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Induction of CD1-Restricted Immune Responses in Guinea Pigs by Immunization with Mycobacterial Lipid Antigens

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Group 1 CD1 molecules have been shown to present lipid and glycolipid Ags of mycobacteria to human T cells. However, a suitable animal model for the investigation of this component of antimycobacterial immunity has not yet been established. Previously, we found that guinea pigs express multiple isoforms of group 1 CD1 proteins that are homologous to human CD1b and CD1c. In this study, we show that CD1-restricted T cell responses can be generated in guinea pigs following immunization with lipid Ags from Mycobacterium tuberculosis. Splenic T cells from lipid Ag-immunized guinea pigs showed strong proliferative responses to total lipid Ags and partially purified glycolipid fractions from M. tuberculosis. These lipid Ag-reactive T cells were enriched in CD4-negative T cell fractions and showed cytotoxic activity against CD1-expressing guinea pig bone marrow-derived dendritic cells pulsed with M. tuberculosis lipid Ags. Using guinea pig cell lines transfected with individual CD1 isoforms as target cells in cytotoxic T cell assays, we found that guinea pig CD1b and CD1c molecules presented M. tuberculosis glycolipid Ags to T cells raised by mycobacterial lipid immunization. These results were confirmed using a T cell line derived from M. tuberculosis lipid Ag-immunized guinea pigs, which also showed CD1-restricted responses and cytolytic activity. Our results demonstrate that CD1-restricted responses against microbial glycolipid Ags can be generated in vivo by specific immunization and provide support for the use of the guinea pig as a relevant small animal model for the study of CD1-restricted immune responses to mycobacterial pathogens.


The human CD1 gene family consists of five nonpolymorphic genes, CD1A, CD1B, CD1C, CD1D, and CD1E, which map to a locus on chromosome 1 (1). The proteins encoded by this gene family are transmembrane glycoproteins expressed in association with β2-microglobulin on the surface of dendritic cells and other APCs. The CD1 proteins show limited but significant homology to MHC class I and II Ag-presenting molecules that present peptide Ags to T cells (2). This homology of structure provided a clue to the possible function of CD1 proteins in the presentation of foreign Ags to T cells. However, CD1 proteins are distinct from MHC-encoded molecules by the marked abundance of hydrophobic amino acids in the membrane distal domains. This finding suggested that CD1 proteins might have a different type of Ag binding site than the peptide binding groove found in MHC molecules (3). This prediction has been confirmed by the crystal structure of a mouse CD1 protein, which shows a deep internal cavity formed by the α1 and α2 domains which is lined almost entirely by hydrophobic amino acid side chains (3).

The unique structure of the Ag binding groove of CD1 suggested that CD1 binds and presents something other than peptides. Direct evidence now confirms this hypothesis, as several studies have shown that the human group 1 CD1 proteins (i.e., the group composed of CD1a, CD1b, and CD1c) mediate specific T cell recognition of mycobacterial lipid and glycolipid Ags (4). Ags identified to date include mycolic acid, glucose monomycolate, lipoarabinomannan, and other phosphoglycolipids (5–8). T cells recognizing mycobacterial lipid Ags presented by CD1 can release IFN-γ and show cytolytic activity upon contact with lipid Ag-pulsed target cells and mycobacteria-infected macrophages. Furthermore, CD8+ CD1-restricted T cells have also been recently shown to mediate a direct microbialidal effect on intracellular microorganisms by granulysin-mediated killing (9). These activities suggest a proinflammatory role for these T cells in type 1 cellular immune responses that are critical for the elimination of intracellular pathogens such as Mycobacterium tuberculosis (MTb) and Mycobacterium leprae (10).

Whereas previous work establishes the function of CD1 in Ag recognition by T cells, the critical question of the role of this system in host defense against infection in vivo has not been examined. Evaluation of the in vivo role of the CD1 system in the protective immune response to pathogens such as mycobacteria will require the development of a suitable animal model in which such responses are generated and can be studied. In the case of MTb infection, such an animal model would ideally be one that accurately replicates the important immunopathological and clinical features of human tuberculosis and also must have a CD1 system comparable to that which is present in humans.

Abbreviations used in this paper: MTb, Mycobacterium tuberculosis; MPL, monophosphoryl lipid A; BM-DC, bone marrow-derived dendritic cell; DN, double negative.
rattus are probably not suitable animal models in this case because they express only CD1d-like molecules and lack obvious homologs of the human group1 CD1 proteins (i.e., CD1a, b, and c) which are all known to present microbial lipid Ags (4). In contrast, our previous studies suggested that the guinea pig may prove to be an excellent animal model for studying group1 CD1-dependent immune responses in MTb infection (11).

We have already reported that the guinea pig genome contains an extended family of CD1 genes, with at least eight distinct CD1 homologs (11). Based on analyses of predicted amino acid sequences, it was possible to classify each guinea pig homologs (11). Based on analyses of predicted amino acid sequences, it was possible to classify each guinea pig CD1 gene as a homolog of one specific human CD1 isoform. This revealed that the guinea pig has genes that encode multiple homologs of human CD1b (i.e., guinea pig CD1B1, B2, B3, and B4) and CD1c (CD1C1, C2, and C3), in addition to at least one gene encoding a CD1e-like protein. In the current study, we demonstrate the existence of CD1-restricted nonpeptide lipid Ag-reactive T cells in guinea pigs. These CD1-restricted T cell responses were shown to be induced in vivo following immunization with MTb lipid Ags and led to the generation of cytotoxic T cell responses restricted by several different members of the guinea pig group1 CD1 family. These findings provide further justification for the use of the guinea pig as a relevant small animal model for study of the in vivo role of CD1-mediated immune responses to lipid Ags in host defense against MTb and other microbial pathogens.

Materials and Methods

Animals

Outbred female Hartley guinea pigs were purchased from Charles River Breeding Laboratories (Wilmington, MA). Inbred strain 2 guinea pigs were obtained from Tenevus Research Laboratory (Southampton, U.K.) and bred in our animal facility at the Dana-Farber Cancer Institute. Animals were housed under specific pathogen-free conditions and all animal experiments were performed according to the Guidelines on Animal Welfare approved by the committee on animal use at Harvard Medical School and the Brigham and Women’s Hospital.

Monoclonal Abs

CT5 (anti-pan guinea pig T cell), CT6 (anti-guinea pig CD8), and CT7 (anti-guinea pig CD4) were obtained from Serotec (Raleigh, NC). CD1F2/6B5 (mouse IgG1), reactive with all of the currently known isoforms of guinea pig group 1 CD1, was produced in our laboratories from a hybridoma cell line generated from splenocytes of a mouse hyperimmunized with guinea pig CD1 proteins (12). A hybridoma line producing mAb IVA12 (anti-human MHC class II monomorphic determinant and cross-reactive to guinea pig MHC class II) was obtained from the American Type Culture Collection (Manassas, VA).

Purification of lipid Ags from MTb

Lipid Ags were purified as previously described (13). Briefly, total sonicates (10 mg dry weight bacteria/ml of MTb strain 331) in PBS were extracted with four volumes of chloroform/methanol (2:1, v/v) according to the method of Folch et al. (14). After phase separation, the organic phase was collected, dried by rotary evaporation, and resuspended in the original volume of distilled water for storage. Further subfractions of MTb total lipids, silica column chromatography was used (5). After evaporation of the solvent from the MTb total lipid extract, the lipids were resuspended in hexane and passed over open acid silica columns to which were added by adding an appropriate volume of PBS and passing three times through a 25-gauge needle.

Immunization of guinea pigs with lipid Ags

Specific pathogen-free outbred female Hartley strain guinea pigs (250–300 g) or inbred strain 2 guinea pigs (250–300 g) were immunized s.c. with purified lipid Ags of MTb in liposomes with or without adjuvant (QS-21 or MPL). For each immunization, animals received liposomes containing a mixture of some or all of the MTb lipid Ags (glycolipids, neutral lipids, phospholipids, and mycolic acids) as indicated. In each case, the amount of immunogen used per animal contained 500–600 μg of each lipid component, and the dose of QS-21 or MPL applied as adjuvant was 50 or 300 μg per immunization, respectively (19, 20). In some experiments, liposomes containing OVA were made by adding soluble OVA (900 μg) during the rehydration step. Liposome suspensions were lypophilized for storage at −20°C and immediately before use were resuspended by adding an appropriate volume of PBS and passing through a 25-gauge needle.
The results showed that the in vivo immunization with BM-DCs pulsed with MTb lipid Ags induced strong CTL responses against MTb lipid Ags. In contrast, immunization of guinea pigs with liposomes containing MTb lipids plus either QS-21 (Fig. 2A) or MPL (Fig. 2B) resulted in weak proliferative responses to the glycolipid, neutral lipid, or mycolic acid fractions. In contrast, immunization of guinea pigs with liposomes containing MTb lipids plus either QS-21 (Fig. 2A) or MPL (Fig. 2B) resulted in weak proliferative responses to the glycolipid, neutral lipid, or mycolic acid fractions.
proliferative responses to MTb glycolipid and phospholipid fractions. The high level of background proliferation observed in this case (medium alone) was most likely due to the simultaneous sensitization of alloreactive T cells, since Hartley guinea pigs are not fully inbred. To rectify this potential problem, we conducted analogous experiments using inbred strain 2 guinea pigs as the BM-DC donor and as the recipient immunization with BM-DCs pulsed with MTb lipid Ag. As shown in Fig. 2C, in vivo immunization of an inbred strain 2 guinea pig with MTb lipid Ag-pulsed BM-DCs of strain 2 origin also induced significant proliferative responses of splenic T cells in vitro, as well as a strong response to the phospholipid fraction and a weak but significant response to the glycolipid fraction. In vivo sensitization of strain 2 guinea pigs using MTb lipid Ag-pulsed BM-DCs was repeated three times, showing consistent levels of T cell proliferation to total MTb lipid Ags and phospholipids (stimulation index of 3.2–5.5 compared with medium alone) and a lesser level of proliferation to glycolipids (stimulation index, 1.7–2.6), with minimal or no responses to neutral lipids. In similar experiments conducted in parallel, we also observed that guinea pigs immunized with splenic DCs pulsed with the total MTb lipid Ag extract showed proliferative responses to MTb lipid Ags upon in vitro challenge (data not shown). These results indicated that BM-DCs and splenic DCs expressing CD1 could present mycobacterial lipid Ags to T cells in vivo, thus leading to lipid Ag-specific recall responses that could be detected by T cell proliferation ex vivo.

Ex vivo CTL responses from guinea pigs immunized with mycobacterial lipid Ags

In humans, the majority of group 1 CD1-restricted T cell lines reactive with mycobacterial lipid Ags that have been studied to date exhibit cytotoxic activity against lipid Ag-pulsed DCs or CD1-expressing transfectant cells along with proliferative responses and IFN-γ production. Therefore, we next examined whether the MTb lipid Ag-reactive T cells arising after immunization of guinea pigs showed cytotoxic activity against MTb lipid Ag-pulsed target cells. Based on the results described in Fig. 1, we immunized naïve strain 2 or Hartley guinea pigs with MTb lipid Ags (mixture of glycolipid, neutral lipid, and mycolic acid fractions) entrapped in liposomes with the combination of QS-21 and MPL as adjuvants. Effector T cells were prepared from immunized guinea pigs by in vitro stimulation of splenic T cells with MTb total lipid extract (10 μg/ml) and glycolipid-enriched fraction (30 μg/ml) for 6 days. We first tested CTL responses against lipid Ag-pulsed syngeneic strain 2 BM-DCs, which we have shown to express high levels of multiple different guinea pig CD1 proteins (12). As shown in Fig. 3A, the effector T cells derived from the MTb lipid Ag-immunized guinea pigs lysed the target BM-DCs...
pulsed with MTb glycolipids or total lipids significantly above the background level of target cells that were not pulsed with MTb lipids. Similar to the proliferative responses observed after MTb lipid Ag immunization using the liposome vehicle with QS-21 or MPL as adjuvant (Fig. 1), effector T cells in cytolytic assays showed the strongest reactivity to the MTb total lipid Ag preparation and the glycolipid-enriched fraction, but also showed a trend toward weak recognition of BM-DCs pulsed with the neutral lipid-enriched fraction.

Next, we examined these cytotoxic T cell responses for their dependence on the expression of particular CD1 molecules by the target cells. As described elsewhere, we generated a new mAb (CD1F2/6B5) against guinea pig CD1 proteins which recognized all seven of the currently identified isoforms of guinea pig group 1 CD1 proteins (12). As shown in Fig. 3B, the cytolytic activity of effector T cells obtained from lipid Ag-immunized guinea pigs against BM-DCs pulsed with glycolipids or total lipids were significantly inhibited by anti-CD1 mAb, but not by isotype-matched control mAb (P3, mouse IgG1). These results strongly suggested that mycobacterial lipid Ag-reactive cytotoxic T cells elicited in guinea pigs by immunization with MTb lipid Ags were at least partially CD1 restricted.

To address which of the multiple different group 1 CD1 proteins of the guinea pig were involved in CTL responses evoked by immunization with MTb lipid Ags, we used a panel of guinea pig cell lines stably transfected with one of six different forms of guinea pig CD1 as target cells in CTL assays. Subclones of the guinea pig cell line 104C1 each transfected with a construct encoding one of six different guinea pig CD1 proteins (i.e., guinea pig CD1b1, b2, b3, b4, c2, or c3) or with empty vector alone (mock transfected cells) (12) were pulsed with the MTb glycolipid fraction and used as target cells for CTL assay (note that guinea pig CD1c1 was not included in this analysis because we were unable to achieve stable high levels of expression of this isoform by transfecting 104C1 cells). The MTb glycolipid fraction was used for this analysis because this fraction was consistently active in preliminary CTL experiments (Fig. 3A and additional data not shown) and represented a more purified form of lipid Ag without significant protein or peptide contamination. As shown in Fig. 3C, the CTL responses of either inbred strain 2 or Hartley guinea pigs immunized with MTb lipid Ags in liposomes plus MPL and QS-21 showed consistent recognition of MTb glycolipid-pulsed transfectants expressing either guinea pig CD1b1 or guinea pig CD1c2. Note that one animal (Hartley guinea pig H0408) was immunized with lipid Ag three times and sacrificed 6 mo after the final immunization to investigate the long-term memory responses of lipid Ag-reactive CD1-restricted CTLs in this system. The appearance of similar levels of CTL activity in this animal that was rested for an extended period of time compared with the animals that had been more recently boosted strongly suggested that a long-term CD1-restricted memory CTL response can be induced following MTb lipid Ag immunization.

A more detailed analysis of the effects of key parameters of the CTL reaction (glycolipid Ag concentration used for pulsing targets, and E:T ratio) was conducted for CTL recognition of targets expressing guinea pig CD1b1 and guinea pig CD1c2 (Fig. 4). As shown in Fig. 4, guinea pig CD1b1 and c2 but not mock-transfected 104C1 were recognized by effector T cells from strain 2 guinea pigs immunized twice with lipid Ags in liposomes with QS-21 plus MPL, and the level of lysis varied directly with the MTb glycolipid Ag concentration and the E:T ratio. Furthermore, to exclude the possibility of random variations between different 104C1 transfectant clones accounting for the observed CTL lysis, we analyzed the CTL responses using multiple independently derived subclones of 104C1-expressing guinea pig CD1b1 (two clones: clone 16 and clone 18) or guinea pig CD1c2 (three clones: clone 5, clone 11, and clone 12). All of these subclones behaved similarly as target cells in these assays, yielding specific lysis values that were significantly greater than the background level obtained with the mock transfected cells (data not shown). Taken together, these results indicated that immunization with MTb lipid Ags elicited MTb glycolipid-specific CTL responses in vivo that...
were restricted by at least two different group 1 guinea pig CD1 proteins.

Phenotype of MTb lipid Ag-specific T cells elicited in immunized guinea pigs

To gain further insight into the types of CD1-restricted T cells that arise following MTb lipid Ag immunization in vivo, we examined the phenotype of the lipid-reactive T cells with respect to expression of CD4 and CD8 coreceptor molecules. Since the T cells showed CTL activity, we reasoned that at least a fraction of them would belong to the CD8 single-positive fraction or possibly to the CD4-CD8- double-negative (DN) fraction which has also been associated with CTL effector activity (13, 22). Thus, to confirm that MTb lipid Ag-reactive T cells reside in the CD4-negative fraction (i.e., among CD8 single-positive or DN T cells), we performed the following experiments. Hartley guinea pigs were s.c. immunized with OVA and a mixture of MTb lipid Ags (glycolipids, neutral lipids, and phospholipids) incorporated into liposomes with QS-21 and MPL. Two weeks after the immunization, splenic T cells were purified and further stimulated with MTb total lipids (10 μg/ml) and glycolipid-enriched fraction (30 μg/ml) for 6 days in the presence of irradiated splenic adherent cells as APCs and subsequently tested for cytotoxic activity against lipid-pulsed BM-DCs as target cells. In the experiment shown in B, mAb CD1F2/6B5 against guinea pig CD1 proteins or isotype-matched control mAb (P3) were tested for inhibition of the CTL responses. Asterisks indicate statistically significant difference compared with medium-only control (A) or to isotype-matched control mAb (B) (*, p = 0.035; **, p < 0.001). C, CTL responses of guinea pig T cells from MTb lipid Ag-immunized guinea pigs against guinea pig CD1 transfectant cells. Guinea pigs were immunized with MTb lipid Ag (mixture of glycolipid, neutral lipid, and phospholipid fractions) in liposomes along with QS-21 and MPL. Guinea pig fibroblast cell line 104C1 transfected with each isoform of guinea pig CD1 gene were labeled with 51Cr, pulsed with glycolipids, and used as target cells in CTL assay. Effector T cells were prepared by 6 days of in vitro stimulation of splenic T cells with a mixture of MTb glycolipids (30 μg/ml) and total lipids (10 μg/ml) in the presence of autologous APCs. Effector splenic T cells were from strain 2 guinea pigs immunized once (animal S2007/8, sacrificed 4 wk after a single immunization) or twice with MTb lipids (animal S2-003, sacrificed 12 wk after the second immunization) and from a Hartley guinea pig immunized three times (animal H0408, sacrificed 6 mo after the third immunization). Asterisks indicate statistically significant difference compared with lysis of mock transfectant (*, p < 0.001).

FIGURE 3. Primary ex vivo cytotoxic T cell responses to mycobacterial lipid Ags. A and B, CTL activity against MTb lipid Ag-pulsed BM-DCs. A strain 2 guinea pig (S2-005) was immunized with MTb lipid Ags (mixture of glycolipid, neutral lipid, and phospholipid fractions) entrapped in liposomes with QS-21 and MPL. Two weeks after the immunization, splenic T cells were purified and further stimulated with MTb total lipids (10 μg/ml) and glycolipid-enriched fraction (30 μg/ml) for 6 days in the presence of irradiated splenic adherent cells as APCs and subsequently tested for cytotoxic activity against lipid-pulsed BM-DCs as target cells. In the experiment shown in B, mAb CD1F2/6B5 against guinea pig CD1 proteins or isotype-matched control mAb (P3) were tested for inhibition of the CTL responses. Asterisks indicate statistically significant difference compared with medium-only control (A) or to isotype-matched control mAb (B) (*, p = 0.035; **, p < 0.001). C, CTL responses of guinea pig T cells from MTb lipid Ag-immunized guinea pigs against guinea pig CD1 transfectant cells. Guinea pigs were immunized with MTb lipid Ag (mixture of glycolipid, neutral lipid, and phospholipid fractions) in liposomes along with QS-21 and MPL. Guinea pig fibroblast cell line 104C1 transfected with each isoform of guinea pig CD1 gene were labeled with 51Cr, pulsed with glycolipids, and used as target cells in CTL assay. Effector T cells were prepared by 6 days of in vitro stimulation of splenic T cells with a mixture of MTb glycolipids (30 μg/ml) and total lipids (10 μg/ml) in the presence of autologous APCs. Effector splenic T cells were from strain 2 guinea pigs immunized once (animal S2007/8, sacrificed 4 wk after a single immunization) or twice with MTb lipids (animal S2-003, sacrificed 12 wk after the second immunization) and from a Hartley guinea pig immunized three times (animal H0408, sacrificed 6 mo after the third immunization). Asterisks indicate statistically significant difference compared with lysis of mock transfectant (*, p < 0.001).

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proliferative responses to the MTb total lipid Ag (Fig. 5B). This suggested that the T cell fraction responding to MTb lipid Ags was mainly or exclusively composed of CD4−CD8− and/or CD8+ T cells. This finding was confirmed by preliminary experiments designed to determine optimal conditions for establishing CD1-restricted MTb lipid Ag-specific T cell lines using CD4-depleted splenic T cells from inbred strain 2 animals similarly immunized with MTb lipid Ags. As shown in Fig. 5C, CD4-depleted T cell fractions from immunized strain 2 guinea pigs again responded to MTb whole lipid extract and to the glycolipid-enriched Ag fraction. These results provided further support for the conclusion that CD4-negative T cells were involved in the proliferative responses to these Ags.

**Establishment of a CD1-restricted MTb lipid Ag-reactive T cell line from an immunized guinea pig**

The ability to detect proliferative and cytotoxic T cell responses to MTb lipid Ags prompted us to establish CD1-restricted MTb lipid Ag-specific T cell lines to facilitate more detailed in vitro analyses. Since the CD4 depletion studies indicated that CD1-restricted T cells primed by MTb lipid immunization of guinea pigs may be predominantly CD4 negative, we used CD4-depleted splenic T cells from strain 2 guinea pigs immunized with liposome-entrapped purified MTb lipid Ags along with QS-21 and MPL and boosted with lipog-Ag-pulsed BM-DCs to initiate cultured T cell lines. These T cells were cultivated in the presence of CD1+ BM-DCs from inbred strain 2 guinea pigs and pulsed with an MTb whole lipid extract. To prevent possible overgrowth of any residual CD4+ T cells during the culture, the CD4 depletion was repeated three times during the course of culture. After two stimulations using syngeneic CD1+ BM-DCs, the third and fourth stimulations were conducted with CD1−BM-DCs from allogeneic outbred Hartley guinea pigs to favor presentation of lipid Ags by nonpolymorphic Ag-presenting molecules such as CD1. After the sixth restimulation, one T cell line was obtained (designated as S2-1031) that had expanded sufficiently to permit detailed analysis. FACS analysis revealed that line S2-1031 was composed predominantly of CD4−CD8− and CD8+ T cells, with only 5.2% CD4+ T cells (Fig. 6A).

The specificity and restriction of T cell line S2-1031 was examined by [3H]thymidine incorporation assay and mAb blocking. The T cell line proliferated strongly to CD1+ BM-DCs pulsed with MTb total lipid Ags. Attempts to characterize the specific lipid Ag recognized by line S2-1031 were unsuccessful, as this T cell line responded to MTb total lipid extract but not to any of the standard subfractions derived from it (i.e., purified mycolic acids or silicic acid column fractions enriched in MTb glycolipids, neutral lipids, or phospholipids; data not shown). The CD1 restriction of this proliferative response to MTb lipids was shown by adding the CD1-specific blocking mAb (CD1F2/6B5.1) to the proliferation assay. The mAb (CD1F2/6B5.1) blocked the MTb lipid Ag-specific response of line S2-1031 by >90%, whereas the same mAb did not affect the proliferative responses of an alloreactive CD4+ guinea pig T cell line (Fig. 6B). Isotype-matched control mAb (mAb P3, mouse IgG1) had no effect on these proliferative responses (data not shown).

To further dissect the CD1 restriction of this MTb lipid Ag-reactive T cell line, CTL responses were determined using transfectants of cell line 104C1 that selectively expressed each of the cloned guinea pig CD1 isoforms. T cell line S2-1031 lysed MTb lipid Ag-pulsed 104C1 cells stably transfected with guinea pig CD1b1, -b2, and -c3, but not mock transfectants in an Ag dose-dependent manner, as shown in Fig. 7. Consistent with the findings obtained in the proliferation assays with this T cell line, we could not clearly detect significant CTL responses (i.e., >10% specific lysis) to more highly purified lipid Ag fractions (purified mycolates or the MTb glycolipid, neutral lipid, and phospholipid-enriched fraction; data not shown). Thus, although the precise lipid Ag specificity of line S2-1031 cannot yet be determined, the results provided clear evidence for the restriction of its MTb-specific CTL activity by at least three members of the guinea pig group 1 CD1 family.

**Discussion**

To test the possible role of group 1 CD1 molecules in host defense, it is necessary to develop an appropriate animal model for assessment of the functions of group 1 CD1-restricted T cell responses in vivo. Mice and rats are inadequate for this purpose since the genomes of these rodents lack all group 1 CD1 genes. As previously reported, we have analyzed the guinea pig CD1 gene family and proposed that guinea pigs could serve as a suitable small animal model to test the role of group 1 CD1 proteins in host defense (11). In the current study, we have extended our previous findings on this animal model by demonstrating that guinea pigs can mount CD1-restricted T cell responses following in vivo immunization with MTb lipid Ags. This is the first demonstration to our knowledge of group 1 CD1-restricted lipid Ag-specific T cell responses in a nonhuman species and also the first successful demonstration...
of CD1-restricted recall responses following in vivo immunization with lipid Ags.

Evidence supporting the hypothesis that MTb lipid Ag-specific CD1-restricted T cells play a role in host defense against microbial pathogens comes from the recent finding that human CD1c-restricted T cells specific for a mycobacterial isoprenoid glycolipid Ag are seen in individuals previously infected with MTb, but not in naive donors (8). CD1-restricted T cells specific for mycobacterial Ags have also been derived from the active skin lesions of leprosy patients, and effective cell-mediated immunity is correlated with expression of group 1 CD1 molecules by the DCs in leprosy skin lesions (23, 24). Furthermore, these CD1-restricted mycobacterial lipid Ag-specific T cells have been found to be biased toward secretion of Th1 type cytokines and can kill CD1⁺ target cells pulsed with lipid Ags while concurrently producing the bactericidal protein granulysin (25). Together, these data provide strong circumstantial evidence that CD1-restricted T cells specific for foreign lipid Ags may play a role in host defense against bacterial infections.

In humans, CD1-restricted responses were initially found among CD4⁻ CD8⁻ DN T cells, although subsequent reports also confirmed the existence of CD1-restricted T cells that were CD4⁺ (23) or CD8⁺ single positive (13). In our analysis of MTb lipid-immunized guinea pigs, we found that CD1-restricted lipid Ag-specific T cells were prominent in the CD4⁻ fraction of splenic T cells, consistent with CD1 restriction being mainly a feature of CD8⁺ or DN T cells in this experimental setting. These CD4⁻ negative CD1-restricted T cells were associated with both proliferative and cytotoxic activities, similar to what has been described for human CD1-restricted T cells reactive with mycobacterial lipid Ags. In the case of human MTb lipid-reactive T cells, Ag recognition has typically been associated with production of IFN-γ, a key cytokine in the immune response to pathogenic mycobacteria.

We are not yet able to test whether this is a property of the CD1-restricted T cells that develop after MTb lipid immunization in the guinea pig because of the current lack of reagents for the specific detection of IFN-γ or other cytokines in this species.

Although our current study established the priming of Ag-specific T cell responses following immunization with suitably adjuvanted and reconstituted mycobacterial lipid preparations, it is also of interest to determine whether such responses develop during

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**FIGURE 5.** Enrichment of MTb lipid Ag-specific cells in CD4-depleted T cell fractions. A, Proliferative responses over a range of Ag doses to OVA (▲) or MTb total lipids (■) of unfractionated splenic T cells from a Hartley guinea pig immunized with a mixture of MTb lipid Ags and OVA in liposomes with QS-21 plus MPL. APCs were irradiated autologous nylon wool-adherent splenocytes. B, Effect of CD4 T cell depletion on reactivity to MTb lipid Ags and OVA. A Hartley strain guinea pig was immunized with MTb lipid Ags (mixture of glycolipid, neutral lipid, and phospholipid fractions) with OVA in liposomes along with QS-21 and MPL. The animal was sacrificed 4 wk after immunization, and splenic T cells were purified and used as responders in proliferation assays with irradiated autologous splenic nylon wool-adherent cells as APCs. Some portions of splenic T cells were further treated with anti-CD4 mAb, followed by depletion of CD4⁺ cells using magnetic beads. Whole splenic T cells and CD4-depleted splenic T cells were compared for reactivity to 12.5 μg/ml MTb total lipid Ag extract or OVA. C, CD4-depleted splenic T cells obtained from inbred strain 2 guinea pigs immunized as in B were tested for proliferative responses to MTb total lipid extract (12.5 μg/ml) or glycolipid-enriched fraction (12.5 μg/ml) using irradiated syngeneic splenic nylon wool-adherent cells as APCs. Two representative experiments obtained from separate individual guinea pigs are shown. Asterisks indicate statistically significant difference compared with medium-only control (*, p < 0.001).
actual infections with mycobacterial pathogens. Our previous studies on PBL from human donors with previous mycobacterial infection strongly support the view that CD1-restricted T cell responses to specific lipid Ags do develop as a result of natural infections with MTb (8). In the guinea pig model, preliminary experiments on PBL from human donors with previous mycobacterial infection strongly support the view that CD1-restricted T cell responses to specific lipid Ags do develop as a result of natural infections with MTb (8). In the guinea pig model, preliminary

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Generation of a CD1-restricted T cell line specific for MTb lipid Ag from an immunized guinea pig. A, FACS analysis showing phenotype of lipid Ag-specific guinea pig T cell line S2-1031. The T cell line was stained with anti-guinea pig pan-T cell (mAb CT5), anti-guinea pig CD4 (mAb CT7), anti-guinea pig CD8 (mAb CT6), or isotype-matched control Ab (P3). B, CD1 restriction of T cell line S2-1031. T cells were stimulated with MTb total lipid Ags in the presence of syngeneic BM-DCs. Anti-CD1 mAb (CD1F2/6B5) was added to a final concentration of 20 μg/ml. An alloreactive CD4+ T cell line derived from a Hartley guinea pig by stimulating with BM-DCs from a strain 2 guinea pig was also stimulated with strain 2 BM-DCS with or without anti-CD1 mAb. Results shown are representative of three independent experiments. Asterisk indicates statistically significant difference (*, p = 0.0066).

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Restriction of MTb lipid Ag-specific T cell line by individual guinea pig CD1 molecules. Guinea pig fibroblast cell line 104C1 cells transfected with different isoforms of guinea pig CD1 were used as target cells in CTL assay. Transfectant cells were first labeled with 51Cr and pulsed for 18 h with either 10 or 20 μg/ml MTb total lipid Ags or in medium alone. The CD1-restricted lipid Ag-specific T cell line S2-1031 was used for the effector cells. CTL activity at an E:T ratio of 40:1 is shown. Asterisks indicate statistically significant difference compared with lysis of cells not pulsed with MTb lipid Ag (*, p < 0.05; ***, p < 0.01).
three distinct patterns of intracellular localization (12). Given our current findings on the ability of several guinea pig CD1 proteins to present MTb lipid Ags to T cells, it will be of interest to determine in future studies whether the different guinea pig CD1 molecules involved in these responses acquire their lipid Ags in distinct subcellular compartments of APCs.

Considerable work remains to be done to identify the specific MTb lipids that are recognized by guinea pig CD1-restricted T cells. As shown in the current study, MTb lipid Ag-immunized guinea pigs frequently gave positive T cell responses to the MTb glycolipid-enriched fraction after immunization with a broad mixture of mycobacterial lipid Ags, whereas highly purified mycolic acids and a neutral lipid-enriched fraction appeared not to be well recognized in most cases. Analysis of amino acid content of the various lipid preparations that we have used does indicate a possibility of low levels of residual protein contamination in some of these, since up to 6.4% amino acid content on a weight basis was detected in the total lipid extract and phospholipid-enriched fractions. However, analysis by SDS-PAGE and ultrasensitive silver staining failed to reveal any detectable protein contaminants in our lipid Ag preparations, and the profile of amino acids present suggests that most of these are derived from peptidoglycan rather than from proteins. Thus, although we cannot completely rule out at this point the possibility that trace amounts of highly immunogenic protein Ags in our lipid preparations could contribute to some of the T cell responses we have observed, this appears to be unlikely. It is also noteworthy that in several cases we demonstrated guinea pig CD1-restricted recognition of the MTb glycolipid-enriched fraction, which represents material eluted from acetone with a silica acid column and contains only trace amounts of detectable amino acids. Finally, the restriction of these responses by guinea pig CD1 proteins provides further evidence in favor of lipid rather than protein Ag targets, since work on human and murine CD1 has strongly linked these proteins to the binding and presentation of lipids rather than proteins or peptides.

Our current data provide further support for the possibility of using the guinea pig as an animal model in which to test the hypothesis that augmentation of group 1 CD1-restricted mycobacterial lipid Ag-specific T cell responses can be used as a component of an effective vaccination strategy against MTb infection (29). This appears to us to be an appealing strategy, given that CD1 genes are largely nonpolymorphic and that the CD1 target ligands (i.e., complex lipids and glycolipids) are essential components of bacterial cells that may be less variable in their structures than the more highly mutable peptide Ags that are targets of MHC-restricted T cells. Currently, Mycobacterium bovis bacillus Calmette-Guérin is the only available vaccine against tuberculosis, and its efficacy had been disputed despite extensive use in humans and testing in the field. CD1-mediated presentation of lipid Ags may provide a completely new vaccination strategy that could offer advantages to new protein subunit vaccines or live attenuated mycobacterial vaccines that are currently under development. Thus, the guinea pig model may provide valuable opportunities for testing the efficacy of lipid Ag vaccination in a small animal model that includes the potential for a prominent group 1 CD1-restricted T cell response.

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