IL-12 and IL-18 synergistically induce the bactericidal activity of murine peritoneal cells against *M. leprae*

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We examined the effect of IL-12 and IL-18 on bactericidal activities of mouse peritoneal cell (PC) against *Mycobacterium leprae* (*M. leprae*). We demonstrated that IL-12 and IL-18 synergistically induced the NO-dependent bactericidal activity of PC by stimulating Natural Killer (NK) cells and T-cells through IFN-γ production. IL-12 and IL-18 induced host cell death through NK-cells and T-cells. Therefore, IL-12 and IL-18 play an important role on direct killing of intracellular *M. leprae* and on indirect killing of them through inducing host cell death.

INTRODUCTION

*M. leprae* is an intracellular pathogen residing preferentially in macrophage and Schwann cells. The ability of macrophage to cope with *M. leprae* is an issue central to understand the mechanism of host resistance to leprosy1)-4). The contributions of cytokines have been studied for this issue. IL-12 secretion by antigen presenting cell (APC) play a critical role for the development of protective Th1-type T lymphocytes in mice infected with *mycobacterium*2). Recently, IL-18 has been identified as an IFN-γ inducing factor (IGIF) by Okamura H et al3). IL-18 shares some biological activities with IL-12, although no significant homologies between the IL-12 and IL-18 have been identified at the protein level. Ottenhoff TH et al. reported that IL-2 and IL-12 acted in synergy to overcome antigen-specific T cell unresponsiveness in leprosy patients12), and Modlin RL et al. reported that IL-18 promoted type 1 cytokine, IFN-γ production from NK and T cells in leprosy patients5). Zhang T et al reported that IL-12 and IL-18 synergistically induced the fungicidal activity of murine peritoneal exudate cells against *C. neoformans* through production of IFN-γ by NK cells 6). We have previously demonstrated using RT-PCR that mRNA for IL-12 and IL-18 were expressed in peritoneal macrophages that were stimulated with/ without proteose peptone or infected with *M. leprae*.
The expression was stronger in *M. leprae*-treated macrophages than with/without proteose peptone\(^7\). These findings may suggest that IL-12 together with IL-2 or IL-18 play an important role in host defense by inducing bactericidal activity against *M. leprae*. And *M. leprae* infection induce IL-12 and IL-18 production. In the present study, we examined the bactericidal activity of murine peritoneal cells (PC) against *M. leprae* by cytokines IL-12 and IL-18 as one of the experimental model for leprosy.

**MATERIALS AND METHODS**

**Bacilli.** *M. leprae* (Thai 53 strain), derived from foot-pads of BALB/cA-nu/nu mice, was kindly given by Dr. M. Matsuoka, Leprosy Research Center, NIID.

**Cell culture.** PC (2 x 10⁵/well) of female BALB/cA mice (6-7 weeks of age) were cultured on the Thermonax coverslip (Nunc., Inc. Illinois, USA) in a 24 well-plate (Corning, New York, USA) for 3 hours with DMEM containing 15% FBS and penicillin 100U/ml, and they were stimulated with *M. leprae*, recombinant (r) IL-12 (10ng/ml, R&D Systems, Minneapolis, MN) or rIL-18 (10ng/ml) which was prepared as described by Okamura H et al\(^4\).

The depletion of NK cells and T cells from PC culture was done by using rabbit anti-asialo GM1 (anti-ASGM1) polyclonal Ab (100 μg/10⁶ cells, Wako Pure Chemical Industries, Ltd, Tokyo, Japan), anti-CD4 monoclonal (M) antibody or anti-CD8 MAb (10 μg/10⁶ cells, Seikagaku corp., Tokyo, Japan), and they were followed by addition with low-toxicity guinea pig complement (20 μl/10⁶ cells, Cederlane Lab. Ontario, Canada).

The following neutralizing antibodies were used; anti-mouse IL-18 rabbit serum prepared by Okamura. (20 μl/ml), anti-mouse IL-12 antibody (2 μg/ml, R&D Systems) and anti-mouse IFN-γ MAb (50 μg/ml, Endogen, Cambridge, MA). N⁰Monomethyl-L-arginine (L-NMMA), a competitive inhibitor of nitric oxide (NO) synthesis (500 μM) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich Chem. Milw., WI), a free-radical scavenger (1mM)\(^8\) were also used.

**Measurements of IFN-γ and NO.** The supernatants of the PC cultures were harvested on day 3 and the protein concentrations for IFN-γ were measured using commercially available ELISA kit (ENDOGEN, Cambridge). On day 4 - 7, NO produced by PC was measured using Griess Reagent\(^6\).

**Assessment of number of bacilli and adherent host cells.** Intracellular bacilli were counted as follows; coverslips were washed with PBS, suspended in one ml of water, sonicated for one minute to separate the bacilli from the host cells, and the number of bacilli were counted under microscope by Shepard's method\(^9\). The adherent cells infected with bacilli were also counted as follows; the plastic coverslips were washed well with PBS, fixed with methanol for 10 min., and stained with Ziehl-Neelsen method. The number of the adherent cells, stained blue color by methylene blue, was expressed as the mean cell number/field by counting 10 fields randomly selected under microscope (x 400).

**FDA/EB staining.** The bacterial suspensions separated from host cells were centrifuged at 10,000rpm for 10 min and the pellets were re-suspended with 50 μl of PBS and kept in 4°C for a few days. FDA/EB test was done as described by Kinomoto M et al\(^10\). Observation was done with Olympus Fluorescent Microscope using blue filter. Viable bacilli were stained green and non-viable ones were stained orange. Percent viability was calculated from 20 fields randomly selected microscopically (x 1000).

**RESULTS**

IL-12 and IL-18 synergistically enhance the bactericidal activity of PC. PC were cultured with *M. leprae* for 15 days, and their bactericidal activities were determined by FDA/EB test. As shown in Fig. 1, 45% of the intracellular bacilli have spontaneously been killed during 15 days of cultivation. There is no enhancing effect of single use of IL-12 and IL-18 on bactericidal activity by PC infected with
M. leprae. However, when both IL-12 and IL-18 were added to the PC culture, this bactericidal activity increased: i.e., 77% of the bacilli were killed. It is known that PC are capable of producing IL-12 and IL-18. Therefore, we examined the role of the cytokines produced by M. leprae-infected PC. To this end, we checked the viability of intracellular bacilli in the presence of neutralizing MAb s to IL-12 and IL-18. Because the titer of IL-18 was impossible to assay in vitro. Around 90% of the bacilli were viable when PC were cultured with anti-IL-12 Ab or anti-IL-18 Ab, or both. Anti-hsp65 MAb s (B2C, 50 E g/ml, each) showned no effect on M. leprae viability (not shown), and rabbit IgG also no effect for fungicidal activity as described by T. Zhang et al.

These results suggest that IL-12 and IL-18, may be produced upon bacterial infection, have the bactericidal activity. The bactericidal activity induced by IL-12 and IL-18 was observed in PC after 5 days of cultivation (data not shown).

Production of IFN-γ and NO in vitro. Production of IFN-γ and NO by PC culture in the presence of IL-12 + IL-18 was assessed (Fig. 2). A significant dose of IFN-γ and NO were produced when M. leprae-infected PC were treated with both IL-12 and IL-18. However, when either IL-12 or IL-18 were used, minimum doses of IFN-γ and no NO were generated (not shown). To examine the involvement of CD4+ T and NK cells, which are known to be involved in host defense by producing IFN-γ, these populations were depleted from PC. The depletion reduced the production of both IFN-γ and NO from M. leprae-infected PC. More significant reduction was observed when NK cells were depleted. While L-NMMA, a NO synthesis inhibitor, suppressed only NO production, anti-IFN-γ MAb suppressed not only IFN-γ but also NO production.

To define the role of IFN-γ, NO and H2O2 on bactericidal activity of M. leprae-infected PC, we examined the effects of neutralizing anti-IFN-γ MAb, L-NMMA and Trolox (Fig. 3). The anti-IFN-γ and L-NMMA inhibited the bactericidal activity of PC stimulated with IL-12 and IL-18. These results suggest that the bactericidal activity of PC is chiefly mediated by NO through producing IFN-γ from PC infected with M. leprae and stimulated with both IL-12 and IL-18. In addition to NO, we examined H2O2 effect on bactericidal activity using Trolox, a H2O2 scavenger. Trolox also inhibited the bactericidal activity of M. leprae-infected PC stimulated with IL-12 and IL-18 (Fig. 3).
Effects of IL-12 and IL-18 on the viability of host cell. When PC were infected with M. leprae in the presence of IL-12 and IL-18 for more than 10 days, the host cell death appeared. On day 11 of cultivation, the viability of adherent host cells have decreased as shown in Fig. 4. However, no deduction was induced by either IL-12 or IL-18 alone (not shown). The depletion of NK, CD4⁺, CD8⁺ and CD4⁺ plus CD8⁺ T cells from PC inhibited the deduction of viable cell number, and the highest inhibition was observed when NK cells were depleted. The apoptotic cell death was examined using DNA ladder and in situ Apoptosis Kit (Trevigen, Inc. MD USA), but no apoptosis was observed (not shown).

DISCUSSION

We demonstrated here that IL-12 and IL-18 synergistically (10 μg/ml, each) induced the NO-dependent bactericidal activity of PC by stimulating NK and T-cells to produce IFN-γ (Fig. 1,2,3), even though the minimum dose of these cytokines for bactericidal activity remained to be examined. In addition to the action of NO, H₂O₂ may be involved in killing intracellular M. leprae. Akita T et al. reported that intracellular growth of M. tuberculosis in macrophage was accelerated by treatment of macrophages with either reactive nitrogen or oxygen intermediates scavengers. In cell-free systems, the free fatty acids that were induced in macrophage by inoculation of M. tuberculosis were essential for antimicrobial effects in addition to the reactive nitrogen or oxygen. Therefore, factors, other than NO and H₂O₂, may be produced in macrophages by IL-12 + IL-18 in accordance with M. leprae infection and may be necessary for bactericidal activity.

In addition to the bactericidal activity, IL-12 and IL-18 activated T cells and/or NK cells which led to the production of IFN-γ and NO by PC infected with M. leprae (2 x 10⁶) stimulated with IL-12 and IL-18. PC (2 x 10⁶ cells/well), CD4⁺ T cells or NK cells-depleted PC were cultured in the presence of IL-12 and IL-18. The concentration of IFN-γ (3 days after culture) and NO (7 days after culture) in the supernatant was assayed. Each column (IFN-γ) and dot (NO) represents the mean of triplicate cultures. The SD was less than 10% of the mean.

Fig. 2. Production of IFN-γ and NO by PC infected with M. leprae (2 x 10⁶) stimulated with IL-12 and IL-18. PC (2 x 10⁶ cells/well), CD4⁺ T cells or NK cells-depleted PC were cultured in the presence of IL-12 and IL-18. The concentration of IFN-γ (3 days after culture) and NO (7 days after culture) in the supernatant was assayed. Each column (IFN-γ) and dot (NO) represents the mean of triplicate cultures. The SD was less than 10% of the mean.

Fig. 3. Effects of IFN-γ, NO and H₂O₂ on the bactericidal activity of PC infected with M. leprae and stimulated with IL-12 and IL-18. PC (2 x 10⁶) infected with M. leprae (2 x 10⁶) were cultured with anti-IFN-γ, L-NMMA and Trolox together in the presence of IL-12 and IL-18 for 5 days. The dead/live bacilli ratio was expressed.
host cell death (Fig. 4). The cell death may be associated with necrosis, because we could not detect any evidence suggesting apoptosis. We examined the effects of neutralizing MAbs to IFN-γ, and TNF-α, and L-NMMA on IL-12 + IL-18-mediated host cell damages. However, no apparent activities of them were observed. (data not shown). As reported by Nakada Y. et al., NK cell-mediated cytotoxicity may be associated with the production of radicals, especially hydroxyl radicals, we might have to determine the effect of hydroxyl radicals on host cell damages in future. Thus, the mechanisms for bactericidal activity induced by IL-12 and IL-18 synergistically against M. leprae were impossible to explain only by cellular immunity through IFN-γ, but also need other cascades inducing H₂O₂ and host cell damages.

Taken together, the induction of IL-12 and IL-18 is important for inducing direct killing of intracellular M. leprae, and also inducing the killing of bacilli indirectly by inducing host cell death. These findings also may be useful to understand the mechanisms of leprosy reactions such as reversal (Type 1) and erythema nodosum leprosum (ENL, type 2) in leprosy patients immunologically.

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IL-12およびIL-18で刺激されたマウス腹腔内培養細胞の抗らい菌効果

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IL-12およびIL-18によるマウス腹腔内培養細胞におけるらい菌の殺菌に及ぼす効果について検討した。IL-12およびIL-18の同時作用が、NKおよびT細胞の関与によりIFN-γの産生を促進し、それがNO依存性らい菌の殺菌につながることを明らかにした。さらに、IL-12およびIL-18の同時作用は、宿主細胞の障害をも誘導した。

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