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Induction of persistent in vivo resistance to *Mycobacterium avium* infection in BALB/c mice injected with interleukin-18-secreting fibroblasts

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Abstract

Interferon-gamma (IFN-γ) is closely associated with the generation of cell-mediated immunity and resistance to intracellular parasites. Interleukin-18 (IL-18) is known to strongly induce IFN-γ production by T cells and natural killer (NK) cells. To determine whether the paracrine secretion of IL-18 can efficiently stimulate the resistance to *Mycobacterium avium* complex (MAC) infection, 3T3 fibroblasts were stably transfected to secrete bioactive IL-18 and their effects on MAC infection were investigated in genetically susceptible BALB/c mice, compared with that of free recombinant IL-18. Immunization with IL-18-secreting fibroblasts (3T3/IL-18) during intranasal infection with MAC resulted in a significant decrease in bacterial load of lung during the entire 8-week observation period, while rIL-18 reduced the bacterial load at initial 1 week but not by 8 weeks postinfection. Immunization with the 3T3/IL-18 cells induced and maintained significantly higher levels of cytotoxic activity and nitric oxide production by lung cells than those of rIL-18 immunization. Furthermore, lung cells in mice injected with the 3T3/IL-18 cells showed persistent production of IFN-γ throughout the 8-week period, suggesting that the 3T3/IL-18 cells induced the resistance to MAC infection via IFN-γ production. This work suggests that IL-18-secreting fibroblasts may serve as a vehicle for paracrine secretion of IL-18 in immunotherapy of MAC infection.

Keywords: *Mycobacterium avium*; IL-18-secreting fibroblast; Immunity

1. Introduction

Bacteria of the *Mycobacterium avium* complex (MAC) are facultative intracellular pathogens and the most common cause of disseminated bacterial infection in AIDS patients. Acquisition of a MAC infection significantly shortens the life span of these patients compared with that of patients with the same T-cell counts [1]. Control of MAC infection requires the presence of activated $CD4^+$ T cells which produce an array of cytokines, including interferon-gamma (IFN-γ), involved in activating macrophage bactericidal activity. Studies involving IFN-γ gene and IFN-γ receptor gene knockout mice showed that IFN-γ, produced by activated $CD4^+$ T cells and natural killer (NK) cells, played an essential role in protective cellular immunity against mycobacteria [2,3].

Interleukin-18 (IL-18), first designated as an IFN-γ inducing factor, is a newly identified cytokine of Th1 type, and the cDNAs encoding murine and human IL-18 have recently been cloned [4,5]. IL-18 has been known to strongly induce IFN-γ production by both $CD4^+$ T cells and NK cells, and to stimulate naive T cells to promote the development of Th1 (IFN-γ-producing) cells [6]. The development of a Th1 response and IFN-γ production is central to eradication of various pathogens including *Cryptococcus neoformans* [7], *Leishmania major* [8], and *Mycobacterium leprae* [9]. IL-18 knockout mice were susceptible to the infection of the parasite *L. major* and *Staphylococcus aureus*, while the wild-type mice were highly resistant to the infection of the parasites [10]. The infected IL-18 knockout mice produced significantly lower levels of IFN-γ and larger amounts of IL-4 compared with similarly infected wild-type mice. IL-18, therefore, has been known to play a decisive role in host defense against intracellular infectious microorganisms.

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Recombinant cytokines have been clinically used in the treatment of human diseases including cancer and infectious diseases [11,12]. However, a single injection is not sufficient for a protective or therapeutic effect [13,14] and recombinant cytokines should be highly purified before use. In some case, a cytokine injection is effective only if a cytokine is administered either before or at the time of parasite inoculation [15]. Sato et al. [16] reported that although IFN-γ-treated macrophages killed *M. avium* during the initial 3 days of culture, regrowth of the intracellular organisms was subsequently observed [16]. Repeated injections of recombinant cytokines at relatively high doses showed side effects such as fever, nausea, leukopenia, and abnormal liver function tests [17].

Alternatively the cytokine concentration necessary for a therapeutic effect could be secured by administration of genetically engineered cell lines containing an inserted cytokine gene, and constitutively producing cytokine [18]. Previous reports showed that fibroblasts were genetically modified and used for obtaining the paracrine secretion of cytokines in tumor models [19,20]. Immunization with cytokine gene-transfected fibroblasts delayed tumor formation and promoted antitumor immunity [21], and clinical studies using cytokine-secreting fibroblasts are under investigation in cancer patients [22].

In this study we investigated the effects of a long-lasting IL-18 concentration maintained by IL-18-secreting fibroblasts on MAC infection, and compared with those of free rIL-18. We demonstrate here that IL-18-secreting fibroblasts are more efficient than free rIL-18 in inducing in vivo persistent bactericidal properties during MAC infection, leading to the reduction of bacterial loads in MAC-infected mice for prolonged periods.

2. Materials and methods

2.1. Reagents, mAbs and animals

Middlebrook 7H10 agar, Bacto Middlebrook OADC enrichment solution and Middlebrook 7H9 broth were purchased from Difco Laboratories (Detroit, MI). Anti-mIFN-γ mAbs (R46A2 and XMG1.2) were purified from ascitic fluids by ammonium sulfate precipitation followed by DEAE-Sephacel chromatography (Sigma, St. Louis, MO). MAb-secreting hybridomas, BALB/3T3 cells, P815 cells and L929 cells were obtained from the ATCC (Rockville, MD). The cells were maintained at 37 °C in a humidified 5% CO_2 in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (growth medium). Six to 8-week-old female BALB/c mice were obtained from the Charles River Laboratories (Wilmington, MA). The mice were maintained in pathogen-limited conditions and treated according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Preparation of IL-18-secreting fibroblasts (3T3/IL-18 cells)

Murine mature form of IL-18 gene was subcloned into a retroviral vector pMFG.puro (Hanyang University, Seoul). The pMFG.puro vector contains the bacterial puromycin-resistant gene under control of the Moloney leukemia virus long terminal repeat and an immunoglobulin kappa leader sequence. The mature IL-18 cDNA was inserted in frame with the immunoglobulin kappa leader sequence, to allow for secretion of the translated protein. 3T3 fibroblasts were cotransfected with these expression plasmids using Superfect according to the manufacturer's protocol (Qiagen, Valencia, CA). To obtain stably transfected clones, transfected cells were grown in growth medium containing puromycin (2 μg/ml) for approximately 14 days, and resistant clones propagated separately, with subsequent confirmation of IL-18 gene expression by RT-PCR, Western blot and biological assay. Control 3T3 cells containing only the puromycin-resistant gene were prepared by transfection with pMFG.puro vector using the same procedure.

2.3. RT-PCR, SDS-PAGE, and Western blotting

Total RNA was prepared from the cells and reverse-transcribed into cDNA, and then PCR amplification of the cDNA was performed. The sequences of PCR primers are as follows: IL-18 (sense, 5′-ACT GTA CAA CCG GAG TAA TAC GG-3′; antisense, 5′-AGT GAA CAT TAC AGA TTT ATC CC-3′), and puromycin-resistant gene (sense, 5′-TGC GCC TCG CCA CCC GCG AC-3′; antisense, 5′-TCA TGC ACC AGG TGC GCG GT-3′). The PCR reactions were run for 35 cycles of 94 °C (30 s), 58 °C (45 s), 72 °C (30 s) using a MJ thermal cycler (Watertown, MA). After the amplification, 5 μl of the RT-PCR products were separated in 1.2% (w/v) agarose gels and stained with ethidium bromide.

The expression of IL-18 protein by puromycin-resistant clones was identified by Western blot analysis using biotinylated anti-murine IL-18 mAb (RDI Inc., NJ). Recombinant mouse IL-18 (MBL Co. Ltd., Nagoya, Japan) was used as a positive control. The cells were lysed in lysis buffer (50 mM Tris buffer, pH 7.5 containing 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml PMSF) by incubation on ice for 30 min. Lysates were then centrifuged at 13,000 × *g* at 4 °C for 10 min and protein concentrations of the lysates were determined using the Coomassie protein assay reagent (Bio-Rad, Richmond, CA). Following electrophoresis, the protein was transferred into nitrocellulose by electroblotting using a semi-dry blotter. The blot was then immunostained with biotinylated anti-mIL-18 mAb and developed by ECL system (Amersham Life Sciences, Arlington Height, IL).

2.4. Immunization of MAC-infected mice with the 3T3 cell constructs

Mice were infected intranasally with 10^5 MAC organisms inoculated onto the external nares with a micropipette. Afterward, the mice were injected intraperitoneally (i.p.) with each of fibroblast cell constructs as described in the legends of tables and figures. The virulent MAC strain 101 was used throughout the study (obtained from Dr. P. Gangadharam, University of Illinois, IL). Single cell suspensions from transparent colonies were obtained and the number of microorganisms was confirmed by colony counting techniques as previously described [23].

2.5. Determination of the number of microorganism

At intervals after infection, five mice per group were sacrificed and the lungs were removed from each mouse aseptically, and homogenized in 5 ml of sterile phosphate-buffered saline with a tissue homogenizer. Suitable dilutions were placed on Middlebrook 7H11 agar for viable counts, and the plates were incubated in a humidified container at 37 °C for 7 days before counting.

2.6. Preparation of lung cell suspensions

The lungs were taken from each mouse aseptically and the single cell suspension was prepared as previously described [24]. In brief, the lungs were washed with DMEM medium and sliced finely with a multiblade slicer. The lung tissue was incubated for 40 min at 37 °C in 10 ml of DMEM medium containing 0.25% dispase (Boehringer, Mannheim, Germany). Afterwards, the tissue supernatant was centrifuged at $1000 \times g$ for 5 min to pellet cells. The remaining undigested tissue was subjected to digestion two more times, and any remaining tissue was then forced through a fine-mesh sieve and centrifuged. The viability of the cells was generally greater than 85%, assessed by trypan blue exclusion. For cytokine assay, 5×10^5 lung cells were incubated in RPMI 1640 medium containing 10^7 MAC. After 48 h, supernatants were removed from each culture for measurement of IFN-γ.

2.7. In vitro depletion of T-cell subsets

For in vitro depletion of either $CD8^+$ T cells or $CD4^+$ T cells, mAbs for each T-cell subset were used as previously described [20]. In brief, lymph node cells from immunized mice were incubated with anti-CD4 (L3T4) or anti-CD8 (Lyt-2.2) mAbs on ice for 30 min, followed by addition of low-toxicity rabbit complement (Pel-Freez, Rogers, AR) at 37 °C for 45 min. The antibodies were titered such that the concentrations used were five times the minimal amount required to saturate the specific binding sites of liver cells from naive BALB/c mice, as determined by cytofluorometric analysis of serially diluted antibodies. After depletion of the specific cell types, the remaining cells were washed with serum-free medium, and incubated with MAC for 48 h. Immunofluorescent analysis of lung cells after mAb treatment indicated that there was >95% depletion of specific T-cell subsets with no decrease in the frequency of the other subsets.

2.8. Cytokine assays

The biological activity of IL-18 produced by transfectants was determined by the ability to stimulate IFN-γ production in spleen cells in vitro as previously described [25]. Results were extrapolated from a standard curve using a defined dose of rIL-18. 2×10^6 spleen cells were cultured in 2 ml of cell culture medium in 12-well plates in the presence of transfectants' supernatants. IFN-γ levels in the supernatants were determined by ELISA as previously described [26]. The mAbs for coating the plates and the biotinylated second mAbs were rat anti-mouse IFN-γ (HB170) and biotinylated rat anti-mouse IFN-γ (XMG1.2), respectively. The lower limit of detection was 125 pg/ml for IFN-γ.

2.9. NO_2^- assay

Cultures of 5×10^5 lung cells pooled from five mice per group with or without stimulation by 10^7 live MAC were incubated in 200 μl of DMEM-10% fetal bovine serum in 96-well microtiter plates for 72 h. The supernatants were then harvested and assayed for NO by the Griess reaction [27]. Briefly, culture supernatants (50 μl) were mixed with 100 μl of 1% sulfanilamide (Sigma) and 100 μl of 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in 2.5% polyphosphoric acid at room temperature for 5 min. A_{540} was measured and NO_2^- was quantified by comparison to $Na(NO_2)$ as a standard.

2.10. In vitro cytotoxic assay

The cytotoxicity measurements were performed using a standard 4 h ^{51}Cr release assay as previously described [28]. The percentage of specific cytolysis was calculated as:

$$\frac{\text{test cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

where cpm is counts per minute.

2.11. In vivo administration of anti-IFN-γ mAb

In some experiments, the course of infection was modulated by i.p. administration of a neutralizing anti-mIFN-γ antibody (XMG1.2, rat IgG1) or isotype control antibody (rat IgG1). The Ab treatment started 1 day before MAC infection and continued for 6 days every other day and then once a week until sacrifice.

2.12. Statistics

The Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical differences between values of the various experimental and control groups. The *P*-values < 0.05 considered statistically significant.

3. Results

3.1. Stable expression of IL-18 by fibroblasts transfected with a retroviral vector carrying a mature IL-18 cDNA

IL-18-secreting 3T3 fibroblasts were constructed as described in Section 2. The culture supernatants obtained from 40 puromycin-resistant colonies were tested for IL-18 bioactivity. Among these colonies, colony 7 (3T3/IL-18 cells) was chosen for the use in the experiments because of its much higher expression (12 ng/10^7 cells/48 h; 1 ng/ml induces approximately 20 IU/ml of IFN-γ) compared with the others (Fig. 1A). This activity, measured by the ability to stimulate IFN-γ production by spleen cells in vitro, could be inhibited by a neutralizing anti-IL-18 mAb (rat IgG1) but not by a control isotype mAb (anti-IL-6, rat IgG1), suggesting that the activity was IL-18 specific. Furthermore, approximately equivalent amount of IL-18 was formed after in vitro continuous passage more than 2 months (10 ng/ml), suggesting that the IL-18-secretion by the 3T3/IL-18 cells was stable. The 3T3 cells transfected with pNFG/puro as a control vector (3T3/puro cells) did not form detectable amounts of IL-18. The expression of IL-18 gene by the genetically modified fibroblasts was further confirmed by the RT-PCR using primers specific for mIL-18 or *puro*^{r} gene (Fig. 1B), and by the Western blot analysis using anti-mIL-18 mAb (Fig. 1C).

3.2. Inhibition of bacterial growth in MAC-infected mice by IL-18-secreting fibroblasts (3T3/IL-18 cells)

To test the effect of IL-18-secreting fibroblasts on the resistance to MAC infection, BALB/c mice were infected with MAC, immediately followed by the immunization with 1×10^7 3T3/IL-18 cells, or with 3T3/puro cells as a control. We also included a group of mice injected with free recombinant IL-18 (50 ng) to investigate the importance of long-lasting IL-18 concentrations maintained by the 3T3/IL-18 cells. At 1, 4 and 8 weeks postinfection, mice were sacrificed and lungs were collected for bacterial analysis. As seen in Table 1, during the first 1 week of infection, a significant reduction in infection with MAC was seen in mice injected with the 3T3/IL-18 cells or rIL-18 (50 ng) plus 3T3/puro cells. The numbers of bacteria in lung culture were approximately 1.6–15.8-fold lower in the 3T3/IL-18-treated mice than those in the 3T3/puro-treated mice. The levels were not significantly different between the 3T3/IL-18- and rIL-18-treated mice during the first 1 week of infection. However, by the 4 and 8 weeks postinfection, bacterial numbers sharply increased in rIL-18-treated mice, whereas did not increase in mice treated with the 3T3/IL-18 cells. The numbers of bacteria were approximately 10-fold lower in

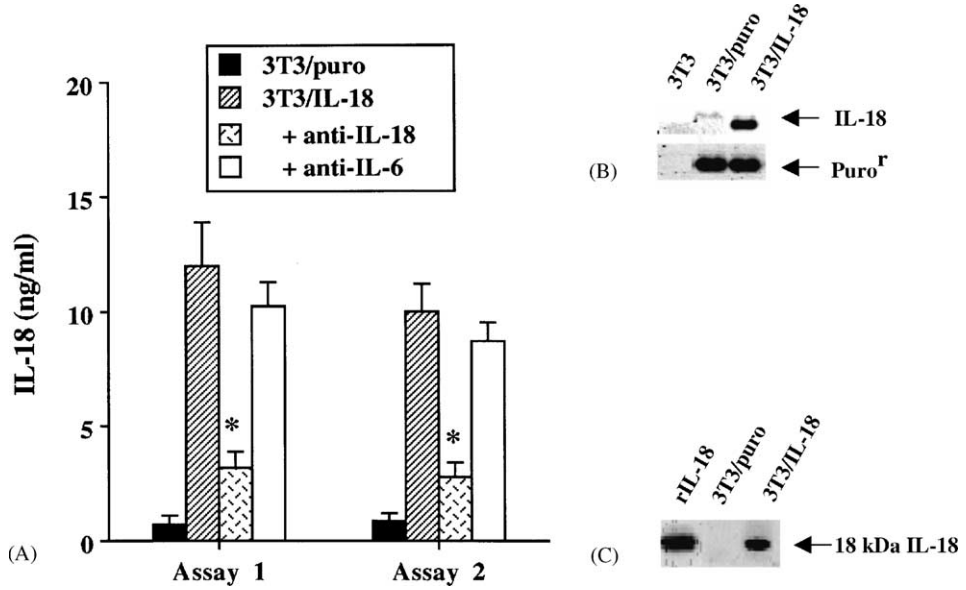

Fig. 1. Expression of bioactive IL-18 protein from 3T3 cells transfected with the pMFG.IL-18 plasmid. (A) The supernatants were collected after 48 h culture of 10^7 cells of each cell type, and the biological assay was determined by the ability to stimulate IFN-γ production by spleen cells in vitro in the absence or presence of anti-mIL-18 or anti-IL-6 as a control isotype. The assay was done immediately after the selection of the transfected cells in puromycin (Assay 1), and after continuous culture of more than 2 months in CO_2 incubator (Assay 2). The values represent the means ± standard errors of three independent experiments. (B) RNA was prepared from the 3T3 cells transfected with either pMFG.puro or pMFG.IL-18 plasmid, and RT-PCR was conducted. (C) For Western blot analysis, IL-18 protein was immunoprecipitated from culture supernatants of the transfected 3T3 cells, electrophoresed under non-reducing condition, blotted, and detected by anti-mIL-18 mAb.

Table 1
Effect of IL-18-secreting fibroblasts on bacterial growth in MAC-infected mice

Treatment[a]	Number of bacteria in lung		
	1 week infected	4 weeks infected	8 weeks infected
Untreated	5.4 ± 0.3[b]	6.1 ± 0.2	7.5 ± 0.2
3T3/puro (1×10^7)	5.3 ± 0.3	ND[c]	7.6 ± 0.2
3T3/IL-18 (1×10^7)	4.7 ± 0.2[d]	5.2 ± 0.1	6.3 ± 0.2[e]
rIL-18 (10 ng) + 3T3/puro (1×10^7)	5.1 ± 0.3	6.3 ± 0.1	7.6 ± 0.3
rIL-18 (50 ng) + 3T3/puro (1×10^7)	4.8 ± 0.1[d]	6.1 ± 0.2	7.5 ± 0.4
3T3/puro + 3T3/IL-18			
8.0×10^6 (80%), 2.0×10^6 (20%)	5.0 ± 0.2[d]	5.0 ± 0.2	6.5 ± 0.3
9.0×10^6 (90%), 1.0×10^6 (10%)	5.2 ± 0.2	ND	6.7 ± 0.2
9.8×10^6 (98%), 0.2×10^6 (2%)	5.6 ± 0.3	6.4 ± 0.1	7.7 ± 0.2

[a] Mice were intranasally infected with 10^5 MAC, followed by injection with 1×10^7 3T3/IL-18 cells or 3T3/puro cells, or 3T3 cells consisting of varying percentages of the 3T3/puro and 3T3/IL-18 cells.
[b] Data are the mean log bacterial numbers ± standard deviations of five mice. The experiment was repeated twice with similar results.
[c] Not determined.
[d] $P < 0.05$, relative to groups treated with 3T3/puro cells or untreated.
[e] $P < 0.001$, relative to groups treated with mixtures of rIL-18 and 3T3/IL-18 cells.

the 3T3-IL-18-treated mice than those in the rIL-18-treated mice at 8 weeks after MAC infection. In addition to lung, significant infection with MAC was seen in spleen and liver, and Ziehl–Neelsen staining and viable counts on Middlebrook 7H11 agar showed a large decrease of infected intracellular bacteria in these organs of 3T3/IL-18-treated mice compared with those of 3T3/puro- or rIL-18-treated mice. In spleen the number of bacteria was three–five folds lower in the 3T3-IL-18-injected mice than that in the rIL-18-treated mice at 8 weeks after MAC infection (data not shown).

Furthermore, to determine if the 3T3/IL-18 cells stimulate the resistance to MAC in a dose-dependent manner, MAC-infected mice were inoculated with 1×10^7 3T3 cells (3T3/puro + 3T3/IL-18) containing varying percentages of 3T3/puro cells and 3T3/IL-18 cells. As shown in Table 1, animals inoculated with 10% or more 3T3/IL-18 cells showed a significant reduction in bacterial load. Animals with 2% 3T3/IL-18 cells showed only a marginal reduction.

We next investigated the effect of the 3T3/IL-18 cells in established MAC-infected mice. Mice were first infected with MAC and, 5 or 10 days later, treated with the 3T3/IL-18 cells, 3T3/puro cells or rIL-18, or untreated. The bacterial numbers of lungs in mice were determined at 8 weeks after MAC infection. As shown in Fig. 2, the bacterial load in lungs was significantly reduced in mice treated with the 3T3/IL-18 cells, even if the cells were administered into MAC-infected mice at 10 days postinfection. The inhibitory effect of the 3T3/IL-18 cells on bacterial load was still observed at 14 days postinfection (data not shown).

3.3. IL-18-secreting fibroblasts efficiently induced and maintained the bactericidal properties of lung cells during MAC infection

The production of NO is one mechanism used by infected cells to kill invading bacteria. To determine the effect of IL-18-secreting fibroblasts on the NO production, BALB/c mice were infected with MAC, immediately followed by the immunization with 1×10^7 3T3/IL-18 cells, or with 1×10^7 3T3/puro cells or 50 ng rIL-18 as controls. At 1 and 8 weeks postinfection, mice were sacrificed and lung cells were tested for the NO production. As seen in Table 2, during the first 1 week of infection, the levels of NO production by lung cells from 3T3/IL-18- and rIL-18-treated mice showed a significant increase compared with those of 3T3/puro-treated mice. The levels were not significantly different between

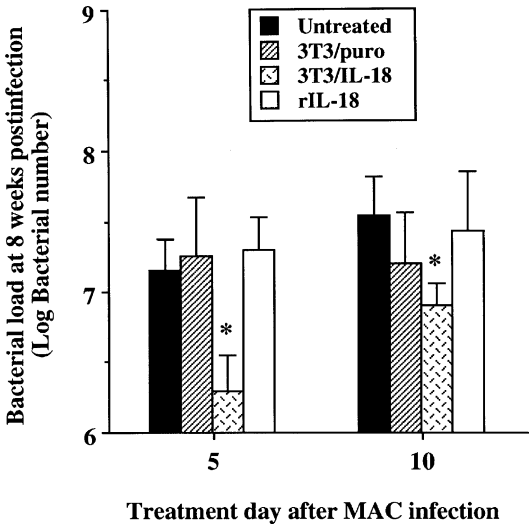

Fig. 2. Effect of IL-18-secreting fibroblasts on bacterial growth in mice previously infected with MAC. Mice were intranasally infected with 10^5 MAC. Five and 10 days later, the infected mice were i.p. injected with 10^7 3T3/IL-18 or 3T3/puro cells, or 50 ng rIL-18, or untreated. Eight weeks after the infection, the bacterial numbers in lung were determined. Data represent the mean log bacterial numbers ± standard deviations of five mice. The experiment was repeated twice with similar results. (*) $P < 0.01$, relative to groups treated with the 3T3/puro cells or rIL-18.

Table 2
Effect of IL-18-secreting fibroblasts on NO production by lung cell cultures following MAC infection

Treatment[a]	NO_2^- level (μM)[b]	
	1 week infected	8 weeks infected
Uninfected	<5.0	<5.0
Intact infected	40.5 ± 5.1	72.2 ± 9.3
3T3/puro	45.5 ± 4.7	59.4 ± 10.2
3T3/IL-18	108.4 ± 7.2[c]	295.5 ± 10.5[d]
rIL-18	110.5 ± 8.0[c]	93.7 ± 15.2

[a] Mice were intranasally infected with 10^5 MAC followed by i.p. injection of 1×10^7 3T3/puro or 3T3/IL-18 cells, or 50 ng rIL-18.

[b] Cultures of 5×10^5 lung cells were incubated for 72 h in 200 μl volumes in a 96-well plate, and the culture supernatants were harvested and assayed for nitrate levels. The data are the means ± standard deviations of triplicate determinations from cultures pooled from five mice infected for 1 or 8 weeks. The experiment was repeated twice with similar results.

[c] $P < 0.001$, relative to a group treated with 3T3/puro cells.

[d] $P < 0.001$, relative to groups treated with rIL-18 or 3T3/puro cells.

the 3T3/IL-18- and rIL-18-treated mice during the first 1 week of infection. However, by the 8 weeks postinfection, the levels of NO production by lung cells sharply increased in the 3T3/IL-18-treated mice, whereas did not increase in mice treated with the rIL-18-treated cells.

Furthermore, lung cells were used in an in vitro cytotoxicity assay to test their ability to lyse the macrophage-sensitive cell line P815. Lung cells from 3T3/IL-18-treated mice showed significantly higher levels of cytotoxicity than those in mice treated with the 3T3/puro cells or rIL-18 at 1 week postinfection (Fig. 3). Importantly, the higher levels of cytotoxicity were maintained during the entire 8-week observation period in mice treated with the 3T3/IL-18 cells. Therefore, IL-18-secreting fibroblasts induced in vivo persistent anti-mycobacterial activity in MAC-infected mice, while free rIL-18 did not.

3.4. IFN-γ production by lung cells from MAC-infected mice injected with IL-18-secreting fibroblasts

Experiments were performed to determine whether or not the endogenous production of IFN-γ by lung cells were similar or differentially regulated in mice injected with the 3T3/IL-18 cells or rIL-18, or with the 3T3/puro cells. At 1 and 8 weeks after MAC infection, the lung cells from each group were cultured in vitro with MAC for 48 h and the level of IFN-γ in culture supernatants were determined by an IFN-γ-specific ELISA. As shown in Fig. 4A, lung cells from mice injected with the 3T3/IL-18 cells produced more IFN-γ than those injected with the 3T3/puro cells at both time points.

To determine which cells are involved in IFN-γ production, $CD4^+$ or $CD8^+$ T cells were depleted from MAC-infected lung cells by treatment with anti-CD4 or anti-CD8 mAb plus complement, followed by stimulation in vitro with MAC. Afterward, the IFN-γ levels in the depleted cell cultures were compared with those of cell cultures incubated with either growth medium or complement alone, followed by in vitro stimulation with MAC. As indicated (Fig. 4B), in vitro re-stimulation of MAC-infected liver cells induced significant levels of IFN-γ, which were reduced to the background levels after depletion of $CD4^+$ T cells not $CD8^+$ T cells, indicating that $CD4^+$ T cells were the primary cell type responsible for the production of IFN-γ in MAC-infected cells.

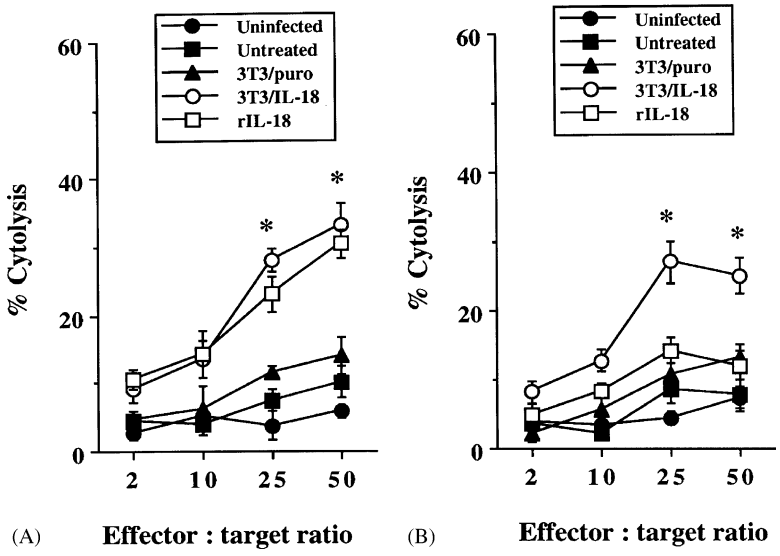

Fig. 3. Specific lysis of P815 cells by lung cells from MAC-infected mice treated with 3T3/IL-18 cells, 3T3/puro cells, or free rIL-18. Mice (five per group) were intranasally infected with 10^5 MAC 1 week (A) or 8 weeks (B) prior to sacrifice. The data are the means ± standard deviations of triplicate cultures. Experiments were repeated three times with similar results. (*) $P < 0.001$, relative to groups treated with the 3T3/puro cells or rIL-18.

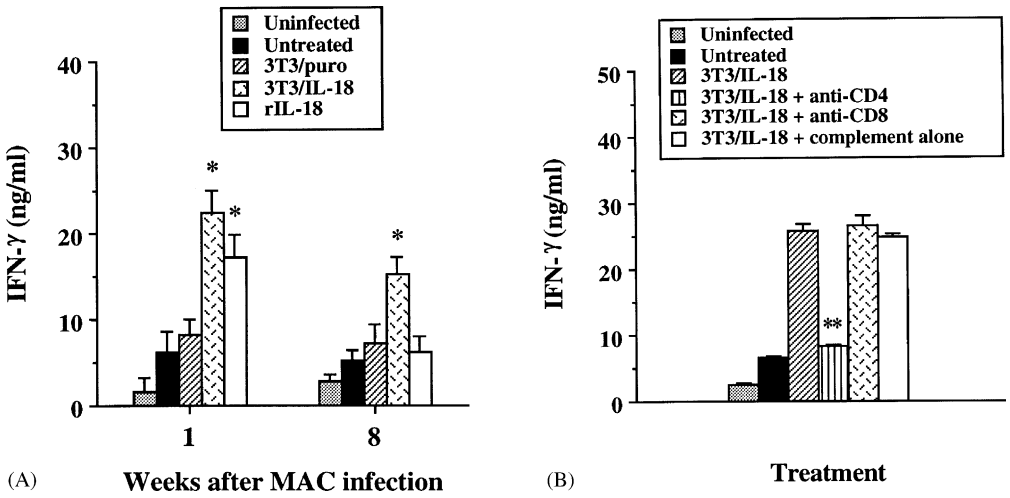

Fig. 4. In vitro production of IFN-γ by $CD4^+$ T cells from MAC-infected mice injected with IL-18-secreting fibroblasts. Lung cell suspensions (5×10^5) were immediately stimulated with 10^7 MAC for 48 h (A), or first depleted of specific cell type using mAb and complement, followed by in vitro stimulation with MAC (B). Culture supernatants were collected and assayed for IFN-γ by a sandwich ELISA. The values represent the means ± standard deviations of triplicate determinations. Experiments were repeated two times with similar results. (*) $P < 0.01$, relative to groups treated with the 3T3/puro cells or rIL-18. (**) $P < 0.001$, relative to a 3T3/IL-18-injected group treated with complement alone.

3.5. Treatment with a neutralizing anti-IFN-γ mAb abrogated the resistance to MAC infection in mice injected with the 3T3/IL-18 cells

To further determine whether the increased IFN-γ production resulted in the induction of anti-mycobacterial activity, the mice were injected with the 3T3/IL-18 cells immediately followed by treatment with anti-IFN-γ mAb or isotype control mAb as described in Section 2. One day later, the mice were infected with MAC, and the anti-mycobacterial activities were determined at 3 weeks after MAC infection.

As shown in Table 3, treatment with anti-IFN-γ mAb significantly decreased the anti-mycobacterial activity induced with the 3T3/IL-18 cell vaccination. The numbers of bacteria in the 3T3/IL-18 cell-injected mice with anti-IFN-γ mAb treatment were significantly higher than those in the 3T3/IL-18 cells injected mice with isotype Ab treatment. In addition, the levels of nitric oxide production and cytotoxic activity against P815 cells by lung cells were significantly low in the treated mice with anti-IFN-γ mAb, compared with those in the treated mice with isotype control mAb (Table 3).

Table 3
Effect of anti-mIFN-γ mAb treatment on anti-mycobacterial activity in mice injected with IL-18-secreting fibroblasts

Treatment[a]	Number of bacteria in lung	NO_2^- levels (μM)	Cytolysis (%) at 50:1 (E:T)
3T3/puro	6.2 ± 0.3	58.6 ± 4.8	14.8 ± 2.8
Anti-IFN-γ mAb	5.9 ± 0.2	32.3 ± 5.7	9.1 ± 2.1
Isotype mAb	6.0 ± 0.3	51.8 ± 6.4	13.8 ± 1.8
3T3/IL-18	5.2 ± 0.1	145.8 ± 5.5	31.7 ± 3.5
Anti-IFN-γ mAb	6.2 ± 0.3[b]	36.2 ± 5.1[b]	10.9 ± 4.2[b]
Isotype mAb	5.3 ± 0.2	128.7 ± 6.7	32.0 ± 3.6

[a] Mice were injected with 1×10^7 3T3/puro or 3T3/IL-18 cells and either anti-IFN-γ mAb or isotype mAb, as described in Section 2. One day later, the mice were intranasally infected with 10^5 MAC. The bacterial numbers in lung (the mean log bacterial numbers ± standard deviations of three mice), and nitrate levels and cytotoxic activity in lung cells were determined. The values represent the mean ± standard deviations of triplicate determinations from cultures pooled from three mice infected for 3 weeks. The values are representative of three experiments.

[b] $P < 0.001$, relative to a 3T3/IL-18 cell-injected group treated with isotype mAb.

4. Discussion

IL-18 has been shown to play a central role in the development of immunity against pathogens, by acting on Th1 cell differentiation, cell-mediated cytotoxicity, and inflammation via IFN-γ production. Its role in immunity to intracellular parasites, including *Salmonella thyphimurium*, *Mycobacterium tuberculosis*, *Shigella flexneri*, and *Chlamydia trachomatis* has been well documented [29]. In this report we have demonstrated that the paracrine secretion of IL-18 by the IL-18-gene-transfected fibroblasts induced in vivo persistent anti-mycobactericidal activity, leading to the efficient stimulation of the resistance to MAC infection than free rIL-18. This was the case if the fibroblasts were administered into genetically susceptible BALB/c mice immediately after MAC infection, or at 5 or 10 days postinfection. The efficacy was dependent on the injection dose of the IL-18-secreting cells.

The precise reason for the efficiency with which the 3T3/IL-18 cells induce persistent anti-mycobacterial activity compared with free rIL-18 is not clear. One possibility is that IL-18-transduced fibroblasts may function as an effective vehicle to produce IL-18 for prolonged period.

In our studies, the transfected 3T3 cells were observed in mouse cavity even at 2 weeks postinjection, and the 3T3/IL-18 cells recovered from the cavity still secreted biologically active IL-18. Furthermore, immunization with the 3T3/IL-18 cells did not show toxicities including high fever, weight loss, etc. Lymphoid hyperplasia or tissue necrosis was also not noted in liver, spleen, or lungs in mice receiving the 3T3/IL-18 cells (data not shown). Previous reports showed that fibroblasts genetically engineered to secrete GM-CSF, IFN-γ, IL-2 or IL-12 could suppress tumor growth and induced anti-tumor immunity with minimal toxicity [30–32].

The higher effectiveness of the 3T3/IL-18 cells to induce the resistance to MAC infection, compared with free rIL-18, is closely correlated with the higher levels of nitric oxide production and IFN-γ production, and of cytotoxic activity for prolonged periods in lung cells from the immunized mice with the 3T3/IL-18 cells. Prolonged activation of macrophages by the 3T3/IL-18 cells is likely responsible for the more efficient bacterial killing and resistance to MAC infection because cytotoxic activity and release of reactive nitrogen intermediates are increased in activated macrophages [33–35], and MAC organisms proliferate less rapidly or may be killed by activated macrophages. However, optimal treatment of MAC infection with IL-18 may necessitate the paracrine secretion of IL-18, or need additional adjuvants including other cytokines such as TNF-α [36]. It is of interest to note that plasmid encoding IL-18 alone does not protect against *M. tuberculosis* infection [37], suggesting that a cell vaccine may be a better way of IL-18 delivery in mice than DNA vaccine. In addition, the protective efficacy of IL-18 vaccination against mycobacteria may be different depending on species of mycobacterial pathogens.

Because of IL-18 involvement in the early stage of the cytokine cascade leading to a Th1 type immune response, IL-18 may constitute an ideal cytokine candidate to be used for directing the immune response toward the Th1 type. IL-18 acts in synergy with IL-12 to induce the production of IFN-γ. Since infection with mycobacteria usually results in the secretion of IL-12 [38], the administration of IL-18-secreting cells during mycobacterial infection may result in a synergistic effect leading to high levels of IFN-γ production.

Studies are currently underway to elucidate the role of TNF-α on in vivo persistent anti-mycobacterial activity induced with the 3T3/IL-18 cells because anti-bacterial effects of IFN-γ on intracellular pathogens including mycobacteria were known to primarily mediated by IFN-γ-induced TNF-α [39,40]. It is also under investigation to determine an optimal condition for inducing therapeutic anti-mycobacterial activity in mice using IL-18-secreting fibroblasts (injection times, injection dose, treatment time after MAC infection, etc.).

In summary, IL-18-secreting fibroblasts strongly induce and maintain anti-mycobacterial activity for prolonged period and seem to serve as a safe and efficient means to deliver IL-18 for immunotherapy of MAC infection.

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