Highly Purified Mycobacterial Phosphatidylinositol Mannosides Drive Cell-Mediated Responses and Activate NKT Cells in Cattle

Chris Pirson, Regina Engel, Gareth J. Jones, Thomas Holder, Otto Holst, H. Martin Vordermeier

Mycobacterial lipids play an important role in the modulation of the immune response upon contact with the host. Using novel methods, we have isolated highly purified phosphatidylinositol mannoside (PIM) molecules (phosphatidylinositol dimannoside [PIM₂], acylphosphatidylinositol dimannoside [AcPIM₂], diacyl-phosphatidylinositol dimannoside [Ac₂PIM₂], acylphosphatidylinositol hexamannoside [AcPIM₆], and diacylphosphatidylinositol hexamannoside [Ac₂PIM₆]) from virulent Mycobacterium tuberculosis to assess their potential to stimulate peripheral blood mononuclear cell (PBMC) responses in Mycobacterium bovis-infected cattle. Of these molecules, one (AcPIM₆) induced significant levels of gamma interferon (IFN-γ) in bovine PBMCs. Three PIM molecules (AcPIM₂, Ac₂PIM₂, and Ac₂PIM₆) were shown to drive significant proliferation in bovine PBMCs. AcPIM₆ was subsequently used to phenotype the proliferating cells by flow cytometry. This analysis demonstrated that AcPIM₆ was predominantly recognized by CD3⁺ CD335⁺ NKT cells. In conclusion, we have identified PIM lipid molecules that interact with bovine lymphocyte populations, and these lipids may be useful as future subunit vaccines or diagnostic reagents. Further, these data demonstrate, for the first time, lipid-specific NKT activation in cattle.

Members of the mycobacterial genus are renowned for their waxy, lipid-rich outer envelope. Under physiological conditions, this outer layer is likely to be the first point of contact between the bacterial cell and the host’s immune system, and the outcome of this interaction is pivotal in the establishment of infection. One of the most important groups of membrane bound lipids consists of the phosphatidylinositol mannoses (PIMs). Interest in PIMs was stimulated since it was shown that phosphatidylinositol dimannoside (PIM₂) forms the phosphoglycerolipid anchor which tethers a large array of glycolipids and lipoglycans, including lipomannan (LM) and lipoarabinomannan (LAM), to the cellular membrane (1). PIMs have been shown to interact with a variety of immune components and mediate significant effects on the host. Even since the realization that NKT cells could respond to lipid antigens (2–4) and the subsequent discovery of the CD1d-restricted lipid antigen α-galactosylceramide (α-GalCer) (5, 6), much research effort has been concentrated on understanding lipid antigens. Although work was initially focused on invariant NKT cells, it has since been shown that great diversity exists in the lipid-responsive T cell receptor (TCR) repertoire (7–9) and that these diverse NKT cells contribute to the Th1/Th2 balance (7, 10). It has been shown that CD1d-restricted NKT cells are capable of recognizing a variety of lipid antigens, including phospholipids (11). More recently, a CD1b-restricted subset of T cells has been found (12). Similarly to CD1d-restricted invariant NKT (iNKT) cells, the CD1b-restricted variant cells require CD1B for their development and produce proinflammatory cytokines in response to CD1b-expressing dendritic cells (DCs) (12). It is clear that NKT-like cells have a significant role to play in lipid-mediated responses, and the hunt for their antigens has continued (13–15).

Given the ability of lipid molecules to generate responses in peripheral blood mononuclear cells (PBMC), we decided to assess the ability of individual, highly purified natural PIMs to activate bovine lymphocytes. In this study, we developed a novel extraction method which allowed us to extract and highly purify a variety of PIM molecules from virulent Mycobacterium tuberculosis H37Rv. The ability of these molecules to induce lymphocyte responses in Mycobacterium bovis-infected cattle was investigated by measuring lymphocyte proliferation and gamma interferon (IFN-γ) production. Furthermore, flow cytometry techniques were utilized to characterize responding cell populations.

MATERIALS AND METHODS

**Extraction of PIMs.** Using the novel methodology outlined below, highly pure phosphatidylinositol dimannoside (PIM₂), acylphosphatidylinositol dimannoside (AcPIM₂), diacyl-phosphatidylinositol dimannoside (Ac₂PIM₂), acylphosphatidylinositol hexamannoside (AcPIM₆), and diacylphosphatidylinositol hexamannoside (Ac₂PIM₆) were successfully isolated. Individual PIM molecules were analyzed by electrospray ionization mass spectrometry (ESI-MS) to confirm identity and purity as shown in Fig. 1. Up to 1 g of dry bacterial mass of Mycobacterium tuberculosis H37Rv was suspended in 20 to 30 ml of H₂O and ruptured utilizing a French press at a minimum of 20,000 kPa. This procedure was performed five times, and the combined sample was lyophilized.

Up to 0.5 g of this lyophilized material was extracted three times according to the method of Bligh and Dyer (16). The dry mass was suspended in 4 ml of H₂O and washed twice in an additional 2 ml of H₂O.
before being transferred into a 100-ml Erlenmeyer flask. To this sample was added 30 ml of CHCl₃·CH₃OH (1:2, vol/vol), and the sample was sonicated (Branson Sonifier 250; output 2, 40% duty cycle, 20 min). Then, 10 ml of CHCl₃ was added, and the sample was sonicated for a further 5 min. Subsequently, an additional 10 ml of H₂O was added, and the sample was sonicated for a final 5 min. The sample was decanted evenly into between two and four 50-ml Nalgene Teflon tubes and centrifuged for 30 min at 10,000 g to generate three phases (a water phase, a CHCl₃ phase, and an interphase). The water phases (containing LAM and LM) were removed, combined, and lyophilized, while the CHCl₃ phases were transferred into a single 100-ml pear-shaped flask. The total yield of material after three extractions was about 11% of the bacterial dry mass.

Since the remaining interphases contained a lot of AcPIM6, they were suspended in 8 ml of H₂O and combined into 30-ml Kimble high-speed glass tubes before being lyophilized. These phases were then extracted overnight with 30 ml of CHCl₃·MeOH (1:2, vol/vol) using a shaker. The sample was then centrifuged for 30 min at 10,000 × g, and the CHCl₃·MeOH phase was removed. This phase was dried under an N₂ gas stream before being resuspended in equal parts of CHCl₃·MeOH, filtered through a 0.2-μm-pore-size polytetrafluoroethylene (PTFE) filter, and dried under N₂. This extraction was repeated two times until no PIMs could be identified (total yield of about 2% of the bacterial dry mass). The obtained PIMs were further purified with silica gel 60 chromatography (see below), as were the CHCl₃ phases of the Bligh and Dyer extraction.

**Purification of PIMs.** All samples were separated on a column (7 by 1 cm) of silica gel 60 (0.04 to 0.063 mm) which was successively eluted with (i) 80 ml of CHCl₃·MeOH (8:2, vol/vol), (ii) 60 ml of CHCl₃·MeOH (1:1, vol/vol), and finally (iii) 40 ml of CHCl₃·MeOH·H₂O (10:10:3, vol/vol/vol). Coextracted cardiolipin and other lipids eluted in the first mobile phase, most PIMs eluted in the second, and the rest of the PIMs eluted in the third. Fractions ii and iii were dried under N₂, resuspended in 10 ml of CHCl₃·MeOH (8:2, vol/vol), and passed through a 0.2-μm-pore-size PTFE filter before being analyzed by high-performance thin-layer chromatography (HPTLC). HPTLC was performed using glass-backed 10- by 10-cm silica gel 60 plates (Merck KGAA, Darmstadt, Germany) run in CHCl₃·MeOH·H₂O (10:8:2, vol/vol/vol) and stained with Hanessian's stain (0.5 g of ceric sulfate and 25 g of ammonium molybdate in 470 ml of water supplemented with 30 ml of sulfuric acid with stirring) and visualized at 150°C.

Fractions ii and iii from both of the CHCl₃ phases of the Bligh and Dyer extraction and the PIMs extracted from the interphase were then further separated by high-performance liquid chromatography (HPLC) using 5-μm Kromasil 100 C₁₈ columns (250 by 20 mm) eluted with eluent A (CHCl₃·MeOH·H₂O [240:1:140:620, vol/vol/vol/vol] containing 10 mM NH₄CH₃CO₂) and eluent B (CHCl₃·MeOH [1,400:600, vol/vol] containing 50 mM NH₄CH₃CO₂). The initial eluent B gradient was 15% for 60 min, followed by 20% for 140 min, 40% for 80 min, and finally 100% for 60 min at 4 ml min⁻¹. Samples were detected by a light-scattering detector (Sedex; nitrogen pressure, 2 × 10⁵ Pa; temperature, 50°C; split, 1:70). Samples were applied in 200 μl of CHCl₃·MeOH (8:2, vol/vol). For analytical runs, 10 μg of sample was injected while 10 mg was injected for preparative separations.

Since PIM2 and AcPIM6 coeluted on the C₁₈ column, they were separated by HPLC on 5-μm Prontosil 200-5-C₁₈ reverse-phase columns (250 by 4.6 mm) using the same elution reagents. The initial eluent B gradient was 5% for 5 min, followed by 10% for 15 min, 15% for 50 min, and finally 100% for 10 min at 0.8 ml min⁻¹. Samples were injected as a mixture of 0.6 mg in 80 μl of CHCl₃·CH₃OH·H₂O (10:10:3, vol/vol/vol) and detected by the light-scattering detector described above.
Cattle. Blood samples were obtained from 10 naturally infected, single intradermal comparative cervical tuberculin test-positive reactors (between 6 and 36 months of age). Animals were sourced from herds with confirmed bovine tuberculosis breakdowns in Devon, Herefordshire, or Worcestershire and were housed at the Animal Health and Veterinary Laboratories Agency (AHVLA) at the time of blood sampling. Infection was confirmed by necropsy and *M. bovis* culture in all animals. All procedures involving animals were carried out under a project license granted by the Home Office of Great Britain under the Animals (Scientific Procedures) Act 1986. This project was approved by the local VLA Animal Ethics Committee prior to submission to the Home Office.

**Isolation of bovine PBMC from whole blood.** Whole blood was mixed in equal parts with sterile Hanks balanced salt solution (HBSS) containing 10 U ml⁻¹ heparin. This mixture was overlaid onto Histopaque 1077 (Sigma-Aldrich) and centrifuged at 800 × g for 40 min. The PBMC interface was removed using a pastette and washed twice in HBSS containing heparin. Live cells were identified via trypan blue exclusion and enumerated using a hemocytometer.

**Preparation of lipid antigen suspensions.** Briefly, lipids were suspended in an aqueous phase for use in cell culture experiments after removal of CHCl₃·CH₃OH by evaporation using an N₂ gas stream. Cell culture medium was added to the dried lipid, and the mixture was subjected to two cycles of heating at 80°C and then sonication for 5 min. Lipids were used to stimulate cells in vitro at 20 μg ml⁻¹ in all assays.

**Lymphocyte proliferation assay.** Bovine PBMC were prepared from all 10 animals as described above and were cultured in complete cell culture medium (RPMI 1640 medium containing 25 mM HEPEs, 10% fetal calf serum [FCS], 1% nonessential amino acids [NEAA], 5 × 10⁻⁵ mM β2-mercaptoethanol, 100 U ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin [Gibco Life Technologies, Paisley, United Kingdom]) at 37°C in 5% CO₂ for 5 days in the presence of antigen at 2 × 10⁶ cells well⁻¹. After 5 days, cells were pulsed with 1 μCi well⁻¹ of [³H]thymidine overnight, before being harvested using a Harvester 96 Mach III (TomTec, Inc., Pullman, Washington, USA). Finally, labeled cells were washed in flow cytometry buffer and resuspended in 150 μl of 2% paraformaldehyde (Cytofix; BD Biosciences, Oxfordshire, United Kingdom), and Alexa Fluor 647 (CC63; AbD Serotec, Oxfordshire, United Kingdom), and Alexa Fluor 488-Zenon-labeled (Invitrogen Life Technologies, Paisley, United Kingdom) mouse anti-bovine CD3 (MM1A; WSU Monoclonal Antibody Centre, Pullman, Washington, USA), mouse anti-bovine CD4 conjugated to Alexa Fluor 488 (CC63; AbD Serotec, Oxfordshire, United Kingdom), and Alexa Fluor 488-Zenon-labeled (Invitrogen Life Technologies, Paisley, United Kingdom) mouse anti-bovine y6-TCR1 (GB21a; WSU Monoclonal Antibody Centre, Pullman, Washington, USA). Finally, labeled cells were washed in flow cytometry buffer and resuspended in 150 μl of 2% paraformaldehyde (Cytofix; BD Biosciences, Oxfordshire, United Kingdom) for at least 30 min at 4°C before analysis on a CyAn ADP analyzer. For capture and analysis, initial gating was on single, NIRDV⁺/IgG2α (live) cells in a subsequent lymphocyte gate before gating on CellTrace violet⁺ cells.

**Data and statistical analysis.** All data representation and statistical analysis were performed using GraphPad Prism, version 5.04, and GraphPad InStat, version 3.06 (GraphPad Software, La Jolla, CA, USA). Statistical analysis of IFN-γ and lymphocyte proliferation data was performed using a nonparametric repeated-measures analysis of variance (ANOVA; Friedman test) with a Dunn’s multiple comparisons posttest.

**RESULTS**

Highly purified PIM molecules can be isolated from virulent mycobacteria. PIM molecules differing in the number of acyl and mannose residues were highly purified from *M. tuberculosis*. Five distinct PIM molecules were isolated: PIM₁, AcPIM₂, AcPIM₆, AcPIM₉, and AcPIM₁₀. In total, these lipids constituted 3% of the bacterial dry mass after silica gel 60 separation. The ability of the purification method to isolate highly pure PIMS is shown in Fig. 1, in which the structures and purity of the different PIM molecules were confirmed by ESI-MS. In addition, bands corresponding to AcPIM₂ molecules (that differed only in the number of carbon atoms in the acyl chains) were clearly resolved by thin-layer chromatography (TLC) analysis (see Fig. S1 in the supplemental material).

Purified PIM molecules activate lymphocytes from *M. bovis*-infected cattle. In order to assess the ability of purified PIMS to induce in vitro immune responses in cattle, PBMC from 10 naturally *M. bovis*-infected cattle were cultured for 5 days in the presence of each PIM molecule, and the level of IFN-γ was measured by ELISA (Fig. 2A). Both the frequency and strength of IFN-γ responses differed depending on the nature of the PIM molecule. AcPIM₁ was least recognized, inducing responses in only 2 of the 10 animals. PIM₂, AcPIM₉, and AcPIM₁₀ were more frequently recognized, with responses detected in 3 (PIM₂) and 4 (AcPIM₂ and AcPIM₁₀) out of 10 animals. AcPIM₆ was most frequently recognized, inducing responses in half of the animals studied. Furthermore, AcPIM₉ was the only PIM molecule to induce significantly greater levels (*P < 0.01*) of IFN-γ overall than nonstimulated controls.

In addition to measuring IFN-γ production, we also investigated the ability of the PIM molecules to induce PBMC proliferative responses in the same animals. Again, the frequency of responding animals differed depending upon the nature of the PIM molecule (Fig. 2B). PIM₁ failed to induce a proliferative response in any of the animals studied, while AcPIM₂ induced responses in only 3 out of 10 animals. In contrast, AcPIM₂, AcPIM₉, and AcPIM₁₀ were more frequently recognized, inducing proliferative responses in 6 (AcPIM₂ and AcPIM₉) and 7 (AcPIM₁₀) out of 10 animals. Overall, significantly greater PBMC proliferation was de-
tected in the Ac2PIM3, AcPIM6, and Ac2PIM6 treatment groups than in nonstimulated controls, with median values tending to be greater following AcPIM6 stimulation.

Phenotyping of AcPIM6-responsive proliferating cells by flow cytometry. As AcPIM6 was the only PIM molecule to generate significantly increased levels of IFN-γ (Fig. 2A) and produced the greatest increase in the median proliferative response (Fig. 2B), we used this antigen to stimulate PBMC from an M. bovis-infected animal to characterize the proliferating cell populations by flow cytometry. Purified protein derivative from M. bovis (PPD-B) was used as a control antigen. CellTrace violet-labeled cells were incubated for 5 days with antigen before being harvested and labeled for flow cytometric analysis. After stimulation with either PPD-B or AcPIM6, three populations of proliferating cells were identified based on cell surface phenotyping: (i) CD4+ T cells (CD3+ CD4+), (ii) CD8+ T cells (CD3+ CD8+), and (iii) NKT-like cells (CD3+ CD335+). An example of the gating strategy for identifying proliferating NKT cells is highlighted in Fig. 3A, which demonstrates a greater level of proliferating cells in response to stimulation with AcPIM6 (58.29%) than in the non-antigen-stimulated control (29.87%).

The effects of stimulation with either PPD-B or AcPIM6 on the three different cell populations are summarized in Fig. 3B. Stimulation with PPD-B drove antigen-specific proliferation of approximately 60% of the CD4+ T cells (CD3+ CD4+). Similarly, an antigen-specific proliferative response was seen in approximately 15% of the CD8+ T cells (CD3+ CD8+) to PPD-B. A slight increase in NKT cell (CD3+ CD335+) proliferative responses (approximately 20%) was also seen to these antigens (Fig. 3B).

Stimulation with AcPIM6 induced only limited proliferation of CD4+ T cells (approximately 5%) and no proliferation of CD8+ T cells above the background (Fig. 3B). In contrast, approximately 30% of the NKT cell population mounted a proliferative response after stimulation with AcPIM6 (Fig. 3B). Little or no proliferation above the unstimulated negative control was seen in the CD3− CD335+ cells or in the CD3+ γδ-TCR+ populations (data not shown).

DISCUSSION

Mycobacterial lipids have long been implicated in the induction of responses in both the innate and adaptive cell-mediated immune responses (17–21). Although one strategy has been published which allows the isolation of PIMs from the avirulent M. bovis BCG strain (18, 22–24) and M. tuberculosis H37Rv (22) and certain synthetic molecules (17, 19, 21), in this study we successfully developed a novel method for extracting and subsequently highly purifying and characterizing individual PIM species from the pool of fraction of virulent M. tuberculosis H37Rv. The method developed here improves upon the previously published protocols primarily by using a French press to disrupt the bacterial cells, thereby increasing PIM yield. Other refinements include the removal of the hot acetone incubation and the use of different reverse-phase conditions for PIM purification. Our strategy allowed us to isolate a greater yield of more highly purified PIMs, as confirmed by ESI-MS, that could be subsequently assayed for their ability to generate responses in lymphocytes.

To assess the ability of these highly purified PIMs to drive immune responses, the individual molecules were used to stimulate peripheral lymphocytes isolated from M. bovis-infected cattle. Only AcPIM6 drove significant levels of IFN-γ from PBMC (Fig. 2A). Interestingly, when whole blood taken from the same animals was stimulated overnight with the PIM molecules as previously described (25–27), no IFN-γ could be measured, and this was not due to a lack of viability as stimulation with pokeweed mitogen (PWM) generated high levels of IFN-γ (data not shown). Although no IFN-γ production was seen from the whole-blood assay, AcPIM6 was able to drive significant production of IFN-γ from PBMC incubated for 5 days (Fig. 2A).

There are very few studies showing the effect of mycobacterial lipids in short-incubation, whole-blood assays. Cell-mediated immune responses to lipid antigens are more commonly assessed by enzyme-linked immunosorbent spot (ELISpot) assay, with incubation times of at least 48 h required before measurable responses become apparent (24, 28). Further, the requirement for antigen processing and presentation of specific PIMs has been demonstrated previously (20); perhaps the most likely explanation for the discrepancy between whole-blood and PBMC IFN-γ responses is that the frequencies of lipid-responsive cells are low and that an extended incubation allows for expansion of these cells. This is supported by our demonstration of strong proliferative responses induced after stimulation of PBMC with PIMs (Fig. 2B).
A recent study has shown that bovine NKT cells are present only at low frequencies (0.1% to 1.7%) (29).

Previous work using mice has shown that the ability of PIMs to generate cell-mediated responses is dependent on the acyl structures of the molecules. Early work performed using PIM2 and PIM6 demonstrated that the acyl chain was essential for NKT cell recruitment while the complexity of the mannose residues did not alter the response (23); however, it was subsequently shown that the second acyl chain of PIM4 enhances binding to murine CD1d but that the polar mannose head was essential for antigen recognition, proliferation, and IFN-γ production (28). As well as the number and location of acyl chains, their degree of unsaturation

![Flow cytometry gating strategy](FIG 3)

(A) Flow cytometric gating strategy. Single, live CD3⁺CD335⁺ lymphocytes were assessed for CellTrace violet labeling, and cells expressing low levels of CellTrace violet were gated for phenotyping. Numbers represent the percentages of proliferating cells in response to each antigen. FS Lin, forward scatter (linear). (B) Proliferation of CD3⁺CD4⁺, CD3⁺CD8⁺, or CD3⁺CD335⁺ cells in response to either PPD-B or AcPIM₆. Each bar represents the percentage of cells proliferating after subtraction of the unstimulated control.
and cis, but not trans, stereochemistry are critical in determining antigenicity (14, 30).

The larger and more complex PIM molecules tested here (Ac2PIM2, Ac2PIM6, and AcPIM6) drove significant levels of proliferation. AcPIM6 also drove proliferation in 3 animals while PIM2 generated no positive responses.

As AcPIM6 was the only molecule to drive significant IFN-γ responses in our study (Fig. 2A) and one of the most potent inducers of proliferation (Fig. 2B), we decided to use AcPIM6 to characterize the proliferative response. Stimulation with AcPIM6 induced higher levels of proliferation in NKT cells than in CD4+ or CD8+ T cells (Fig. 3). However, from these data it is not possible to tell if the proliferative CD4+ or CD8+ cells also coexpress CD335 as our flow cytometric labeling panels do not allow the discrimination; however, this is a distinct possibility.

Although well characterized in humans and mice, the presence of NKT cells in cattle has been a controversial issue (31–34). Nevertheless, studies have shown that the bovine CD1D gene is expressed and translated in vivo (35), and recent work has identified a subset of cattle lymphocytes that express both T cell (CD3) and NK cell (NKP46) markers, suggesting the presence of an NKT cell population in bovine peripheral blood (29). Furthermore, bovine NKT cells have been shown to express both αβ- and γδ-TCRs, to have a broad TCR repertoire, and to have fully functional NKP46, CD16, and CD3 signaling pathways (29). Interestingly, these cells require ligation of their CD3 molecules to produce IFN-γ. While this may initially suggest that a CD3 binding component may be present in our PIM preparations, it is worth noting that we have not identified the cytokine-producing cells.

The identification of AcPIM6 as a potent immunostimulatory molecule is of great interest both as a potential vaccine candidate (21), an adjuvant formulation (36), or a target for attenuation in the development of novel live vaccines (37). PIMs have also been used previously as diagnostic reagents for both tuberculosis and leprosy, although with limited success (38).

In conclusion, we present here the ability to extract and selectively purify PIMs to a high level of purity. These molecules could have shown antigen-specific NKT activation in cattle.

ACKNOWLEDGMENTS

This study was funded by the Department for Environment, Food and Rural Affairs, United Kingdom (EMIDA-funded project Mycobactdiag).

We sincerely appreciate the staff of the Animal Services Unit at AHVLA for their dedication to the welfare of test animals. We also thank Florence Dufreneix for her technical assistance and Buko Lindner (Research Centre Borstel) for ESI-mass spectrometry.

We have no competing interests.

REFERENCES

1. Khoo KH, Dell A, Morris HR, Brennan PJ, Chatterjee D. 1995. Structural definition of acylated phosphatidylinositol mannosides from Mycobacterium tuberculosis: definition of a common anchor for lipomannan and lipoarabinomannan. Glycobiology 5:117–127. http://dx.doi.org/10.1093/glycob/5.1.117.

2. Beckman EM, Melian A, Behar SM, Sieling PA, Chatterjee D, Furlong ST, Matsumoto R, Rosat JP, Modlin RL, Porcelli SA. 1996. CD1c restricts responses of mycobacteria-specific T cells. Evidence for antigen presentation by a second member of the human CD1 family. J Immunol 157:2795–2803.

3. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. 1994. Recognition of a lipid antigen by CD1-restricted ab+ T cells. Nature 372:691–694. http://dx.doi.org/10.1038/372691a0.

4. Sieling PA, Chatterjee D, Porcelli SA, Prigozy TJ, Mazzocco Rj, Soriano T, Bloom BR, Brenner MB, Kronenberg M, Brennan PJ. 1995. CD1d-restricted T cell recognition of microbial lipoglycan antigens. Science 269:227–230. http://dx.doi.org/10.1126/science.7542404.

5. Kawanoto T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koski H, Taniguchi M. 1997. CD1d-restricted and TCR-mediated activation of Va14 NKT cells by glycosylceramides. Science 278:1626–1629. http://dx.doi.org/10.1126/science.278.5343.1626.

6. Spada FM, Koezuka Y, Porcelli SA. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. J Exp Med 188:1529–1534. http://dx.doi.org/10.1084/jem.182.4.993.

7. Behar SM, Podrebarac TA, Roy CJ, Wang CR, Brenner MB. 1999. Diverse TCRs recognize murine CD1. J Immunol 162:161–167.

8. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. 1995. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. J Exp Med 182:993–1004. http://dx.doi.org/10.1084/jem.182.4.993.

9. Chiu YH, Jayawardena J, Weiss A, Lee D, Park SH, Dauntry-Varsat A, Bendelac A. 1999. Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments. J Exp Med 189:110–110. http://dx.doi.org/10.1084/jem.189.1.103.

10. Yssel T, Bendelac A, Watson C, Hu-Li J, Paul WE. 1995. Role of NK1.1+ T cells in a Th2 response and in immunoglobulin E production. Science 270:1845–1847. http://dx.doi.org/10.1126/science.270.5243.1845.

11. Gumperz JE, Roy C, Makawaska A, Lum D, Sugita M, Podrebarac T, Koezuka Y, Porcelli SA, Cardell S, Brenner MB, Behar SM. 2000. Murine CD1d-restricted T cell recognition of cellular lipids. Immunity 12:211–221. http://dx.doi.org/10.1016/S1074-7613(00)80174-0.

12. Li S, Choi HJ, Felio K, Wang CR. 2011. Autoreactive CD1b-restricted T cells: a new innate-like T-cell population that contributes to immunity against infection. Blood 118:3870–3878. http://dx.doi.org/10.1182/blood-2011-03-341941.

13. Kinjo Y, Kronenberg M. 2005. Va14 iNKT cells are innate lymphocytes that participate in the immune response to diverse microbes. J Clin Immunol 25:522–533. http://dx.doi.org/10.1007/s10875-005-8065-5.

14. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benschia MB, Zajonc DM, Ben-Menachem G, Ainge GD, Painter GF, Khurana A, Hoebel K, Behar SM, Beutler B, Wilson IA, Tsuji M, Sellati TJ, Wong CH, Kronenberg M. 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nat Immunol 7:978–986. http://dx.doi.org/10.1038/ni380.

15. Skold M, Behar SM. 2003. Role of CD1d-restricted NKT cells in microbial immunity. Infect Immun 71:5447–5455. http://dx.doi.org/10.1128/IAI.71.14.5447-5455.2003.

16. Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917. http://dx.doi.org/10.1139/o59-099.

17. Ainge GD, Parlane AD, Denis M, Dyer BS, Harer A, Hayman CM, Larsen DS, Painter GF. 2007. Phosphatidylinositol mannosides ether analogues: syntheses and interleukin-12-inducing properties. J Org Chem 72:5291–5296. http://dx.doi.org/10.1021/jo070639m.

18. Doz E, Rose S, Court N, Front Vasseur SV, Charbon S, Gilleron M, Pupo G, Fremeaux I, Delneste Y, Erard F, Ryffel B, Martin OR, Quesniaux VF. 2009. Mycobacterial phosphatidylinositol mannosides negatively regulate host Toll-like receptor 4, MyD88-dependent proinflammatory cytokines, and TRIF-dependent co-stimulatory molecule expression. J Biol Chem 284:23187–23196. http://dx.doi.org/10.1074/jbc.M109.037846.

19. Court N, Ross E, Bourguet M-L, Front S, Martin OR, Dowling JK, Kenny EF, O’Neill L, Erard F, Quesniaux VF. 2011. Mycobacterial PIMs inhibit host inflammatory responses through CD14-dependent and CD14-independent mechanisms. PLoS One 6:e24631. http://dx.doi.org/10.1371/journal.pone.0024631.

20. De Paspe D, Layne R, Giacometti G, Garcia-Alles LF, Mori L, Hanau D, de Libero G, de la Salle H, Pupo G, Gilleron M. 2012. Deciphering the role of CD1e protein in mycobacterial phosphatidylinositol-myo-inositol mannosides (PIM) processing for presentation by CD1b to T lymphocytes. J Biol Chem 287:31494–31502. http://dx.doi.org/10.1074/jbc.M111.386300.

21. Parlane NA, Compton B, Hayman CM, Painter GF, Basaraba RJ,
Heiser A, Buddle BM. 2012. Phosphatidylinositol di-mannoside and derivatives modulate the immune response to and efficacy of a tuberculosis protein vaccine against Mycobacterium bovis infection. Vaccine 30:580–588. http://dx.doi.org/10.1016/j.vaccine.2011.11.055.

22. Gilleron M, Quesniaux VF, Puzo G. 2003. Acylation state of the phosphatidylinositol hexamannosides from Mycobacterium bovis bacillus Calmette Guerin and Mycobacterium tuberculosis H37Rv and its implication in Toll-like receptor response. J Biol Chem 278:29880–29889. http://dx.doi.org/10.1074/jbc.M303446200.

23. Gilleron M, Ronet C, Mempel M, Monsarrat B, Gachelin G, Puzo G. 2001. Acylation state of the phosphatidylinositol mannosides from Mycobacterium bovis bacillus Calmette Guerin and ability to induce granuloma and recruit natural killer T cells. J Biol Chem 276:34896–34904. http://dx.doi.org/10.1074/jbc.M103908200.

24. Sprott GD, Dicaire CJ, Gurnani K, Sad S, Krishnan L. 2004. Activation of dendritic cells by liposomes prepared from phosphatidylinositol mannosides from Mycobacterium bovis bacillus Calmette-Guerin and adjuvant activity in vivo. Infect Immun 72:5235–5246. http://dx.doi.org/10.1128/IAI.72.9.5235-5246.2004.

25. Cockle PJ, Gordon SV, Lavalvi A, Buddle BM, Hewinson RG, Vordermeier HM. 2002. Identification of novel mycobacterium tuberculosis antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. Infect Immun 70:6996–7003. http://dx.doi.org/10.1128/IAI.70.12.6996-7003.2002.

26. Ewer K, Cockle PJ, Gordon SV, Mansoor H, Govaerts M, Walravens K, Marche S, Hewinson RG, Vordermeier HM. 2006. Antigen mining with iterative genome screens identifies novel diagnostics for the mycobacteria tuberculosis complex. Clin Vaccine Immunol 13:90–97. http://dx.doi.org/10.1128/CVI.13.1.90-97.2006.

27. Jones GJ, Gordon SV, Hewinson RG, Vordermeier HM. 2010. Screening of predicted secreted antigens from Mycobacterium bovis reveals the immunomodulation of the ESAT-6 protein family. Infect Immun 78:1326–1332. http://dx.doi.org/10.1128/IAI.01246-09.

28. Fischer K, Scotet E, Niemeyer M, Koebenick H, Zerrahn J, Maillet S, Hurvitz R, Kursar M, Bonneville M, Kaufmann SH, Schable UE. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. Proc Natl Acad Sci U S A 101:10685–10690. http://dx.doi.org/10.1073/pnas.0403787101.

29. Connelley TK, Longhi C, Burrells A, Degnan K, Hope J, Allan AJ, Haemmond JA, Storset AK, Morrison WI. 2014. NKp46+ CD3+ cells: a novel nonconventional T cell subset in cattle exhibiting both NK cell and T cell features. J Immunol 192:3868–3880. http://dx.doi.org/10.4049/jimmunol.1302464.

30. Rauch J, Gumperz J, Robinson C, Skold M, Roy C, Young DC, Lafleur M, Moody DB, Brenner MB, Costello CE, Behar SM. 2003. Structural features of the acyl chain determine self-phospholipid antigen recognition by a CD1d-restricted invariant NKT (iNKT) cell. J Biol Chem 278:47508–47515. http://dx.doi.org/10.1074/jbc.M308089200.

31. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. 1997. Impaired NK1.1 T cell development and early IL-4 production in CD1-deficient mice. Immunity 6:459–467. http://dx.doi.org/10.1016/S1074-7613(00)80289-7.

32. Gapin I, Matsuda JL, Surh CD, Kronenberg M. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. Nat Immunol 2:971–978. http://dx.doi.org/10.1038/ni710.

33. Loorinig van Beeck FA, Reinink P, Hermsen R, Zajonc DM, Laven MJ, Fun A, Troskie M, Schoemaker NJ, Morar D, Lenstra JA, Verveelde L, Rutten VP, van Eden W, Van Rhijn I. 2009. Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants. Mol Immunol 46:1424–1431. http://dx.doi.org/10.1016/j.molimm.2008.12.009.

34. Van Rhijn I, Koets AP, Im JS, Piebides D, Reddington F, Besra GS, Porcelli SA, van Eden W, Rutten VP. 2006. The bovine CD1 family contains group 1 CD1 proteins, but no functional CD1D. J Immunol 176:4888–4893. http://dx.doi.org/10.4049/jimmunol.176.8.4888.

35. Nguyen TK, Koets AP, Vordermeier M, Jervis PJ, Cox LR, Graham SP, Santema WJ, Moody DB, van Calenbergh S, Zajonc DM, Besra GS, Van Rhijn I. 2013. The bovine CD1D gene has an unusual gene structure and is expressed but cannot present α-galactosylceramide with a C26 fatty acid. Int Immunol 25:91–98. http://dx.doi.org/10.1093/intimm/dxs092.

36. Wedlock DN, Denis M, Painter GF, Ainge GD, Vordermeier HM, Hewinson RG, Buddle BM. 2008. Enhanced protection against bovine tuberculosis after coadministration of Mycobacterium bovis BCG with a Mycobacterial protein vaccine-adjuvant combination but not after coadministration of adjuvant alone. Clin Vaccine Immunol 15:765–772. http://dx.doi.org/10.1128/CVI.00034-08.

37. Mahon RN, Rojas RE, Fulton SA, Franko JL, Harding CV, Boom WH. 2009. Mycobacterium tuberculosis cell wall glycolipids directly inhibit CD4+ T-cell activation by interfering with proximal T-cell-receptor signaling. Infect Immun 77:4574–4583. http://dx.doi.org/10.1128/IAI.00222-09.

38. Mullins RJ, Fournie JJ, Moloney B, Baumgart K, Jones P, Brown P, Basten A. 1992. Serological response to purified mycobacterial phosphatidylinositol mannoside in healthy controls and in patients with tuberculosis and leprosy. Int J Lepr Other Mycobact Dis 60:353–367.
Erratum for Pirson et al., Highly Purified Mycobacterial Phosphatidylinositol Mannosides Drive Cell-Mediated Responses and Activate NKT Cells in Cattle

Chris Pirson, Regina Engel, Gareth J. Jones, Thomas Holder, Otto Holst, H. Martin Vordermeier
TB Research Group, Animal Health and Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, United Kingdom; Division of Structural Biochemistry, Research Centre Borstel, Leibniz-Centre for Medicine and Biosciences, Borstel, Germany

Volume 22, no. 2, p. 178–184, 2015. Page 180, column 1, final line: “IgG2a” should read “IgG1.”