Modulation of the Cytokine Response in Human Monocytes by *Mycobacterium leprae* Phenolic Glycolipid-1

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Leprosy is a chronic but treatable infectious disease caused by the intracellular pathogen *Mycobacterium leprae*. *M. leprae* cell wall is characterized by a unique phenolic glycolipid-1 (PGL-1) reported to have several immune functions. We have examined the role of PGL-1 in the modulation of monocyte cytokine/chemokine production in naive human monocytes. PGL-1 in its purified form or expressed in a recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) background (rBCG-PGL-1) was tested. We found that PGL-1 selectively modulated the induction of specific monocyte cytokines and chemokines and, when used as prestimulus, exerted priming and/or inhibitory effects on the induction of selected cytokines/chemokines in response to a second stimulus. Taken together, the results of this study support a modulatory role for PGL-1 in the innate immune response to *M. leprae*. Thus, PGL-1 may play an important role in the development of the anergic clinical forms of disease and in tissue damage seen in lepromatous patients and during the reactional states of leprosy.

Introduction

LEPROSY, A HUMAN DISEASE caused by infection with *Mycobacterium leprae*, is treatable but requires a long period of multidrug therapy for cure (Scollard and others 2006). *M. leprae* is an intracellular pathogen infecting primarily phagocytes (dendritic cells, monocytes, macrophages) and Schwann cells. In leprosy patients, the bacilli grow slowly, primarily in the cooler extremities and regions of poor circulation (nasal passages and eye brows). *In vitro*, in cultured monocytes and macrophages, *M. leprae* thrives optimally but does not grow at 33°C (Truman and Krahenbuhl 2001; Scollard and others 2006). As the organisms do not replicate in culture, the 2 animal models of infection that support bacillary growth, the armadillo and mouse footpads, are used extensively for leprosy research, to study host pathogen interactions and as a source of *M. leprae* for experimental infection as well as biochemical analysis (Scollard and others 2006).

Leprosy manifests in patients as a spectrum of clinical states. At one pole lies tuberculoid or paucibacillary leprosy, characterized by robust production of proinflammatory cytokines and a strong Th1 immune response. At the opposite pole lies lepromatous or multibacillary leprosy, characterized by a weak Th1-type T-cell response, a strong Th2-type humoral response, and numerous cutaneous intracellular bacilli (Ridley and Jopling 1966). During the chronic course of the disease, acute immunologically mediated episodes (reactions)

may occur, leading to impairment of nerve function and disability. The 2 main types of reactions are reversal reaction (RR) and erythema nodosum leprosum (ENL). RR often develops at the beginning of treatment, probably because of lysis of the microorganisms and release of *M. leprae* antigens that drive the immunologic response (Lockwood and others 2002), with enhancement of Th1 type cytokines and tumor necrosis factor alpha (TNF- α). ENL, which occurs almost exclusively in the lepromatous form of disease, is characterized by elevated blood levels of TNF- α and the formation of immune complexes (Yamamura and others 1992).

We and others have reported that *M. leprae* is a poor *in vitro* activator of monocytes and dendritic cells (Suzuki and others 1993; Murray and others 2007; Sinsimer and others 2010). More recently, we have shown that, in addition to being a poor inducer of proinflammatory cytokines, *M. leprae* elicits high levels of the negative regulatory molecules monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-1Ra from monocytes. Used as a prestimulus, *M. leprae* primes monocytes for increased production of TNF- α and IL-10 and inhibits the induction of IL-1 β in response to a second stimulus (Sinsimer and others 2010).

M. leprae cell wall is characterized by a unique structure rich in lipid and similar in composition to other mycobacterial cell walls (Vissa and Brennan 2001; Scollard and others 2006). As much as 2% of the *M. leprae* cell wall is comprised of phenolic glycolipid-1 (PGL-1), characterized by an

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antigenically specific trisaccharide unique to *M. leprae* (Hunter and Brennan 1981; Hunter and others 1982), differing from the BCG PGL, so-called mycoside B, which contains a monosaccharide (Daffé and others 1988; Chatterjee and others 1989). Several functions have been attributed to PGL-1. It facilitates binding of the bacilli to macrophages and Schwann cells (Schlesinger and Horwitz 1991; Ng and others 2000) and promotes the survival of *M. leprae* in human macrophages (Neill and Klebanoff 1988; Vachula and others 1989, 1990a, 1990b). It suppresses the proliferative response to mitogens of murine and human T cells (Mehra and others 1984; Prasad and others 1987; Nomaguchi and others 1989; Hashimoto and others 2002). PGL-1 has also been reported to play a role in the induction of TNF- α release (Charlab and others 2001; Dhungel and others 2008) and to specifically inhibit the release of TNF- α and IL-1 β from human peripheral blood mononuclear cells in response to stimulation with lipopolysaccharide (LPS) (Silva and others 1993). Tabouret and others (2010) showed that PGL-1 expressed in recombinant *M. bovis* BCG can suppress the secretion of TNF- α in human macrophages in response to BCG.

We examined whether the modulation of monocyte cytokine production by *M. leprae* (Sinsimer and others 2010) may be attributable to PGL-1. Naive monocytes isolated from healthy blood donors were exposed to exogenous purified PGL-1 or to PGL-1 expressed endogenously in recombinant BCG (rBCG PGL-1), and the cytokine and chemokine expression profiles were analyzed. We also examined whether preexposure of monocytes to PGL-1 could modulate cytokine and chemokine induction profiles in response to a second stimulus.

Materials and Methods

Reagents

PGL-1 was obtained in the dried form through the NIH Biodefence and Emerging Infections Research Resources Repository, NIAID, NIH: *Mycobacterium leprae* PGL-1, NR-19342. As recommended by the provider, PGL-1 was stored at room temperature until use, reconstituted in methanol, and stored in small single-use aliquots in screw-capped vials with o-rings at -20°C . Dilutions in culture medium were prepared immediately before addition to the cell culture. Recombinant *M. bovis* BCG expressing PGL-1 (r-BCG PGL-1) was constructed as previously described (Tabouret and others 2010). BCG containing an empty vector (r-BCG) was used as control. Stocks were prepared by growing the bacteria in 7H9 broth medium (Difco) and stored at -80°C until use.

Isolation and stimulation of human monocytes

Human blood (buffy coat) was obtained from anonymous healthy donors (New Jersey Blood Center). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque separation and plated at a density of $3\text{--}5 \times 10^6$ per well in 24-well tissue culture plates (Corning, Inc.). Monocytes were allowed to adhere for 2 h and then nonadherent cells were removed by several washings. Adherent monocytes were cultured in RPMI 1640 supplemented with 20% human serum (R20). Monocytes were infected with r-BCG or r-BCG PGL-1 at a multiplicity of infection (MOI) of 1:1 (bacilli:monocyte). Intracellular growth was determined by disrupting monocytes by probe sonication (Manca and others

FIG. 1. Cytokine production by monocytes stimulated with PGL-1 or LPS. Naive human monocytes from healthy donors were stimulated with purified PGL-1 (50 $\mu\text{g}/\text{mL}$) or with LPS (100 ng/mL) or left unstimulated (UN). Cell supernatants were collected at 24 h poststimulation and analyzed by Luminex for the presence of the cytokines TNF- α , IL-1 β , IL-10 (A), VEGF, MCP-1, and IL-1Ra (B). Data for TNF- α , IL-1 β , and VEGF are presented as scatter plots and are the means of 7 experiments (representing 7 independent donors). Results for MCP-1, IL-1Ra, and IL-10 are the means of 5 experiments (representing 5 independent donors). PGL-1, phenolic glycolipid-1; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor alpha; IL, interleukin; VEGF, vascular endothelial growth factor; MCP, monocyte chemotactic protein.

1999) and plating serial dilutions on Middlebrook 7H10 (Beckton Dickinson). In the priming experiments, monocytes were preincubated for 5 h with PGL-1 (50 $\mu\text{g}/\text{mL}$) or culture medium alone and then stimulated with LPS (100 ng/mL ; InvivoGen) for a further 19 h. Controls received PGL-1 only or culture medium alone. Culture supernatants were isolated at 24 h from the unstimulated cells or from monocytes exposed to the stimulants and stored at -80°C for later cytokine analysis.

Detection of cytokines and chemokines in the culture supernatants

Cytokine levels were determined by probing the culture supernatants with a multiplex human cytokine/chemokine

Luminex panel (Bio-Rad) according to the manufacturer's instructions. The Luminex panel used for the analysis of supernatants included TNF- α , IL-6, IL-1 β , IL-10, IL-1Ra, IL-12p70, MCP-1, and vascular endothelial growth factor (VEGF). Results were analyzed with a Bio-Plex™ 200 system (Bio-Rad) and data and statistical analyses were carried out using GraphPad Prism 4 program (GraphPad).

Thin-layer chromatography analysis

PGL-1 production by r-BCG PGL-1 was visualized and confirmed by thin-layer chromatography (TLC) analysis. Similar amounts (400 μ g) of total lipid extracts from r-BCG PGL-1 and the control r-BCG grown for 4 weeks in Sauton medium were spotted onto a silica gel 60 thin-layer chromatography plate (20 $\times$ 20 cm; Merck), and as previously described by Tabouret and others (2010), the plate was run in CHCl₃/CH₃OH (95:5, v/v). PGL-1 and mycoside B were visualized by spraying the plate with 0.2% anthrone solution in concentrated H₂SO₄, followed by heating. Lipids were quantified with a CAMAG TLC scanner using the Win CATS v1.4.3 software.

Statistical analysis

Data analysis was done using a 2-tailed paired *t*-test and *P* < 0.05 was chosen to indicate statistically significant difference.

Results

Effect of PGL-1 on cytokine and chemokine production by human monocytes

To assess whether PGL-1 has a role in *M. leprae* induction of cytokines and chemokines *in vitro*, we exposed native human monocytes isolated from healthy individuals to purified PGL-1 and measured selected cytokine and chemokine levels in the culture supernatant at 24 h poststimulation. LPS, a potent monocyte stimulant, was used as a positive control and elicited high levels of all the cytokines tested. Similar to the results previously obtained with *M. leprae* (Sinsimer and others 2010), PGL-1 elicited very low levels of the proinflammatory cytokines TNF- α (18.9 ± 17.8 pg/mL), IL-1 β (32.6 ± 28.3 pg/mL), and IL-10 (3 ± 0.7 pg/mL) (Fig. 1A). Levels of IFN- γ in these cell culture supernatants were below the limit of detection in both the test and control wells (data not shown). In the same set of experiments, PGL-1 elicited the negative regulatory molecules MCP-1 and IL-1Ra at levels comparable to those induced by LPS (Fig. 1B), again consistent with our published results for *M. leprae* (Sinsimer and others 2010). We also observed that PGL-1 stimulation induced VEGF (Fig. 1B). Thus, the cytokine profile elicited by PGL-1 is consistent with that induced by *M. leprae*, suggesting that PGL-1 may be an important contributor to the response of human monocytes to *M. leprae* infection.

FIG. 2. Cytokine production by monocytes prestimulated with PGL-1. Monocytes from healthy human donors were exposed for 5 h to PGL-1 or R20 alone followed by LPS stimulation. Cell supernatants were collected at 24 h and proinflammatory cytokines (A), VEGF, MCP-1, and IL-1Ra levels (B) were analyzed by Luminex. Results are the means $\pm$ standard error of 4–6 experiments (representing 4–6 independent donors) performed in duplicate. A 2-tailed paired *t*-test was used for statistical analysis (**P* < 0.05, compared with cells exposed to R20 for 5 h prior to LPS stimulation).

Effect of PGL-1 prestimulation on the monocyte response to LPS

In our previous study using whole bacteria, we found that *M. leprae* primes monocytes for increased production of TNF- α and IL-10 and inhibits the induction of IL-1 β in response to a second stimulus (Sinsimer and others 2010). Here we investigated whether the priming of monocyte cytokine/chemokine production by *M. leprae* can be attributed to PGL-1. Monocytes were preincubated for 5 h with PGL-1 or culture medium (R20) alone and then stimulated with LPS for a further 19 h. Controls received PGL-1 or culture medium alone. Levels of cytokines and chemokines were measured at 24 h. Similar to our results with *M. leprae* (Sinsimer and others 2010), PGL-1 prestimulation resulted in a significant increase in the TNF- α level in response to LPS. We also saw a priming effect by PGL-1 prestimulation on IL-10 induction in response to LPS. This effect was not seen in our earlier study using *M. leprae* as a prestimulus for LPS, but was seen when BCG was used as second stimulus (Fig. 2A) (Sinsimer and others 2010). Induction of IL-6 by LPS was also increased by PGL-1 prestimulation. Although *M. leprae* prestimulation blocked the induction of IL-1 β by LPS, the molecule primed the monocytes for increased LPS-induced IL-1 β release.

Interestingly, PGL-1 prestimulation blocked LPS induction of VEGF (Fig. 2B). MCP-1 levels were unaffected, whereas for IL-1Ra there was an additive effect of PGL-1 and LPS stimulation. Thus, despite the fact that PGL-1 alone induced very low levels of the proinflammatory cytokines, when used as a prestimulus prior to LPS it resulted in a dramatic increase of the levels of these cytokines. In contrast, the molecules that were strongly induced by PGL-1 alone appeared to be unaffected or even reduced by PGL-1 prestimulation prior exposure of the cells to LPS. Taken together, these observations suggest that PGL-1 contributes to *M. leprae* modulation of monocyte cytokine/chemokine release in response to later exposure to other stimuli.

Effect of PGL-1 expressed in recombinant BCG on cytokine release in monocytes

We next investigated whether PGL-1 expressed endogenously in recombinant BCG has a modulatory effect on cytokine release. We used a strain of BCG genetically engineered to express PGL-1 from a chromosomally integrated plasmid (r-BCG PGL-1) and, as a control, BCG containing the unmodified plasmid vector (r-BCG) (Tabouret and others 2010). Production of PGL, PGL-1 by the r-BCG PGL-1 and mycoside B by the control r-BCG, was visualized by TLC analysis (Fig. 3). The same amount of total lipids for r-BCG PGL-1 and r-BCG were loaded. As described by Tabouret and others (2010), the amount of PGL-1 was estimated to be 20% of the total PGL in the r-BCG PGL-1. Chatterjee and others (1989) estimated the amount of mycoside B per unit weight of BCG to be in the order of 19–25 mg/g, representing between 2% and 2.5% of the cell dry weight. On this basis we estimate that PGL-1 represents 3.8–5 mg/g corresponding to 0.4%–0.5% of the dry weight of r-BCG PGL-1.

We infected fresh human monocytes with the recombinant strains at an MOI of 1:1 (bacilli:monocyte) and analyzed the intracellular bacillary growth and the pattern of cytokines/chemokines released at 24 and 48 h postinfection. The intracellular growth of r-BCG and r-BCG PGL-1 was determined

FIG. 3. Thin-layer chromatography (TLC) analysis of recombinant BCG strains. Same amount of lipids extracted from r-BCG PGL-1 and r-BCG were dissolved in CHCl₃ (at a concentration of 20 mg/mL) and 20 μ L of these extracts were loaded on the TLC and run in CHCl₃/CH₃OH (95:5, v/v). Glycolipids were visualized by spraying the TLC plate with 0.2% anthrone (w/v) solution in concentrated H₂SO₄ followed by heating; BCG, Bacillus Calmette-Guérin.

over 4 days in these cultures by cfu. At 24 and 48 h postinfection the cfu counts for the 2 strains were comparable. At 96 h postinfection, the cfu counts for r-BCG PGL-1 were higher than for the r-BCG control (Fig. 4A), and the doubling times from day 2 to 4 postinfection were 36 ± 5.2 h for r-BCG PGL-1 and 65 ± 26.8 h for r-BCG. At similar bacillary loads, the levels of TNF- α and IL-12p70 induced by r-BCG PGL-1 were significantly higher than those induced by r-BCG control (Fig. 4B). We also measured IL-6, IL-1 β , IL-10, MCP-1, and macrophage inflammatory protein (MIP)-1 β in the same culture supernatants. Comparable levels of these cytokines/chemokines were measured for both r-BCG PGL-1 and r-BCG control, whereas VEGF was undetectable for both strains (data not shown).

Effect of prestimulation with r-BCG PGL-1 on the monocyte response to LPS

We next evaluated whether the priming effect of purified PGL-1 was also seen in cells preexposed to r-BCG PGL-1. Monocytes were incubated for 5 h with r-BCG PGL-1 or culture medium alone, before exposure to LPS for an additional 19 h. Control cells were incubated for 24 h with r-BCG PGL-1 or medium alone. Levels of TNF- α , IL-1 β , IL-10, VEGF, and MCP-1 were determined in the culture medium at 24 h. Similar to the results with purified PGL-1, pre-exposure of monocytes to r-BCG PGL-1 primed the cells for increased induction of IL-1 β and IL-10 by LPS (Fig. 4C). In

FIG. 4. Modulation of cytokine production in monocytes stimulated with PGL-1 expressed in a recombinant BCG (r-BCG PGL-1). Fresh human monocytes from healthy donors were infected with r-BCG and r-BCG PGL-1 at an MOI of 1:1 (bacilli:cell). Growth of r-BCG and r-BCG PGL-1 within monocytes was evaluated over 4 days of culture by cfu (**A**) and supernatant was analyzed at 24 h postinfection for the presence of TNF- α and IL-12p70 (**B**). Monocytes were exposed for 5 h to r-BCG PGL-1 or R20 alone followed by LPS stimulation for 19 h. Cell culture supernatants were collected at 24 h and analyzed for the presence of IL-1 β , IL-10 (**C**), VEGF, and MCP-1 (**D**) by Luminex. Results are the means $\pm$ standard error of 4–6 experiments (representing 4–6 independent donors) performed in duplicate. A 2-tailed paired *t*-test was used for statistical analysis (**P* < 0.05, compared with cells exposed to R20 for 5 h prior to LPS stimulation).

contrast, priming did not affect TNF- α induction (data not shown). Prestimulation with r-BCG PGL-1 completely blocked VEGF induction by LPS and partially blocked MCP-1 induction by LPS (Fig. 4D). These results are consistent with those obtained using purified PGL-1 and may help to understand the role of PGL-1 in the interaction between *M. leprae* and the infected host.

Discussion

In this study, we sought to understand the role of *M. leprae* PGL-1 in the *in vitro* induction of cytokines and chemokines by human peripheral blood-derived monocytes using 2 different approaches. We have exposed monocytes to PGL-1 as a purified molecule or in the context of BCG, using a recombinant BCG strain expressing endogenous PGL-1. Using LPS as a positive control, we showed that purified PGL-1 was a relatively weak stimulator of TNF- α , IL-1 β , and IL-10, while

yielding relatively high levels of MCP-1 and IL-1Ra, 2 negative regulatory molecules reported to be associated with suppression of IL-12 and IL-1 β , respectively (Dinarello and Thompson 1991; Flores-Villanueva and others 2005; Sinsimer and others 2010). These results are similar to our findings using whole *M. leprae* to stimulate monocytes (Sinsimer and others 2010) and consistent with the results of other studies showing that purified PGL-1 has a modulatory effect on cytokine release by human cells and is a poor inducer of TNF- α (Charlab and others 2001; Dhungel and others 2008), IL-1 β , and IL-6 (Silva and others 1993). This selective modulation of specific cytokines and chemokines produced by monocytes supports the hypothesis that PGL-1 is a key component in *M. leprae*'s control of the monocyte immune response.

We have previously shown that, although simultaneous exposure of monocytes to *M. leprae* plus LPS or BCG did not alter the response to either of the later 2 stimuli, preexposure of the cells to *M. leprae* had a profound stimulatory effect on

the release of TNF- α and a stimulatory or inhibitory effect on IL-10 and IL-1 β , depending on the second agonist used, that is, LPS or BCG (Sinsimer and others 2010). For this reason, we investigated the impact of preexposure of monocytes to PGL-1 followed by LPS stimulation on the cytokine response. In monocytes preexposed to PGL-1, the cells were primed, leading to increased levels of TNF- α , IL-6, IL-1 β , and IL-10, in response to LPS stimulation (Fig. 2A). In our previous study, preexposure of monocytes to whole *M. leprae* followed by LPS stimulation showed a similar enhancement of TNF- α production. Although *M. leprae* followed by LPS did not enhance IL-10 production, the use of BCG as the second stimulus showed a priming effect on this cytokine. These results indicate that purified PGL-1 can selectively modify the monocyte response to subsequent stimuli in a manner that shows some similarities with that obtained with whole *M. leprae*. The differences in cytokines profiles observed using PGL-1 or *M. leprae* are likely due to the greater antigenic complexity of the whole organism. This antigenic diversity of the intact bacilli may result in activation and/or deactivation of multiple intracellular signaling pathways controlling cytokine production and release. Other studies have shown that the addition of PGL-1 together with *M. leprae* to stimulate whole blood, PBMC, or THP-1 cell cultures resulted in enhancement of TNF- α release (Charlab and others 2001; Dhungel and others 2008). In a separate study using PBMC, PGL-1 together with LPS was shown to inhibit induction of TNF- α , IL-1 β , and IL-6 by LPS (Silva and others 1993). Taken together, these studies support a modulatory role for PGL-1 in the innate immune response to *M. leprae*, while highlighting the diversity of its effect, depending upon the cell type and the nature of the second stimulus to which the cells are exposed.

In our present studies, production of TNF- α and IL-12p70 in response to r-BCG PGL-1 was enhanced, relative to that seen in response to the control r-BCG strain. In contrast, Tabouret and others (2010) have reported that human macrophages released lower levels of TNF- α in response to r-BCG PGL-1, compared with the response to the control BCG, whereas IL-12 (p40 and p70) and IL-10 were poorly induced by both r-BCG PGL-1 and control BCG. It is possible that the differences in our observations and those of Tabouret and others (2010) are attributable to the difference in mononuclear phagocyte differentiation and maturation in culture (macrophages versus monocytes) used by their group and ours. When r-BCG PGL-1 was used as a prestimulus in human monocytes, we observed enhanced LPS induction of IL-1 β and IL-10 but not TNF- α . Thus, PGL-1 in the context of recombinant BCG retained some of the properties of purified PGL-1, but again showed some differences reflective of the antigenic background of the whole organism.

Interestingly, in this study we observed that purified PGL-1 preferentially induced VEGF, a growth factor known to promote angiogenesis. VEGF has been found to be elevated in patients at the lepromatous end of the leprosy spectrum; these patients are also characterized by elevated bacterial load and PGL-1 levels (Bhandarkar and others 2007). Overexpression of VEGF has also been found in tissue samples from leprosy patients with RR (Fiallo and others 2002). We observed that preexposure of monocytes to purified PGL-1 or to r-BCG PGL-1 dramatically blocked LPS induction of VEGF and inhibited LPS induction of MCP-1. The mechanism responsible for this effect remains unknown and will be further investigated.

Taken together, our observations suggest that PGL-1 plays a key role in the active regulation of the monocyte cytokine and chemokine response to *M. leprae*. Thus, PGL-1 may play an important role in the development of anergic clinical forms of disease and in the tissue damage seen in lepromatous patients and in leprosy patients experiencing reactional states (Yamamura and others 1992; Misch and others 2010).

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Author Disclosure Statement

No competing financial interests exist.

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