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Differential potentiation of anti-mycobacterial activity and reactive nitrogen intermediate-producing ability of murine peritoneal macrophages activated by interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α)

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SUMMARY

The anti-mycobacterial activities of IFN-γ and TNF-α-treated murine peritoneal macrophages were determined. Resident macrophages pretreated with IFN-γ or TNF-α for 2 days were infected with test organisms and subsequently cultured for up to 7 days. First, the early-phase growth of *Mycobacterium tuberculosis* (days 0–3) was strongly suppressed in IFN-γ-treated macrophages, and progressive bacterial elimination was subsequently observed. Although TNF-α treatment of macrophages did not affect the early phase growth of organisms, bacterial killing was observed in the later phase of cultivation. Second, although IFN-γ-treated macrophages killed *M. avium* during the first 3 days of culture, regrowth of the intracellular organisms was subsequently observed. TNF-α treatment of macrophages did not influence the mode of intracellular growth of *M. avium*. Third, IFN-γ but not TNF-α enhanced production of reactive nitrogen intermediates (RNI) by macrophages infected with *M. tuberculosis* or *M. avium*, whereas both cytokines increased macrophage release of reactive oxygen intermediates (ROI). The present findings therefore show that IFN-γ and TNF-α potentiated the anti-mycobacterial activity of murine peritoneal macrophages in different fashions. They also suggest that RNI played more important roles than did ROI in the expression of macrophage anti-mycobacterial, particularly anti-*M. avium*, activity.

Keywords macrophages mycobacteria interferon-gamma tumour necrosis factor-alpha reactive nitrogen intermediates reactive oxygen intermediates

INTRODUCTION

Multidrug-resistant tuberculosis and disseminated *Mycobacterium avium* complex (MAC) infections are frequently encountered in AIDS patients. Host resistance to mycobacterial organisms is largely dependent on bactericidal and/or bacteriostatic activity of macrophages induced by IFN-γ [1]. Other proinflammatory cytokines, including TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and combinations of these two have been reported to potentiate the anti-mycobacterial activity of host macrophages [1–3]. Although IFN-γ is a representative macrophage activator, its ability to up-regulate the anti-mycobacterial activity of human and murine macrophages remains controversial. Although IFN-γ treatment is generally effective in augmenting anti-mycobacterial functions of murine macrophages [1,3,4], macrophage anti-*M. lepraemurium* activity is down-regulated by this cytokine [5]. Moreover, in previous studies IFN-γ failed to potentiate the anti-mycobacterial activity of human macrophages [1,6–8]. IFN-γ has occasionally been found to cause accelerated growth of *M. tuberculosis* in human macrophages [1,9]. TNF-α is known to play a role in host resistance to mycobacterial infections by potentiating macrophage microbicidal activity [1,2,10–12]. However, the TNF-α-mediated potentiation of macrophage anti-mycobacterial activity is time-dependent, and TNF-α is incapable of augmenting the anti-MAC activity of macrophages phagocytizing a large number of organisms [2,13].

The antimicrobial functions of macrophages are generally mediated by effector molecules, including reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI), and cationic bactericidal proteins [14–18]. However, the precise mechanisms by which macrophages exert their anti-mycobacterial effects are not clearly understood. Previous studies demonstrated the roles of RNI in expression of anti-*M. tuberculosis* activity by murine macrophages [14–18]. However, the roles played by RNI in macrophage-mediated host defence mechanisms against MAC

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remain unclear [19–22]. We observed no relationship between RNI susceptibility of various MAC strains and their virulence in mice [22]. Dumarey *et al*. [20] reported that RNI played no significant role in the expression of anti-*M. avium* activity of human monocytes, as a result of an intrinsic defect of inducible nitric oxide (NO) synthase in human macrophages [23]. Second, the roles played by ROI in macrophage anti-mycobacterial activity are also obscure [10,15,24–26]. For instance, a J774 cell line mutant unable to produce ROI exhibited the same level of anti-*M. tuberculosis* activity as that of the parent cells [15]. Moreover, the anti-MAC activities of macrophages from MAC-resistant (Bcg^r) and MAC-susceptible (Bcg^s) strain mice did not parallel their ROI production.

Therefore, in order to determine the roles of RNI and ROI in macrophage-mediated anti-mycobacterial activity, we examined the effects of IFN-γ and TNF-α on anti-*M. tuberculosis* and anti-*M. avium* activity of murine peritoneal macrophages as related to their production of RNI and ROI. We found that macrophage anti-mycobacterial activity, particularly anti-*M. avium* activity, was differentially regulated by IFN-γ and TNF-α. We also found that the anti-*M. avium* activity of and RNI production by murine peritoneal macrophages were concomitantly regulated by these cytokines, indicating that RNI are important antimicrobial effectors of macrophage anti-*M. avium* antimicrobial activity.

MATERIALS AND METHODS

Organisms

Mycobacterium tuberculosis $H_{37}Rv$ and *M. avium* N-425 grown in Middlebrook 7H9 medium (Difco Labs, Detroit, MI) were used. The MAC strain was isolated by us from a patient with pulmonary MAC infection. It produced smooth, transparent, irregularly shaped colonies, which are characteristic of virulent colonial variants of MAC, on 7H11 agar plates. The cultured organisms were collected by centrifugation at 1700 ***g*** for 30 min and the pellet suspended in distilled water. After sonication (Handy Sonic Model UR-20P; Tomy Seiko Co., Tokyo, Japan) at maximum power for 20 s, the resultant bacterial suspension was again centrifuged at 180 ***g*** for 5 min to remove bacterial clumps, and the upper layer (2/3 from meniscus) was used as a bacterial source.

Mice

Female BALB/c mice (8–12 weeks old) purchased from Japan Clea Co. (Osaka, Japan) were used for preparation of peritoneal macrophages.

Medium

Macrophages were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% (v/v) fetal bovine serum (FBS) (Bio Whittaker Co., Walkersville, MD).

Special agents

Murine recombinant IFN-γ and human recombinant TNF-α were obtained from Genzyme Co. (Cambridge, MA) and Dai-Nippon Pharmaceutical Co. (Tokyo, Japan), respectively. One unit of IFN-γ is defined as the amount required to protect 50% of the indicator cell population (L929 cells) from viral (VSV) destruction. One unit of TNF-α is defined as the amount required to mediate half-maximal cytotoxicity of L929 cells. Cytochrome C and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St Louis, MO) and Wako Pure Chemical Industry Co. (Osaka, Japan). These cytokine preparations were essentially free from lipopolysaccharide (LPS) contamination in the Limulus J Single Test (Wako). Test cytokines were initially dissolved in 10% FBS–RPMI 1640 medium at a concentration of 10^5 U/ml and kept at −80°C until use. The pooled cytokine solutions were diluted with the medium immediately before use.

Preparation of peritoneal macrophages

Peritoneal cells (PC) were harvested with Hanks' balanced salt solution (HBSS) containing 2% FBS. After washing once with 2% FBS–HBSS by centrifugation at 180 ***g*** for 5 min, the PC were suspended in 10% FBS–RPMI 1640 medium and seeded into microculture wells (96 wells, flat-bottomed) (Becton Dickinson, Lincoln Park, NJ) at a density of 3×10^5 cells/well. After 2 h incubation at 37°C in a CO_2 incubator (5% CO_2–95% humidified air), the wells were gently rinsed with 2% FBS–HBSS to remove non-adherent cells. The resultant monolayer cell cultures were used as peritoneal macrophages.

Anti-mycobacterial activity of macrophages

Monolayer cultures of peritoneal macrophages were precultured in 0·2 ml of the medium with or without the addition of 100 U/ml each of IFN-γ or TNF-α (preliminary experiments showed this dose was optimum for up-regulation of macrophage anti-mycobacterial functions by these cytokines) at 37°C in a CO_2 incubator for 2 days. After washing with 2% FBS–HBSS, the macrophage culture was incubated in the medium (0·1 ml) with or without the addition of *M. tuberculosis* $H_{37}Rv$ (3×10^5 colony-forming units (CFU)/ml) or *M. avium* N-425 ($1{\cdot}5\times10^6$ CFU/ml) at 37°C in a CO_2 incubator for 2 h. These infection doses gave similar levels of multiplicity of infection in both of *M. tuberculosis*- and *M. avium*-infected macrophages. After washing the macrophages with 2% FBS–HBSS to remove unphagocytosed bacteria, the resultant macrophages were further cultured in fresh medium (0·2 ml) at 37°C in a CO_2 incubator for up to 7 days. At intervals, 80 μl of 0·23% SDS solution were added to the macrophage culture and the resultant macrophage lysate (0·28 ml) was mixed with 0·12 ml of 20% bovine serum albumin (BSA) in PBS, and the number of bacterial CFU in the macrophage lysate was counted on 7H11 agar plates. Nearly the same number of viable cells was recovered from monolayer cultures of cytokine-treated or untreated macrophages during the whole period of chase incubation after MAC infection.

RNI-producing ability of macrophages

RNI production of macrophages was measured in terms of nitrite (NO_2^-) accumulation into the culture fluids of test macrophages. The macrophage monolayer cultures prepared by seeding 2×10^6 PC onto 16-mm wells (Becton Dickinson) were cultivated in 1·0 ml of 10% FBS–RPMI 1640 medium with or without the addition of IFN-γ or TNF-α at 37°C for 2 days in a CO_2 incubator. The resultant macrophages were washed with 2% FBS–HBSS and incubated in the medium (0·5 ml) with or without the addition of *M. tuberculosis* (4×10^5 CFU/ml) or *M. avium* (2×10^6 CFU/ml) at 37°C for 2 h. After washing with 2% FBS–HBSS to remove unphagocytosed organisms, the macrophage cultures were further cultivated for up to 5 days. At intervals, culture supernatants were withdrawn and allowed to react with Griess reagent, and the nitrite (NO_2^-) content was quantified by measuring the absorption at 550 nm.

ROI-producing ability of macrophages

ROI production of macrophages was measured in terms of superoxide anion (O_2^-) release from macrophages triggered by cell–cell contact with test mycobacteria. Macrophage monolayers on 16-mm culture wells were pretreated with TNF-α or IFN-γ as described above. After washing with HBSS, 0·5 ml of phenol red-free HBSS containing 80 μM cytochrome C and either *M. tuberculosis* or *M. avium* at the concentration of the optical density (540 nm) of 0·5 (ca. $2 \sim 8 \times 10^8$ CFU/ml) was added into the wells and incubated at 37°C for 60 min in a CO_2 incubator. The amount of O_2^- released was measured on the basis of increase in absorbance at 550 nm of the culture supernatant due to O_2^--mediated reduction of cytochrome C. In control experiments, 1000 U/ml SOD completely inhibited the macrophage-mediated colour change of the substrate, cytochrome C. This confirmed that the observed reduction of cytochrome C was essentially due to O_2^- released from macrophages.

RESULTS

Effects of IFN-γ and TNF-α on macrophage anti-mycobacterial functions

Figure 1 shows profiles of intracellular growth of *M. tuberculosis* in murine resident peritoneal macrophages pretreated with IFN-γ or TNF-α before infection. In this experiment, priming of macrophages with test cytokines did not significantly affect the ability of macrophages to ingest the organisms, since the numbers of organisms recovered from cytokine-treated macrophages immediately after bacterial phagocytosis (time 0) did not significantly differ from those recovered from untreated control macrophages (Fig. 1). In IFN-γ-treated macrophages, early-phase growth (days 0–3) of *M. tuberculosis* was significantly suppressed, and progressive bacterial killing was subsequently observed during days 3–7. Second, in TNF-α-treated macrophages, deceleration of the early-phase growth (days 0–3) of organisms was not observed, although bacterial elimination was observed later, as for IFN-γ-treated macrophages. This suggests that different types of macrophage antimicrobial mechanisms were mobilized against *M. tuberculosis* in a bimodal fashion, i.e. in the early (days 0–3) and later (days 3–7) phases of macrophage cultivation.

Figure 2 shows profiles of intracellular growth of *M. avium* in macrophages pretreated with IFN-γ or TNF-α. In this experiment, priming of macrophages with test cytokines did not affect the ability of macrophages to phagocytose *M. avium*, since the numbers of organisms recovered from cytokine-treated macrophages at time 0 were essentially the same as those recovered from untreated control macrophages (Fig. 2). In IFN-γ-treated macrophages, temporary elimination of intracellular *M. avium* was observed in the early phase of cultivation (days 0–3), although regrowth of the organisms was observed from day 5 to day 7. Notably, in TNF-α-treated macrophages, growth of *M. avium* was not reduced, but was in fact slightly increased during the first 5 days of culture.

These findings demonstrate significant differences between the modes of intracellular growth of *M. tuberculosis* and

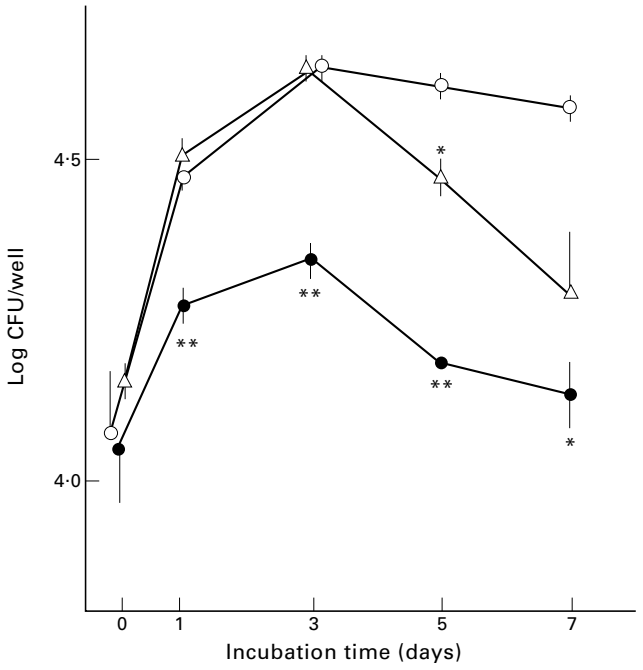

Fig. 1. Profiles of intracellular growth of *Mycobacterium tuberculosis* phagocytosed in mouse peritoneal macrophages treated with IFN-γ or TNF-α. Resident peritoneal macrophages were precultured in the medium in the absence (○) or presence of 100 U/ml each of IFN-γ (●) or TNF-α (△) for 2 days. After washing, the macrophages were allowed to phagocytose *M. tuberculosis* (3×10^4 colony-forming units (CFU)/well) for 2 h, then thoroughly rinsed, and further cultivated in the fresh medium for up to 7 days. Each bar indicates the mean ± s.e.m. ($n = 3$). Significantly different from the value of the control macrophages: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; Student's *t*-test.

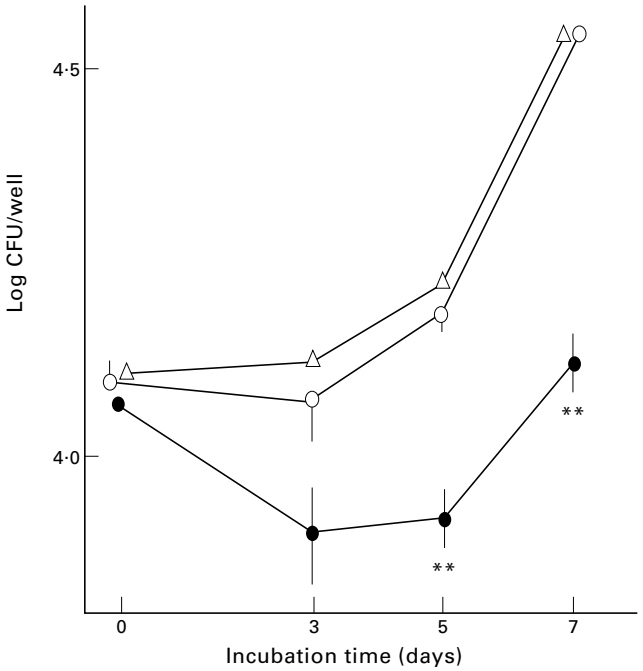

Fig. 2. Profiles of intracellular growth of *Mycobacterium avium* phagocytosed in mouse peritoneal macrophages treated with IFN-γ or TNF-α. Resident periotneal macrophages were precultured in the medium in the absence (○) or presence of 100 U/ml each of IFN-γ (●) or TNF-α (△) for 2 days. After washing, the macrophages were allowed to phagocytose *M. avium* ($1{\cdot}5 \times 10^5$ colony-forming units (CFU)/well) for 2 h, then thoroughly rinsed, and further cultivated in the fresh medium for up to 7 days. Other details are as in Fig. 1.

M. avium in macrophages, and suggest that IFN-γ and TNF-α modulate macrophage antimicrobial activity against these organisms in different fashions. Notably, TNF-α did not significantly potentiate macrophage anti-*M. avium* activity, although it enabled macrophages to eliminate *M. tuberculosis* in the later phase of macrophage cultivation. On the other hand, IFN-γ potentiated macrophage antimicrobial activity against both *M. avium* and *M. tuberculosis*. In separate experiments, TNF-α was less efficacious than IFN-γ in potentiating macrophage antimicrobial activity against other atypical mycobacterial strains, *M. intracellulare* N-260 and *M. kansasii* ATCC 12478 (unpublished observations).

Effects of IFN-γ and TNF-α on the RNI- and ROI-producing ability of macrophages

In order to determine the roles played by ROI and RNI in the expression of anti-mycobacterial activity by IFN-γ- or TNF-α-primed macrophages, we studied the effects of these cytokines on production of RNI and ROI by murine peritoneal macrophages, in terms of NO_2^- and O_2^- production, respectively. As shown in Table 1, both *M. tuberculosis*- and *M. avium*-infected macrophages generated larger amounts of RNI than uninfected macrophages. When macrophages were treated with IFN-γ for 2 days, RNI production by macrophages with or without bacterial infection was markedly enhanced, being 3·9–7·7-fold of that by untreated macrophages. In contrast, TNF-α treatment did not significantly affect macrophage RNI production. As shown in Table 2, IFN-γ and TNF-α both significantly enhanced O_2^- release by macrophages triggered by cell–cell contact with *M. tuberculosis* or *M. avium*. Notably, IFN-γ and TNF-α had nearly the same efficacy in potentiating macrophage O_2^- production.

Table 1. Effects of treatment of peritoneal macrophages with IFN-γ and TNF-α on their reactive nitrogen intermediate (RNI)-producing ability after infection with *Mycobacterium tuberculosis* or *M. avium**

Pretreatment with	Infection with	NO_2^- production (nmol)† Day 3	Day 5
–	–	2·8 ± 0·2 (100)	3·6 ± 0·2 (100)
IFN-γ	–	16·4 ± 0·7 (588)	14·2 ± 0·3 (393)
TNF-α	–	2·7 ± 0·2 (96)	3·6 ± 0·2 (100)
–	*M. tuberculosis*	4·8 ± 0·3 (173)	5·6 ± 0·2 (156)
IFN-γ	*M. tuberculosis*	20·0 ± 0·2 (717)	25·7 ± 0·6 (712)
TNF-α	*M. tuberculosis*	5·0 ± 0·3 (177)	5·6 ± 0·3 (156)
–	*M. avium*	4·0 ± 0·6 (142)	4·2 ± 0·1 (116)
IFN-γ	*M. avium*	26·9 ± 1·0 (964)	32·5 ± 0·2 (900)
TNF-α	*M. avium*	4·0 ± 0·1 (144)	4·1 ± 0·3 (114)

* Macrophages cultured in the medium with or without addition of 100 U/ml of IFN-γ or TNF-α for 2 days were allowed to phagocytose *M. tuberculosis* $H_{37}Rv$ (2×10^5 colony-forming units (CFU)/well) or *M. avium* N-425 (1×10^6 CFU/well). After washing, the resultant macrophages were further cultivated in the fresh medium for up to 5 days. At intervals, NO_2^- accumulation in the culture fluids was measured (see Materials and Methods).

† The amounts of NO_2^- produced per macrophage culture on a 16-mm dish at days 3 and 5 (mean ± s.e.m.; $n = 3$) are indicated. In parentheses, the relative RNI production (%) is indicated.

Table 2. Effects of treatment of peritoneal macrophages with IFN-γ and TNF-α on their reactive oxygen intermediate (ROI)-producing ability in response to triggering with *Mycobacterium tuberculosis* or *M. avium**

Cytokine treatment	Triggering with	O_2^- production (nmol)†	Relative O_2^- production (%)
–	*M. tuberculosis*	0·88 ± 0·05	100
IFN-γ	*M. tuberculosis*	1·69 ± 0·09	192
TNF-α	*M. tuberculosis*	1·70 ± 0·10	193
–	*M. avium*	0·95 ± 0·04	100
IFN-γ	*M. avium*	1·82 ± 0·07	191
TNF-α	*M. avium*	1·85 ± 0·10	195

* Macrophages cultured in the medium with or without addition of 100 U/ml of IFN-γ or TNF-α at 37°C for 2 days were measured for their ROI-producing abilities in terms of O_2^- production in response to triggering with the indicated stimulants (see Materials and Methods).

† O_2^- released per macrophage monolayer culture on a 16-mm dish in 60 min incubation (mean ± s.e.m.; $n = 3$ or 4). Superoxide dismutase at 1000 U/ml completely inhibited the macrophage-mediated colour change of the substrate (cytochrome C).

DISCUSSION

In the present study, pretreatment of murine resident peritoneal macrophages with IFN-γ and TNF-α before infection potentiated the anti-mycobacterial activity of murine peritoneal macrophages. However, these cytokines displayed different modes of action in modulating macrophage anti-mycobacterial activity. IFN-γ potentiated macrophage antimicrobial activities against *M. tuberculosis* and *M. avium*, while TNF-α augmented macrophage anti-*M. tuberculosis* but not anti-*M. avium* activity. In addition, TNF-α potentiated the anti-*M. tuberculosis* activity of macrophages only during the late period of macrophage cultivation (days 3–7), unlike IFN-γ, which enhanced macrophage function during both the early and late phases of cultivation. Thus, in the case of TNF-α-treated macrophages, a time lag of about 3 days may exist prior to full mobilization of anti-*M. tuberculosis* microbicidal mechanisms.

Mycobacterium tuberculosis rapidly grew in cytokine-untreated macrophages in the early phase of cultivation (days 0–3), but its growth ceased in the later phase of cultivation. In contrast, intracellular growth of *M. avium* was not observed in the early phase, but rapid bacterial growth subsequently occurred (Figs 1 and 2). The same profiles of intracellular growth of *M. avium* were observed by other investigators in human monocytes [27]. This suggests that the macrophage antimicrobial mechanism mobilized in the early phase of cultivation (designated the type 1 antimicrobial mechanism) might be preferentially effective in bacteriostasis of *M. avium*, whereas that mobilized in the later phase of macrophage cultivation (designated the type 2 antimicrobial mechanism) might instead be effective in inhibiting the intracellular growth of *M. tuberculosis*. It appears that TNF-α selectively potentiated the type 2 antimicrobial mechanism of macrophages, while IFN-γ activated both type 1 and type 2 antimicrobial mechanisms. Vodovotz *et al*. [28] found that LPS, a co-inducer of inducible nitric oxide synthase (iNOS), caused murine peritoneal macrophages to inactivate IFN-γ-induced iNOS about 3 days after onset of induction. Since *M. tuberculosis* and *M. avium* both contain a mycobacterial LPS, lipoarabinomanan [29], similar depression of iNOS activity might also occur in macrophages infected with these

organisms in the early phase of cultivation. This may be one of the reasons for the expression of differential modes of macrophage anti-mycobacterial activity, depending on the phase of cultivation after infection.

Previous studies have indicated that ROI are insufficient alone for effective killing or inhibition of mycobacterial pathogens [6,15,17,30,31]. We also found that a xathine oxidase-acetaldehyde system, which enzymatically generates ROI, was unable to kill mycobacteria [32], suggesting that ROI alone are not sufficient for macrophage anti-mycobacterial activity. These findings are consistent with the present one that ROI production by IFN-γ- and TNF-α-treated macrophages did not parallel their anti-*M. avium* activity (compare Table 2 and Fig. 2). Although the H_2O_2-mediated halogenation system is capable of effectively killing mycobacteria [33], the anti-*M. avium* activity of the H_2O_2-halogenation system was almost completely eliminated when this system was combined with other microbicidal effector molecules, including RNI and free fatty acids [33]. This may be why ROI molecules do not play crucial roles in mediating macrophage anti-mycobacterial activity.

RNI are important effectors of the antimicrobial activity of murine macrophages against mycobacteria, particularly *M. tuberculosis* [1,15–17]. The following findings of the present study confirm that RNI play roles as anti-mycobacterial effectors of murine peritoneal macrophages. First, the production of RNI by macrophages treated with test cytokines was correlated with their anti-mycobacterial, particularly anti-*M. avium*, activity (Table 1). IFN-γ- primed macrophages producing large amounts of RNI were capable of effectively killing and/or inhibiting *M. avium*, while TNF-α-activated macrophages, which produced small amounts of RNI, were not (compare Fig. 2 and Table 1). However, it appears that RNI alone are not sufficient in host defence mechanisms against MAC *in vivo*, since no significant relationship was found between the degree of resistance of MAC strains to RNI and their virulence in mice [22]. Therefore, collaboration of RNI with other antimicrobial effector molecules appears to be required for the manifestation of macrophage-mediated host resistance against MAC organisms. This hypothesis is supported by our recent finding that the anti-MAC activity of RNI was significantly potentiated by combination with free fatty acids, one of the major antimicrobial effectors of macrophages [33]. Second, it appeared that the deceleration of the early phase growth of *M. tuberculosis* in IFN-γ-treated macrophages, illustrated in Fig. 1, was in part mediated by RNI, since a marked increase in RNI production was observed for IFN-γ-primed macrophages responding to *M. tuberculosis* infection, but not for TNF-α-primed macrophages, in which the early-phase growth of the organisms was unaffected by priming (compare Fig. 1 and Table 1). Further detailed studies of the roles of collaboration between RNI and other antimicrobial effectors in the expression of macrophage antimicrobial activity and *in vivo* host defence mechanisms against *M. tuberculosis* and MAC are currently underway.

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