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Evidence for Human CD4⁺ T Cells in the CD1-Restricted Repertoire: Derivation of Mycobacteria- Reactive T Cells from Leprosy Lesions

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Both the CD4⁺ CD8⁻ (double negative) and CD4⁺ CD8⁺ T cell lineages have been shown to contain T cells which recognize microbial lipid and glycolipid Ags in the context of human CD1 molecules. To determine whether T cells expressing the CD4 coreceptor could recognize Ag in the context of CD1, we derived CD4⁺ T cell lines from the lesions of leprosy patients. We identified three CD4⁺ Mycobacterium leprae-reactive, CD1-restricted T cell lines: two CD1b restricted and one CD1c restricted. These T cell lines recognize mycobacterial Ags, one of which has not been previously described for CD1-restricted T cells. The response of CD4⁺ CD1-restricted T cells, unlike MHC class II-restricted T cells, was not inhibited by anti-CD4 mAb, suggesting that the CD4 coreceptor does not impact positive or negative selection of CD1-restricted T cells. The CD4⁺ CD1-restricted T cell lines produced IFN-γ and GM-CSF, the Th1 pattern of cytokines required for cell-mediated immunity against intracellular pathogens, but no detectable IL-4. The existence of CD4⁺ CD1-restricted T cells that produce a Th1 cytokine pattern suggests a contributory role in immunity to mycobacterial infection.


Until recently, Ag presentation to T cells was defined by a limited number of proteins encoded within the MHC locus. It is now clear that additional proteins within the MHC locus, as well as proteins encoded outside the MHC locus, can also present Ags to T cells. The best studied of these non-MHC-encoded proteins is the CD1 family (3, 4). The pathway of CD1-presented Ags diverges from that of classical MHC processing, indicating that the CD1 Ag presentation may be complementary to the MHC pathways. The most surprising finding of the CD1 Ag-presenting system is that the Ags presented by CD1 are not peptide, but rather lipid or glycolipid in nature (5, 6).

The CD1 proteins have been classified into two subgroups based on sequence similarity: The group 1 proteins which include human CD1a, CD1b, and CD1c molecules are much more closely related to one another than they are to the fourth human protein, CD1d. Group 2 proteins include human CD1d and murine CD1. Most of our knowledge about group 1 CD1-restricted T cells and their role in microbial infection derives from studies investigating the host response to mycobacteria. Immunity to mycobacterial infection requires both MHC class I- and class II-restricted T cells (7, 8) which are directed against peptide Ags. However, mycobacteria are characterized by a complex lipid and glycolipid envelope, which has potent immunostimulatory activity for B cells and monocytes (9, 10). Previous studies from our laboratories have shown that this complex mycobacterial lipid envelope is a rich source of CD1-restricted T cell Ags as well (4, 6, 11). The general structural features of these T cell Ags are a hydrophobic region (fatty acid chains) coupled to a polar or charged hydrophilic end. The large hydrophobic pockets identified in murine CD1 (12) are thought to be capable of accommodating the fatty acid portions of these Ags. Direct binding studies have confirmed that the lipid portion of CD1 ligands bind to CD1b (13).

As an initial step toward defining the in vivo relevance of CD1 Ag presentation, we have studied the involvement of CD1 in the immune response to Mycobacterium leprae, the causative agent of leprosy. Infection with this organism provides an opportunity to study the human immune response to a mycobacterial pathogen in situ, since much of the pathology is localized to the skin where the relevant lesions can be easily sampled. Furthermore, leprosy presents as a spectrum in which clinical disease correlates with different levels of responsiveness to M. leprae. At one pole of the spectrum are patients with strong cell-mediated immunity to M. leprae and a localized form of disease, which constitutes tuberculous leprosy. At the opposite pole are patients with lepromatous leprosy, who lack effective cell-mediated immunity and suffer from a more disseminated form of the disease. The existence of this spectrum provides the opportunity to assess immunoregulatory mechanisms that may operate in vivo in humans to determine the ultimate outcome of the immune response to infection.
Previous studies have shown that only CD4+ (CD8+ and double negative (DN))<sup>4</sup> T cells recognize mycobacterial Ags in the context of CD1 (4, 6, 14, 15). However, CD4<sup>+</sup> T cells predominate in the lesions of tuberculoid leprosy (16) and CD4 can be expressed on group 2 CD1-restricted T cells (17). To determine whether T cells expressing the CD4 coreceptor could be selected by group 1 CD1 proteins, we derived CD4<sup>+</sup> T cell lines from the lesions of leprosy patients. We identified three CD4<sup>+</sup> M. leprae-reactive, CD1-restricted T cell lines: two that recognize mycobacterial Ags in the context of CD1b and one that recognizes an as yet unidentified lipid Ag in the context of CD1c. Monoclonal Abs that prevent CD4<sup>+</sup> HLA-DR-restricted T cells from responding to Ag had no effect on CD4<sup>+</sup> or DN CD1-restricted T cells, indicating that the CD4 coreceptor does not impact positive or negative selection of CD1-restricted T cells. The CD4<sup>+</sup>, CD1-restricted T cell lines produced IFN-γ and GM-CSF, the Th1 pattern of cytokines required for cell-mediated immunity against intracellular pathogens, but no detectable IL-4. Therefore, the existence of CD4<sup>+</sup> CD1-restricted T cells. The CD4<sup>+</sup>, CD1-restricted T cell lines produced IFN-γ and GM-CSF, the Th1 pattern of cytokines required for cell-mediated immunity against intracellular pathogens, but no detectable IL-4. Therefore, the existence of CD4<sup>+</sup> CD1-restricted T cells that produce a Th1 cytokine pattern suggests a contributory role in immunity to mycobacterial infection.

Materials and Methods

Patients and clinical specimens

Leprosy patients were recruited on a volunteer basis from the ambulatory population seen at the Dermatology Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and the University of Miami Medical Center. Clinical classification of patients with Dermatologic Clinics at the University of Southern California Medical Center and

Ags and Abs

Extracts of M. leprae, M. tuberculosis (strain H37Rv), and M. phlei were prepared by probe sonication (19). To prepare non-peptide Ags, sonicated mycobacteria were treated with proteinase K (0.7 mg/ml, Boehringer Mannheim, Indianapolis, IN) for 30 min at 60°C, and the enzyme was heat-inactivated for 10 min at 70°C. Purified mycobacterial lipids and lipoglycans were isolated as described previously (5, 11, 20, 21). The following Abs were used for flow cytometry: IgG controls (Sigma, St. Louis, MO), OKT6 (anti-CD1a (22)), BCD1b3.1 (anti-CD1b (23, 24)), F10/21A3 (anti-CD1c (25)); OKT3 (anti-CD3; American Type Culture Collection (ATCC), Manassas, VA); OKT4 (anti-CD4; ATCC); OKT8 (anti-CD8; ATCC), BMA 031 (anti-TCRab; Caltag, Burlingame, CA); 5A6.E9 (anti-TCRγδ; Caltag); HP-3D9 (anti-CD94; PharMingen, San Diego, CA); DX12 (anti-CD161/NK-PIA; PharMingen).

In vitro culture of CD1-expressing monocyte-derived dendritic cells

CD1 expression on monocytes was induced in vitro with a combination of recombinant human GM-CSF (200 U/ml) and recombinant human IL-4 (100 U/ml) for 72 h as described elsewhere (4, 26). Cells were harvested using incubation in PBS/0.5 mM EDTA to detach adherent cells and analyzed for CD1 expression by flow cytometry (4) or irradiated (5000 rad) and used as APCs.

T cell lines and proliferation assays

T cell lines were derived from leprosy lesions and the blood of healthy donors as described previously (6, 28), although CD4 and CD8 T cells were not depleted. Briefly, cells were extracted from lesions with a tissue sieve and lymphocytes were isolated by density gradient centrifugation. HLA-DR-restricted T cell lines were initiated in the presence of irradiated autologous PBMC and mycobacterial extract, whereas CD1-restricted T cells were cultured in the presence of irradiated autologous CD1<sup>+</sup> APCs and extract (4). T cell lines were maintained by serial antigenic stimulation in recombinant IL-2 (1 nM; Chiron Diagnostics, Norwood, MA) supplemented medium. For measurement of Ag-specific proliferation, T cells (1 × 10<sup>5</sup>) were cultured with varying numbers (usually 1 × 10<sup>5</sup>) of irradiated (5000 rad) heterologous CD1<sup>+</sup> APCs in culture medium (0.2 ml) in the presence or absence of bacterial Ags for 3 days in microtiter wells (in triplicate) at 37°C in a 7% CO2 incubator. Cells were pulsed with [3H]thymidine (1 μCi/well; ICN Biomedicals, Costa Mesa, CA) and harvested 4–6 h later for liquid scintillation counting. To determine CD1 restriction of the T cell lines, neutralizing CD1 Abs were added 30 min before the addition of T cells. Alternatively, THP-1 cells expressing distinct isoforms of CD1 were used as APCs (29). To determine the role of CD4 accessory interactions, T cells were cultured with a neutralizing CD4 Ab (clone QS4120; Caltag, San Diego, CA) or isotype-matched control Ab for 30 min, cells were washed to remove unbound Ab, and T cells were added to APCs and Ag. Cytokine release from T cells was measured by ELISA after stimulation with CD1-positive APCs and Ag or media for 24 h. ELISAs (IFN-γ and IL-4; Endogen, Woburn, MA; GM-CSF, PharMingen, San Diego, CA) were performed according to the instructions of the manufacturers.

Results

CD4<sup>+</sup> CD1-restricted T cells in leprosy lesions

We derived T cell lines from the granulomatous lesions of patients with tuberculoid leprosy in the presence of CD1<sup>+</sup> APCs and an extract of M. leprae. Flow cytometry and T cell proliferative assays revealed that three cell lines (LCD4.1 (Fig. 1), LCD4.2, and LCD4.3) from three different patients expressed CD4 and TCRαβ and the ability to respond to M. leprae Ag in the presence of heterologous CD1<sup>+</sup> APCs. These three cell lines were therefore selected for further study.

CD4<sup>+</sup> T cells derived from tuberculoid leprosy lesions have been shown to produce a Th1 cytokine pattern (30, 31). These T cell lines were peptide-reactive HLA-DR-restricted and have generally been thought to contribute to immunity against M. leprae. To determine the cytokine production from CD4<sup>+</sup> T cells derived by culture with CD1<sup>+</sup> APCs, we stimulated the cell lines with CD1<sup>+</sup> APCs and M. leprae. The T cell lines produced IFN-γ, a potent stimulus for MHC class II expression, but no detectable IL-4 (Fig. 2). We have previously shown that both GM-CSF and CD1 are elevated in tuberculoid as compared with lepromatous lesions (32, 33). CD1 expression in vitro is dependent on cytokines, notably GM-CSF (4, 26). The CD4<sup>+</sup> T cell lines also

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<sup>4</sup> Abbreviations used in this paper: DN, double negative; LAM, lipoarabinomannan; GMM, glucose monomycoborate; PIM, phosphatidyl monomyl cinnamide.
produced GM-CSF in response to Ag. Together, these data suggest that CD4 T cell lines stimulated under conditions to activate CD1-restricted T cells produce cytokines that would enhance Ag presentation through both the MHC class II and CD1 pathways.

To determine whether the T cell lines recognized *M. leprae* in the context of CD1, we used neutralizing Abs to the three isoforms of CD1 present on dendritic cells in leprosy lesions, CD1a, CD1b, and CD1c (33). Fig. 3, A and B, shows that Abs to CD1c blocked the proliferation and IFN-γ production of the CD4+ T cell line LCD4.1, indicating that LCD4.1 recognized *M. leprae* Ag in the context of CD1c. The CD1c restriction of LCD4.1 was confirmed using the monocytic cell line, THP-1, transfected with distinct CD1 isoforms. CD1c-expressing THP-1 cells induced IFN-γ production from LCD4.1 in the presence of *M. leprae* (Fig. 3C), whereas CD1b-transfected THP-1 did not induce IFN-γ production. Similarly, LCD4.1 was activated to produce IFN-γ in the presence of *M. leprae*-pulsed CD1c, but not CD1b-transfected HeLa cells (data not shown).

In contrast to LCD4.1, which was CD1c restricted, Abs to CD1b inhibited the proliferation and IFN-γ production of LCD4.2, indicating that CD1b presented *M. leprae* Ag to LCD4.2 (Fig. 4, A and B). Interestingly, THP-1 cells transfected with CD1a, CD1b, or CD1c did not induce IFN-γ production from LCD4.2 (data not shown). The third CD4+ T cell line, LCD4.3, produced IFN-γ, which was inhibited by neutralizing Ab to CD1b, but not by anti-CD1a or anti-CD1c (Fig. 4C), indicating CD1b restriction. Finally, to demonstrate that this population of T cells was present in the pool of peripheral blood T cells, we also derived CD1-restricted CD4+ T cells from the blood of a healthy donor (BCD4.1, Fig. 4D). We conclude therefore that CD4+ T cells belong to the repertoire of T cells that recognize microbial Ags in the context of group 1 CD1 proteins.

**Expression of NK receptors on CD4+ CD1-restricted T cells derived from leprosy lesions**

Murine CD1-restricted T cells are characterized by their expression of NK receptors, in particular NK1.1 (34), and therefore are often referred to as NK T cells. Activation of NK T cells through a scinitillation counter. B, T cell supernatants (24 h) prepared as in Fig. 2 were measured for IFN-γ production by ELISA. C, T cells (2 x 10⁶) were cultured with THP-1 cells (2 x 10⁶) transfected with distinct CD1 isoforms in the presence or absence of *M. leprae* (1:1000 dilution of extract). Supernatants were harvested at 24 h and IFN-γ was measured by ELISA. Values (means ± SEM) shown are representative of at least three independent experiments.

CD4+ CD1-restricted T cells recognize non-peptide Ags

To date, human CD1-restricted T cells have been shown to recognize lipid and glycolipid Ags (5, 6, 11, 19), thus we investigated the nature of the Ags that activated CD4+ CD1-restricted T cells. LCD4.1 T cells were activated by extracts from *M. leprae*, *M. tuberculosis*, and *M. phlei*, (Fig. 6A), suggesting that the Ag was...
FIGURE 4. CD1b-restricted CD4⁺ T cell lines LCD4.2 and LCD4.3. A, T cells were cultured as described in Fig. 2 and pulsed with [³H]thymidine (4 h) after 72 h of culture. Cells were harvested, and [³H] incorporation was measured by a scintillation counter. B, T cell supernatants prepared as in Fig. 2 were measured for IFN-γ production by ELISA. C, IFN-γ production by CD4⁺ T cell line LCD4.3. D, CD4⁺ CD1b-restricted T cell line BCD4.1, derived from the peripheral blood of a healthy donor. Values (means ± SEM) shown are representative of three or more independent experiments.

structurally conserved in different mycobacterial species. To determine whether the Ag was a protein, *M. tuberculosis* extract was treated with proteinase K. Fig. 6B demonstrates that proteinase K treatment of *M. tuberculosis* had no effect on the ability to stimulate LCD4.1, whereas proteinase K abrogated the ability of *M. tuberculosis* to activate an HLA-DR-restricted T cell line LDR-2 (Fig. 6C), indicating that LCD4.1 recognizes a non-peptide Ag. To determine whether the Ags previously shown to stimulate CD1b- and CD1c-restricted T cells could activate CD4⁺, li-


duced IFN-γ from LCD4.2 by 82%, whereas anti-CD1c inhibition was weak by comparison. As indicated above, THP-1 cells transfected with CD1b did not stimulate LCD4.2, probably due to the requirement for the mannose receptor for uptake of LAM and PIM for CD1b Ag presentation (41). Taken together, with the data from Fig. 4, we conclude that T cell line LCD4.2 recognizes PIM in the context of CD1b.

To determine the Ag responsiveness of the CD1b-restricted T cell line LCD4.3, we also tested the known mycobacterial CD1 Ags. LCD4.3 responded to *M. leprae* and mycolic acid (Fig. 7C), a previously recognized CD1 Ag (5), but exhibited no response to two other CD1b Ags, LAM (6) and GMM (11). The data indicate that all three CD1-restricted CD4⁺ T cell lines derived from lep-


destined T cells to mycobacterial Ag. CD4 Ab had minimal effect on the response of LCD4.1 or LCD4.3 to *M. leprae* (Fig. 8, A and B), whereas the Ab inhibited the proliferation of an HLA-DR-restricted T cell line, D103-5 (45, 46), by 83% (Fig. 8C). These data indicate that the CD4 accessory interaction between the T cell and APC is not required for the response of CD4⁺ CD1-restricted T cells.

Discussion

The established paradigm for Ag presentation has been one of MHC-encoded molecules presenting peptide Ags to T cells. The paradigm holds that CD8⁺ T cells recognize 8–10 aa peptides presented by MHC class I molecules and CD4⁺ T cells recognize 13–17 aa peptides presented by MHC class II molecules. Recent studies involving the non-MHC-encoded family of CD1 proteins challenges this view and indicates that T cells recognize lipid Ags in the context of CD1 Ag-presenting molecules. The first human CD1-restricted T cells described were CD4⁺ CD8⁻ DN expressing either αβ or γδ TCRs (47). DN T cells represent a minor subset of
the T cell repertoire, suggesting CD1-restricted T cells might play a minor role in immune responses. However, recent studies indicate that human CD1-restricted T cells can also express CD8αβ heterodimers (14, 15). The present study provides evidence that T cells expressing CD4 and TCRαβ may recognize foreign Ags in the context of CD1 molecules. Three CD4+ T cell lines were derived from leprosy lesions that recognized non-peptide Ags in the context of CD1b or CD1c. These T cell lines produced IFN-γ and GM-CSF, but no detectable IL-4, and the response of the T cells did not require CD4 accessory interaction, in contrast to a CD4+ HLA-DR-restricted T cell line. The existence of human CD4+ TCRαβ+ T cells that are CD1 restricted suggests that the T cell response to Ag presented by CD1 encompasses the complete repertoire of T cells.

One unresolved question regarding CD1-restricted T cells is their representation in different subsets of the T cell repertoire. Our earlier studies demonstrated that CD1-restricted T cells can express CD8 or be CD4 and CD8 negative (DN) (4, 6, 14, 47). The...
present study now indicates that CD1-restricted T cells can express CD4\(^+\) coreceptors. Furthermore, both CD4\(^+\) and CD4\(^-\) T cells have the capacity to recognize the same ligand (PIM) in the context of CD1b (6). In this study, we demonstrated that CD4 accessory interaction is not required for T cell recognition, thus one might conclude that CD4 has no contributory role to the ability of CD1-restricted T cells to recognize Ag or an inhibitory role in preventing such recognition. These findings, however, do not preclude that membrane reorganization and signaling of CD4 could occur in a normal fashion (48–50).

Our data may provide some insight into the role of coreceptors in selection of CD1-restricted T cells. Most of our understanding of selection of CD1-restricted T cells derives from studies in mice. CD1-restricted T cell selection does not require MHC class II molecules (17, 51) and although a role for MHC class I has not been ruled out, the ligand responsible is most likely CD1 itself (52). Since CD1-restricted T cells 1) can be CD4\(^+\) or DN (34), 2) can recognize the same glycolipid Ag regardless of CD4 expression (6), and 3) do not require CD4 accessory interactions, our findings indicate that MHC class II is not required for selection of human CD1-restricted T cells and that CD4 is not required for positive selection. The existence of CD4\(^+\) CD1-restricted T cells further suggests that CD4 does not negatively select against CD1 responses.

Using leprosy as a model of intracellular infection to evaluate the role of CD1 in human immune responses, we have derived CD4\(^+\) CD1-restricted T cells from lesions of patients with tuberculous leprosy, those patients able to localize the infection and mount T cell responses against the pathogen. CD4\(^+\) T cells predominate in the lesions of tuberculous patients (16) and CD4\(^+\) T cell clones derived from these lesions produce cytokines that promote cell-mediated immune responses (30). We have also found a strong correlation between the expression of three forms of CD1 (i.e., CD1a, CD1b, and CD1c) known to be involved in the presentation of mycobacterial lipid Ags and the presence of effective host immunity to M. leprae (33). CD1 expression in tuberculous lesions was shown to be primarily restricted to a mature dendritic cell population, and in vitro functional studies revealed that dendritic cells bearing the phenotype found in tuberculous lesions were strikingly efficient at presenting mycobacterial lipid Ags to CD1b-restricted T cells. Since all three group I CD1 proteins can present lipid Ags to T cells (5, 14, 19), these results support the hypothesis that lipid Ag presentation by the CD1 system plays a beneficial role in host immunity to microbial pathogens in vivo and that CD4\(^+\) CD1-restricted T cells participate in the local immune response.

Upon activation by mycobacterial Ag, CD4\(^+\) CD1-restricted T cells produced IFN-\(\gamma\) and GM-CSF similar to DN and CD8\(^-\) T cells. IFN-\(\gamma\) is a critical cytokine for immunity to mycobacterial infection as evidenced by the greater susceptibility to infection in IFN-\(\gamma\) knockout mice and the greater IFN-\(\gamma\) expression seen in tuberculous leprosy (32, 53, 54). GM-CSF induces CD1 on monocytes, which would lead to a greater frequency of Ag presentation to CD1-restricted T cells. Together, our data suggest that CD1\(^+\) dendritic cells at the site of disease present lipid Ags to CD4\(^+\) T cells, leading to the Th1 cytokine response and increased CD1 expression. In this manner, CD4\(^-\) CD1-restricted T cells can contribute to cell-mediated immune responses for host defense against intracellular pathogens. In summary, our findings extend the universe of CD1-restricted T cell repertoire to include CD4\(^+\) TCR\(\alpha\)\(\beta\) T cells and identifies these cells as part of the immune response that combats infection.

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References


