Major T Cell Response to a Mycolyl Glycolipid Is Mediated by CD1c Molecules in Rhesus Macaques

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Human CD1b molecules contain a maze of hydrophobic pockets and a tunnel capable of accommodating the unusually long, branched acyl chain of mycolic acids, an essential fatty acid component of the cell wall of mycobacteria. It has been accepted that CD1b-bound mycolic acids constitute a scaffold for mycolate-containing (glyco)lipids stimulating CD1b-restricted T cells. Remarkable homology in amino acid sequence is observed between human and monkey CD1b molecules, and indeed, monkey CD1b molecules are able to bind glucose monomycolate (GMM), a glucosylated species of mycolic acids, and present it to specific human T cells *in vitro*. Nevertheless, we found, unexpectedly, that *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)-vaccinated monkeys exhibited GMM-specific T cell responses that were restricted by CD1c rather than CD1b molecules. GMM-specific, CD1c-restricted T cells were detected in the circulation of all 4 rhesus macaque monkeys tested after but not before vaccination with BCG. The circulating GMM-specific T cells were detected broadly in both CD4⁺ and CD8⁺ cell populations, and upon antigenic stimulation, a majority of the GMM-specific T cells produced both gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α), two major host protective cytokines functioning against infection with mycobacteria. Furthermore, the GMM-specific T cells were able to extravasate and approach the site of infection where CD1c⁺ cells accumulated. These observations indicate a previously inconceivable role for primate CD1c molecules in eliciting T cell responses to mycolate-containing antigens.

Group 1 CD1 molecules bind a variety of lipidic antigens (Ags) and present them to specific T cells. In humans, three group 1 CD1 molecules, namely, CD1a, -b, and -c, exist that have evolved mutually distinct Ag-binding grooves. Therefore, a group of microbial Ags with unique lipid tails may bind preferentially to a particular CD1 isoform. The lipid species essential for the cell wall architecture of mycobacteria include a family of α -alkyl- β -hydroxy fatty acids with an extremely long acyl chain, termed mycolic acids, which Beckman and colleagues identified as a CD1b-presented Ag (1). Subsequently, glucose monomycolate (GMM), a glucosylated species of mycolic acid, was also shown to be presented by human CD1b molecules (2), and the crystal structure of the GMM-CD1b complex underscored that the acyl chain of GMM fitted tightly in a maze of pockets and a tunnel elaborated in human CD1b molecules (3). Furthermore, glycerol monomycolate can also be presented by human CD1b molecules, leading to the assumption that CD1b-bound mycolic acids constitute a scaffold for mycolate-containing (glyco)lipids stimulating CD1b-restricted T cells (4). A potential link between GMM and glycerol monomycolate and the active and latent phases of human tuberculosis, respectively, has been proposed (4, 5), and studies of immune responses to these Ags in experimental animals are now important for future advances in this field.

Mice and rats have lost all the group 1 CD1 genes, and the reconstitution of human group 1 functions in mice by gene transfer has provided significant insights (6); however, it is unclear whether the CD1-restricted T cell response generated in transgenic mice faithfully represents that naturally elicited in humans. Alternatively, animals, such as guinea pigs (7) and cows (8), that are naturally equipped with the group 1 CD1 system have been utilized, but the number and the expression patterns of the group 1 CD1 isoforms differ significantly from those in humans. Obviously, a fair prediction would be that nonhuman primates will

serve as reliable animal models, and indeed, our previous work has indicated that the group 1 CD1 system is highly conserved between humans and rhesus macaque monkeys (9). Furthermore, monkey CD1b molecules were capable of binding GMM and presenting it to T cells expressing GMM-specific, human CD1b-restricted T cell receptors (9).

To extend this work further in an *in vivo* system, the current study was initially designed to monitor GMM-specific T cell responses in *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)-immunized monkeys. During the course of the study, we found that a major T cell response to GMM in these animals was restricted by CD1c molecules. Upon antigenic stimulation, the GMM-specific T cells produced host protective cytokines. Furthermore, GMM-specific T cells were recruited to the site of infection where CD1c⁺ cells aggregated, suggesting their role in host defense against mycobacterial infections.

MATERIALS AND METHODS

Animals and vaccination. The rhesus macaques (*Macaca mulatta*) used in this study were treated humanely in accordance with institutional regulations, and experimental protocols were approved by the Committee for Experimental Use of Nonhuman Primates at the Institute for Virus

Received 17 August 2012 Returned for modification 12 October 2012

Accepted 30 October 2012

Published ahead of print 6 November 2012

Editor: J. L. Flynn

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doi:10.1128/IAI.00871-12

FIG 1 GMM-specific T cell responses induced in BCG-immunized monkeys. (A) Four monkeys were vaccinated intradermally with BCG and GMM liposome. In some cases, BCG was administered via an intratracheal route and the GMM liposome was injected along with the Pam3Cys-SKKKK adjuvant. At the indicated time points, PBMCs were obtained, and the number of GMM-specific cells was determined by IFN- γ ELISPOT assays. The numbers of the GMM-specific spots per 1×10^6 PBMCs are plotted. (B and C) Preimmune PBMCs and those obtained after the third BCG immunization were incubated with either the GMM liposome (GMM +) or empty liposome (GMM -), followed by detection of IFN- γ -producing cells in an ELISPOT assay. Statistical analysis was performed using a one-way analysis of variance (ANOVA). Error bars show standard deviations.

Research, Kyoto University. The Tokyo 172 strain of BCG (Japan BCG Laboratory, Tokyo, Japan) was grown in 7H9 medium and harvested at its mid-log-phase growth as described previously (7). GMM was purified from cultured mycobacteria and integrated into stearylated octaarginine-containing liposomes as described previously (7). Four rhesus macaque monkeys were vaccinated intradermally with 1×10^8 CFU of BCG and challenged with 50 μ g of the GMM liposome at various intervals from 6 to 20 weeks as shown in Figure 1A. The dose of BCG was determined on the basis of our previous studies with guinea pigs (7). In some cases, BCG was administered via an intratracheal route using a bronchoscope, and the GMM liposome was injected along with 100 μ g of Pam₃Cys-SKKKK (EMC Microcollections, Tuebingen, Germany).

IFN- γ ELISPOT assays. Gamma interferon (IFN- γ) enzyme-linked immunosorbent spot (ELISPOT) assays were performed using a human/monkey IFN- γ ELISPOT kit (Mabtech, Nacka Strand, Sweden) as described previously (10), with slight modifications. In blocking experiments, cells were preincubated with 5 μ g/ml of anti-CD1a (10H3), CD1b (b3.1), CD1c (M241), or negative control (P3) antibodies (Abs) for 20 min before Ag exposure. In some experiments, responder T cells were purified from peripheral blood mononuclear cells (PBMCs) by negative selection with anti-CD1c (M241), CD14 (M5E2), CD16 (3G8), CD20 (2H7), and CD56 (B159) Abs, and ELISPOT assays were performed using the LLC-MK2 rhesus macaque kidney epithelial cell line expressing rhesus macaque CD1a, CD1b, or CD1c (9) as Ag-presenting cells (APCs).

Derivation of T cell lines and T cell assays. GMM-specific T cell lines were established independently from MM552 and MM553. Peripheral blood mononuclear cells (PBMCs) (1.1×10^7 /well) were cultured with the GMM liposome (1 μ g/ml), and antigenic stimulation was repeated every 2 weeks in the presence of irradiated autologous PBMCs. The cul-

ture medium used was RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 3 nM interleukin-2 (IL-2), 2-mercaptoethanol (Invitrogen, Carlsbad, CA), penicillin, and streptomycin. For T cell assays, the cells (5×10^4 /well) were incubated with the GMM liposome (1 μ g/ml) in the presence of LLC-MK2 cell transfectants (5×10^4 /well) expressing rhesus macaque CD1a, CD1b, or CD1c using a 96-well flat-bottom microtiter plate. After 24 h, aliquots of the culture supernatants were collected, and the amount of IFN- γ released into the medium was measured using a human/monkey IFN- γ enzyme-linked immunosorbent assay (ELISA) kit (Mabtech).

Flow cytometry. Freshly isolated PBMCs (5×10^5 /well) were stimulated with either empty liposome or the GMM liposome (1 μ g/ml) for 6 h in 96-well flat plates and then treated with brefeldin A for an additional 6 h. Subsequently, the cells were harvested and labeled with anti-CD8 Ab (RPA-T8; phycoerythrin [PE]-Cy7) and anti-CD4 Ab (OKT4; eFluor 450). This was followed by cell fixation and permeabilization. The cells were then labeled with anti-IFN- γ Ab (4S.B3; PE) and anti-tumor necrosis factor alpha (TNF- α) Ab (monoclonal antibody 11 [MAb11]; fluorescein isothiocyanate [FITC]) and analyzed by flow cytometry using a BD FACS CantoII flow cytometer (BD Biosciences).

Adoptive transfer. Cells (1×10^7) of the MM552-derived, GMM-specific T cell line were labeled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37°C. The labeled cells were suspended in 10 ml of glucose lactated Ringer's solution and injected intravenously into the MM552 monkey from which the T cells were derived. At the same time, the MM552 monkey received an intradermal inoculation with BCG (1×10^8 CFU). After 4 days, the monkey was sacrificed, and skin samples were collected. The excised skin was fixed

FIG 2 The GMM-specific T cell response was dependent on CD1c molecules. (A and B) Postimmune PBMCs from the monkeys were subjected to IFN- γ ELISPOT assays in the presence or absence of anti-CD1 MAbs, and the numbers of GMM-specific spots per 1×10^6 PBMCs are shown (A). The percentage of inhibition by anti-CD1 Abs in each monkey was plotted, and statistical analysis was performed using a one-way ANOVA (B). (C) Postimmune PBMCs were isolated from MM553, and negative selection with Abs to CD1c, CD14, CD16, CD20, and CD56 was performed. Subsequently, the cells were subjected to a IFN- γ ELISPOT assay using LLC-MK2 cell transfectants expressing monkey CD1a, CD1b, or CD1c molecules as APCs. (D) Cells of the GMM-specific monkey T cell line established from MM553 were incubated with the LLC-MK2 cell transfectants in the presence or absence of the GMM liposome, and the amount of IFN- γ released into the medium was measured by a IFN- γ ELISA. neg. cont., negative control. Error bars show standard deviations.

with 4% paraformaldehyde and deep-frozen in optimal cutting temperature (OCT) compound, and the cryosections were mounted with Vectashield mounting medium with DAPI (4',6'-diamidino-2-phenylindole; Vector Laboratories) and viewed under a fluorescence microscope. Some cryosections were processed for immunohistochemistry as described previously (11) and labeled with M241 or P3. Signals were visualized with the standard protocol using a Vector Elite ABC kit.

RESULTS

GMM-specific T cell responses were induced in BCG-vaccinated monkeys. We previously detected GMM-specific T cell responses in BCG-vaccinated guinea pigs (7). To see if such T cell responses were also induced in nonhuman primates, four rhesus macaque monkeys (MM552, MM553, MM556, and MM559) received intradermal inoculation with BCG 3 times during the course of the study (Fig. 1A), and MM552 and MM556 were additionally challenged with BCG via an intratracheal route. We also attempted to sensitize the animals by intradermal injection of the GMM liposome either with or without the Pam3Cys-SKKKK lipopeptide adjuvant. At the time points indicated in Figure 1A, PBMCs were obtained from the monkeys and tested for their ability to respond to GMM in IFN- γ ELISPOT assays. The GMM-specific response was only marginal after the first intradermal vaccination with BCG in all cases, but the response was more prominent after the second and the third vaccinations. We did not see such a robust response elicited by the administration of BCG via an intratracheal route (MM552 and MM556). Furthermore, no implications

were obtained for the potential of the GMM liposome in terms of inducing GMM-specific T cell responses (Fig. 1A).

Preimmune PBMCs and those after the third BCG vaccination were obtained from each subject, and a IFN- γ ELISPOT assay was performed in parallel (Fig. 1B). Numerous spots were detected when PBMCs obtained after the third BCG vaccination were stimulated *in vitro* with the GMM liposome, but not with empty liposome, whereas preimmune PBMCs failed to respond to GMM (Fig. 1B, postimmune, + and -, and preimmune, +, respectively). The increase in the number of GMM-specific spots after BCG vaccination was statistically significant (Fig. 1C). Therefore, as in guinea pigs, GMM-specific T cell responses were elicited by BCG vaccination in rhesus macaque monkeys.

The GMM-specific response was restricted by CD1c molecules. Given that monkey CD1b molecules present GMM to specific T cells *in vitro* (9), we initially predicted that CD1b molecules would function as the restriction element for the GMM-specific response induced *in vivo* by BCG vaccination. However, blocking studies with a panel of anti-CD1a (10H3), anti-CD1b (b3.1), and anti-CD1c (M241) MAbs that were known to specifically block human T cell responses and cross-react to the corresponding monkey CD1 isoforms (9) indicated that CD1c rather than CD1b molecules restricted the GMM-specific response in all 4 monkeys (Fig. 2A). The inhibitory effect of anti-CD1c but not anti-CD1b Abs was statistically significant (Fig. 2B). To confirm that the GMM-specific T cell response was restricted by CD1c molecules,

peripheral blood T cells were purified from a BCG-vaccinated monkey and tested for their ability to respond to GMM when LLC-MK2 cell transfectants expressing rhesus macaque CD1a, CD1b, or CD1c molecules were used as APCs. In a IFN- γ ELISPOT assay, significant numbers of GMM-specific T cell spots were detected only in the presence of CD1c⁺ LLC-MK2 cells (Fig. 2C). Finally, a GMM-specific T cell line established from the MM553 monkey responded to GMM by producing IFN- γ when LLC-MK2 cell transfectants expressing CD1c but not those expressing the other CD1 isoforms were used as APCs (Fig. 2D). Taken together, these lines of evidence indicate an outstanding ability of rhesus macaque CD1c molecules to mediate GMM-specific T cell responses.

GMM-specific T cells were detected broadly in CD4⁺ and CD8⁺ T cell populations. Initial studies of group 1 CD1-restricted T cell lines (1, 12), as well as our previous study of BCG-vaccinated humans (13), suggested that group 1 CD1-restricted T cells were enriched in the CD4⁺ T cell population. However, recent evidence obtained from human cases with tuberculosis indicated that a majority of GMM-specific, CD1b-restricted T cells was CD4⁺ (14). To determine the phenotype of GMM-specific T cells detected in our monkey model, PBMCs derived from the 4 BCG-vaccinated monkeys were stimulated *in vitro* with either the GMM liposome or empty liposome and subjected to a multicolor flow cytometric analysis for intracellular cytokines and surface markers. In all cases, a T cell population expressing both IFN- γ and TNF- α was readily detectable when cells were stimulated with the GMM liposome (Fig. 3; boxed in bold lines). The GMM-reactive T cell population thus identified was further separated, based on the cell surface expression of CD4 and CD8 α glycoproteins, into CD4 single-positive (SP), CD8 SP, and CD4 CD8 double-positive (DP) cells (Fig. 3). We found that the GMM-reactive T cells were detected broadly in CD4 SP, CD8 SP, and DP populations except for MM553, in which the GMM-reactive T cells were detected predominantly in CD4 SP and DP populations. It was not surprising that Ag-specific DP T cells were detected in this study because such T cells are known to comprise a significant population of memory T cells in the peripheral blood of monkeys (15). Upon antigenic stimulation, the GMM-reactive T cells did not upregulate transcription of IL-17A and IL-17F (data not shown), suggesting that these T cells were distinct from Th17 cells.

GMM-specific T cells were recruited to the site of infection. Circulating GMM-specific T cells are detectable in patients with tuberculosis (14), but it remains to be examined if these cells are able to extravasate and gain access to the site of infection. To address this, the GMM-specific T cell line derived from the MM552 monkey was labeled with CFSE and injected intravenously back into the same subject, i.e., MM552. At the time of injection with CFSE-labeled cells, BCG was inoculated in the skin, and after 4 days, the BCG-infected skin was examined for infiltration by the CFSE-labeled cells. The local accumulation of CD1c⁺ cells was prominent in the infected tissue (Fig. 4A, bottom right), and CFSE-labeled T cells (Fig. 4B, bottom left, indicated with arrowheads) penetrated deeply into the granulomatous cell aggregates formed at the site of BCG infection. Such cellular responses were undetectable in uninfected skin areas (Fig. 4A and B, top). Taken together, these observations indicated that, in response to BCG vaccination, GMM-specific, CD1c-restricted T cells could expand in the circulation with the potential for mobilization to the site of mycobacterial infection.

FIG 3 Profiles for surface marker expression on monkey GMM-specific T cells. PBMCs obtained from the BCG-vaccinated monkeys were incubated for 6 h with either empty liposome or the GMM liposome, and then brefeldin A was added to the culture. After an additional 6-h incubation, the cells were fixed, permeabilized, and labeled with Abs to CD4, CD8, IFN- γ , and TNF- α . This was followed by a multicolor flow cytometric analysis. The IFN- γ ⁺ TNF- α ⁺ cell population (boxed in bold lines) was further separated based on the expression of CD4 and CD8 α molecules. The percentage of cells present in indicated regions is shown for each panel.

DISCUSSION

The present study provided evidence that monkey CD1c molecules were capable of functioning as a restriction element for GMM-specific T cells *in vitro* and eliciting GMM-specific T cell responses *in vivo* (Fig. 1). The crystal structure of the human CD1b-GMM complex indicated that the T' tunnel connecting the A' and F' pockets is essential for accommodating the long meromycolate chain (3). The tunnel structure constructed in human CD1b molecules is obstructed in human CD1a and CD1c isoforms by replacement of the glycine residue at position 98 (CD1b) with valine (CD1a and CD1c). Although the crystal structure has not yet been elucidated, monkey CD1c molecules exhibit much higher homology with human CD1c molecules (90.4% in the amino acid sequence) than with human CD1b (58.9%) and have valine at position 98, making it unlikely that the meromycolate chain-accommodating T' tunnel detected for human CD1b is constructed in monkey CD1c. The crystal structure of human CD1c molecules was resolved recently, indicating significant dif-

FIG 4 Recruitment of GMM-specific T cells to the site of infection. The CFSE-labeled, GMM-specific T cells were injected into the circulation of the donor. At the same time, BCG (1×10^8 CFU) was inoculated into the skin. After 4 days, samples of the infected (bottom, BCG-positive) or uninfected (top, BCG-negative) skin were obtained and examined for infiltration by CD1c⁺ cells (A) and CFSE-labeled cells (B). The tissue sections were also counterstained with DAPI (B). Arrowheads indicate CFSE-positive cells, and dashed lines indicate the area of granulomatous macrophage aggregation. The amorphous fluorescence-positive structure (indicated with an asterisk) appeared to represent the necrotic center of the granuloma. neg. cont., negative control. Scale bars, 100 μ m.

ferences between human CD1b and CD1c molecules (16). The human CD1c molecule contains two deep grooves, each of which has a portal (D' portal and E' portal) at the bottom. As the two portals are located in close proximity, one potential mechanism for interaction between monkey CD1c and the long meromycolate chain of GMM is that the fatty acyl could exit one portal and immediately enter the other, minimizing its uncomfortable exposure to the aqueous external milieu. The crystallographic structure of monkey CD1c molecules is under investigation and should provide valuable insights into how monkey and, possibly, human CD1c molecules bind GMM.

In the present study, we performed an adoptive transfer experiment, which is a common approach in studies with genetically identical experimental rodents but is challenging in nonhuman primates. This is probably the first direct demonstration that group 1 CD1-restricted T cells are recruited from the circulation to the site of infection (Fig. 4). These T cells produce IFN- γ and TNF- α , representative cytokines critical for host defense against mycobacterial infections (Fig. 3). Furthermore, inoculation of purified GMM into the skin of BCG-vaccinated monkeys resulted in elicitation of delayed-type hypersensitivity (DTH), as in guinea pigs (7), that was associated with the expression of microbicidal agents, such as granulysin (data not shown). Given that GMM is produced in tissues by utilizing host-derived glucose as a substrate for mycolyltransferases (5), GMM-specific T cells may be particularly important for eliminating metabolically active, replicating microbes.

Monkey studies are often recognized as a surrogate for human studies. On the other hand, monkey studies may occasionally provide compelling new insights that have never been noted in a full range of human studies. Despite significant advances in the biology of group 1 CD1 molecules over the past 2 decades, the GMM-specific, CD1c-restricted T cell response in monkeys was unexpected and is stimulating enough to address whether such responses may exist in humans and how CD1b and CD1c play distinct roles.

ACKNOWLEDGMENTS

This work was supported by a grant from the Japan Society for the Promotion of Science (grant-in-aid for Scientific Research [B]; grant

number 24390255 [to M.S.]). It was also supported by a grant from the Ministry of Health, Labor and Welfare (Research on Emerging and Reemerging Infectious Diseases, Health Sciences Research Grants) and by the Cooperation Research Program of the Primate Research Institute, Kyoto University. D.M. is a Research Fellow (PD) of the Japan Society for the Promotion of Science.

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