IL-18 Promotes Type 1 Cytokine Production from NK Cells and T Cells in Human Intracellular Infection[1]

Verónica E. García,* Koichi Uyemura,* Peter A. Sieling,* María T. Ochoa,* Craig T. Morita,† Haruki Okamura,‡ Masashi Kurimoto,§ Thomas H. Rea,¶ and Robert L. Modlin[2]*‖

We investigated the role of IL-18 in leprosy, a disease characterized by polar cytokine responses that correlate with clinical disease. In vivo, IL-18 mRNA expression was higher in lesions from resistant tuberculoid as compared with susceptible lepromatous patients, and, in vitro, monocytes produced IL-18 in response to *Mycobacterium leprae.* rIL-18 augmented *M. leprae*-induced IFN-γ in tuberculoid patients, but not lepromatous patients, while IL-4 production was not induced by IL-18. Anti-IL-12 partially inhibited *M. leprae*-induced release of IFN-γ in the presence of IL-18, suggesting a combined effect of IL-12 and IL-18 in promoting *M. leprae*-specific type 1 responses. IL-18 enhanced *M. leprae*-induced IFN-γ production rapidly (24 h) by NK cells and in a more sustained manner (5 days) by T cells. Finally, IL-18 directly induced IFN-γ production from mycobacteria-reactive T cell clones. These results suggest that IL-18 induces type 1 cytokine responses in the host defense against intracellular infection. ***The Journal of Immunology,* 1999, 162: 6114–6121.**

Development of an effective immune response to an invading pathogen is achieved by the complex interaction of a variety of immunocompetent cells including T cells, B cells, NK cells, and macrophages. Cytokines produced during the immune response play an important role in host defense, regulating the interaction of these immunocompetent cells and acting as effector molecules in antimicrobial immunity. The pattern of cytokines released by the T cells is pivotal in determining the course of the immune response to microbial pathogens. Type 1 T cells release IL-2 and IFN-γ and are generally associated with resistance to intracellular infection, whereas type 2 T cells release IL-4, which is associated with progressive disease (1–3). In this context, cells from the innate immune response, dendritic cells, monocytes, and NK cells, shape the nature of the subsequent adaptive T cell response by influencing the cytokine pattern (4). In particular, IL-12, a cytokine produced by dendritic cells and monocytes, plays an obligatory role in the generation of type 1 cells (5, 6), promotes NK cell stimulation (7), and induces IFN-γ production from NK and T cells (8, 9).

Recently, IL-18 has been shown to influence both the innate and adaptive immune response by enhancing type 1 cytokine responses of NK and T cells, respectively (10, 11). Macrophages have been shown to produce IL-18 as well as IL-12 (12). IL-18 has been found to have a variety of biological actions, including stimulating proliferation of activated T cells, enhancing NK cell activity, and inducing type 1 cytokine responses (11–14). Several investigators have demonstrated that IL-12 has a synergistic effect with IL-18 on the production of IFN-γ by anti-CD3-activated T cells (11, 14) and anti-CD40-activated B cells (15). Moreover, a recent study has shown that exogenously administrated IL-12 and IL-18 acted synergistically to protect mice against cryptococcal infection (16). Additional studies have indicated a role for IL-18 in immune responses to infection, both in mouse models and human disease (17–21).

Leprosy provides an ideal model to investigate the regulation and role of IL-18 in human infectious disease, because the various clinical manifestations of leprosy have been shown to lie on an immunological spectrum, according to the level of cell-mediated immunity (CMI)[3] to the pathogen, *Mycobacterium leprae* (22). At one extreme, tuberculoid patients are able to restrict the growth of the pathogen, mount strong T cell responses to *M. leprae*, and locally produce the type 1 cytokine pattern (2, 3). In contrast, lepromatous patients manifest disseminated infection, their T cells weakly respond to *M. leprae*, and their lesions express the type 2 cytokines, typical of humoral responses and suppression of CMI (3, 23). In this study, we provide evidence that IL-18 contributes to CMI in human infectious disease.

Materials and Methods

Patients

Patients with leprosy were evaluated at the Los Angeles County Hansen's Disease Clinic and classified according to the criteria of Ridley and Jopling (22). Peripheral blood was collected in heparinized tubes from patients with tuberculoid (T-Lep) and lepromatous (L-Lep) leprosy. After receiving informed consent, skin biopsies from leprosy patients were obtained, embedded in OCT medium (Ames, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −70°C.

*Division of Dermatology and ‖Department of Microbiology and Immunology, University of California, Los Angeles, School of Medicine, Los Angeles, CA 90095; †Division of Rheumatology and Immunology, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115; ‡Hyogo College of Medicine, Mukogawa-cho, Nishinomiya, Japan; §Fujisaki Institute, Hayashibara Biochemical Labs, Fujisaki, Okayama, Japan; and ¶Section of Dermatology, University of Southern California School of Medicine, Los Angeles, CA 90033

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[2] Address correspondence and reprint requests to Dr. Robert L. Modlin, Division of Dermatology, 52–121 CHS, University of California, Los Angeles, School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail address: rmodlin@medicine.medsch.ucla.edu

[3] Abbreviations used in this paper: CMI, cell-mediated immunity; T-Lep, tuberculoid leprosy; L-Lep, lepromatous leprosy; RT, room temperature; IPP, isopentenyl pyrophosphate; HuIGIF, human IFN-γ-inducing factor.

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M. leprae

M. leprae was provided by Dr. Patrick Brennan (Colorado State University, Ft. Collins, CO) and prepared by probe sonication (24). The level of LPS in the *M. leprae* sonicate was measured quantitatively with the *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD) and found to be <1.0 endotoxin U/mg *M. leprae* sonicate. A similar extract was prepared from the virulent *Mycobacterium tuberculosis* strain H37Rv. Stimulation of cells throughout the present study was performed with these sonicated preparations of mycobacteria.

Cell preparations

PBMCs were isolated from heparinized blood by density gradient centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ) and cultured in 24-well plates at 10^6 cell/ml with RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 2 mM glutamine (Life Technologies), 0.1 mM sodium pyruvate (Life Technologies), and 10% of heat-inactivated FBS (HyClone Laboratories, Logan, UT) for 24 or 48 h according to the experiment. Human rIL-18 (Hayashibara Biochemical Laboratories, Okayama, Japan) was obtained from *Escherichia coli* and purified by chromatography as described (12). Human rIL-12 (a generous gift from Hoffman-La Roche, Nutley, NJ) was added to some cultures at final concentration of 1 nM.

To determine which cell population(s) respond to IL-18, $CD3^+$ or $CD56^+$ cells were depleted from PBMCs by two rounds of negative selection using magnetic beads (Dynal, Oslo, Norway). The negative cells were washed, counted, and depletion was evaluated by flow cytometry. Cells were then cultured in 96-well plates at 10^6 cell/ml in RPMI 1640 supplemented as described above, in the presence of *M. leprae* with or without increasing concentrations of IL-18 as shown in the figures. After 24 h, cell-free supernatants were collected, and IFN-γ concentration was determined by ELISA.

Purified monocytes were obtained as described previously (25). Briefly, blood was collected into heparinized syringes containing Plasmagel (Laboratoire Roger Bellon, Neuille, France), and red cells were permitted to sediment to the bottom of the syringe. The cellular supernatant was collected, and the leukocytes were incubated in hypertonic (1.54 M NaCl) PBS. The monocytes resist a change in density in the presence of high salt concentrations as compared with other leukocytes that rapidly become denser under such conditions. Monocytes were harvested by density centrifugation in hypertonic Ficoll-Hypaque (Pharmacia). This protocol routinely gives a monocyte purity of 73 ± 8% monocytes and a yield of 3.4×10^5 monocytes/ml blood. Purified monocytes (1×10^6 cell/ml; 79.6% monocytes) were stimulated in the presence or absence of *M. leprae* for 16 h before RNA isolation.

PBMCs from T-Lep and L-Lep patients were isolated, and adherent cells were prepared as previously described (26). Adherent cells were plated (1×10^6/ml) with *M. leprae* (5 μg/ml) or without Ag at 37°C in a CO_2 incubator with RPMI 1640 and 10% FCS for 3, 16, and 36 h, and total RNA was isolated.

The human myelomonocytic leukemia cell line U-937 was maintained in culture in RPMI 1640 supplemented with 10% FCS. Cells were cultured in 24-well plated at 10^6 cell/ml with *M. leprae*, *M. tuberculosis*, or LPS.

Detection of IL-18 mRNA

PCR for IL-18 mRNA in leprosy lesions and purified monocytes was performed as described (3, 26, 27). RNA was isolated by guanidinium isothiocyanate lysis, phenol-chloroform extraction, and ethanol precipitation. DNA was removed using RNase-free DNase (10 U; Promega, Madison, WI). First strand cDNA was synthesized using Superscript reverse transcriptase (Life Technologies Life Science, Bethesda, MD) and oligo dT priming (Pharmacia). cDNA samples were amplified with IL-18-specific PCR primers and *Taq* polymerase (Perkin-Elmer, Norwalk, CT) in a DNA thermocycler (Perkin-Elmer). The PCR conditions consisted of 40 cycles of denaturation (94°C for 30 s) and annealing-extension (65°C for 45 s). For comparison of IL-18 mRNA levels among different samples, cDNA concentrations were normalized to yield equivalent β-actin PCR products. PCR products were electrophoresed on 1.5% agarose gels, transferred to nylon membranes (Amersham), probed with a ^{32}P-labeled IL-18 oligonucleotide internal to the PCR primers, and visualized by autoradiography. The sequences of primer pairs, 5′ and 3′, were as follows: 5′-GCTT GAATCTAAATTATCAAGTC, 3′-GAAGATTCAAATTGCATCTTAT. The IL-18 probe sequence was as follows (5′ to 3′): TGACTGTA GAGATAATGCACCCCGGACC.

Blocking experiments

PBMCs from patients were cultured with *M. leprae* alone or in the presence of neutralizing anti-IL-18 mAb (10 μg/ml), anti-IL-12 mAb (10 μg/ml; Endogen, Cambridge, MA), or isotype control mouse IgG (Becton Dickinson, San Jose, CA) for 48 h and then assayed for IFN-γ production by ELISA. In these series of experiments, cells were incubated with the neutralizing mAb for 2 h before the addition of the Ag. Anti-IL-18 mAb (BALB/c mouse IgG1κ, purity 90%) was obtained from Hayashibara Biochemical Laboratories (mAb human IFN-γ-inducing factor (HuIGIF) 125-2H) and used at 10 μg/ml according to the reported activity (1 μg/ml mAb-HuIGIF 125-2H neutralizes 5ng/ml rHuIGIF).

T cell clones and T cell lines

The T cell clones and cell lines used in this study have been described (28, 29). $\gamma\delta$ T cells (HF.2, 1×10^5) were plated in microtiter plates and stimulated with isopentenyl pyrophosphate (IPP; 3 μM; Sigma, St. Louis, MO) in the absence of APC. We used the HF.2 $\gamma\delta$ T cell clone that specifically recognizes IPP, a metabolite found in prokaryotic and eukaryotic cells (30). The CD1b-restricted T cell line LDN4 was cultured with Ag lipoarabinomannan in the presence of $CD1^+$ APC as described (29). rIL-18 was added to the $\gamma\delta$ T cells or to the double-negative T cells at different concentrations as indicated in the legend to Fig. 9. After overnight incubation, supernatants were collected, and IFN-γ levels were measured by ELISA.

ELISA for cytokines

The concentration of IL-18 in the culture supernatants was measured by ELISA as described (31). In brief, 96-well ELISA plates (Corning Glass Works, Corning, NY) were coated with mAb 125-2H (20 μg/ml in PBS) at room temperature (RT) for 3 h and blocked with PBS containing 1% BSA at 4°C overnight. Standard human IL-18 (Hayashibara Biochemical Laboratories) (Ref. 12) and samples were added and incubated at RT for 2 h. Peroxidase-conjugated anti-IL-18 (159-12B, 0.5 μg/ml) was added to each well, and the plate was incubated at RT for 2 h. Peroxidase substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and the plates were read in an ELISA reader (Cambridge Technology, Watertown, MA) at a wavelength of 405 nm. Washing steps (PBS containing 0.1% BSA and 0.05% Tween 20) were included between each step of the ELISA. A standard curve was made by plotting the duplicate readings for the standards vs the concentration of the standards, and regression analysis was applied. The IL-18 concentration of each sample was calculated by regression analysis using the mean absorbance (average of triplicate readings) of the sample.

IFN-γ (Endogen) ELISA was performed according to the manufacturer's instructions. Briefly, flat-bottom 96-well microtiter plates were coated with mouse anti-human IFN-γ mAb (M-700A; Endogen) at 5 μg/ml in sodium carbonate buffer, pH 9.6, and incubated overnight at 4°C, followed by blocking with PBS containing 1% BSA for 1 h at RT. Samples and IFN-γ standard (human rIFN-γ, R-IFN-γ-50; Endogen) were serially diluted and incubated at RT for 3 h. Subsequently, biotinylated anti-IFN-γ mAb (M-701-B; Endogen) was added at 2 μg/ml for 1 h at RT. Avidin-peroxidase conjugate (A-3151; Sigma) was then added at 2 μg/ml for 30 min at RT. Peroxidase substrate solution (Kirkegaard & Perry Laboratories) was added, and the plates were read in an ELISA reader (Cambridge Technology) at a wavelength of 405 nm. Washing steps (PBS containing 0.1% BSA and 0.05% Tween 20) were included between each step of the ELISA. A standard curve was made and regression analysis was applied. The IFN-γ concentration of each sample was calculated by regression analysis using the mean absorbance (average of triplicate readings) of the sample. The sensitivity of this assay was 10 pg/ml.

Intracellular analysis of cytokine production

Intracellular cytokine staining was used to determine the IFN-γ production at the single cell level as previously described (32). Briefly, to induce the intracellular accumulation of newly synthesized proteins, monensin (2 μM; Calbiochem, La Jolla, CA) was added for 2 h to the cells in culture. Cells were then harvested and stained for surface expression with mAbs specific for CD56 (Becton Dickinson) and CD3 (Becton Dickinson). After washing with PBS-2% FCS, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at RT. Cells were washed with PBS-2% FCS and permeabilized with 0.5% (w/v) saponin (Sigma) in PBS during 30 min at RT. PE-conjugated anti-IFN-γ (used at the manufacturer's recommended concentrations; PharMingen, San Diego, CA) was added to the permeabilized cells and allowed to bind for 30 min. Cells were then washed with PBS-0.5% saponin and finally with PBS/2% FCS to allow membrane closure. Samples were analyzed on a FACScan flow cytometer, and data were analyzed using LYSIS software (Becton Dickinson). Negative control samples were

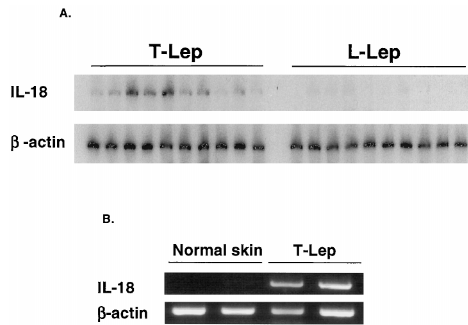

FIGURE 1. *A*, IL-18 mRNA in leprosy lesions. RNA was isolated from leprosy lesions by guanidinium isothiocyanate lysis, phenol-chloroform extraction, and ethanol precipitation. First strand cDNA was synthesized and cDNA samples were amplified using IL-18-specific PCR primers (see *Materials and Methods*). For comparison of IL-18 mRNA levels among different samples, cDNA concentrations were normalized to yield equivalent β-actin PCR products. Electrophoresed PCR products for β-actin and IL-18 were probed with a radiolabeled oligonucleotide internal for each primer. *B*, IL-18 mRNA in skin biopsies from healthy donors. PCR for IL-18 mRNA in skin biopsies was performed as described above. Ethidium bromide-stained agarose gels are shown.

incubated with irrelevant isotype-matched Abs in parallel with all experimental samples.

Statistical analysis

Nonparametric statistical analysis by the Signed rank test for paired samples was used for comparison of cells after culture. Values of $p < 0.05$ were considered significant.

Results

Expression of IL-18 mRNA in leprosy lesions and in monocytes

To determine the relative distribution of IL-18 in leprosy patients, we performed PCR on RNA derived from leprosy lesions. cDNAs were synthesized and were normalized to amounts of β-actin PCR products as a measure of total cellular RNA. Our results showed that IL-18 mRNA could be detected in the majority of the T-Lep patients (7 of 10), whereas it was undetectable in the L-Lep patients (Fig. 1*A*). Therefore, the distribution of IL-18 in leprosy, parallels the level of CMI to *M. leprae*.

As IL-18 mRNA is constitutively expressed in various human tissues (12), and given our finding that T-Lep patients expressed IL-18 mRNA in lesions and L-Lep patients did not, we wanted to determine the levels of IL-18 mRNA in skin biopsies from healthy donors. Our results showed that the levels of IL-18 mRNA in skin from normal individuals were undetectable as compared with T-Lep patients (Fig. 1*B*).

It has been shown that IL-18 mRNA could be detected in the total RNA of human unstimulated PBMCs, with no significant changes of IL-18 mRNA expression after mitogen stimulation (12). Because human IL-18 is known to be produced by cells of the monocyte lineage (12), we investigated whether *M. leprae* could stimulate detectable levels of IL-18 mRNA in purified human monocytes compared with nonstimulated cells. RT-PCR analysis demonstrated a significant induction of IL-18 mRNA expression in the presence of *M. leprae* (Fig. 2*A*).

We next performed experiments to investigate the levels of IL-18 mRNA in monocytes from leprosy patients after *M. leprae* stimulation. We found low levels of IL-18 mRNA in unstimulated PBMCs from T-Lep patients, while a considerable induction of IL-18 mRNA was achieved by *M. leprae* stimulation. Undetectable or very low levels of IL-18 mRNA were measured in PBMCs from L-Lep patients, and *M. leprae* stimulation did not induce IL-18 mRNA in L-Lep patients (Fig. 2*B*). These results correlate with the expression of IL-18 mRNA in lesions.

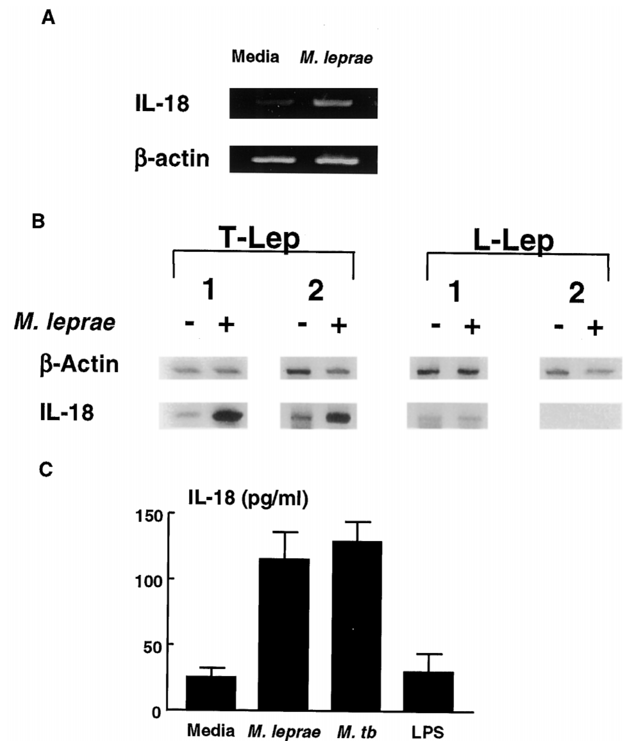

FIGURE 2. *A*, IL-18 mRNA detection in human monocytes. cDNA were derived from monocytes cultured with or without *M. leprae* for 16 h and normalized to yield equivalent β-actin PCR products. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The shows one representative experiment of three. *B*, IL-18 mRNA detection in adherent cells from leprosy patients. PBMCs from T-Lep and L-Lep patients were isolated, and adherent cells were prepared as previously described (26). Adherent cells were plated with *M. leprae* (5 μg/ml) or without Ag at 37°C in a CO_2 incubator with RPMI 1640 and 10% FCS for 16 h, and total RNA was isolated. PCR for β-actin and IL-18 was performed as described above. Southern blots probed with IL-18 or β-actin are shown. *C*, *M. leprae* induces IL-18 production. Cells from the human leukemia cell line U-937 were stimulated with *M. leprae* (5 μg/ml), *M. tuberculosis* (2 μg/ml) or LPS (1 μg/ml). Cell-free supernatants were collected at 20 h and assayed for IL-18 by ELISA. Values are expressed as mean of triplicate determinations.

M. leprae-*stimulated IL-18 release*

We next investigated whether IL-18 protein was released upon stimulation with *M. leprae*. To investigate this possibility, we stimulated human monocytic cell lines, monocytes, and PBMCs with LPS or with mycobacteria. Stimulation of the monocytic cell line U937 with mycobacteria showed that IL-18 was barely detectable (limit of detection = 10 pg/ml) in cultures grown in the absence of mycobacteria. In contrast, *M. leprae* and *M. tuberculosis* stimulated detectable IL-18 protein, while stimulation with LPS also induced IL-18 (Fig. 2*C*). *M. leprae* also induced considerable levels of IL-18 after stimulation of THP-1 cells (THP-1 mycobacterial stimulation, 450 pg/ml). While specific Ag stimulation of human cell lines induced considerable levels of IL-18 protein, low levels of IL-18 were detected after stimulation of either monocytes (LPS stimulation, 90 pg/ml; mycobacterial stimulation, 30 pg/ml) or PBMCs (LPS stimulation, 25 pg/ml; mycobacterial stimulation, 35 pg/ml). Other investigators have studied the production of IL-18 after microbial infection (21, 33) with variable results; the low

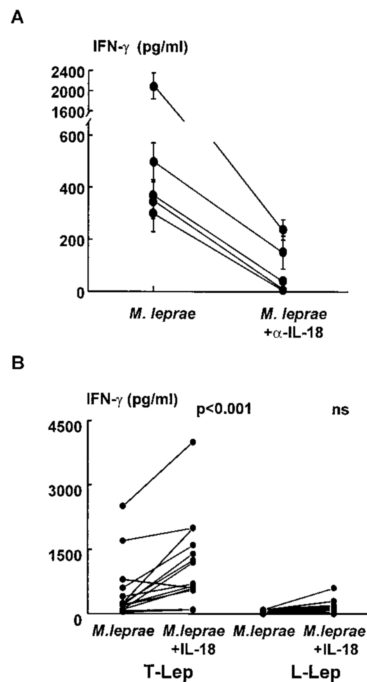

FIGURE 3. Effect of IL-18 on *M. leprae*-induced IFN-γ production. *A*, Anti-IL-18 inhibition of *M. leprae*-induced IFN-γ production. Representative results from five T-Lep patients showing inhibition of IFN-γ production to *M. leprae*. Neutralizing anti-IL-18 mAb (10 μg/ml) was added to PBMCs cultures in the presence of *M. leprae*, and IFN-γ production was measured by ELISA after 48 h. Isotype-matched control mAb (mouse IgG1, 10 μg/ml) showed no inhibition of *M. leprae*-induced IFN-γ production. Values are expressed as mean ± SEM of triplicates determinations. *B*, *M. leprae*-specific IFN-γ production from leprosy patients in the presence of IL-18. PBMCs from T-Lep and L-Lep patients were stimulated with *M. leprae* in the presence or absence of IL-18 (10 ng/ml). Cell-free supernatants were collected at 20 h and assayed for IFN-γ by ELISA. Values are expressed as the mean of triplicate determinations. The *p* values shown were calculated using the Signed rank test for paired samples by comparing the median of the levels of IFN-γ measured in the *M. leprae*-stimulated group of patients vs the median of the levels of IFN-γ measured in the *M. leprae* plus IL-18-stimulated group of patients.

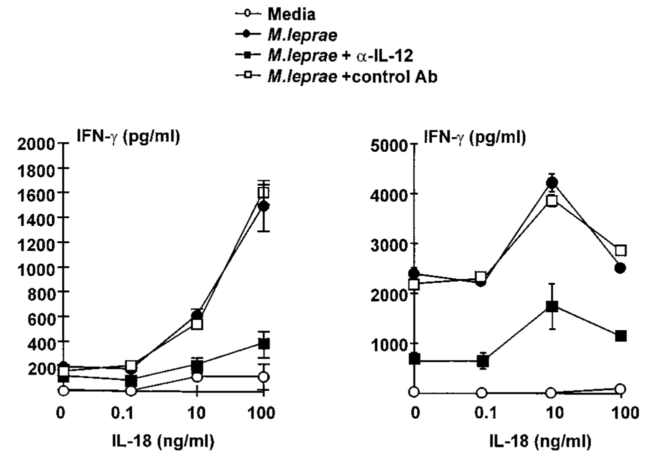

FIGURE 4. Effect of anti-IL-12 mAb on *M. leprae*-specific IFN-γ production in the presence of IL-18. PBMCs from two T-Lep patients were stimulated with *M. leprae* plus increasing amounts of IL-18, in the presence or absence of anti-IL-12 mAb (10 μg/ml) or isotype control Ab (10 μg/ml), and IFN-γ production was measured by ELISA after 48 h. Values are expressed as the mean ± SEM of triplicates determinations.

level of IL-18 protein may be explained by absorption by T and B cells in the culture (20).

In a separate set of experiments, neutralizing anti-IL-18 Abs were used to determine whether *M. leprae*-induced IFN-γ production was dependent on endogenous IL-18 production. PBMCs from T-Lep patients were stimulated with *M. leprae* in the presence or absence of neutralizing anti-IL-18 Ab or an isotype control Ab. As shown in Fig. 3*A*, the IFN-γ levels induced in response to Ag were inhibited by 80–99% in T-Lep patients, demonstrating that endogenous IL-18 production contributes to *M. leprae*-induced IFN-γ production.

Effect of IL-18 on the specific response to M. leprae

It has been reported that human IL-18 showed significant IFN-γ-inducing activity when human PBMCs were stimulated with anti-CD3, ConA, or IL-2, but not when unstimulated PBMCs were used (12). We next examined whether IL-18 could modify the response of human PBMCs to a specific Ag. To address this question, we stimulated PBMCs from a large group of leprosy patients in the presence or absence of rIL-18. Fig. 3*B* illustrates that IL-18 significantly increased *M. leprae*-induced IFN-γ production from T-Lep patients ($p < 0.001$). No effect of IL-18 on IFN-γ production was observed in the absence of Ag (data not shown).

Given the dependence of *M. leprae*-induced T cell proliferation on IL-12, we investigated whether IL-18 could enhance Ag-induced T cell responses in L-Lep patients. Our results showed that, in marked contrast with the effect of IL-12 on *M. leprae*-specific T cell responses, IL-18 did not augment Ag-induced IFN-γ production from L-Lep patients (Fig. 3*B*). The addition of rIL-18 had no effect on IL-4 production, either from T-Lep or from L-Lep patients (data not shown).

In an attempt to investigate the interaction of IL-18 and IL-12 in the immune response to *M. leprae*, we first studied the effect of neutralizing anti-IL-12 Ab on the *M. leprae*-induced IFN-γ production in the presence of IL-18. PBMCs from T-Lep patients were stimulated with *M. leprae* and IL-18 in the presence or absence of anti-IL-12 Ab or an isotype control Ab. The results showed that IFN-γ production to *M. leprae* in the presence of IL-18 was inhibited by 50–80% by anti-IL-12 (Fig. 4), suggesting that IL-18 and IL-12 collaborate in the immune response against *M. leprae*.

We next performed experiments using both neutralizing anti-IL-12 and anti- IL-18 mAbs to further investigate the interaction of these cytokines on IFN-γ production after stimulation with *M. leprae*. PBMCs from T-Lep patients were stimulated with Ag in the presence or absence of anti-IL12 Ab, anti-IL-18 Ab, or anti-IL-12 plus anti-IL-18 Abs. The results showed that both neutralizing mAbs considerably inhibited *M. leprae*-induced IFN-γ production, indicating that the two cytokines are involved in the immune response against *M. leprae* (Fig. 5). Moreover, when IL-12 and IL-18 were added together to PBMCs from T-Lep patients in culture with *M. leprae*, a significant increase in the IFN-γ production was observed (Fig. 6). These data suggest that IL-12 and IL-18 act together to enhance IFN-γ production in response to *M. leprae*.

IL-18 rapidly induces NK cell IFN-γ production to M. leprae

To identify the population(s) of cells that respond to IL-18, depletion of $CD3^+$ cells or $CD56^+$ cells were performed from PBMCs of T-Lep patients. After the depletion, the cells were stimulated with *M. leprae* in the presence or absence of IL-18 for 24 h. *M. leprae*-stimulation of the CD3-depleted population induced high levels of IFN-γ in response to IL-18 (Fig. 7). In contrast, *M. leprae*-stimulation of CD56-depleted cells did not induce detectable

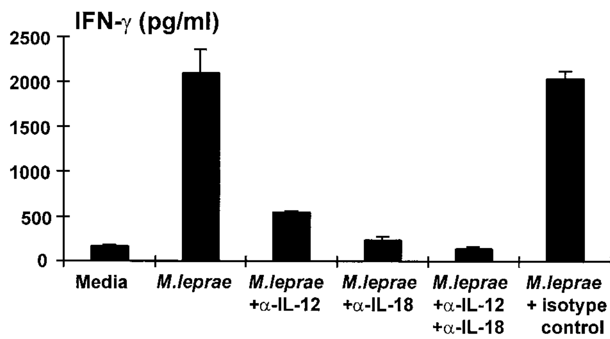

FIGURE 5. Comparison of the effect of neutralizing anti-IL-12 mAb and anti-IL-18 mAb on *M. leprae*-specific IFN-γ production. PBMCs from T-Lep patients were stimulated with *M. leprae*, *M. leprae* plus anti-IL-12, *M. leprae* plus anti-IL-18, *M. leprae* plus anti-IL-12 plus anti-IL-18, or *M. leprae* plus isotype control Ab. Cells were cultured for 48 h, and supernatants were collected and assayed for IFN-γ by ELISA. The data show the results from one representative experiment of three. Values are expressed as the mean ± SEM of triplicates determinations.

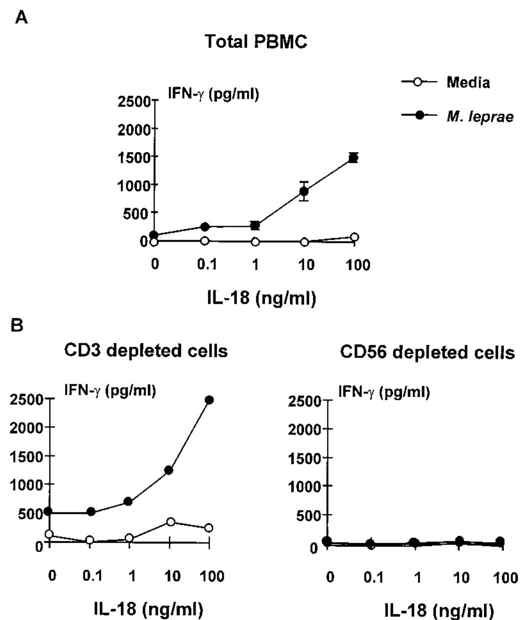

FIGURE 7. IL-18 stimulates *M. leprae*-induced IFN-γ production from NK cells. *A*, PBMCs from a T-Lep patient were stimulated with IL-18 in the presence or absence of *M. leprae*, and IFN-γ production was measured by ELISA after 24 h. Values are expressed as the mean ± SEM of triplicates determinations. *B*, Negative selection with cells from the same patient was performed with specific Abs as described in *Material and Methods*, and the purified populations were stimulated with IL-18 in the presence or absence of *M. leprae*. IFN-γ production was measured by ELISA after 24 h. The results show one representative experiment of three.

levels of IFN-γ in response to IL-18 (Fig. 7). These results suggest that NK cells were involved in the *M. leprae*-induced IFN-γ production in response to IL-18 after 24 h of stimulation. To further confirm the role of NK cells in the observed response, additional experiments using triple-color flow cytometry were performed with PBMCs from T-Lep patients. PBMCs were stimulated with *M. leprae* in the presence or absence of IL-18. After 24 h of incubation, surface staining for CD3 and CD56 and intracellular staining for IFN-γ was performed. As shown in this representative donor, 20% of $CD56^+$ cells were positive for intracellular IFN-γ (Fig. 8*A*, *lower panel*), whereas $<$8% of the $CD56^-$ cells produced IFN-γ (Fig. 8*A*, *lower panel*), with no increase in the number of $CD3^+$ cells producing IFN-γ (data not shown). These data confirmed that IL-18 induces an early IFN-γ response by NK cells.

IL-18 stimulates T cell-IFN-γ production to M. leprae

In an attempt to determine whether IL-18 induces an IFN-γ response in *M. leprae*-stimulated T cells, Ag-stimulated PBMCs from T-Lep patients were cultured in the presence or absence of IL-18 for 5 days. Triple-color flow cytometry for CD3, CD56, and intracellular IFN-γ was then performed. Fig. 8*B* illustrates the results from one representative donor of five. After 5 days of Ag-stimulation, IL-18 induced a clear increase in the percentage of IFN-γ-producing cells bearing the CD3 marker (27%), whereas no changes in the levels of IFN-γ produced by the $CD3^-$ population were detected ($<$7%; Fig. 8*B*). Moreover, when we analyzed the $CD56^+$ population that responded to the Ag in the presence or absence of IL-18, our results showed that 10% of $CD56^+$ cells produced IFN-γ^+ ($CD56^+$ IFN-γ^+) upon stimulation with *M. leprae,* but no change was detected in response to *M. leprae* plus IL-18 (Fig. 8*B*). As $CD56^+$ cells include two populations, NK cells and T cells, which can be differentiated by the expression of CD3, our results showed that, after 5 days of Ag-stimulation, IL-18 induced no change in the percentage of NK cells producing IFN-γ, but clearly increased the percentage of IFN-γ-producing cells bearing the CD3 marker.

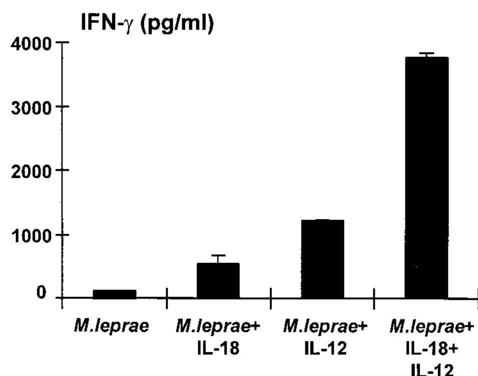

FIGURE 6. Synergistic effect of IL-18 and IL-12 on *M. leprae*-specific IFN-γ production. PBMCs from T-Lep patients were cultured with *M. leprae*, *M.leprae* plus rIL-18, *M. leprae* plus rIL-12, or *M. leprae* plus rIL-18 plus rIL-12, and IFN-γ production was measured by ELISA after 24 h. The data show the results from one representative patient of five. Each bar represents the mean of triplicates determinations ± SEM.

To confirm the effect of IL-18 on Ag-stimulated T cells, we stimulated T cell lines with Ag, in the presence or absence of IL-18, for 12 h, and IFN-γ levels were measured. In these experiments, γδ T cells and double-negative αβ T cells were used. Stimulation of the γδ T cell clone HF.2 with increasing concentrations of IL-18 in the presence of IPP induced high levels of IFN-γ as compared with stimulation in the absence of Ag (Fig. 9*A*). Moreover, stimulation of double-negative cells from the T cell line LDN4 with IL-18 in the presence of lipoarabinomannan induced higher levels of IFN-γ as compared with stimulation with the cytokine in the absence of Ag (Fig. 9*B*). These data show that IL-18 considerably enhanced the IFN-γ production from Ag-stimulated T cells.

Discussion

In the present study, we investigated the role of IL-18 in the generation of type 1 responses to mycobacterial infection. We found that *M. leprae* induced IL-18 release from human monocytic cell lines in vitro. Using leprosy as a model, we found that IL-18

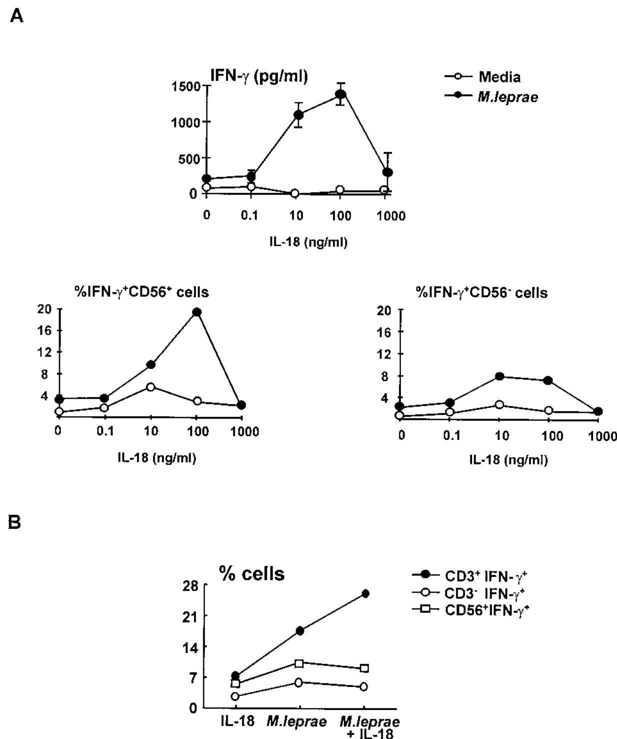

FIGURE 8. IFN-γ expression by PBMCs from a T-Lep patient in response to IL-18 in the presence or absence of *M. leprae*. *A*, PBMCs from a T-Lep patient were stimulated as described for 24 h, and IFN-γ was measured by ELISA. Values are expressed as the mean ± SEM of triplicate determinations (*upper panel*). After 12 h of stimulation as described, surface staining for CD56 and CD3 mAbs was performed, and cells were fixed and stained with mAb against IFN-γ (*lower panel*). The data show the results of one representative experiment of five. *B*, Intracellular IFN-γ expression by PBMCs from a T-Lep patient in response to IL-18 in the presence or absence of *M. leprae* after 5 days of stimulation. After stimulation as described, surface staining for CD56 and CD3 mAbs was performed, and cells were fixed and stained with mAb against IFN-γ. The data show the results of one representative experiment of five.

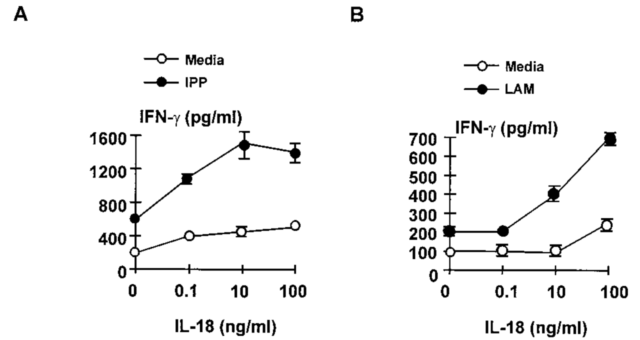

FIGURE 9. Effect of IL-18 on Ag-stimulated T cells. *A*, γδ T cells (clone HF.2) were stimulated with increasing concentrations of IL-18, in the presence or absence of IPP as described in *Materials and Methods*, and IFN-γ measured by ELISA after 12 h. *B*, Double-negative T cells (LDN4) were stimulated with lipoarabinomannan (LAM) in the presence of APC as described in *Materials and Methods* and IFN-γ measured by ELISA after 12 h. Values are expressed as the mean ± SEM of triplicate determinations. The data represents one representative experiment of six.

mRNA expression was higher in lesions from resistant T-Lep patients as compared with susceptible L-Lep patients. Furthermore, high levels of IL-18 mRNA were induced in PBMCs from T-Lep patients after *M. leprae* stimulation, while low IL-18 mRNA expression was detected in Ag-stimulated PBMCs from L-Lep patients. We showed that the addition of rIL-18 augmented *M. leprae*-induced IFN-γ (but not IL-4) production in T-Lep patients as compared with L-Lep patients. A combined effect of IL-12 and IL-18 in promoting *M. leprae*-specific type 1 responses was also demonstrated. Finally, we characterized the cell populations involved in the early and late response to *M. leprae* in the presence of exogenous IL-18, demonstrating an important role of NK cells in the early response (1 day), followed by a delayed (5 day) response by T cells. Taken together, these data indicate that IL-18 is part of the matrix of proinflammatory cytokines produced by monocytes, which contributes to CMI against infection in humans.

IL-18 mRNA was induced by *M. leprae*, although protein expression was low. However, IL-18 was locally detected in leprosy lesions from T-Lep patients, the group of individuals with greater CMI to *M. leprae.* The local production of IL-18 in tuberculoid lesions, along with the production of IL-12 (3), would be expected to facilitate the type 1 cytokine response necessary for CMI against infection by the pathogen. Conversely, low levels of IL-18 mRNA were found in L-Lep patients, the group of individuals with weak CMI to *M. leprae*. The low levels of IL-18, along with the low levels of IL-12 in these patients, likely contribute to the weak IFN-γ response in this group. Recent studies have suggested that IL-18 play a role in the host defense against a range of microbial pathogens (16–18, 21, 34). Regarding bacterial infection, it was suggested that IL-18 might participate in the clearance of *Yersinia* infection (18) and in the protection against experimental *M. leprae* infection in mice (19). In humans, several strains of nonpathogenic *Lactobacillus* and pathogenic *Streptococcus* were shown to induce IL-18 production in PBMCs (20). Our present data suggest that the local production of IL-18 in leprosy appears to have an integral role in determining the nature of the host immune response.

Previously we have demonstrated that IFN-γ production in response to microbial Ag is enhanced by IL-12 and IL-15 (35). Our present data also indicated that IL-18 augmented IFN-γ production in response to *M. leprae*. To elucidate the mechanism for IFN-γ production by IL-18, we studied the cell populations involved in this response. We found that upon stimulation with *M. leprae*, IL-18 induced an early IFN-γ response from NK cells, mediators of innate immunity, and a later IFN-γ response from T cells, mediators of adaptive immunity. The effect of IL-18 on Ag-stimulated T cells was further confirmed using T cell lines. For this purpose we stimulated αβ and γδ T cell lines with specific Ags. The innate immune response pertains to those cells preprogrammed to respond to non-self stimuli, in contrast to the adaptive response that involves the selection and expansion of immune cells, such as T and B cells, with the development of immunologic memory (4). The production of IL-18 from cells of both the innate and adaptive immune responses would allow these two arms of the immune system to act in concert, to cooperate in the induction of IFN-γ release to generate CMI against the pathogen.

Specific unresponsiveness of T cells to *M. leprae* is the primary immunologic characteristic of L-Lep patients. It has been shown that rIL-12 augmented *M. leprae*-specific T cell proliferation in L-Lep patients, increasing T cell IFN-γ production (23). Because IL-18 was demonstrated to share some of its biological activities with IL-12 (12, 14), and given the dependence of *M. leprae*-induced T cell proliferation on IL-12, we investigated whether IL-18 could enhance Ag-induced T cell responses in L-Lep patients. However, while IL-18 increased the type 1 cytokine response from T-Lep patients, L-Lep patients showed no response to IL-18, demonstrating that, in marked contrast with the effect of IL-12 on *M. leprae*-specific T cell responses, L-Lep patients are unresponsive

to IL-18 stimulation. The mechanism for this unresponsiveness requires further investigation. However, it was recently demonstrated that IL-12 and IL-18 act differentially to activate IFN-γ gene transcription in primary human CD4$^+$ T cells (36). It was shown that while both AP-1 and STAT4 are required for IL-12-dependent IFN-γ promoter activation, IL-18 causes direct promoter activation via AP-1. It has been suggested that this differential activation of the IFN-γ promoter gives further insights into molecular pathways governing Th1 T cell development and differentiation (36). Thus, the mechanism whereby IL-18 enhances *M. leprae*-specific IFN-γ production might be through direct activation of AP-1 site in the IFN-γ promoter (36).

Recent studies have demonstrated that IL-18 and IL-12 synergistically induced the production of IFN-γ by NK, T, and B cells (10, 11, 13, 15). In addition, exogenously administrated IL-12 and IL-18 acted synergistically to protect mice against cryptococcal infection (16). These findings may suggest that the two IFN-γ-inducing cytokines synthesized in vivo collaborate and strengthen their protective activities against infection by inducing the production of IFN-γ. Using double knockout mice lacking both IL-18 and IL-12, these cytokines have been shown to induce NK activity as well as in vivo Th1 development (37). Moreover, Robinson et al. showed marked synergy between IL-18 and IL-12 in inducing IFN-γ production from differentiating and committed Th1 cells from mice, suggesting that both IL-12 and IL-18 are required for expression of the Th1 phenotype (38). Our studies demonstrated that IL-18 and IL-12 collaborate in modulating Ag-specific responses by inducing IFN-γ.

In conclusion, we investigated the role of IL-18 in the immune response to *M. leprae* in the hope of developing a model to explain the profound cytokine differences observed in the polar manifestation of leprosy. The local production of the type 1 cytokine pattern in T-Lep patients would promote CMI responses to *M. leprae*, allowing them to control the growth of the bacteria and to respond to the infection. On the contrary, the absence of type 1 cytokine responses in L-Lep patients would contribute to the weak or absent CMI responses to the pathogen, resulting in progressive infection. We have demonstrated that IL-18 can up-regulate NK and T cell functions against *M. leprae*, providing evidence that IL-18 is an important mediator in generation of the type 1 cytokine response in human infectious disease.

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