Preformed Membrane-associated Stores of Interleukin (IL)-12 Are a Previously Unrecognized Source of Bioactive IL-12 That Is Mobilized within Minutes of Contact with an Intracellular Parasite

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Abstract

The prevailing paradigm is that production of the interleukin (IL)-12 p70 heterodimer, a critical T helper cell type 1 (Th1)-inducing cytokine, depends on the induced transcription of the p40 subunit. Concordant with this paradigm, we found that dendritic cells (DCs) produced IL-12 p70 only after at least 2–4 h of stimulation with lipopolysaccharide plus interferon γ. However, using several complementary experimental approaches, including electron and confocal microscopy, we now show that resting murine and human myeloid cells, including macrophages/DCs and DC-rich tissues, contain a novel source of bioactive IL-12 that is preformed and membrane associated. These preformed, membrane-associated IL-12 p70 stores are released within minutes after in vitro or in vivo contact with Leishmania donovani, an intracellular pathogen. Our findings highlight a novel source of bioactive IL-12 that is readily available for the rapid initiation of Th1 host responses to pathogens such as Leishmania species.

Key words: IL-12 p70 • dendritic cells • Th1/Th2 • Leishmania • membrane

Introduction

IL-12 is central to the orchestration of both innate and acquired immune responses (1). IL-12 is produced primarily by phagocytic cells and, among its multiple activities, it is a potent inducer of IFN-γ from T and NK cells (1). As such, it is critical for the development of Th1 responses that are required for generating protective cell-mediated inflammatory responses against invading intracellular microorganisms. The importance of IL-12 in generating protective Th1 responses is illustrated by the finding that humans and experimental murine models with genetic mutations that block IL-12 or IFN-γ-dependent signaling pathways have marked sensitivity to intracellular infections such as the Mycobacterium and Leishmania species (2–5).

There are two general paradigms regarding the mechanisms that control IL-12 production by APCs such as dendritic cells (DCs) and macrophages. First, in all systems where IL-12 regulation has been molecularly defined, it has been found to be heavily transcriptionally dependent (1, 6, 7). Biologically active IL-12 (p70) is a heterodimeric protein composed of the covalently linked products of p40 and p35, two separate genes that are regulated independently (1). Although both p35 and p40 are highly regulated proteins, there is general consensus that new synthesis of IL-12 p70 depends primarily on the induced transcription of the p40 gene (1, 6–8). Notably, the induced transcription of the p40 gene is delayed compared with other proinflammatory cytokines such as TNF-α; stimulation of phagocytic cells with LPS plus IFN-γ or Staphylococcus aureus (both potent inducers of IL-12) leads to the accumulation of IL-12 p40 mRNA within ~2–4 h (1). Because the accumulation of p40 mRNA occurs only after at least 2 h of APC activation, the general consensus is that bioactive IL-12 is available to induce Th1 differentiation only after this time period (1).

A second emerging paradigm is that certain intracellular pathogens may exploit a mechanism of IL-12 regulation that involves cross-linking of surface receptors on macrophages (9). Through receptor-mediated mechanisms, micro-
organisms such as the measles virus, HIV, and Leishmania major have been shown to inhibit the production of IL-12, whereas others, such as M yobacterium tuberculosis, C pytoxoccus neoformans, and T oxoplasma gondii, induce IL-12 production (9–13). The basis for this differential host–microbe interaction remains unclear. Nevertheless, the studies that have examined the Leishmania-mediated suppression of IL-12 production from APCs have generally been restricted to the determination of IL-12 production after 2 h of contact between the microbe and a distinct subset of APCs, namely macrophages/macrophages (10–12).

DCs differ from monocytes/macrophages in their ability to serve as potent activators of naive T cells, thus acting as critical initiators of the primary specific immune response (14). Given the central role of DC-derived IL-12 in inducing Th1 differentiation and the notion that the factors that influence Th1–Th2 skewing should be operative soon after contact with a pathogen (14–18), we hypothesized that there is a preformed store of IL-12 that is readily available for the rapid initiation of a Th1 immune response shortly after contact with specific microorganisms.

Materials and Methods

Materials. All reagents for cell culture were obtained from Life Technologies; recombinant growth factors were from R&D Systems; ELISA reagents and antibodies were from PharMingen Life Technologies; recombinant growth factors were from R&D Systems; GM-CSF (50 ng/ml) and IL-4 (1 ng/ml) as previously described (14). Given the central role of DC-derived IL-12 in inducing Th1 differentiation and the notion that the factors that influence Th1–Th2 skewing should be operative soon after contact with a pathogen (14–18), we hypothesized that there is a preformed store of IL-12 that is readily available for the rapid initiation of a Th1 immune response shortly after contact with specific microorganisms.

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**D C L y s a t e s .** M urine D Cs (10⁷) were resuspended in complete lysis buffer composed of 700 μl of 1× Relaxation buffer-EGTA (1,000 mM KCl, 30 mM NaCl, 35 mM MgCl₂, 10 mM ATP[Na], 12.5 mM EGTA, 100 mM Pipes, pH 6.8; reference 24) prepared in distilled water, 100 μl of 1 mg/ml leupeptin, 100 μl of 1 mg/ml of pepstatin (all Sigma-Aldrich), 100 μl of 5% NP-40 (Polysciences), and 10 μl of 100 mM PMSF (Sigma-Aldrich) in isopropanol. The D C lysates were centrifuged at 2,200 rpm at 4°C. Supernatants were collected and used in an ELISA for IL-12 p70.

**D C P l a s m a M e m b r a n e E x t r a c t i o n .** The methods of M a e d a et al. (25) were used to isolate plasma membranes from 5 × 10⁷ bone marrow-derived D Cs. In brief, D Cs were resuspended in 5 ml of the homogenization buffer described by M a e d a et al. and homogenized using a Polytron homogenizer (Ika Labortechnics). The homogenate was layered over 10 ml of 41% sucrose in the complete lysis buffer described above and then used as the plasma membrane fraction (25). Using this method for cell fractionation, the pellet below the interface was used as the nonplasma membrane fraction (25).

**P r o t e i n E x t r a c t i o n f r o m T i s s u e s .** M ouse and human spleens, thymus, and lymph nodes, and mouse ears were homogenized. Ears from BALB/c mice were dissected, the dermal and epidermal sheets were separated and cut into small pieces with scissors, and the pieces were then homogenized between the frosted ends of two microscope slides. The homogenized ear tissue was sonicated for 15 s on ice and rested for 1–2 min on ice, and the sonication was repeated three more times. Single-cell suspensions were prepared from mouse spleen, thymus, and lymph nodes by homogenizing the tissue between the frosted ends of two microscope slides. Erythrocytes in these cell suspensions were lysed with red cell lysis buffer (Sigma-Aldrich); the cell pellet was washed twice with PBS and then resuspended in 500 μl of complete lysis buffer (described above). The cell lysates from the aforementioned tissues were centrifuged at 2,200 rpm at 4°C, and an ELISA was used to measure IL-12 p70 in these supernatants. Frozen human tissues (courtesy of Dr. D. Troyer, Pathology and Laboratory Medicine, University of Texas Health Science Center at San Antonio) were homogenized and resuspended in the complete lysis buffer and centrifuged, and IL-12 p70 levels in the supernatants were measured by ELISA. All of the aforementioned procedures were conducted at 4°C or on ice.

**H u m a n L e u k o c y t e I s o l a t i o n a n d C e l l L y s a t e s .** Leukocytes were isolated from 50 ml of heparinized venous blood obtained from normal volunteers. Equal volumes of blood and a solution containing 3% dextran (Amersham Pharmacia Biotech) and 0.85% NaCl were mixed, and erythrocytes were sedimented for 18 min at 1,800 rpm. Supernatants were collected and used in an ELISA for IL-12 p70. The local review boards of the University of Texas Health Science Center at San Antonio approved all protocols.

**R e s u l t s**

Preformed, Membrane-associated IL-12 p70. Based on the homology between the IL-12 p40 subunit and the extracellular domain of the IL-6 receptor as well as the finding that p35 encodes an NH₂-terminal sequence resembling that found in some membrane-associated proteins, a theoretical argument was made for a membrane-bound p70-like complex that could initiate responses on cell–cell contact (27). Using a heterodimer-specific anti–IL-12 mAb, Fan et al. (28) provided flow cytometry evidence suggestive of IL-12 on the cell surface of a human monocytic and a murine macrophage cell line. However, direct evidence for a functional membrane-associated, preformed pool of IL-12 is lacking. Given the central role of the DC–IL-12 axis in host immune functions, our initial efforts were focused on determining whether there is a membrane-associated form of IL-12 p70 in this cell type.

By confocal microscopy, IL-12 p70 immunoreactivity was localized to the periphery of nonpermeabilized D Cs (Fig. 1, A and B). By FACS™ analysis, 70% of the identical population of D Cs used for confocal microscopy stained for IL-12 p70 (Fig. 1 C), and after permeabilization there was no increase in fluorescence intensity of IL-12 p70 and only a 4–6% increase in the number of positive-staining cells (data not shown). The immunoreactivity for IL-12 p70 colocalized with that for intercellular adhesion molecule (ICAM)-1 (29, 30). In additional experiments, immunoreactivity for IL-12 p40 or p35 was also detected on the periphery of nonpermeabilized D Cs (data not shown).

The peripheral localization of immunoreactive IL-12 p70 on D Cs was confirmed by EM studies (Fig. 2, A and B). Immunogold reactivity for IL-12 p70 but not the isotype control was present along the entire surface of the D C plasma membrane. The p40 subunit of the IL-12 was also detected on the cell surface by EM (data not shown).

We also used cell fractionation of permeabilized and nonpermeabilized cells to determine the distribution of IL-12 p70 in the plasma membrane and nonplasma membrane fractions of murine D Cs. Based on both biochemical and immunologic criteria, plasma membranes prepared by the methods of M a e d a et al. yield high-purity plasma membrane fractions (25). Using this method for cell fractionation in three separate experiments, we found that the ratio of the IL-12 p70 content in D C plasma membranes/nonplasma membranes was generally 5:1. For example, in a typical experiment, the IL-12 p70 content in the plasma membrane fraction was 50 pg/μg of protein, whereas in the nonplasma membrane fraction it was 11 pg/μg of protein. In the absence of N P-40 in the complete lysis buffer, BALB/c mice. After 20 min, the peritoneal fluid was removed, the cells were pelleted by centrifugation at 2,200 rpm for 5 min, and the supernatants were snap frozen and stored at −70°C until measurement of IL-12 p70. The local review boards of the University of Texas Health Science Center at San Antonio approved all protocols.
IL-12 p70 was not detected in any of the samples examined. Thus, these data complement the microscopic studies (Figs. 1 and 2), and collectively indicate that the bulk of the preformed stores of IL-12 p70 in unstimulated DCs is restricted primarily to the plasma membrane.

Biological Relevance of Preformed, Membrane-associated Stores of IL-12 p70. Given the importance of IL-12 in mediating rapid host responses to invading organisms, we asked the following questions: is this membrane-associated immunoreactive IL-12 p70 released from the cell surface, and is it biologically active? We also surmised that in the context of host–parasite interactions, such as that between L. donovani, an intracellular parasite, and DCs, this source of preformed IL-12 should be available to initiate host immune responses soon after contact with the parasite. For this reason, we limited our analyses to determining the bio-

Figure 1. Plasma membrane localization of immunoreactive IL-12 p70 on resting nonpermeabilized murine DCs. DCs were stained with IL-12 p70 (A) or rat IgG1 isotype Ab (B) and visualized by confocal microscopy. (C) FACS™ analysis showing that ~70% of the DC population shown in A has surface staining for IL-12 p70. (D–F) Fluorescence for IL-12 p70-FITC (D, green), ICAM-1-PE (E, red), or colocalization of IL-12 p70 and ICAM-1 (F, orange). One representative experiment out of three is shown.

Figure 2. EM photomicrographs showing plasma membrane localization of IL-12 p70 (arrows) in DCs. DCs were labeled with IL-12 p70 (A) and isotype control Ab (B) and visualized by EM. One representative experiment out of three is shown.
availability of this source of IL-12 shortly after DCs come into contact with *L. donovani*. The IL-12 p70 produced by unstimulated DCs was below the detection limits of the ELISA; however, after as little as 10 min of contact with stationary phase (or purified metacyclic) *L. donovani* promastigotes, murine DCs released IL-12 p70 (Fig. 3 A). This release was independent of de novo IL-12 synthesis, as pretreatment of DCs with actinomycin D (inhibitor of transcription) or monensin or brefeldin A (inhibitors of Golgi transport) had no effect on the amounts of IL-12 p70 released from DCs (Fig. 3 A).

In contrast to these effects of L. donovani, there was no detectable IL-12 p70 in the DC supernatants stimulated for 10 min with LPS plus IFN-γ (Fig. 3 A). However, consistent with the time required to induce IL-12 p40 transcription (1, 6, 7), the release of IL-12 p70 from DCs after stimulation with LPS plus IFN-γ was detected only after 2–4 h (∼100–300 pg/ml), and this was inhibited by pretreatment of DCs with actinomycin D, monensin, or brefeldin A (data not shown).

Although the ELISA used to measure the IL-12 released from DCs detects the bioactive form for IL-12 p70, we used a bioassay to confirm this (Fig. 3 B). Addition of supernatants from DCs in contact with L. donovani increased the production of IFN-γ from Ag-primed splenocytes, and this increase was partially inhibited by an IL-12-blocking Ab (Fig. 3 B). The increase in IFN-γ that occurred after addition of supernatants from DC–parasite cocultures was similar in magnitude to that observed after addition of rIL-12 p70 to splenocytes. Collectively, these findings indicate that biologically active levels of IL-12 p70 were present in the supernatants of DC–L. donovani cocultures.

We next postulated that if the parasites indeed trigger the rapid release of membrane-associated IL-12, then the peripherally localized immunoreactivity for IL-12 p70 should diminish shortly after DCs come into contact with L. donovani. Concordant with this, membrane-bound immunoreactivity for IL-12 p70 was detected before (Fig. 4 A) and immediately after (Fig. 4 B) addition of L. donovani promastigotes but not after 10 or 30 min (Fig. 4 C) of contact between DCs and parasites. The timing at which this complete loss of immunoreactivity occurred depended on the temperature at which DCs had been labeled. For DCs labeled at room temperature it was after 10 min of contact with parasites (data not shown), whereas for DCs labeled at 4°C the loss was partial at 10 min but complete at 30 min (Fig. 4, A–C). In contrast, there was no loss in IL-12 p70 immunoreactivity in DCs that had been stimulated with LPS plus IFN-γ for 10 min, nor if the DCs were exposed to L. donovani parasites pretreated with cytochalasin D (data not shown).

Concomitant with this loss in the membrane-bound immunoreactivity for IL-12 p70 (Fig. 4, A–C), there should be a corresponding decrease in the total content of IL-12 p70 in DCs that were in contact with parasites. Fig. 4 D shows that the IL-12 p70 content in the lysates of DCs that were in contact with parasites was significantly lower than in unstimulated DCs. The converse was observed in the supernatants of these DC cultures: the levels of IL-12 p70 were significantly higher in the supernatants of DC–*L. donovani* cocultures than in unstimulated DCs (Fig. 4 D). Taken together, the confocal microscopy findings and the pattern of IL-12 content in the supernatants and DC lysates before and after addition of parasites is consistent with the presence of a preformed pool of membrane-associated IL-12 p70 that is biologically active and is readily released after contact with L. donovani.

To extend these findings, observed in an in vitro setting of DC–parasite contact, we determined whether IL-12 p70 is released rapidly in vivo in response to an infectious challenge. In this in vivo model, L. donovani promastigotes or PBS was introduced into the peritoneal cavity of BALB/c mice, and the peritoneal fluid was recovered after 20 min. IL-12 p70 could be detected in the fluid recovered from the peritoneal cavity of mice after a challenge with L. donovani promastigotes (Fig. 4 E), but not in the peritoneal fluid collected after the injection of PBS. In contrast, the amounts of IL-12 p70 in the cell lysates of cells collected from mice injected with parasites resembled those observed in vitro (Fig. 4 D).

**Figure 3.** Rapid release of bioactive, preformed IL-12 p70 after 10 min of contact with L. donovani (Ld) promastigotes. (A) IL-12 p70 was detected in the supernatants of DCs in contact with L. donovani for 10 min but not in the supernatants of DCs stimulated for the same duration with LPS plus IFN-γ. Pretreating (pretreat) DCs with actinomycin (Act), brefeldin A (Bref), or monensin (Mon) had no effect on the amounts of IL-12 p70 released from DCs in contact with L. donovani. (B) Supernatants (Sup) from unstimulated DCs or DCs in contact with L. donovani (DC + Ld) was added to Ag-reactive splenocytes (Spl). A blocking IL-12 Ab but not isotype control Ab reduced the amounts of IFN-γ released after addition of supernatants from DC–*L. donovania* cocultures to Ag-reactive splenocytes. The amounts of IFN-γ released from splenocytes after addition of supernatants from DC–*L. donovani* cocultures to Ag-reactive splenocytes was similar to that released after addition of recombinant IL-12 p70 (300 pg/ml).
Preformed Stores of Bioactive IL-12 from the peritoneal cavity after PBS challenge was higher than that found in the cell lysates of cells collected after the parasite challenge. These in vivo findings mirrored the in vitro findings shown in Fig. 4, A–D. Because macrophages and not DCs are the more common cell type in the peritoneal cavity, it is likely that these APCs are the major contributor of the preformed IL-12 detected.

In Vitro Determinants of IL-12 p70 Release. What are the L. donovani determinants that contribute to the rapid release of bioactive IL-12? Is contact with the parasite sufficient, or is phagocytosis required for IL-12 release? Does contact with innate objects or nonmotile organisms also induce the release of IL-12? To address these questions, we conducted a series of experiments (Fig. 5). Parasites inactivated by heat or other inactivating agents (UV light, formalin, or trypsin), Histoplasma capsulatum (an intracellular nonmotile fungal organism), or latex beads of varying diameters, did not induce the release of IL-12 p70 from DCs.

Infection with Leishmania involves two processes: parasite-dependent attachment and host-dependent internalization, and pretreatment of Leishmania promastigotes with cytochalasin is known to reduce significantly the ability of the parasites to attach to cells (22). We found that Leishmania promastigotes immobilized (but not killed) with cytochalasin D did not induce IL-12 release (Fig. 5 A). In contrast, pretreatment of DCs with cytochalasin D, an inhibitor of phagocytosis, reduced DC infection by 50% but did not inhibit IL-12 p70 release from DCs in contact with Leishmania species. Furthermore, phagocytosed intracellular parasites were only visualized after 30 min (Fig. 5 B), but not after 10 min of contact with DCs (Fig. 5 C). Collectively, these findings indicated that an important determinant for the very early release of preformed membrane-associated DC stores of IL-12 is contact with motile live L. donovani parasites but not phagocytosis.

Distribution of Preformed IL-12 p70 in Murine and Human Leukocytes and Tissues. We next determined whether this preformed membrane-associated IL-12 was specific to murine DCs/macrophages or were they also present in other murine and human cells/tissues. As described in Materials and Methods, special care was taken to avoid stimulating the cells while processing these samples. IL-12 p70 (pg/μg of protein; mean ± SD; n = 12 mice) was present in the cell lysates of skin (4.0 ± 2.8), lymph node (4.9 ± 2.0), and...
the total leukocyte population (Fig. 6).

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and thymus, and myeloid cells such as monocytes and gran-
cells of several human tissues including lymph nodes, spleen,
also contained IL-12 p70.

Preformed IL-12 p70 was also present in the cell lysates
of RAW and J774 murine cell lines; the cell lysates from RAW and J774 murine cell lines
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Discussion

In support of our hypothesis, we discovered a bioactive
IL-12 p70 in myeloid cells, including DCs that are readily
available for rapid release after contact with an intracellular
parasite such as L. donovani. This readily mobilizable source
of IL-12 is independent of the induced transcription of IL-
12 p40 and is membrane associated. IL-12 p70 represents
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lated, native phagocytes would suggest a preassembled store
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The complete repertoire of microbial agents and the pre-
cise microbe- or host-dependent factors that trigger the
rapid release of membrane-associated preformed IL-12 are
not known. In addition to the data presented here for L.
donovani, L. major and Listeria monocytogenes—highly motile
microorganisms—also induced the early release of IL-12
p70 from splenocytes and DCs after 10 min of contact (data
not shown). We found that as early as 10 min after DC–L.
donovani contact, NF-κB proteins were translocated (data
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Our studies provide a mechanism for the observation
that IL-12 is released rapidly from DCs after in vivo infec-
tion with L. major (31) or L. donovani (10), and perhaps
other organisms such as Toxoplasma (13). Our in vitro and
in vivo studies would also suggest that preformed stores of
bioactive IL-12 are also available for rapid release from
monocytes and macrophages. The rapid release of these IL-
12 stores appears to be dependent on contact between DCs
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synthesis of IL-12, at least in monocytes/macrophages, is
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Given the central role of IL-12 in host defenses, the
swiftness with which preformed bioactive IL-12 was mobil-
ized after DC–parasite contact should not be surprising. It
is striking that evolutionarily conserved systems are also in
place for the rapid release via preformed stores of other
proinflammatory cytokines such as TNF-α (32, 33). It is
conceivable that the rapid release of IL-12 from DCs may
act in an autocrine fashion to further stimulate DCs via
constitutively expressed IL-12 receptors (34), and/or serve

Figure 5. Determinants of the IL-12 p70 release by DCs. (A) 10 min after adding infectious or noninfectious agents to resting or cyto-
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DCs or parasites with cytochalasin D. Results

Figure 6. Distribution of IL-12 p70 in human cells/tissues. IL-12 p70 (in pg) detected in protein lysates of total leukocytes (Lu) isolated from whole
blood (values normalized to pg/ml of blood); neutrophils (N), lymphocytes (Ly), or monocytes (Mo) (values normalized to pg/10^6 cells); lymph node
(LN), spleen (Sp), or thymus (Th) (values normalized to pg/g tissue). Note that the scales of the left and right y axes are different and are for the values
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mice; the cell lysates from RAW and J774 murine cell lines
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to the left and right, respectively, of the tick marks on the x axis.
as a first line of defense by activating T cells or NK cells in the immediate vicinity (35). In this scenario, the immediate release of even low levels of membrane-associated IL-12 could trigger a host of critical immune responses, including upregulation of the IL-12 receptor (36) and a consequent increase in cell responsiveness to IL-12, and the induction of an early IFN-γ response. This is highly relevant in the context that immunity against intracellular protozoan Leishmania species is highly dependent on the rapid development of a Th1-biased immune response (17, 37). Indeed, the very rapid release of bioactive IL-12 after parasite–DC contact is consistent with the hypothesis that IL-12 serves as a “match” to ignite the immune response in L. major infection (38). Furthermore, several studies using recombinant IL-12 and anti–IL-12 antibody have documented that the early IL-12 response to infection is critical to the final outcome (39, 40). Whether the amounts of preformed membrane-associated IL-12 in humans are genetically controlled remains to be established, but variability in the amounts stored and/or released could be an important determinant of host susceptibility to various infections.

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