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CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection

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The discovery of the CD1 antigen presentation pathway has expanded the spectrum of T-cell antigens to include lipids[1–4], but the range of natural lipid antigens and functions of CD1-restricted T cells *in vivo* remain poorly understood. Here we show that the T-cell antigen receptor and the CD1c protein mediate recognition of an evolutionarily conserved family of isoprenoid glycolipids whose members include essential components of protein glycosylation and cell-wall synthesis pathways. A CD1c-restricted, mycobacteria-specific T-cell line recognized two previously unknown mycobacterial hexosyl-1-phosphoisoprenoids and structurally related mannosyl-β1-phosphodolichols. Responses to mannosyl-β1-phosphodolichols were common among CD1c-restricted T-cell lines and peripheral blood T lymphocytes of human subjects recently infected with *M. tuberculosis*, but were not seen in naive control subjects. These results define a new class of broadly distributed lipid antigens presented by the CD1 system during infection *in vivo* and suggest an immune mechanism for recognition of senescent or transformed cells that are known to have altered dolichol lipids.

CD1a, CD1b and CD1c, three members of the human CD1 family of major histocompatibility complex (MHC) class I-like cell-surface glycoproteins, present bacterial lipid antigens for recognition by T cells[4–8]. A small number of mycobacterial lipid antigens presented by CD1b have been purified to homogeneity and identified[4–6], and structural studies of these antigens and of the CD1 proteins have led to the proposal of a general molecular mechanism for lipid antigen presentation by CD1 (refs 9, 10). This involves the binding of the alkyl components of the antigens within a hydrophobic groove in

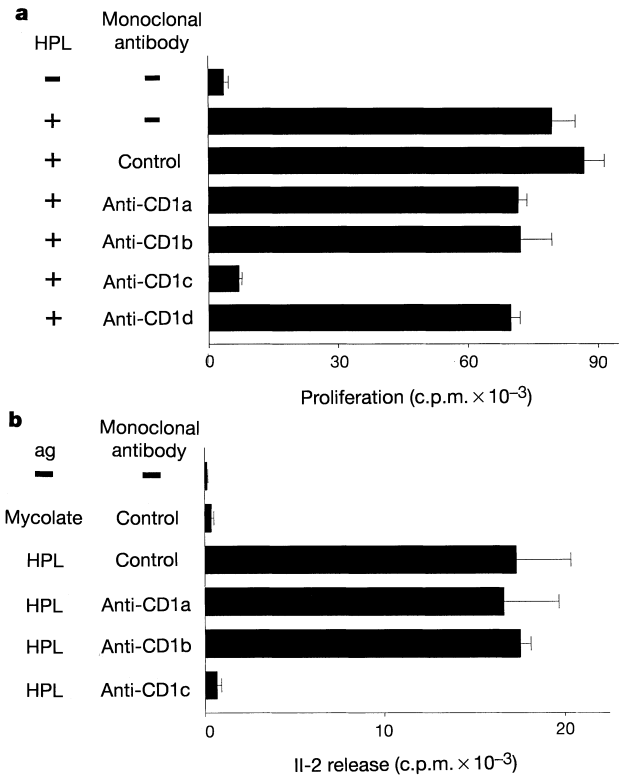

Figure 1 Molecular requirements for T-cell recognition of a hexosyl phospholipid (HPL) antigen. **a**, CD8-1 T cells and irradiated monocyte-derived dendritic cells were cultured for 3 d with the HPL antigen (0.5 μg ml^{-1}) and the monoclonal antibody specific for the indicated CD1 isoform (20 μg ml^{-1}); T-cell proliferation was determined by ^{3}H-thymidine incorporation[7,8]. **b**, The TCR α- and β-chains from T-cell line CD8-1 (TCRAV1S3J17C1, TCRBV2S1J2S7C2) were cloned into the pREP7 and pREP9 expression vectors and transfected into CD3$^-$ J.RT3 T lymphoblastoid cells[13]. J.RT3 transfectants were cultured with irradiated monocyte-derived dendritic cells, antigen (mycolate 50 μg ml^{-1}, HPL 0.5 μg ml^{-1}), blocking antibody (20 μg ml^{-1}) and phorbol myristate acetate (10 ng ml^{-1}; Sigma). Levels of IL-2 released were determined by measuring ^{3}H-thymidine incorporation by HT-2 cells[13]. JRT.3 cells similarly transfected with TCR α- and β-chains from the CD1b-restricted line LDN5 and the CD1a-restricted line CD8-2 did not respond to the *M. avium* HPL (data not shown)[6,7].

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the CD1 protein, and the recognition of their exposed hydrophilic elements by specific T-cell receptors (TCRs)[6,9,11–13]. Information concerning the dominant antigens presented by the CD1 system and evidence for the participation of CD1-restricted T cells in immune responses to human pathogens *in vivo*, however, are currently lacking.

To obtain a broader view of the role of the CD1 system in the human immune response, we isolated and characterized a phospholipid antigen presented by CD1c to CD8-1, a $CD8^+$ TCR $\alpha\beta^+$ T-cell line[7]. Cell walls of *Mycobacterium avium* and *M. tuberculosis*, both of which contained the antigen for CD8-1, were fractionated using several silica-based chromatographic techniques. An extensively purified fraction containing the antigen from *M. avium* reacted with a phosphate-specific molybdenum stain and released mannose and glucose upon hydrolysis as determined by gas chromatography/mass spectrometry (MS) (data not shown). The response of the T-cell line CD8-1 to the purified *M. avium* hexose phospholipid retained the property of CD1c restriction that was observed with responses to whole *M. avium* (Fig. 1a). This response was mediated by the clonotypic TCR, as expression of TCR α- and β-chain complementary DNAs encoding the CD8-1 TCR in J.RT3 T-lymphoblastoid cells conferred CD1c dependent recognition of the hexose phospholipid (Fig. 1b)[13].

A combination of MS experiments gave a detailed structure of the natural mycobacterial phospholipid antigens. In the high-resolution electrospray ionization (ESI) mass spectrum of the *M. avium* antigen, the $[M\text{-}H]^-$ ion appeared at *m/z* 679.4911 corresponding to the elemental formula $C_{36}H_{72}O_9P$. Low-energy collisionally induced dissociation tandem mass spectrometry (CID–MS/MS) yielded a series of abundant product ions that identified the intact antigen as a hexosyl-1-phospholipid with a fully saturated alkyl group of relative molecular mass (M_r) 421 ($C_{30}H_{61}$) (Fig. 2a). High energy CID–MS/MS using a linked scan of electric and magnetic sectors detected a series of product ions separated by 14 units with a 28-unit interval after every fourth member of the series. This pattern indicated the presence of methyl branches at every fourth carbon on the otherwise unbranched alkyl chain, thus showing the isoprenoid nature of the lipid (Fig. 2b). Antigenic fractions from *M. tuberculosis* contained a similar lipid with $[M\text{-}H]^-$ at *m/z* 707.5223 corresponding to $C_{38}H_{76}O_9P$. The fragmentation pattern indicated that this lipid was a structurally related hexosyl-1-phospholipid with a $C_{32}H_{65}$ isoprenoid unit (data not shown).

These two natural hexosyl-phospholipid antigens represented a previously unknown type of fully saturated polyisoprenoid phosphoglycolipid (Fig. 2c). They are structurally related to the evolutionarily conserved family of glycosyl-1-phosphopolyprenols which

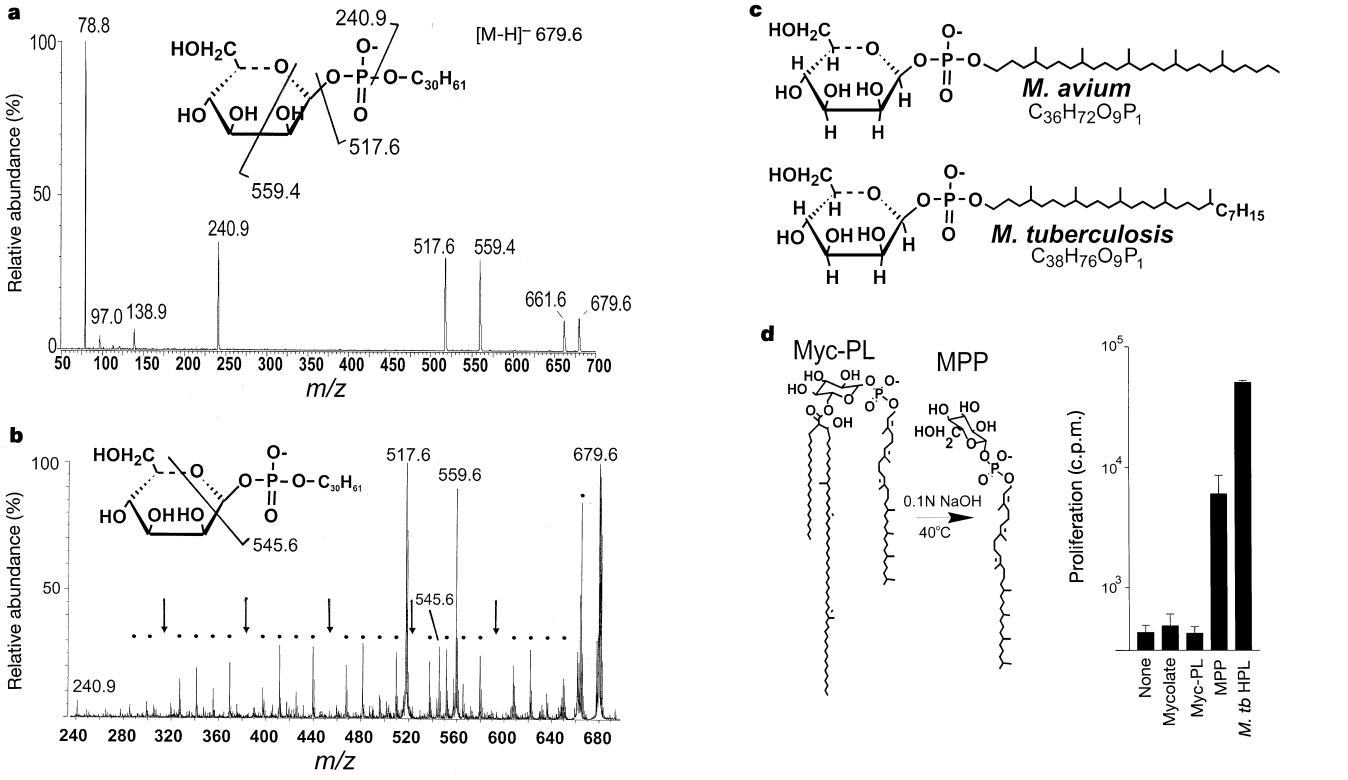

Figure 2 Structures of natural CD1c-presented mycobacterial isoprenoid glycolipids. **a**, Low-energy CID–MS/MS spectrum of the *M. avium* phospholipid parent $[M\text{-}H]^-$ (*m/z* 679.6). Fragments correspond to phosphate (*m/z* 78.8, 97.0), hexosyl phosphate (*m/z* 240.9), phospholipid (*m/z* 517.6), two hexose cross-ring cleavage products (*m/z* 138.9, 559.4) and a dehydration product (*m/z* 661.6). The cross-ring cleavage products presumptively define the phosphate linkage at C_1; this fragment is favoured when the C_2 hydroxyl is *cis* to the C_1 phosphate, suggesting a β1-mannosyl linkage[29]. Similar analysis of the *M. tuberculosis* antigen detected fragments indicative of a hexosyl-1-phospholipid with an alkyl group of M_r 449 ($C_{32}H_{65}$) (data not shown). **b**, Double focusing magnetic sector MS detected a third cross-ring cleavage product at *m/z* 545, consistent with the assignment of phosphate at C_1. A series of product ions (dots), shown at eightfold amplification relative to the parent ion, occurred at intervals of 14 units. The lack of ions at every fifth position in the series (arrows) indicates a methyl branch on every fourth carbon of an otherwise unbranched fully saturated alkyl chain. Analysis of the *M. tuberculosis* antigen revealed a similar pattern indicative of at least five methyl branches (data not shown). **c**, The high-resolution mass determination of the $[M\text{-}H]^-$ ion of the *M. avium* antigen was *m/z* 679.4911, corresponding to $C_{36}H_{72}O_9P$ (calculated *m/z* 679.4914). The *M. tuberculosis* derived antigen was *m/z* 707.5223, corresponding to $C_{38}H_{76}O_9P$ (calculated *m/z* 707.5227). Thus, the antigenic structures are both saturated hexosyl-1-phosphoisoprenoid lipids, new members of a family of mycobacterial glycosyl-1-phosphopolyprenols[18,30]. **d**, CD8-1 also recognized purified natural *M. smegmatis* derived β-D-mannopyranosyl-1-phosphoheptaprenol (MPP) released by treatment of mycolated phospholipid (Myc-PL) with 0.1 N NaOH at 40 °C for 1 hour[18]. Antigens (5 μg ml^{-1}) were presented by monocyte-derived dendritic cells.

are obligate carbohydrate donors in glycan synthesis pathways, such as *N*-linked protein glycosylation in primitive bacteria and eukaryotes and cell-wall assembly in prokaryotes[14]. Although found in all cells, long-chain acyclic isoprenoids differ among various phyla with regard to their length, saturation, phosphorylation and glycosylation. For example, in eubacteria the isoprene unit most proximal to the alcohol (α-unit) is unsaturated, whereas in eukaryotes it is saturated. These α-saturated isoprenoids, generally referred to as dolichols, vary in prenyl length among different organisms, with multicellular organisms having the longest dolichols (C_{90-100}) compared with shorter structures found in fungi (C_{70-90}) and protozoa (C_{50-65})[15,16]. Unusual features of the newly discovered mycobacterial isoprenoids reported here included the complete saturation of their alkyl chains, and a deviation from the repeating five carbon prenyl unit at the proximal and distal ends of these chains. Nevertheless, the unusual lipids of these natural CD1c-presented phospholipids were reminiscent of fully saturated archaebacterial isoprenoid lipids and the known distally saturated

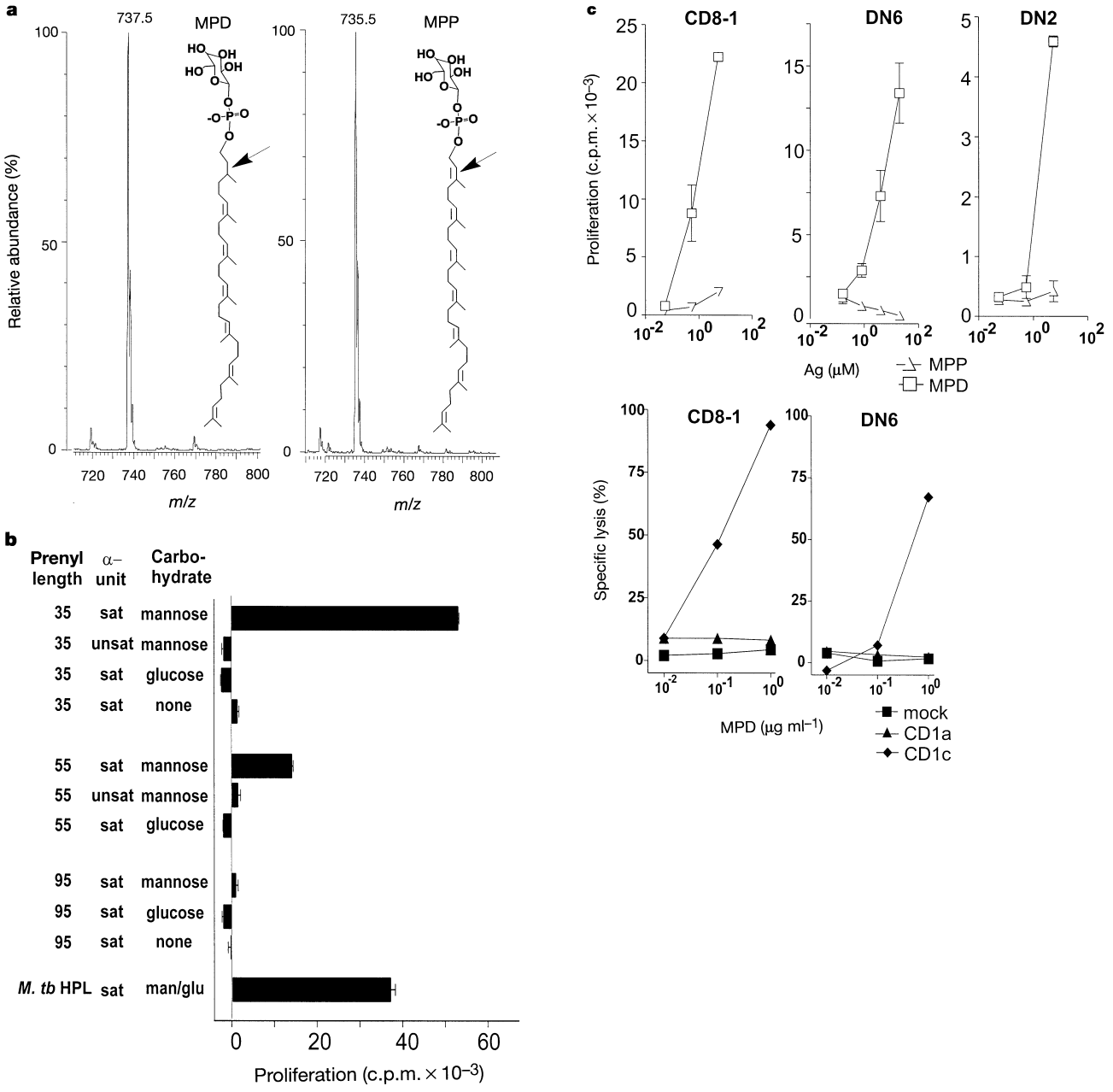

Figure 3 Fine specificity of human CD1c-restricted T-cell lines for mannosyl phosphodolichol. **a**, Hexose dinucleotides were enzymatically coupled with a β1 linkage to pure di-*trans* poly-*cis* phosphopolyprenols that varied in prenyl length and saturation of the α-isoprene unit[27]. ESI–MS spectra of two products had $[M-H]^-$ ions at *m/z* 735.5 and *m/z* 737.5, corresponding to the mannosyl phosphopolyprenol (MPP) and the mannosyl phosphodolichol (MPD), respectively, which differ in structure only in the saturation of the α-isoprene unit (arrow). **b**, Semi-synthetic antigens (20 μM) were tested in duplicate (bars, mean c.p.m. - c.p.m. in the absence of antigen; error bars, range) for stimulation of CD8-1 presented by monocyte-derived dendritic cells. **c**, Three independently derived human T-cell lines that were reactive to *M. tuberculosis* and previously shown to be restricted by CD1c (CD8-1, DN2 and DN6) proliferated in response to MPD but not the α-unsaturated MPP analogue presented by monocyte-derived dendritic cells[8,18]. Both CD8-1 (effector:target, 12:1) and DN6 (effector:target, 10:1) lysed MPD-treated, ^{51}Cr-labelled C1R lymphoblastoid cells transfected with CD1c, but not CD1a- or mock-transfected cells.

mannosyl-β1-phosphoheptaprenol (MPP) portion of the mycolate carrier, Myc-PL, from *Mycobacterium smegmatis*[17,18].

In fact, CD8-1 also responded to the *M. smegmatis* MPP, although the lower magnitude of this reponse, as compared to the *M. tuberculosis* hexosyl-1-phosphoisoprenoid (Fig. 2d), indicated that the T cells were sensitive to structural differences between these compounds such as variations in the lipid saturation, prenyl chain length or identity of the hexose sugar. To gain further insight into the features of isoprenoid glycolipids that affected their recognition by CD1c-restricted T cells, semi-synthetic β1-linked isoprenoid compounds were produced by coupling monosaccharides to synthetic phosphopolyprenols (Fig. 3a)[19]. CD8-1 recognized the mannosylated analogues, but not analogues containing glucose or no carbohydrate (Fig. 3b), indicating that mannose was probably the hexose sugar present in the natural antigens. This result also demonstrated a fine specificity for the hydrophilic head group of this lipid antigen similar to that seen for antigens presented by other CD1 proteins[6,11]. In addition, recognition of semi-synthetic analogues required a saturated α-prenyl unit, a finding that probably explains the stronger response to the fully saturated natural antigens of *M. avium* and *M. tuberculosis* compared with the α-unsaturated MPP of *M. smegmatis* (Fig. 2d). Among the α-saturated dolichol analogues, the T-cell response was inversely proportional to prenyl length. T-cell line CD8-1 recognized the semi-synthetic mannosyl phosphodolichol (MPD) with a C_{35} dolichol more strongly than the MPD analogue with a C_{55} dolichol typical of protozoal pathogens (Fig. 3b)[20]. A self MPD containing a C_{95} dolichol purified from human liver was not recognized at a concentration of 20 μM (Fig. 3b), although weak responses were seen at higher concentrations in some experiments (data not shown).

Recognition of MPD could also be shown for a variety of other T-cell populations in addition to CD8-1. Two other previously described CD1c-restricted T-cell lines specific for mycobacterial lipid were tested for responses to semi-synthetic MPD and showed significant reactivity (Fig. 3c)[8]. In contrast, neither peripheral blood lymphocytes (PBL) nor T-cell lines specific for other defined antigens presented by CD1a, CD1b or MHC class II responded to MPD under identical conditions (data not shown). These findings suggested that MPD recognition may be common among CD1c-restricted T-cell populations that were stimulated *in vitro* with mycobacterial antigen, and led us to determine whether MPD-reactive T cells might be expanded in humans recently infected with *M. tuberculosis*.

To assess the possibility that MPD reactive T cells are generated during *M. tuberculosis* infection *in vivo*, lymphocytes from human subjects with exposure to *M. tuberculosis* and documented positive tuberculin (PPD) skin tests were tested for their responses to MPD and compared with lymphocytes from naive PPD^- control subjects. Overall, the proliferative responses to MPD of PBL from *M. tuberculosis* infected subjects were significantly higher than those of controls (P = 0.0003; Fig. 4a). Sixty-five per cent of *M. tuberculosis* immune subjects compared with zero per cent of control subjects had significantly elevated stimulation indices. The responses of five infected subjects were tested in the presence of blocking monoclonal antibodies, and in all five cases, a monclonal antibody against CD1c significantly blocked the response to MPD

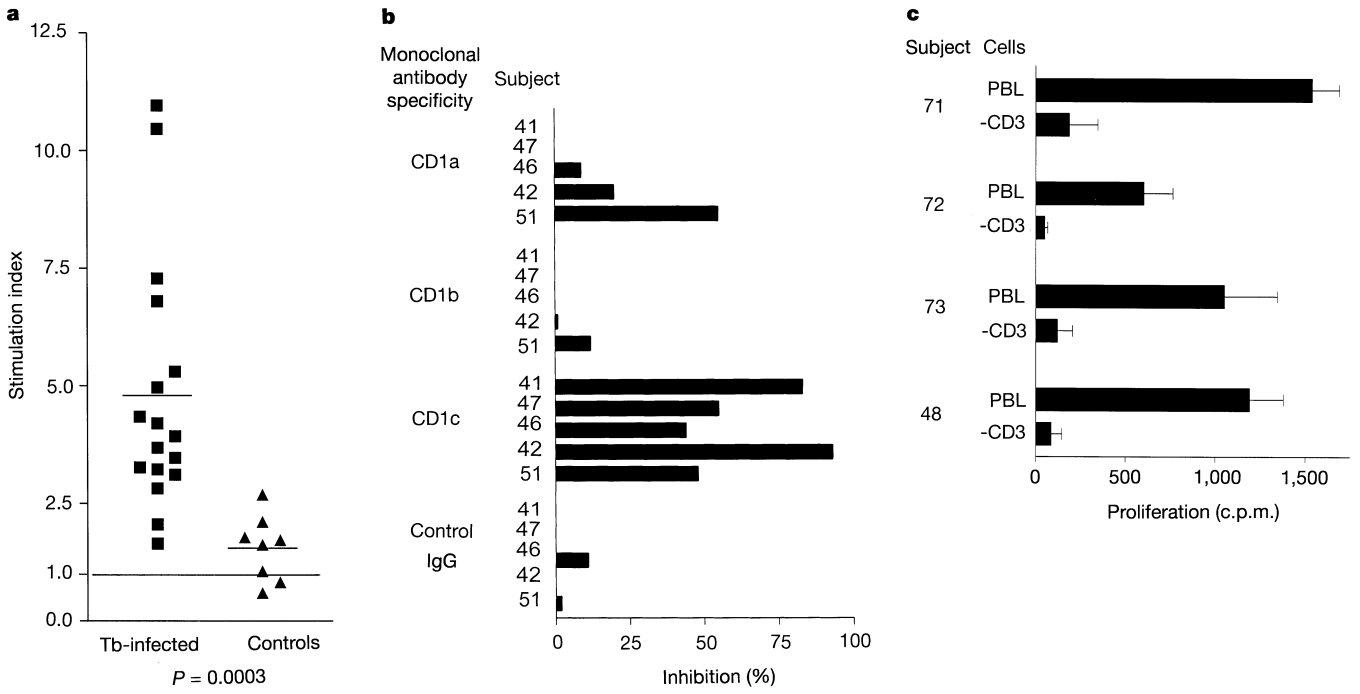

Figure 4 CD1c-restricted MPD-specific immune responses occur in humans with *M. tuberculosis* infection. **a**, Subjects with documented exposure to *M. tuberculosis* and who had positive intradermal tuberculin (PPD) tests within the previous month were phlebotomized. PBL were tested for proliferative responses to the semi-synthetic C_{35} β1-MPD (10 μM) presented by irradiated (5,000 R) autologous monocyte-derived dendritic cells. The stimulation indices were compared with age- and gender-matched PPD^- healthy control subjects and evaluated by the two-tailed Mann–Whitney test (P = 0.0003). The mean stimulation indexes in response to total mycobacterial lipid and PPD were 8.6 (n = 9) and 17.9 (n = 17), respectively, in infected subjects. **b**, Strong inhibition of proliferative responses (see Methods) to semi-sythetic C_{35} β1-MPD of PBL from five infected subjects was observed with addition of anti-CD1c monoclonal antibody (20 μg ml^{-1}). Because anti-CDLa monoclonal antibody 10H3.9.3 did not show significant cross-reactivity with CD1c in flow cytometry studies (data not shown), these results suggest that CD1a may also present the MPD antigen in some cases. **c**, PBL from four infected subjects were depleted of T cells using OKT3 (anti-CD3ε)-coated magnetic beads. Cells lacking CD3 antigen were viable, as assessed by trypan blue exclusion and mitogen responses (not shown), but showed significantly reduced responses to MPD as compared with total PBL. Bars show mean (± s.d.) c.p.m. - c.p.m. in the absence of antigen.

(Fig. 4b). The PBL from five responding subjects were depleted of $CD3^+$ cells, and in all cases the lipid-antigen-specific proliferative responses were significantly or completely inhibited, indicating that T cells mediated these responses (Fig. 4c). These results indicated that isoprenoid glycolipid antigens were active as recall antigens in the human immune response to *M. tuberculosis*, providing the first direct evidence for CD1-mediated, lipid-specific T-cell responses in the natural history of an infectious disease.

In contrast to the nine known lipids presented by other CD1 isoforms that share a common motif of two straight aliphatic hydrocarbon tails connected by a central hydrophilic cap[4–6,11,21–23], the CD1c-presented lipids that we have described here have a single alkyl chain tail composed of one isoprenoid lipid. These differences in antigen structure, along with the inability of CD1b-specific monoclonal antibody with known blocking ability to inhibit polyclonal responses to MPD (Fig. 4b), suggest that the hydrophobic CD1c groove may be structurally specialized to present an array of lipids that is different from those presented by other CD1 isoforms. The current studies directly implicate MPD specific T-cell responses in human mycobacterial infection; however, the ability of CD1c-restricted T cells to also recognize polyunsaturated, di-*trans* poly-*cis* MPDs that are necessary for *N*-linked glycosylation and are broadly distributed in eukaryotes suggests that dolichol recognition by T cells may have other roles. The semi-synthetic C_{55} MPD (Fig. 3b) corresponds to the structure of natural protozoal MPD found in trypanosomes and leishmania[20]. This finding, and the description of CD1d-presented glycosyl phosphatidylinositol anchors[21], suggests that protozoal parasites can be targets of CD1-mediated immune responses. These foreign MPDs are shorter but otherwise similar to a known family of self MPDs[14,15]. Dolichols are scarce in normal cells but shorten in length or increase in abundance depending on the age and growth rate of cells[24–26]. Thus, T-cell activation by recognition of dolichols such as MPD offers a mechanism for immune recognition of changes in the level or structure of isoprenoid glycolipids that occur as a result of infection, ageing or transformation. □

Methods

Lipid antigens

Hexosyl phosphoisoprenoids were purified from $CHCl_3$:CH_3OH extracts of *M. tuberculosis* H37Ra and *M. avium* serovar 4 using an open silica column eluted sequentially with chloroform, acetone and methanol as described[6,7]. Methanol-eluting fractions were further purified using one-dimensional preparative thin layer chromatography (1D TLC) developed with $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:35:7.2:0.8) and 2D TLC developed with $CHCl_3$:CH_3OH:H_2O (60:30:6) followed by $CHCl_3$:CH_3COOH:CH_3OH:H_2O (40:25:3:6) with final yields estimated by charring with cupric acetate in comparison to standards[6]. Semi-synthetic β1-linked isoprenoid phosphoglycolipids were prepared by transfer of mannose or glucose from the corresponding dinucletoides (GDP-mannose or UDP-glucose; Sigma) to pure polyprenol phosphates from plants or human liver (Polish Academy of Sciences, Institute of Biochemistry and Biophysics) using mannosyltransferase from *M. smegmatis* or *Micrococcus luteus* or glucosyltransferase from chick oviduct membranes and extracted as described[27]. Unglycosylated polyprenol phosphates were removed by preparative TLC.

Electrospray ionization (ESI) mass spectrometry

ESI and low-energy CID–MS/MS were carried out using a Quattro II, triple quadrupole mass spectrometer. The double focusing magnetic sector mass spectrometer (JEOL MStation) was operated with high collision energy (5kV) in the B/E linked scan mode.

Human subjects and bioassays

M. tuberculosis infected subjects (mean age 35 yr; 35% female) had all of the following: (1) a history of clinical exposure to active tuberculosis; (2) a documented positive intradermal tuberculin (PPD) test (> 15 mm induration, WHO criteria) within one month before entry into the study; and (3) negative serology for human immunodeficiency virus. Subjects were phlebotomized before initiation of isoniazid therapy and responses of their PBL to antigen were compared with PBL obtained from healthy PPD^- controls with no history of *M. tuberculosis* exposure (mean age 31 yr; 38% female). Isolation of fresh PBL and monocytes, and differentiation of monocytes into dendritic cells by 3 d of culture with GM-CSF and IL-4 were done as described[8]. All samples were processed on the day of phlebotomy, and the isolated PBL (with or without depletion of T cells by OKT3 and magnetic beads (MACS, Multenyi Biotec)) were cryopreserved for 3 d to allow the differentiation of monocytes from each donor into dendritic cells. The PBL were then thawed and tested for proliferation in the presence of autologous dendritic cells by ^{3}H-thymidine incorporation with or without addition of semi-synthetic C_{35} β1-MPD and blocking antibodies. Results were reported as stimulation indices (S.I.: c.p.m. (ag) / c.p.m. (no ag)). Responders were defined as subjects for which the S.I. was both > 3 and > 1 + 2 s.d. of the S.I. value. T-cell culture, assays and monoclonal antibodies against CD1a (OKT6, 10H3.9.3), CD1b (BCD1b3), CD1c (F10/21A3) and IgG control (P3) have been described[8,13,28].

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