B10S macrophages and that its effects may be independent of nitric oxide.

Anti-TNF-α and anti-IL-10 differentially modulate activation of caspase 1

Since TNF-α can also promote apoptosis through activation of the caspase cascade (50), we measured the percentage of infected cells expressing activated caspase 1 in parallel with the number of macrophages producing TNF-α or IL-10 after treatment with their respective mAbs. Infection with M. tuberculosis resulted in significantly (p < 0.0001) more B10R (Fig. 5A) than B10S (Fig. 5B) macrophages expressing activated caspase 1. Anti-TNF-α induced a small but significant decrease in the number TNF-α-B10R-positive cells (p < 0.01, Fig. 5A), but there was no significant effect on B10S cells (Fig. 5B). Anti-TNF-α almost completely blocked the expression of activated caspase 1 (p < 0.002) but augmented the percentage of IL-10-positive B10R (p < 0.001 (Fig. 5A)) and B10S (p < 0.02 (Fig. 5B)) macrophages as compared with the isotype treated cells (Fig. 5).

The blockade of IL-10 in B10R (Fig. 5A) and B10S (Fig. 5B)-infected macrophages increased the percentage of TNF-α-positive

above suggest that there are at least two pathways capable of inducing macrophage apoptosis in *M. tuberculosis*-infected macrophages: caspase 1 and nitric oxide.

**TNF-α and IL-10 modulate p53 and Bcl-2 expression in M. tuberculosis-infected macrophages**

Since p53 and Bcl-2 are well-known pro- and anti-apoptotic factors, we tested whether TNF-α and IL-10 affect the expression of Bcl-2 and p53 in infected macrophages. As shown in Fig. 7B, 99% of B10R-uninfected macrophages express Bcl-2, and there was no expression of p53 compared with isotype control (Fig. 7A). After 24 h of infection with *M. tuberculosis*, Bcl-2 was completely down-regulated and there were 75% of cells expressing p53 (Fig. 7C). Treatment of infected cells with anti-TNF-α (Fig. 7D) increased the levels of Bcl-2 expression in 80% of the cells and completely inhibited the expression of p53 compared with infected macrophages. On the other hand, treatment of infected cells with anti-IL-10 (Fig. 7E) resulted in the expression of p53 in all cells. Interestingly, there were 21% double-stained cells, and 78% of them expressed only p53, suggesting that these two molecules are counterbalancing the apoptotic machinery as previously reported in other models (51–55).

**AMG prevents p53 expression in M. tuberculosis-infected macrophages**

Considering that nitric oxide is involved in the induction of apoptosis and correlates with increased expression of p53 in several systems (56–60), we assessed whether *M. tuberculosis*-induced apoptosis simultaneously encompassed nitric oxide production and p53 expression. As shown in Fig. 8, infection with *M. tuberculosis* resulted in 90% of the cells exhibiting DNA breaks, and among them 72% expressed p53 (Fig. 8B). The blockade of nitric oxide production by AMG prevented both the formation of DNA strand breaks and the expression of p53 in infected macrophages (Fig. 8C).

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**FIGURE 5.** Effect of anti-TNF-α and anti-IL-10 on the production of cytokines and activation of caspase 1. B10R (A) and B10S (B) macrophages were infected with *M. tuberculosis*-macrophage (5:1) in the presence of 2.5 μg/ml anti-TNF-α or 2.0 μg/ml anti-IL-10 for 24 h. Thereafter, cells were split for intracellular determination of ICE, TNF-α, and IL-10 by flow cytometry.

**FIGURE 6.** Effect of TNF-α and IL-10 on apoptosis of noninfected macrophages. A, B10R (A) and B10S (B) macrophages (Mφ) were treated with different concentrations of mrTNF-α (5–40 U/ml) for 24 h. Then, the cell viability was determined by trypan blue exclusion. B, B10R macrophages were treated with different concentrations of mrTNF-α (0, 5, 15, or 30 U/ml) for 24 h and the number of hypoploidic cells was determined with PI staining by flow cytometry as described in Materials and Methods. C, B10R macrophages were treated with 30 U/ml mrTNF-α in the presence of growing concentrations of IL-10 (3–150 U/ml) for 24 h. Thereafter, nitrite accumulation, activated ICE, and cell viability were determined as described in Materials and Methods.
The induction of p53 during *M. tuberculosis* infection was also studied by Western blot (Fig. 8D). Noninfected cells did not express p53, whereas infection with *M. tuberculosis* resulted in the expression of the protein that was not modified by anti-IL-10 Abs. Incubation of noninfected cells with TNF-α also induced the expression of p53. *M. tuberculosis*-infected macrophages treated with anti-TNF-α, IL-10, or AMG did not express p53 (Fig. 8D).

**Blockade of caspase 1 and nitric oxide independently rescues the cell from *M. tuberculosis*-induced apoptosis**

Finally, we assessed whether nitric oxide and caspase 1 behave as independent apoptotical factors in B10R-infected macrophages.

For these experiments, we added AMG and caspase 1 (YVAD-acmk) in parallel to *M. tuberculosis*-infected macrophages. While AMG was able to inhibit nitric oxide production (p < 0.001), it had no effect on caspase 1 activation; conversely, YVAD-acmk inhibited caspase 1 activation (p < 0.001) but failed to block nitric oxide production (Fig. 9A). The independence of nitric oxide and caspases during apoptosis of *M. tuberculosis*-infected macrophages was evidenced by kinetic experiments using both inhibitors (Fig. 9B). Although both YVAD and AMG were able to delay cell death compared with *M. tuberculosis*-infected macrophages, only the simultaneous presence of both inhibitors resulted in complete inhibition of apoptosis. These results indicate that although nitric...
oxide and caspase 1 are independent effector mechanisms of apoptosis, both participate (in additive or synergistic manner) in the final apoptotic outcome.

Discussion

Cytokines participate in many physiological processes including the regulation of immune and inflammatory responses. In vivo, these molecules are transient and locally produced, controlling the amplitude and duration of the response. Particularly, cytokines produced by macrophages at the onset of the intracellular infection by *M. tuberculosis*, are thought to be decisive for the outcome of the infection. Our interest was focused on two macrophage-derived cytokines known to have opposite effects under different situations, TNF-α and IL-10.

We have previously found that resistance and susceptibility of macrophages to infection with virulent *M. tuberculosis* correlated with a differential production of nitric oxide (46). Moreover, in our system, resistant (B10R) macrophages are consistently more likely to undergo apoptosis than susceptible (B10S) macrophages after infection with *M. tuberculosis* or stimulation with either PPD or IFN-γ (40). The differences between B10R and B10S macrophages pointed out the importance of nitric oxide as a possible mediator of both phenomena. Inhibition with AMG, a competitive inhibitor of nitric oxide synthase activity (61), or the blockade of TNF-α by mAbs decreased the production of nitric oxide and dramatically reduced the loss of cell viability (40), indicating a critical role of this cytokine in the induction of apoptosis by *M. tuberculosis*.

Our current experiments showing that infection with virulent *M. tuberculosis*, as well as treatment with PPD, led to a higher production of TNF-α by resistant (B10R and Nramp1+/−) compared with susceptible macrophages (B10S and Nramp1−/−) are in agreement with previous reports (38, 39). These findings strongly support the role of Nramp1 in the control of *M. tuberculosis*-induced macrophage apoptosis and validate the use of B10R and B10S macrophages to study the modulation by these cytokines. These macrophage lines behave similarly to splenic macrophages regarding nitric oxide production and the control of *M. bovis* (62) and the fact that they differentially control *M. tuberculosis* (46).

ManLAM was a more potent stimulus for IL-10 production in comparison with *M. tuberculosis* or PPD in B10R macrophages. This observation suggests that IL-10, previously reported as an apoptotic factor (14, 27, 63), may account for some of the ManLAM effects. Furthermore, ManLAM inhibited the *M. tuberculosis* decrease in the expression of Bcl-2 and inhibited the activation of caspases.4

To further explore the biological activities of TNF-α and IL-10 in *M. tuberculosis*-induced apoptosis, we performed blockade experiments with specific mAbs to TNF-α and IL-10. Treatment of infected macrophages with anti-TNF-α increased the cell viability and decreased nitric oxide production, confirming our previous results (40), but it was also able to reduce caspase 1 activation and p53 expression and to reverse the negative regulation of Bcl-2 secondary to *M. tuberculosis* infection. Similar results have been observed in other models. Keane et al. (64) reported that TNF-α has a critical role in *M. tuberculosis*-induced apoptosis of human alveolar macrophages. Addition of exogenous TNF-α enhanced apoptosis and, conversely, treatment with pentoxifylline or anti-TNF-α enhanced macrophage survival (64). On the contrary, treatment with anti-IL-10 diminished cell viability and Bcl-2 expression, while increasing caspase 1 activation, p53 expression, and the number of TNF-α-producing cells. These findings are in agreement with those of Estaquier et al. (65), showing that IL-10 is able to rescue the cells from apoptosis by down-regulating protease cascade rather than by inhibition of nitric oxide production. During infection with *S. choleraesuis*, a significant increment in TNF-α production and apoptosis were observed after treatment with anti-IL-10 mAbs (29). Furthermore, it has been reported that Bcl-2 is down-regulated in human mononuclear phagocytes after infection with *M. tuberculosis* (66). The down-regulation of Bcl-2 was accompanied up-regulation of Bax, which has recognized apoptotic effects (66).

We also wanted to determine whether these cytokines are able to modulate apoptosis independent of the mycobacterial infection. First, we found that TNF-α alone was able to induce similar levels of hypoploidy and decrease cell viability in both B10R- and B10S-noninfected macrophages. This finding indicate that both macrophage cell lines are equally sensitive to the apoptotic effects of TNF-α and that the final effect may be the result of differential production of this cytokine in response to *M. tuberculosis* infection. Moreover, the fact that exogenous IL-10 rescued the cells from TNF-α-induced apoptosis by decreasing the production of nitric oxide and the activation of caspase 1 further supports the notion that the differential effects of mycobacteria on macrophage viability are caused by the contrasted production of TNF-α and IL-10 during the infection.

Treatment with anti-IL-10 in infected B10R and B10S macrophages did not result in higher nitric oxide production. This apparent discrepancy may be explained by the direct stimulatory effect of the mycobacteria on nitric oxide production (46) that resulted in 2–3-fold increase in the concentration of nitrites detected in the supernatants of B10R-infected anti-IL-10-treated macrophages (Fig. 4A) compared with noninfected, TNF-α-stimulated cells (Fig. 6). Another possible explanation is that the concentration of IL-10 attained with the exogenous addition of TNF-α

![FIGURE 9. AMG and ICE inhibitor rescue the cell viability but differentially inhibit nitric oxide production and caspase 1 activation in B10R-infected macrophages. B10R macrophages were infected with *M. tuberculosis*-macrophage (5:1) in the presence of 1 mM AMG or 1 μg/ml YVAD-acmk for 24 h (A), or were infected with 5:1 *M. tuberculosis*-macrophage (5:1) in the presence of 1 mM AMG or 1 μg/ml YVAD for different periods of time (B). Thereafter, the percentage of cell viability was determined by trypan blue exclusion.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
to noninfected B10R macrophages and able to inhibit nitric oxide production (> 6.25 U/ml) was in a pharmacological range well above the endogenous production of IL-10 in M. tuberculosis-infected cells.

Interestingly, during infection with M. tuberculosis, the activation of cysteine proteases and the down-regulation of Bcl-2 were prevented by TNF-α blockade, suggesting a relationship between these cysteine proteases and Bcl-2. The cysteine proteases family has been shown to play a fundamental role in programmed cell death (reviewed in Ref. 16). Bcl-2 inhibits the conversion of pro-caspase 1 into caspase 1 (67–74). Additionally, there is evidence indicating the reciprocity of Bcl-2 and p53 expression (55). While the production of nitric oxide did not affect the expression of caspase 1, nor the blockade of nitric oxide production affect the activation of caspase 1. The independent inhibition of caspase 1 and nitric oxide delayed but did not rescue the cells from apoptosis after infection, suggesting that the convergence between caspase 1 and nitric oxide pathways may take place in the final steps of M. tuberculosis-induced macrophage apoptosis. The differential production of TNF-α and IL-10 by B10R and B10S macrophages in response to infection with M. tuberculosis may be under the control of the Nramp1 gene, and the balance between TNF-α and IL-10 may explain some of the phenotypical differences between resistant and susceptible macrophages, such as the control intracellular microorganisms (45, 46, 62), nitric oxide production (46), and apoptosis (40).

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References


