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Research Article

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Interferon-γ Differentially Regulates Interleukin-12 and Interleukin-10 Production in Leprosy

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Abstract

The ability of monocytes to influence the nature of the T cell response to microbial pathogens is mediated in part by the release of cytokines. Of particular importance is the release of IL-12 and IL-10 by cells of the monocyte/macrophage lineage upon encountering the infectious agent. IL-12 promotes cell mediated immunity (CMI) to intracellular pathogens by augmenting T-helper type 1 responses, whereas IL-10 downregulates these responses. The ability of IFN-γ to modulate the balance between IL-12 and IL-10 production was examined by studying leprosy as a model. In response to *Mycobacterium leprae* stimulation, IFN-γ differentially regulated IL-12 and IL-10 production resulting in upregulation of IL-12 release and downregulation of IL-10 release. Furthermore, we determined that the mechanism by which IFN-γ downregulates IL-10 was through the induction of IL-12. The data suggest a model of lymphocyte–monocyte interaction whereby the relative presence or absence of IFN-γ in the local microenvironment is a key determinant of the type of monocyte cytokine response, and hence the degree of CMI in the host response to infection. (*J. Clin. Invest.* 1997. 99:336–341.) Key words: cytokine • monocytes • infection • mycobacteria • immunity

Introduction

An invading pathogen encounters two systems of immunity in its interaction with a host: the innate immune response and the adaptive immune response. The innate immune response pertains to those cells that are preprogrammed to respond to nonself stimuli, and includes cells of the monocyte/macrophage lineage. This is in contrast to the adaptive response, which involves the selection and expansion of immune cells, such as T and B cells, with the development of immunologic memory. For intracellular pathogens, the pattern of cytokines released by the T cells determines the nature of the immune response. 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).

Cells of the innate immune response not only serve as a first line of defense, but via cytokine release, also shape the nature of the subsequent adaptive immune response by influencing the T cell cytokine pattern. The ability of monocytes to regulate development of the adaptive type 1 and type 2 T cell cytokine patterns is mediated in part by the secretion of IL-12 and IL-10.

IL-12 is produced by activated monocytes as a p70 heterodimeric protein composed of p35 and p40 subunits. It is the p40 subunit which is considered the regulatory component for IL-12 expression (4). IL-12 production in response to microbial pathogens leads to activation of natural killer (NK) cells and T cells with ensuing production of IFN-γ. In the presence of high IFN-γ and low IL-4, there is subsequent development of a type 1 T cell cytokine response and vigorous cell-mediated immunity (CMI)[1] (5–7). In contrast, IL-10 was originally described as a factor secreted by type 2 T cells that inhibits cytokine synthesis and proliferation of type 1 T cells (8). Subsequently, it has been shown that monocytes are also a major source of IL-10 (9). Thus, the balance between monocyte-derived IL-12 and IL-10 is critical to the outcome of T cell cytokine responses.

The regulation and role of IL-12 and IL-10 in human infectious disease can be approached by investigating leprosy as a model. The various clinical manifestations of this disease have been shown to lie on an immunological spectrum, according to the level of CMI to the pathogen, *Mycobacterium leprae* (10). 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). IL-12 p40 mRNA levels and the number of IL-12 p70 protein containing cells are greatest in these lesions while IL-10 mRNA levels are low (11). 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 immunosuppression of CMI. Here, IL-10 expression is dominant while the IL-12 levels are extremely low (2, 11). In this study, we investigated the regulation of IL-12 and IL-10 production in leprosy patients in the hope of developing a model to explain the profound cytokine differences observed in the polar manifestations of this human infectious disease.

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1. *Abbreviation used in this paper:* CMI, cell-mediated immunity.

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 (10). Peripheral blood was collected in heparinized tubes from patients with tuberculoid and lepromatous leprosy, as well as from normal healthy controls.

M. leprae. Mycobacterium leprae was provided by Dr. Patrick Brennan (Colorado State University, Ft. Collins, CO) and prepared by probe sonication. The level of LPS in the *M. leprae* sonicate was measured quantitatively with a Limulus Amoebocyte Lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD) and found to be < 1.0 endotoxin U/mg *M. leprae* sonicate. With each individual's monocytes, LPS-induced levels of IL-12 p40 and IL-10 were measured using a concentration of LPS 50 times greater than the amount present in the *M. leprae* sonicate. In every case, the cytokine levels were either undetectable or $\leq$ 1/10 the *M. leprae*-induced levels (data not shown).

Collection of monocyte supernatants. PBMC were isolated on Ficoll-Paque gradients (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and cultured (5×10^5/well) for 2 h in 96 well plates at 37°C in a CO_2 incubator with RPMI 1640 and 10% heat inactivated FBS. Nonadherent cells were removed and the medium replaced with the addition of *M. leprae* sonicate (5 μg/ml). Cytokines and/or their neutralizing antibodies were added to some cultures; supernatants were harvested 20 h later and stored at −20°C until assayed for cytokine levels by ELISA. All samples were assayed in duplicate.

Addition of cytokines and/or neutralizing antibodies. Human cytokines and/or their neutralizing antibodies were added to some cultures at final concentrations as follows: 200 U/ml IFN-γ, recombinant human (Endogen Inc., Cambridge, MA); 100 U/ml rIL-10 (gift of Dr. Kevin Moore, DNAX Research Institute, Palo Alto, CA); 1 nM rIL-12 (gift of Dr. Maurice Gately, Hoffman–La Roche Inc., Nutley, NJ); 100 ng/ml (neutralizes 100 U IFN γ/ml) neutralizing mouse mAb, anti-IFN-γ (IgG2a, Genzyme Diagnostics, Cambridge, MA); neutralizing rat mAbs, anti-IL-12 24A-1 (IgG2b specific for p40), and 2OC2 (specific for p70) (gift of Dr. Maurice Gately, Hoffman–La Roche Inc.) at 1:1 mix of 5 μg/ml. Addition of the cytokines or their neutralizing antibodies by themselves did not result in any measurable IL-12 p40, p70, or IL-10 release (data not shown).

ELISA for IL-12 p40 and p70. 96 well ELISA plates (Corning Glass Works, Corning, NY) were coated overnight at 4°C with 100 μl of rat anti-human IL-12 mAb (2.5 μg/ml, mAb 2-4A1 [specific for p40] or mAb 2OC2 [specific for p70]; gift of Dr. Maurice Gately, Hoffman–La Roche Inc.). Plates were incubated with 150 μl of 1% BSA (Fisher Biotech, Pittsburgh, PA) in PBS for 1 h at room temperature. 100-μl aliquots of each sample were then added to each well. Samples were incubated at room temperature for 2 h, and standard dilutions for IL-12 p40 or p70 (gift of Dr. Maurice Gately, Hoffman–La Roche Inc.) were also evaluated. Peroxidase-conjugated anti-IL-12 mAb (POD-4D6, 179 ng/ml; gift of Dr. Maurice Gately, Hoffman–La Roche Inc.) was added to each well and incubated for 1 h. Peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used to detect IL-12 p40 and p70. Plates were read in an ELISA reader (Biotech Instruments Ltd., Luton, UK) at a wavelength of 405 nm.

ELISA for IL-10. ELISA plates were coated overnight at 4°C with 40 μl of rat anti-human IL-10 mAb (PharMingen, San Diego, CA) at final concentration of 4 μg/ml. After blocking with PBS + 1% BSA, samples and standard dilutions of rIL-10 (R&D Systems, Inc., Minneapolis, MN) were added to wells in 50 μl aliquots and incubated at room temperature for 2 h. Biotinylated rat anti-human IL-10 mAb (1 μg/ml; PharMingen) was added to each well and incubated for 1 h at room temperature, followed by a 30 min incubation with streptavidin/peroxidase (1:1000 dil Duoset ELISA kit, Genzyme Diagnostics). Peroxidase substrate solution was added as previously noted and optical density read at 405 nm.

Statistics. All values are presented as mean±SEM. Statistical significance of differences after treatment of a group was calculated using a two-tailed paired Student's *t* test. Statistical significance of differences between two groups was calculated using a two-tailed z-test for means.

Results

M. leprae-induced production of IL-12 and IL-10, and their regulation by IFN-γ. Adherent PBMC from leprosy patients and normal controls were incubated with a sonicated extract of *M. leprae* and cytokine levels in the cell free supernatant were measured by ELISA. IL-12 p40 was detected in the *M. leprae*-stimulated cultures from all patients (Fig. 1). The level of IL-12 p40 induced by *M. leprae* in tuberculoid patients was 325±69 pg/ml ($n = 13$). Similar levels were detected in cultures from lepromatous patients (368±84 pg/ml, $n = 16$). IL-12 p40 release was somewhat lower in the normal controls (190±80 pg/ml, $n = 8$). Addition of IFN-γ increased the IL-12 p40 levels in all three groups (Fig. 1) to 2054±366 pg/ml in tuberculoid patients, 1401±236 pg/ml in lepromatous patients, and 2049±317 pg/ml in the normal controls. The increase in *M. leprae*-induced IL-12 p40 release caused by IFN-γ was statistically significant

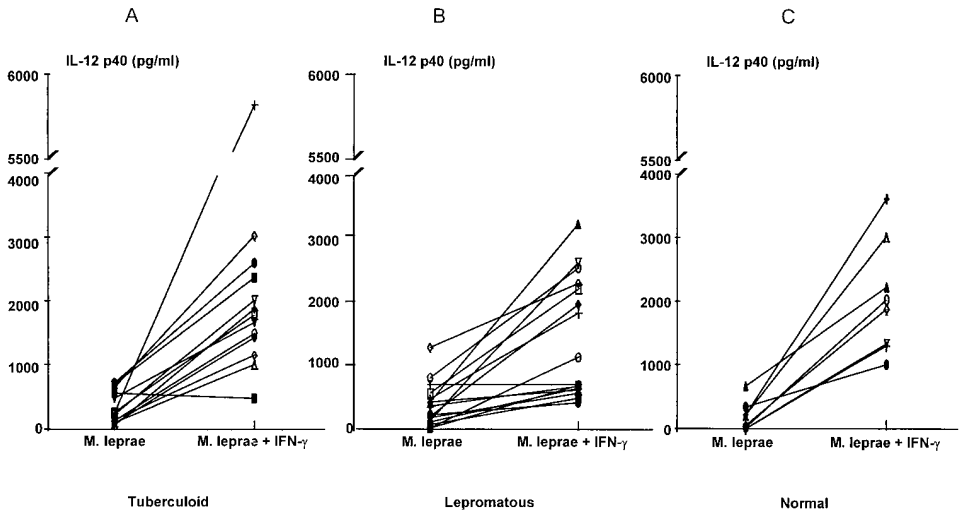

Figure 1. IFN-γ upregulates *M. leprae*-induced IL-12 p40 release. Adherent PBMC from (A) tuberculoid leprosy patients ($n = 10$), (B) lepromatous leprosy patients ($n = 16$), and (C) normal controls ($n = 8$) were stimulated with *M. leprae* sonicate (5 μg/ml) in the presence or absence of IFN-γ (200 U/ml). Cell free supernatants were collected at 20 h and assayed for IL-12 p40 by ELISA. Values are expressed as mean of duplicate determinations.

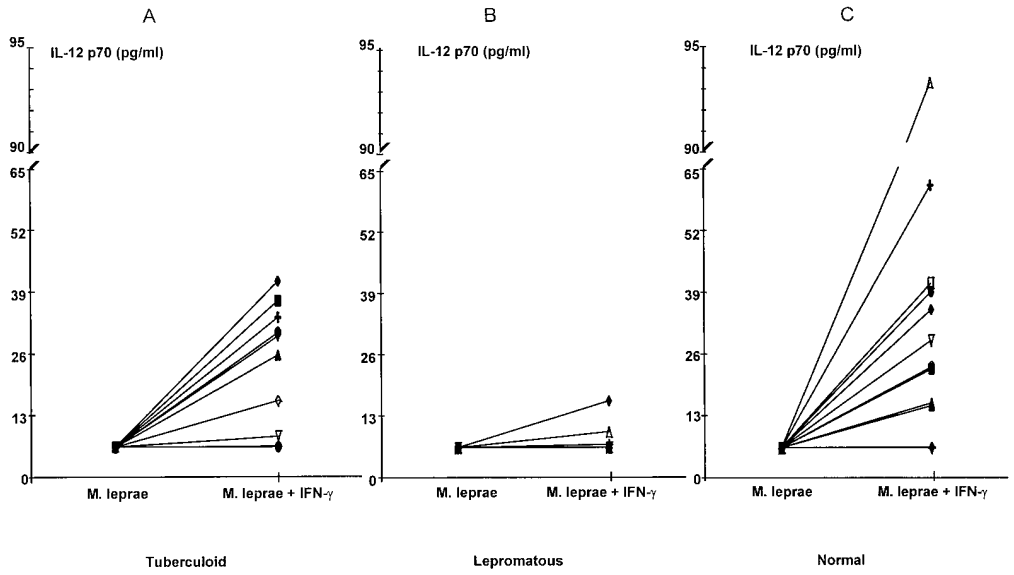

Figure 2. IFN-γ upregulates *M. leprae*-induced IL-12 p70 release. Adherent PBMC from (A) tuberculoid leprosy patients (n = 10), (B) lepromatous leprosy patients (n = 12), and (C) normal controls (n = 12) were stimulated with *M. leprae* sonicate (5 μg/ml) in the presence or absence of IFN-γ (200 U/ml). Cell free supernatants were collected at 20 h and assayed for IL-12 p70 by ELISA. Values are expressed as mean of duplicate determinations.

in each group ($P < 0.001$ for cultures with IFN-γ versus without IFN-γ in each donor group). The level of IL-12 p40 induced by *M. leprae* in the presence of IFN-γ was greatest in tuberculoid leprosy patients and normal controls, and was lower in the lepromatous leprosy group ($P = 0.03$ compared to either tuberculoid leprosy or normal control group).

Since the IL-12 p70 heterodimer is the bioactive form of the cytokine, we measured IL-12 p70 in the cell free supernatants by ELISA. In the adherent PBMC studied, the levels of IL-12 p70 induced by *M. leprae* were near or below the level of detection for the ELISA (6.3 pg/ml) (Fig. 2). However, IFN-γ synergized with *M. leprae* in the induction of IL-12 p70 in tuberculoid patients (23.4±4.2 pg/ml, n = 10, P = 0.003 compared with *M. leprae* alone) and normal controls (32.3±7.2 pg/ml, n = 12, P = 0.004 compared with *M. leprae* alone). However, in the lepromatous leprosy group, IL-12 p70 was near the level of detection, even when cells were stimulated by *M. leprae* and IFN-γ in combination (7.4±0.6 pg/ml, n = 12, $P < 0.001$ compared with either tuberculoid patients or normal controls).

M.leprae induced IL-10 release from adherent PBMC derived from patients with tuberculoid or lepromatous leprosy at levels similar to normal controls (tuberculoid, 852±146 pg/ml, n = 13; lepromatous, 529±158 pg/ml, n = 14; normal controls = 644±194 pg/ml, n = 7) (Fig. 3). In contrast to the effect of IFN-γ on IL-12 release, IFN-γ significantly reduced *M. leprae*–induced IL-10 release in all donor groups (tuberculoid, 105±31 pg/ml, lepromatous, 114±21 pg/ml, normal controls, 143±46 pg/ml, $P \leq 0.03$ for each group of cultures with IFN-γ versus without IFN-γ). These data indicate that IFN-γ differentially regulates the production of IL-12 and IL-10 in the monocytes of leprosy patients and healthy controls, altering the ratio of IL-12 to IL-10. Furthermore, the ability of IFN-γ to augment IL-12 release was significantly weaker in lepromatous patients as compared with tuberculoid patients and normal controls.

Regulation of IL-12 in the presence of IFN-γ. In the milieu of a leprosy lesion, cytokine cross-regulation is key to the modulation of cellular activity. We had previously shown that IL-10 can downregulate *M. leprae* induced IL-12 production (11). We therefore chose to investigate the regulatory role of IL-10

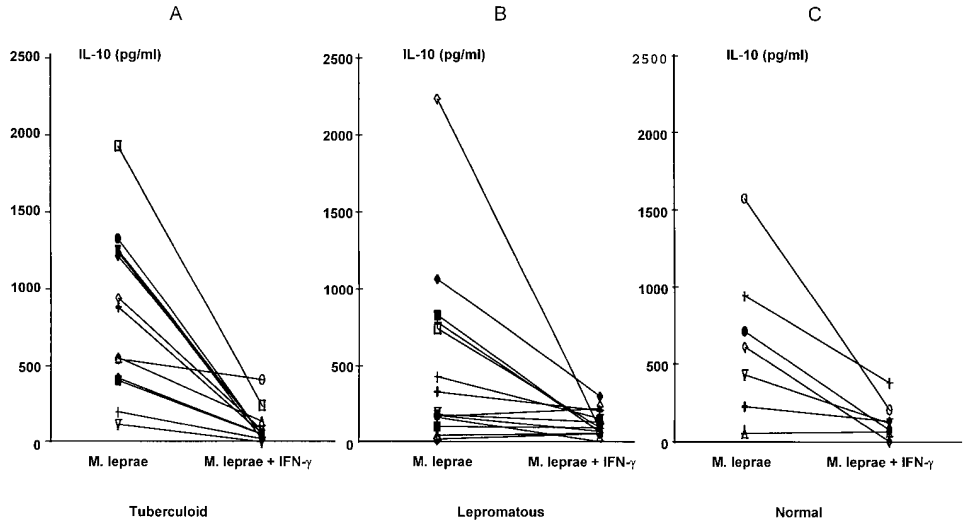

Figure 3. IFN-γ downregulates *M. leprae*-induced IL-10 release. Adherent PBMC from (A) tuberculoid leprosy patients (n = 13), (B) lepromatous leprosy patients (n = 14), and (C) normal controls (n = 7) were stimulated with *M. leprae* sonicate (5 μg/ml) in the presence or absence of IFN-γ (200 U/ml). Cell free supernatants were collected at 20 h and assayed for IL-10 by ELISA. Values are expressed as mean of duplicate determinations.

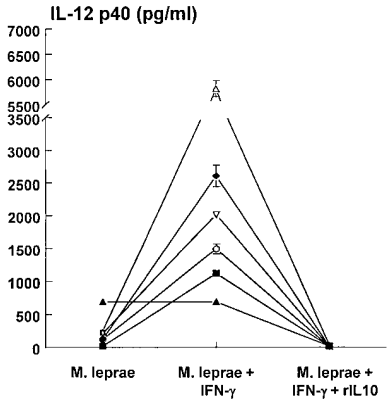

Figure 4. IL-10 downregulates *M. leprae*-stimulated IL-12 release in the presence of IFN-γ. Adherent PBMC from tuberculoid and lepromatous patients ($n = 6$) were stimulated with *M. leprae* sonicate (5 μg/ml) under three conditions: *i*) addition of media alone, *ii*) addition of IFN-γ (200 U/ml), and *iii*) addition of IFN-γ (200 U/ml) + rIL-10 (100 U/ml). Cell free supernatants were collected at 20 h and assayed for IL-12 p40 release by ELISA. Values are expressed as mean±SEM of duplicate determinations. Open symbols, tuberculoid patients; closed symbols, lepromatous patients.

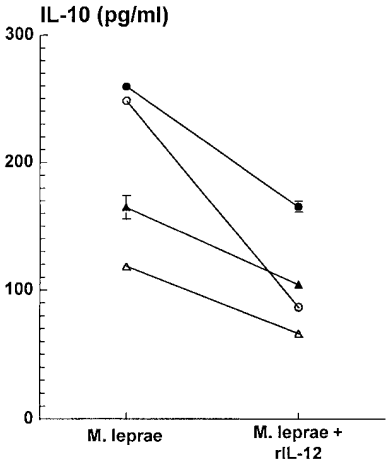

Figure 5. IL-12 downregulates *M. leprae*-stimulated IL-10 release in the absence of IFN-γ. Adherent PBMC from tuberculoid and lepromatous patients ($n = 4$) were stimulated with *M. leprae* sonicate (5 μg/ml) in the presence or absence of rIL-12 (1 nM). All cells were cultured in the presence of neutralizing anti-IFN-γ antibody (100 ng/ml) at a concentration able to neutralize 100 U/ml. In the absence of this neutralizing antibody, trace amounts of IFN-γ were measured in the supernatants, always < 12 U/ml. Cell free supernatants were collected at 20 h and assayed for IL-10 release by ELISA. Values are expressed as mean±SEM of duplicate determinations. Open symbols, tuberculoid patients; closed symbols, lepromatous patients.

on IL-12 release in the presence of IFN-γ in both tuberculoid and lepromatous donors. In all donors, addition of rIL-10 completely abrogated the IFN-γ upregulation of IL-12 p40 release (Fig. 4). The data suggest that in both tuberculoid and lepromatous monocytes, IL-10 exerts a dominant effect over IFN-γ upregulation of IL-12 p40.

IL-10 regulation by IL-12. Next, we investigated the ability of rIL-12 to modulate *M. leprae*-induced IL-10 release. Because trace amounts of IFN-γ were detected in supernatants following *M. leprae* stimulation, neutralizing anti-IFN-γ antibody was added to the adherent PBMC at a concentration able to neutralize 100 U/ml. After the addition of rIL-12, *M. leprae* induced IL-10 was measured from tuberculoid and lepromatous adherent cells ($n = 4$) (Fig. 5). Addition of rIL-12 caused *M. leprae* induced IL-10 production to decrease from 198±34 to 106±21 pg/ml ($P = 0.03$). This points to a negative cross-regulatory cycle between IL-10 and IL-12 and a direct effect of IL-12 on the production of IL-10.

However, in the presence of exogenous IFN-γ, rIL-12 did not add significantly to the downregulation of IL-10 production (Fig. 6 *A*). These data suggest that IFN-γ and rIL-12 downregulate IL-10 production through a common pathway. To ascertain whether IFN-γ downregulation of IL-10 release is mediated by IL-12, we used neutralizing anti-IL-12 antibodies. The addition of neutralizing anti-IL-12 antibodies abrogated the ability of IFN-γ to downregulate IL-10 (Fig. 6 *B*). IFN-γ may exert a direct effect on IL-10 in the absence of IL-12, however this was not addressed in the present or previous studies (12–14). The present data indicate, however, that IFN-γ downregulation of IL-10 release is mediated, at least in part, by IL-12.

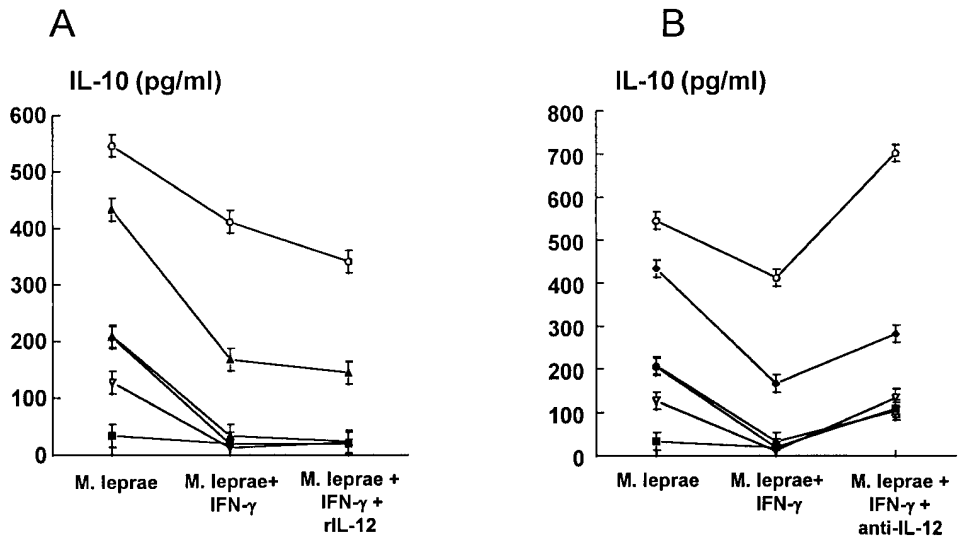

Figure 6. Effect of rIL-12 and anti-IL-12 on IFN-γ induced downregulation of IL-10 release in response to *M. leprae* stimulation. Adherent PBMC from tuberculoid and lepromatous patients ($n = 6$) were stimulated with *M. leprae* sonicate (5 μg/ml) under three conditions: (*i*) addition of media alone, (*ii*) addition of IFN-γ (200 U/ml), (*iii*) addition of IFN-γ (200 U/ml) + rIL-12 (1 nM) or, (*iv*) addition of IFN-γ (200 U/ml) + neutralizing anti-IL-12 Abs (5 μg/ml). Cell free supernatants were collected at 20 h and assayed for IL-10 release by ELISA. A) Addition of rIL-12 does not significantly alter the downregulation of *M. leprae* stimulated IL-10 release by IFN-γ. B) Addition of neutralizing anti-IL-12 antibodies reverses the downregulation of *M. leprae*-stimulated IL-10 release by IFN-γ. Values are expressed as mean±SEM of duplicate determinations. Open symbols, tuberculoid patients; closed symbols, lepromatous patients.

Discussion

In human infectious disease, the varying immunological responses to an invading pathogen determine the clinical manifestations of the infection and the outcome to the host. Nowhere is this more evident than in leprosy, where a clear dichotomy exists between the T cell cytokine pattern and the clinical form of the disease. The type 1 T cell cytokine pattern predominates in self-curing tuberculoid patients and, in contrast, the type 2 T cell cytokine pattern characterizes the progressive disease of lepromatous patients. The development of the T cell cytokine patterns is known to be influenced by the balance between two key monocyte-derived cytokines, IL-12 and IL-10. In this study, we investigated the regulation of IL-12 and IL-10 production in response to *M. leprae.* The results of this study provide new evidence that IFN-γ concomitantly upregulates IL-12 release and downregulates IL-10 release from effector cells in human infectious disease.

Overall, we found that *M. leprae*-stimulated monocytes produced greater amounts of IL-10 than IL-12. IFN-γ altered this ratio by concomitantly increasing IL-12 release and decreasing IL-10 release. In separate studies, IFN-γ similarly modulated IL-12 (15, 16) and IL-10 release (12–14) from monocytes cultured with other microbial stimuli, including *Staphylococcus aureus* Cowan (SAC), *Listeria monocytogenes*, and LPS. The ability of IFN-γ to differentially regulate IL-12 and IL-10 release has relevant implications for the pathogenesis of leprosy. We hypothesize that the strong IL-12 and weak IL-10 expression in tuberculoid lesions is due in part to the local release of IFN-γ (2, 11). The release of IL-12 by monocytes in lesions would further amplify the type 1 cytokine cascade, since our previous experiments demonstrate that IL-12 preferentially stimulates expansion of CD4+ type 1 T cells derived from tuberculoid lesions (11). Furthermore, since IL-10 inhibits *M. leprae* specific T cell proliferation (17), its downregulation by IFN-γ would be expected to further enhance CMI.

One striking finding of this study was that the ability of IFN-γ to augment IL-12 production was significantly lower in lepromatous leprosy patients as compared with tuberculoid leprosy patients and normal controls. On the other hand, there was no difference in the regulation of IL-10 release from lepromatous adherent cells as compared with tuberculoid leprosy and normal donors. In accord with these observations on peripheral blood monocytes, in lepromatous lesions IL-10 expression is strong, whereas IL-12 and IFN-γ expression are weak (2, 11). The relative lack of IFN-γ would allow IL-10 to predominate, downregulating local IL-12 expression, and resulting in the type 2 cytokine pattern. However, our data further indicate that even in the presence of IFN-γ, monocytes from lepromatous patients are not able to mount an appropriate IL-12 response. In vivo, however, treatment of lepromatous patients with rIFN-γ upregulated monocyte hydrogen peroxide formation (18). Further study will enhance our understanding of monocyte unresponsiveness in lepromatous patients.

The mechanism by which IFN-γ augments IL-12 production by monocytes is primarily at the transcriptional level of the p40 gene. IFN-γ has been shown to enhance the binding interaction between NF-κB family members and an 11 nucleotide stretch of the p40 promoter region (19, 20). The IFN-γ induced downregulation of IL-10 is mediated, to a large extent, by IL-12. Previous studies have examined the ability of IL-12 to regulate IL-10 production from T cells (21–23). However, in leprosy, monocytes are the predominant source of *M. leprae*-induced IL-10 (17). The demonstration here of a direct downregulatory effect of IL-12 on IL-10 release from monocytes completes a reciprocal negative feedback loop, as the present and previous data indicate that IL-10 decreases *M. leprae*-induced IL-12 release (11). This feedback loop would add to the polarity of monocyte cytokine response; the result to the host would be the preferential release of either IL-12 or IL-10.

Our data indicate that the relative presence or absence of IFN-γ in the local microenvironment of the lesion is a key determinant of the monocyte cytokine response. Support for this early role of IFN-γ in mycobacterial disease can be found in a murine model of *M. bovis* BCG infection. In a study by Flesch et al., IL-12 production was induced in spleens of immunocompetent mice early during BCG infection, but not in those of mutant mice lacking the IFN-γ receptor (24). However, a critical question remains: what is the initial source of IFN-γ for modulating the monocyte cytokine response? It seems likely that the source of this IFN-γ would be from other members of the innate immune system. Although NK cells are thought to have a role in this process, they are relatively rare in leprosy lesions (25). One prime candidate for such IFN-γ producing cells are the γδ T cells, which can represent up to 35% of the CD3+ cells found in tuberculoid and reversal reaction lesions, but constitute less than 5% of the T cells found in lepromatous lesions (26). A major population of γδ T cells that have rearranged predominantly the Vγ2-Vδ2 genes have been described that are preprogrammed and respond to mycobacterial antigens, specifically isopentenyl pyrophosphate (27). Activation of these T cells results in secretion of high levels of IFN-γ (28, 29).

The balance of IL-12 and IL-10 plays a crucial role in several other human infectious diseases. Notably, in HIV, there is a viral-induced decrease in IL-12 production, and, with progression to AIDS, an increase in IL-10 production (30–32). This dysregulation of IL-12/IL-10 likely contributes to the pathogenesis of common opportunistic infections due to *Toxoplasma gondii* and *Mycobacterium avium.* In visceral leishmaniasis, untreated patients produce IL-10 but no IL-12 p40 in response to *Leishmania donovani* lysate. In contrast, successfully treated (healed) patients produce IL-12 under the same conditions (33).

The data presented here underscore the importance of the innate immune response in determining the course of infection with intracellular pathogens. First, IFN-γ producing cells of the innate immune response are a key determinant of the monocyte cytokine response. Second, the responsiveness of monocytes from patients can also influence the pattern of cytokines released. Further studies to delineate the factors that skew the development of the innate immune response towards the type 1 cytokine pattern should provide new opportunities for therapeutic intervention in a host of infectious diseases.

Acknowledgments

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